Altered Expression of Angiopoietins after Cerebral Trauma

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Laboratory Medicine and Pathobiology
University of Toronto

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Dedication

To my Loving Father

Colonel Keyvan Nourhaghighi
Abstract

Altered Expression of Angiopoietins after Cerebral Trauma

Master of Science Degree Thesis
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University of Toronto

Angiopoietin-1 stabilizes vascular endothelium by activation of the endothelial Tie-2 receptor, and Angiopoietin-2 is a competitive antagonist of Angiopoietin-1/Tie-2 interaction. The role of these ligands in blood-brain barrier breakdown and cerebral angiogenesis post-trauma was studied in the rat cortical cold-injury model by examining their spatial and temporal mRNA and protein expression over a time-course of 6 hours to 6 days.

Increased Angiopoietin-2 relative to Angiopoietin-1 mRNA and immunoreactivity was observed in the early phase post-injury. This suggests a role in blood-brain barrier breakdown and vascular remodeling. Two to 6 days post-injury, the progressive increase in Angiopoietin-1 and decrease in Angiopoietin-2 mRNA coincides with cerebrovascular angiogenesis, maturation of neovessels, and restoration of blood-brain barrier. There was no change in the basal Tie-2 mRNA and immunoreactivity after cold-injury.

These results suggest that Angiopoietins are one of the factors regulating blood-brain barrier integrity and cerebrovascular angiogenesis post-injury.
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## Abbreviations

<table>
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<tbody>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic Day</td>
</tr>
<tr>
<td>HCMC</td>
<td>Human Coronary Microvascular Endothelial Cells</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human Mammary Epithelial Cells</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td>HUVECs</td>
<td>Human Umbilical Vein Endothelial Cells</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1-β</td>
</tr>
<tr>
<td>PI-3</td>
<td>Phosphatidyl Inositol – 3</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein Kinase C</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>rpm</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase – Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-Buffered Saline</td>
</tr>
<tr>
<td>TEK</td>
<td>Tunica interna Endothelial cell Kinase (synonymous with Tie-2)</td>
</tr>
<tr>
<td>Tie</td>
<td>Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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Chapter 1 - Review of Literature

1.1. Introduction

Blood-brain barrier refers to the endothelial interface between the blood and the brain that modulates and selectively restricts the passage of molecules across (Davson 1988). Interaction between cerebrovascular endothelium and peri-endothelial supporting cells is critical for maintaining blood-brain barrier integrity (Arthur et al 1987, Janzer and Raff 1987, and Stewart and Wiley 1981). Disruption of this interaction leads to blood-brain barrier breakdown and extravasation of proteins and fluid into the extravascular space, leading to development of vasogenic cerebral edema which is life-threatening (Klatzo 1967). This breakdown of the blood-brain barrier is observed during various clinical neuropathological conditions (Greenwood 1991), and is associated with varying degrees of vascular proliferation and remodeling (Nag 1996, and Nag et al 1997, 2001). Many factors have been implicated in regulating the sprouting of vessels from pre-existing vessels by a process termed angiogenesis (Folkman 1971).

Angiopoietins are a family of glycoproteins implicated in the development and integrity of blood vessels (Folkman and D’Amore 1996, Patan 1998, and Suri et al 1996). Angiopoietin-1 and Angiopoietin-2 bind to the same receptor, Tie-2, which is ubiquitously expressed in the vascular endothelium (Wong et al 1997). Binding of Angiopoietin-1 to Tie-2 promotes vascular maturation and quiescence by inducing association of endothelium with peri-endothelial supporting cells (Suri et al 1996). Angiopoietin-2 is a putative natural antagonist of Angiopoietin-1, and is involved in vascular remodeling (Maisonpierre et al 1997).

The potential role of Angiopoietins in mediating an interaction between cerebrovascular endothelium and its supporting cells has not been investigated. It is conceivable that
Angiopoietin-1 plays a role in maintaining blood-brain barrier integrity, as it induces a leakage-resistance phenotype in peripheral vessels (Thurston et al 1999). Moreover, Angiopoietin-2 may be involved in blood-brain barrier breakdown and cerebrovascular angiogenesis following brain injury, as it has been implicated in peripheral vascular remodeling and angiogenesis (Cohen et al 2001, and Goede et al 1998). This thesis aims to characterize the temporal and spatial expression of Angiopoietin-1 and Angiopoietin-2 mRNA and proteins in the cerebrovascular system, in normal rat brain and following experimental vasogenic cerebral edema.

1.2. Vascular Development and Remodeling

1.2.1. Vasculogenesis

Vascular development in the vertebrate embryo is initiated by vasculogenesis, which refers to the differentiation of mesoderm-derived hemangioblasts into angioblasts and hematopoietic cells, and the rearrangement of angioblasts into a primitive vascular plexus (Coffin et al 1991, Risau et al 1988, Risau and Flamme 1995, and Wilting and Christ 1996) (Figure 1a). These endothelial tubes become associated with pericytes and vascular smooth muscle cells (Sims 1986). It has recently been suggested that vasculogenesis may not be restricted to embryogenesis, as circulating angioblast-like cells can differentiate into endothelial cells (Asahara et al 1999, Shi et al 1998, and Takahashi et al 1999).

1.2.2. Angiogenesis

Following the formation of a primitive vascular plexus, new vessels arise from pre-existing vessels by a process referred to as angiogenesis (Risau 1997) (Figure 1b). Two proposed mechanisms for angiogenesis are sprouting and non-sprouting (Risau 1997, and Wilting and Christ 1996). During sprouting angiogenesis, new vessels form by budding and extending from
Figure 1. Vasculogenesis vs. Angiogenesis.
(a) Vasculogenesis involves differentiation of hemangioblasts into angioblasts, and their rearrangement into endothelial tubes of the primitive vascular plexus. (b) New vessels form by sprouting from pre-existing vessels (EC-Endothelial Cells, PC-Pericytes, BM-Basement Membrane).

Figure 2. Cerebral Capillary vs. Peripheral Capillary.
Significant differences between a capillary in the brain (a) compared to the periphery (b) include the presence of circumferential tight junctions, decreased pinocytic vesicles, increased mitochondria, and the association of astrocytes (Adapted from Miller 1997).
pre-existing vessels in response to an angiogenic stimulus (Wilting and Christ 1996). Sprouting angiogenesis accounts for the vascularization of previously avascular organs, such as the brain and kidney (Breier 2000, Folkman and D’Amore 1996, Lindahl 1997, Pardanaud et al 1989, Plate 1999, Risau and Wolburg 1990, and Stewart and Wiley 1981). Non-sprouting angiogenesis can be further classified into intussusceptive or intercalated angiogenesis (Wilting and Christ 1996). Intussusceptive angiogenesis refers to splitting of large pre-existing vessels into two smaller capillaries (Patan et al 1996), whereas intercalated angiogenesis refers to incorporation of endothelial cells into an existing vessel to increase both diameter and length of the vessel (Risau 1997).

1.2.3. Cerebral Vascularization

Migration of angioblasts from the splanchnopleuric mesoderm into the head initiates cerebral vascularization (Plate 1999). These angioblasts cover the brain and lead to extracerebral vascularization (Stewart and Hayakawa 1994). Capillaries sprout into the cerebral cortex from the perineural vascular plexus at embryonic day (E) 11.5 in the rat (Plate 1999). At this stage, these new blood vessels are discontinuous (Kniesel et al 1996, and Saunders et al 1999 b). The first known markers of cerebral endothelium appear at E 10.5 in mice (Qin and Sato 1995). There is a disappearance of fenestrations and an appearance of tight junctions between endothelial cells during E 11-13 (Stewart and Hayakawa 1994). Following this initial intracerebral vascularization, vessels disseminate the entire neuroectoderm, and continue to branch for about 2 weeks after birth in the rat (Bar 1980). Vessel maturation then proceeds through the recruitment of pericytes and astrocytic endfeet (Bar 1980). Interaction of cerebral endothelial cells with peri-endothelial cells induces specific characteristics in the endothelium that modulates passage of molecules between the blood and the brain (Arthur et al 1987, and Janzer and Raff 1987).
1.2.4. Post-Natal Vascular Formation


Various events occur during angiogenesis including: (1) disintegration of basement membrane; (2) migration and proliferation of endothelial cells; (3) aggregation to form endothelial channels; (4) formation of a new basement membrane; and (5) maturation of vascular endothelium through association with peri-endothelial supporting cells (Lievens et al 2001, and Tomanek and Schatteman 2000) (Figure 1b).

Numerous factors are involved in regulating angiogenesis, such as vascular endothelial growth factor (VEGF) and Angiopoietins, and the balance between angiogenic promoters and inhibitors determines the state of angiogenesis (Hanahan and Folkman 1996, Iruela-Arispe and Dvorak 1997, Klagsbrun and Moses 1999, and Saaristo et al 2000). Angiopoietins specifically will be discussed in section 1.5.

1.3. Morphology of Mature Cerebral Vessels

1.3.1. Cerebrovascular Endothelium

The cerebrovascular lumen is lined with a monolayer of endothelial cells, which are surrounded by a continuous basement membrane (Audus et al 1992, Broadwell 1989, and
Pugsley and Tabrizchi 2000). These endothelial cells are connected by circumferential tight
junctions and lack fenestrae (Audus et al 1992, Broadwell 1989, Kniesel and Wolberg 1999,
and Nagy et al 1984). They contain fewer pinocytic vesicles (Coomber and Stewart 1985, and
Westergaard 1977) and a higher density of mitochondria (Oldendorf 1977) compared with
peripheral vessels (Joo 1996, and Miller 1999) (Figure 2). Integrity of cerebrovascular
endothelial cells also depends on its unique cytoskeleton (Nag 1995, Nag et al 1978), and a
high luminal negative surface charge (Hardebo and Kahrstrom 1985, and Nag 1984 b).
Furthermore, brain endothelial cells show higher content and activity of enzymes such as p-
glycoprotein, γ-glutamyl transpeptidase, and Na⁺-K⁺-ATPase (Sanchez del Pino et al 1995, and
Vorbrodt et al 1983). For example, the activity of the Na⁺-K⁺-ATPase is approximately 500
times higher than in human umbilical cord endothelial cells (Eisenberg and Sudith 1979).
There are various specific markers for brain endothelial cells including glucose transporter-1
(GLUT-1) and endothelial brain antigen (EBA) (Joo 1996, and Orte et al 1999). The unique
structural and biochemical characteristics of cerebral endothelium create a selective barrier to
the free passage of compounds (Lewandowsky 1900, and Vorbrodt et al 1983).

1.3.1.1. Tight Junctions

Cerebrovascular endothelia are joined by the most complex tight junctions in the vascular
system (Nagy et al 1984, and Schulze and Firth 1992). These tight junctions form
circumferential belts between adjacent endothelium (Brightman 1989, and Brightman and
Reese 1969). Some of the constituents of the cerebral endothelial tight junctions include
occludin (Furuse et al 1993), claudin-1/2 (Furuse et al 1998), ZO-1 (Stevenson et al 1986), ZO-
and cingulin (Citi et al 1989). Changes in the phosphorylation levels of tight junction
constituents and associated factors have been shown to effect the integrity of cerebrovascular

1.3.1.2. Pinocytic Vesicles

Endothelial vesicles have been shown to be involved in shuttling proteins between the luminal side and the abluminal side of vascular endothelium (Palade 1961). Ultrastructural studies suggest that these vesicles fuse to form continuous channels across the endothelium (Bungaard et al 1983, Frokjar-Jensen 1980, Nag 1990, and Wagner 1984). Capillary endothelial cells in the cerebral cortex contain fewer pinocytic vesicles when compared to peripheral capillaries (Audus et al 1992, Brightman and Reese 1969, Broadwell 1989, Connell and Mercer 1974, Joo 1971, and Reese and Karnovsky 1967) (Figure 2). Rat cerebrovascular endothelium contains approximately 5 transport vesicles per μm² (Dux and Joo 1982, and Nag et al 1979).

1.3.2. Association with Astrocytes and Pericytes

Interaction between vascular endothelium and perivascular cells leads to inhibition of endothelial cell migration and proliferation, thus stabilizing the vasculature (Antonelli-Orlidge et al 1989, Orlidge and D’Amore 1987, and Sato and Rifkin 1989). Transplantation experiments by Stewart and Wiley (1981) have demonstrated the importance of peri-endothelial supporting cells in maintaining cerebrovascular integrity. Transplanted gut tissue in the brain was vascularized by brain endothelial cells that lost their cerebrovascular property (Stewart and Wiley 1981). On the other hand, brain tissue transplanted to the gut was vascularized by gut endothelial cells that acquired cerebrovascular characteristics (Stewart and Wiley 1981). These transplantation studies show that the association between cerebral endothelium with the surrounding brain microenvironment is critical in inducing and maintaining integrity of cerebral vessels (Abbott et al 1992, and Stewart and Wiley 1981).
Endothelia of cerebral capillaries are associated with astrocytic endfeet and pericytes (Davson 1967) (Figure 2a).

1.3.2.1. Astrocytes

Astrocytic endfeet surround 99% of the cerebral capillary circumference (Kacem et al 1998), and are situated 20 nm from the endothelium (Paulson and Newman 1987). The electrical resistance across a monolayer of endothelial cells in the absence of astrocytes in vitro is $80 \Omega \cdot cm^2$, which is 100 fold less than the $8000 \Omega \cdot cm^2$ of the mature parenchymal cerebral endothelium in vivo (Smith and Rapoport 1986). Pial vessels in vivo, which lack association with astrocytic endfeet, have an electrical resistance of approximately $1000 \Omega \cdot cm^2$ (Allt and Lawrenson 1997, and Cassella et al 1997). These results together with tissue culture experiments of astrocytes with endothelial cells (Abbott et al 1992, and Rubin et al 1991) suggest that astrocytic association is only partly responsible for maintaining the integrity of vessels in the cerebral cortex (Goldstein 1988).

1.3.2.2. Pericytes

Pericytes cover 20-30% of cerebrovascular surface (Frank et al 1987), and are completely enclosed within the vascular basement membrane (Bar and Budi Santoso 1984). Endothelial cells are in direct physical contact with pericytes through adhesion plaques (Sims 1991), peg and socket contacts (Diaz-Flores et al 1991), and gap junctions (Cuevas et al 1984). In cerebral capillaries, the interaction between endothelium and pericytes is mediated in part by N-cadherin (Gerhardt et al 2000). Pericytes which have a contractile function may decrease permeability of cerebral vessels (Schulze and Firth 1993, and Shepro and Morel 1993). The leakage-resistance inducing effect of pericytes on endothelial cells is evident in the retinal microvasculature which has a 10 fold higher density of pericytes compared to peripheral microvasculature (Frank et al 1987, and Stewart and Tuor 1994). Furthermore, there is
evidence for the role of cerebrovascular pericytes in pinocytosis and phagocytosis, which are important features of macrophages (Kristensson and Olsson 1973, Mato et al 1980, 1982, 1984, and Van Deurs 1976). Interestingly, pericytes express many markers of macrophages such as ED2 and OX-42 (Thomas 1999), and their expression increases following injury (Streit et al 1989) and in disease states (Kosel et al 1997). Therefore, pericytes may have a scavenger function at the interface between the blood and the brain (Broadwell and Salcman 1981, and Mato et al 1996).

1.3.2.3. Endothelial Supporting Cells and Vascular Integrity

Paracrine interaction between endothelial and peri-endothelial supporting cells of quiescent microvessels leads to inhibition of endothelial cell migration (Sato and Rifkin 1989) and proliferation (D’Amore 1992, Hellstrom et al 2001, and Orlidge and D’Amore 1987), thus promoting vascular stability. Growth factors such as platelet-derived growth factor-B (PDGF-B), tumor growth factor-β (TGF-β), basic fibroblast growth factor (bFGF), and VEGF modulate reciprocal interactions between endothelial cells and peri-endothelial supporting cells during vascular remodeling (Antonelli-Orlidge et al 1989, Benjamin et al 1998, Lindahl et al 1997, Sato and Rifkin 1989, Takagi et al 1996, and Watanabe et al 1997).

1.3.3. Circumventricular Organs

Some portions of the brain contain vessels which resemble peripheral vessels rather than cerebrocortical vessels, containing fenestrated endothelium with discontinuous tight junctions and pinocytic vesicles (Baldwin 2000, Brightman 1977, Ganong 2000, and Michel and Curry 1999). These areas are responsible for maintaining systemic homeostasis, and they are situated in close vicinity of the third and fourth cerebral ventricles (Ganong 2000). They are collectively referred to as the circumventricular organs and include: (1) the area postrema; (2) the median eminence; (3) the neurohypophysis (posterior pituitary); (4) the pineal gland; (5) the
organum vasculosum of the lamina terminalis; (6) the subfornical organ; (7) the subcommissural organ; and (8) the choroid plexus (Davson and Segal 1996, and Ganong 2000).

1.4. Blood-Brain Barrier

1.4.1. Introduction

Although molecules can freely traverse the endothelial interface between blood and extravascular tissue in peripheral vessels, the unique ultrastructure and composition of cerebrovascular endothelium limits this passage (Rapoport 1976). The ability of a molecule to cross the blood-brain endothelial interface depends on its lipid solubility and molecular size (Saunders et al 1999 a). This selective barrier to free passage of compounds has been termed the "blood-brain barrier" (Lewandowsky 1900).

Blood-brain barrier refers to the endothelial interface between the blood and the brain which selectively modulates the passage of certain compounds (Davson 1988, Ehrlich 1885, Goldmann 1909, and Rapoport 1976). The major roles of the blood-brain barrier include: (1) protection of the central nervous system against fluctuations in the concentrations of ions, hormones, and growth factors; (2) protection of the brain against neurotoxins and microorganisms; (3) prevention of neurotransmitter escape into the general circulation; and (4) facilitative transport mechanism for certain compounds (Friden 1993).

The integrity of the blood-brain barrier is attributed to: (1) continuous endothelium (Brightman et al 1977, and Reese and Karnovsky 1967); (2) inter-endothelial circumferential tight junctions (Brightman et al 1973, 1977, Brightman and Reese 1969, and Reese and Karnovsky 1967); (3) close association of astrocytic endfeet and pericytes (Balabanov and Dore-Duffy 1998, and Goldstein 1988); (4) facilitative and active transport pumps for specific molecules (Friden 1993, and Pardridge 1994); (5) enzymatic and metabolizing activity (Ghersi-

1.4.2. Blood-Brain Barrier Breakdown to Protein

Experiments with protein-binding dyes such as trypan blue (Tschirgi 1950) and protein tracers such as horseradish peroxidase (HRP) (Reese and Karnovsky 1967) have been used to study the blood-brain barrier to protein. This property is achieved early in development, at the time of tight junction formation between adjacent endothelial cells (Saunders et al 1991, and Saunders and Dziegielewska 1997).

Possible mechanisms of blood-brain barrier breakdown to protein include opening of interendothelial tight junctions and increased pinocytic vesicular transport (Davson and Oldendorf 1967, and Houthoff 1987). A variety of agents can alter tight junctions thereby altering paracellular permeability including cytokines (Walsh et al 2000), toxins (Hecht et al 1992), and leukocytes (Edens and Parkos 2000). Increased pinocytic activity is the principal mechanism of blood-brain barrier breakdown to protein occurring in hypertension, neoplasia, and trauma (Klatzo et al 1981, and Nag et al 1977). These pinocytic vesicles can fuse to form transendothelial channels allowing for the leakage of serum proteins from the cerebrovasculature as shown by electron microscopy (Nag 1984 a, 1990).

Blood-brain barrier breakdown to protein is a hallmark of numerous diseases such as trauma, stroke, hypertension, neoplasia, multiple sclerosis, and AIDS (Greenwood 1991). In these conditions, extravasation of serum proteins and fluid across the disrupted cerebrovasculature into the brain parenchyma leads to vasogenic cerebral edema (Klatzo 1967).
1.4.3. Vasogenic Cerebral Edema

Cerebral edema ("edema" from the Greek "oidema", meaning "swelling") refers to increased brain volume due to elevated water content leading to swelling (Katzman et al 1977, and Reichardt 1905). Types of cerebral edema include vasogenic, cytotoxic, and interstitial (Pappius 1989).

Vasogenic cerebral edema refers to accumulation of fluid in the extravascular space due to a breakdown of the blood-brain barrier to protein (Klatzo 1967). It is the most common form of cerebral edema (Milhorat 1992) and clinically it is the most important since it results in the most significant swelling (Klatzo et al 1981, and Weiss 1985). However, clinical states show presence of several types of edemas which are interrelated (Kimelberg 1995). Cytotoxic cerebral edema refers to an accumulation of fluid within astrocytes, due to a malfunctioning of the Na⁺-K⁺ pump and Ca⁺² transport (Klatzo 1967, Klatzo et al 1981, Norris and Pappius 1970, and Pollay et al 1985) in response to a variety of noxious substances (Blakemore et al 1972, Kesterson and Carlton 1971, and Rizzuto and Gonatas 1974). Cellular damage in later stages of cytotoxic edema leads to extravasation of serum proteins, and development of vasogenic edema (Fujimoto et al 1976). Interstitial cerebral edema refers to accumulation of fluid in the periventricular white matter as observed with hydrocephalus (Fishman 1975). Unlike vasogenic edema, interstitial edema is associated with fluid that is similar to cerebrospinal fluid, and the blood-brain barrier is intact (Fishman 1975).

Since the brain is located within the confined spaces of the cranium, cerebral swelling will cause an increase in intracranial pressure (Grände et al 1997). Such pressure buildup may lead to secondary brain injury and can be fatal (Gade et al 1990, and Schilling and Wahl 1997). Gross examination of human brain in vasogenic cerebral edema shows narrowed sulci and swollen gyri (McComb 1997), indicative of the high intracranial pressure. Microscopy shows
widening of the intercellular spaces of the brain parenchyma and widening of the perivascular spaces (Virchow-Robin) (Kuchiwaki et al 1990 b, and Ohata et al 1990).

Brain herniation is a major outcome of vasogenic cerebral edema (Weiss 1985). Due to the rigidity of the skull and the partitioning of the cranial vault by the falx and tentorial cerebelli, swelling of the brain can lead to displacement into adjacent compartments (Weiss 1985). Herniation may have several consequences: (1) downward herniation may stretch and rupture the basilar artery leading to hemorrhage (Duret hemorrhage); (2) compression of the third cranial nerve leads to pupillary dilation, and later paralysis of extraocular muscles (Third Nerve Palsy); (3) compression of the midbrain may damage the reticular activating system, causing coma; and (4) transtentorial or tonsillar herniation leads to compression of the medulla and may damage the cardiorespiratory centers, causing death (Bakay and Lee 1965).

Vasogenic cerebral edema is present in brain tumors, abscess, hemorrhage, infarction, ischemia, trauma, contusion, and lead encephalopathy (Klatzo 1967, and Manz 1974). Treatment for vasogenic cerebral edema and increased intracranial pressure include: (1) ventricular drainage of cerebrospinal fluid; (2) hyperventilation to decrease carbon dioxide concentration leading to cerebral vasoconstriction; (3) hypothermia; (4) head elevation; (5) surgical removal of edematous area; and (6) administration of mannitol, glucocorticoids, and barbiturates (Ghajar 2000, Grände et al 1997, Schilling and Wahl 1997, and Weiss 1985).

Interestingly, the blood-brain barrier breakdown during vasogenic cerebral edema is associated with varying degrees of vascular proliferation and remodeling, and various angiogenic factors have been implicated (Nag 1996, and Nag et al 1997, 2001). The objective of this thesis is to characterize the expression of a newly described family of angiogenic factors called Angiopoietins in the blood-brain barrier breakdown and angiogenesis during vasogenic brain edema.
1.5. Angiopoietins

1.5.1. Introduction

The interaction between vascular endothelium and perivascular supporting cells is thought to be critical for maintaining vascular stability and integrity (Beck and D’Amore 1997, and Folkman and D’Amore 1996). Angiopoietins regulate vascular development and homeostasis by mediating an interaction between endothelial cells with smooth muscle cells and pericytes (Folkman and D’Amore 1996). Among the Angiopoietin ligands, Ang-1 and Ang-2 have been well studied during vascular development and in the peripheral vasculature (Davis and Yancopoulos 1999).

1.5.2. Angiopoietin-1 and Angiopoietin-2

Angiopoietin-1 (Ang-1) is a 498 amino acid secreted glycoprotein, the gene for which has been localized to human chromosome 8q22.3-q23 (Cheung et al 1998, and Grosios et al 1999). Angiopoietin-2 (Ang-2) is a 496 amino acid secreted glycoprotein with 60% homology with Ang-1, and its gene is located on human chromosome 8p23.1 (Cheung et al 1998, and Grosios et al 1999). Angiopoietins have two homology domains: (1) a coiled-coil domain which is involved in ligand dimerization; and (2) a fibrinogen-like domain which induces auto-phosphorylation of the receptor tyrosine kinase Tie-2 (Procopio et al 1999) (Figure 3a). Ang-2 has been assumed to be the natural endogenous antagonist of Ang-1, since binding of Ang-2 to endothelial Tie-2 does not immediately induce receptor auto-phosphorylation (Maisonpierre et al 1997). However, Ang-2 induction of ectopically expressed Tie-2 on fibroblasts causes receptor phosphorylation (Maisonpierre et al 1997). Furthermore, evidence suggests that prolonged incubation of Ang-2 with Tie-2 also leads to Tie-2 phosphorylation (Teichert-Kuliszewska et al 2001). In addition, Ang-2 at high concentrations can also induce phosphorylation and activation of Tie-2 and lead to endothelial cell survival through the
Figure 3. Angiopoietins.
(a) Angiopoietins have a conserved coiled-coil domain and a fibrinogen-like domain. Ang-1 and Ang-2 share 60% protein sequence homology. The coiled-coil domain is involved in ligand dimerization, and the fibrinogen-like domain induces auto-phosphorylation of the receptor tyrosine kinase Tie-2.
(b) The extracellular portion of Tie-2 consists of two immunoglobulin-like loops separated by three EGF-like repeats that are followed by three fibronectin type III-like repeats. The intracellular portion contains a split-kinase domain.
(c) Binding of Ang-1 to Tie-2 leads to dimerization and cross-phosphorylation of the intracellular tyrosine kinase domains. These domains then bind to adaptor proteins such as Grb2/SOS and p85 and induce signal transduction cascades within the cell.
phosphatidylinositol 3'-kinase/Akt signal transduction pathway (Kim et al 2000 d). Therefore, it is postulated that Ang-2 may not simply be an antagonist of Ang-1, but instead may be transducing a specific signal by acting as a competing agonist (Teichert-Kuliszewska et al 2001).

Various alternatively spliced Ang-1 (Huang et al 2000, and Marziliano et al 1999) and Ang-2 (Kim et al 1999 b, Kim et al 2000 e, and Mezquita et al 1999, 2000) mRNA species have been described. In addition, Ang-3 (Kim et al 1999 a, Nishimura et al 1999, and Valenzuela et al 1999) and Ang-4 (Valenzuela et al 1999) have also been cloned by homology to Ang-1 and Ang-2. Ang-3 in mice acts as an antagonist, and Ang-4 in humans acts as an agonist on the Tie-2 receptor (Valenzuela et al 1999). Furthermore, several Angiopoietin- related genes such as hepatic fibrinogen-related protein (Kim et al 2000 b) and Angiopoietin-related protein (Conklin et al 1999, and Kim et al 1999 b) have been described.

1.5.3. Tie-2

Tie-2 (Tyrosine kinase with immunoglobulin and epidermal growth factor homology domain) is a receptor tyrosine kinase that is a target for Ang-1 and Ang-2 (Davis et al 1996, and Maisonpierre et al 1997). It is ubiquitously expressed in all vascular endothelial cells, including cerebrovascular endothelial cells (Wong et al 1997). Tie-2 has an extracellular domain that is responsible for ligand binding, a transmembrane region for anchoring in the plasma membrane, and an intracellular domain with kinase activity (Procopio et al 1999). The extracellular domain consists of two immunoglobulin-like loops separated by three epidermal growth factor (EGF)-like repeats that are followed by three fibronectin type III-like repeats, and the intracellular part contains a split kinase domain (Iwama et al 1993, and Runting et al 1993) (Figure 3b). Ang-1 binding to Tie-2 induces receptor dimerization and autophosphorylation of the intracellular kinase domains (Procopio et al 1999). This leads to the activation of several
downstream signal transduction cascades such as: (1) binding of p85 activates the phosphotidyl inositol-3 (PI-3) kinase leading to endothelial survival and migration through Akt and Rac / protein kinase C (PKC) respectively; (2) binding of growth factor receptor-bound protein (Grb2) and son-of-sevenless (SOS) leads to activation of the Ras pathway (Fujikawa et al 1999, Jones et al 1999, Kim et al 2000 a, Kontos et al 1998, and Papapetropoulos et al 2000) (Figure 3c). Furthermore, the phosphorylated Tie-2 has been shown to interact with other factors such as Dok-R (Jones and Dumont 1998) and vascular endothelial-protein-tyrosine phosphatase (Fachinger et al 1999).

Tie-1 is 44% homologous to Tie-2 in amino acid sequence, and is highly expressed during vascular development in the embryo (Sato et al 1995). Although Tie-1 knockout mice develop severe edema and hemorrhage due to vascular defects, neither Ang-1 nor Ang-2 has been shown to bind to Tie-1 (Puri et al 1995). A recent study on the interaction of Tie-1 and Tie-2 has shown that Tie-1 exhibits negligible kinase activity, and is found within endothelial cells bound to Tie-2 (Marron et al 2000). It is suggested that Tie-1 is likely an orphan receptor that modulates Tie-2 activity by dimerization (Marron et al 2000).

1.5.4. Angiopoietin-1 and Angiopoietin-2 Expression in Normal Tissue

Whereas Ang-1 is expressed widely in normal adult tissue, Ang-2 has only been observed at sites of vascular remodeling, such as the female reproductive tract (Maisonpierre et al 1997). Ang-1 mRNA expression has been observed in the cerebellum, small intestine, skeletal muscle, prostate, ovary, uterus, and placenta by northern blotting (Maisonpierre et al 1997), and in the lung by reverse transcriptase – polymerase chain reaction (RT-PCR) (Wong et al 2000). Ang-2 expression has been shown in the ovary, uterus, and placenta by northern blotting (Maisonpierre et al 1997), and in the lung by RT-PCR (Wong et al 2000).

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1.5.5. Function of Angiopoietins

1.5.5.1. Role of Angiopoietins in Vasculogenesis and Angiogenesis

Angiopoietins have been localized during placental development (Dunk et al 2000) and at the fetal-maternal interface (Goldman-Wohl et al 2000). During embryonic vascular development, Ang-2 is expressed in vascular endothelial cells, while Ang-1 is expressed in the surrounding smooth muscle cells (Davis et al 1996, and Maisonpierre et al 1997). Ang-1 knockouts displayed defects in remodeling and integrity of the primitive vascular plexus, leading to embryonic lethality (Suri et al 1996). Ultrastructural examination of these defective vessels showed (1) poor association between endothelial cells and the underlying supporting cells such as pericytes, (2) rounded appearance of endothelial cells, and (3) defects in vascular network formation (Suri et al 1996). Furthermore, microvessels in the forebrain of Ang-1 knockouts appear dilated (Suri et al 1996). Tie-2 knockouts displayed a similar phenotype as the Ang-1 knockouts (Patan 1998). The most prominent defects of the Tie-2 knockout mice include lack of capillary sprouts into the neuroectoderm, the failure of the primary plexus to

In the adult, Angiopoietins have been implicated in the physiological angiogenesis of the female menstrual cycle (Geva and Jaffe 2000, Goede et al 1998, and Hazzard et al 1999). Ang-1 has also been shown to induce capillary-like endothelial tubule formation (Hayes et al 1999) and endothelial cell sprouting (Kim et al 2000 c, and Koblizek et al 1998). During tissue culture angiogenesis, Ang-1 is expressed by smooth muscle cells, but not by endothelial cells (Mandriota and Pepper 1998). However, Ang-2 is localized in smooth muscle cells, pericytes, and in endothelial cells at the invading front of vascular sprouts by in situ hybridization (Maisonpierre et al 1997). Ang-1 and Ang-2 expression have been observed in various experimental models of angiogenesis such as cutaneous wound healing (Bloch et al 2000, and Kampfer et al 2001) and retinal neovascularization (Hackett et al 2000).

Ang-2 mRNA is upregulated during non-neoplastic cerebral angiogenesis such as in human arteriovenous malformations (Hashimoto et al 2001) and following hypoxia (Beck et al 2000, and Mandriota et al 2000), whereas Ang-1 is similar or only slightly increased. In situ hybridization has localized the Ang-1 and Ang-2 mRNA upregulation during non-neoplastic

1.5.5.2. Angiopoietins - Vascular Integrity, Permeability, and Survival

Gene knockout studies suggest that the interaction between the endothelium and the perivascular cells is critical for maintaining vascular integrity, and Ang-1 seems to promote this interaction (Patan 1998, and Suri et al 1996). Furthermore, Ang-2 mRNA is down-regulated in response to shear stress in HUVECs (Bongrazio et al 2000).

Transgenic overexpression or acute administration of Ang-1 results in vessels that are resistant to the leakage-inducing effects of inflammatory irritants such as mustard oil (Jain and Munn 2000, and Thurston et al 1999, 2000). The phenotype of transgenic Ang-2 overexpression is similar to the Ang-1 and Tie-2 knockouts, involving severe disruptions in
vascular development (Maisonpierre et al 1997). The mechanism for antipermeability effects of Ang-1 is by targeting of intercellular junctions. Ang-1 localizes proteins such as PECAM-1 into cell junctions between adjacent endothelial cells (Gamble et al 2000). Also, Ang-1 leads to the phosphorylation of PECAM-1 and vascular endothelial cadherin, thereby strengthening inter-endothelial junction (Gamble et al 2000). Ang-1, but not Ang-2, has been shown to promote adhesion of Tie-2 positive hematopoietic stem cells to fibronectin (Takakura et al 1998), providing further evidence for its ability to induce intercellular adhesion. Recently, angiopoietins have been shown to directly associate with integrins, thereby supporting cell adhesion (Carlson et al 2001). This adhesion does not take place through the Tie-2 receptor, as NIH 3T3 cells lacking or ectopically expressing Tie-2 adhere on Ang-1 and Ang-2 coated surfaces (Carlson et al 2001). Furthermore, stimulation of Tie-2 expressing hematopoietic cells with Ang-1 also enhanced adherence to coated fibronectin (Huang et al 1999). Binding of Ang-1 and Ang-2 has been shown in vitro to another extracellular matrix component, vitronectin (Carlson et al 2001). Therefore, Ang-1 may promote adhesion and integrity of the microvascular components (Carlson et al 2001). The role of Angiopoietins in maintaining the blood-brain barrier integrity of cerebral vessels has not been examined.

Ang-1 has been shown to inhibit endothelial apoptosis through the Akt/survivin/PI-3 kinase pathway (Fujikawa et al 1999, Hayes et al 1999, Jones et al 1999, Kim et al 2000 a, Kwak et al 1999, 2000, and Papapetropoulos et al 1999, 2000). On the other hand, Ang-2 is thought to promote apoptosis as it is detected just prior to the onset of apoptosis in vascular cells (Cohen et al 2001, and Zagzag et al 2000). However, Ang-2 at high concentrations has been shown to lead to endothelial cell survival through the PI-3/Akt signal transduction pathway, just like Ang-1 (Kim et al 2000 d). This is in agreement with the results obtained in our laboratory showing phosphorylation of Tie-2 by Ang-2 after extended incubation (Teichert-Kuliszewska et al 2001).
1.5.6. Interactions of Angiopoietins and Vascular Endothelial Growth Factor


mediators of pathological angiogenesis (Siemeister et al 1999). Ang-1 and VEGF lead to growth of vessels with enlarged diameters, and Ang-2 and VEGF induce elongation of vessels, when compared to VEGF alone (Asahara et al 1998). Ang-1 and Ang-2, in the presence of VEGF, lead to proliferation of endothelial cells (Huang et al 1999). VEGF up-regulates Ang-1 expression in human retinal pigment epithelial cells, whereas Ang-2 expression seems unaffected (Hangai et al 2001).

1.5.7. Angiopoietins and Inflammation

Tumor necrosis factor-α (TNF-α) and interleukin-1-β (IL-1-β) are pro-inflammatory cytokines which stimulate the transmigration of leukocytes across the endothelial monolayer lining blood vessels (Furie and McHugh 1989, and Moser et al 1989). Ang-1 inhibits TNF-α-stimulated leukocyte transmigration, and thus may act as an anti-inflammatory agent in HUVECs (Gamble et al 2000). Also, Ang-1 mRNA is down-regulated by IL-1-β (Ristimaki et al 1998). On the other hand, TNF-α induces Ang-2 mRNA and protein expression in a time- and dose-dependent manner (Kim et al 2000 f). Moreover, TNF-α and IL-1-β induce Tie-2 receptor expression in a time- and dose-dependent manner in HUVECs, HMEC, and HCMEC (Willam et al 2000). In addition, the inflammatory mediator phorbol-myristate-acetate enhances Ang-2 mRNA (Krikun et al 2000) and down-regulates Ang-1 mRNA (Enholm et al 1997). Furthermore, Ang-1, Ang-2, and Tie-2 have been implicated in the inflammatory neovascularization of pyogenic granuloma (Yuan et al 2000 b) and psoriasis (Kuroda et al 2001). These observations suggest that Ang-1 is anti-inflammatory, whereas Ang-2 is pro-inflammatory in pathological conditions.

1.5.8. Other Regulators of Angiopoietin-1 and Angiopoietin-2 Expression

Ang-1 expression is down-regulated by serum, PDGF, EGF, and TGF-β (Enholm et al 1997). TNP-470, an antiangiogenic agent, leads to an increase of Ang-1 expression in

1.6. Cortical Cold-Injury

1.6.1. Introduction

The rat cortical cold-injury model is a classic model of vasogenic brain edema that is well established in our laboratory (Nag 1996, and Nag et al 1997). Cortical cold-injury was first introduced approximately 50 years ago in cats (Clasen et al 1953, and Klatzo et al 1955), and has been performed in several other species including rats (Nag 1996), mice (Murakami et al 1999), and sheep (Sheikh et al 1996).

Cold-injury has many neuropathological features of cerebral contusions after trauma including: (1) cytolysis of neurons; (2) petechial hemorrhages; (3) an inflammatory response; and (4) blood-brain barrier breakdown resulting in edema in the lesion penumbra (Clasen et al 1953, and Klatzo et al 1955). Elements of both vasogenic and cytotoxic edema are observed in
cryogenic lesions, however the vasogenic edema is the dominant form (Gazendam et al 1979). Furthermore, recent studies demonstrate that the cold-injury model is an in vivo model of cerebrovascular angiogenesis (Nag 1996, and Nag et al 1997, 2001).

1.6.2. Time-Course of Histological Changes

1.6.2.1. Blood-Brain Barrier Breakdown and Angiogenesis

Evans blue–albumin complex extravasation was maximal 30 minutes after rat cortical cold-injury, and declined sharply thereafter (Chan et al 1983). Extravasation of plasma proteins from disrupted lesion vessels into the interstitium of brain was observed 12 hours post-injury (Nag 1996). This extravasated serum proteins spread into the underlying white matter and diffused into the white matter of the contralateral hemisphere (Nag 1996, and Suzuki et al 1995). Cold-injury resulted in an increase in microvillus formation, endocytosis, and vesicular passage in cerebral capillaries (Trout et al 1986). Permeable vessels, marked with HRP, were mainly arterioles at the margin of the necrotic area (Nag 1996). Water content in the injured hemisphere is shown to gradually increase, peaking at 24 hours post-injury (Murakami et al 1999). This increased water content was especially significant in the white matter (Kuchiwaki et al 1990 a). The composition of the edematous region was similar to that of plasma, showing that most of the edema following cryogenic lesions is due to vasogenic edema (Gazendam et al 1979). Proliferation and reconstruction of cerebral microvascular architecture following experimental cold-injury has been documented using bromodeoxyuridine staining (Orita et al 1988). Many other studies have implicated neovascularization as a component of cryogenic lesions (Cancilla et al 1979, Mitchell et al 1979, Nag et al 1997, 2001, and Olson et al 1987).

1.6.2.2. Other Histological Features

One day post cold-injury, polymorphonuclear leukocytes have been observed within the lesion, and they persist until about 3 days post cold-injury (Nag 1996). Macrophages first
appear within the lesion one day post cold-injury, and their numbers gradually increase to reach a maximum by approximately 4 days post cold-injury (Nag 1996).

Coagulative necrosis was observed 12 hours after cold-injury due to the extravasated serum proteins (Nag 1996). Calcification was noted in the necrotic core at 4-5 days post cold-injury (Nag 1996). At 14 days post-injury, the lesion appeared depressed, and the overlying pial arterioles showed thickening of their walls (Nag 1996). Opening of inter-endothelial tight junctions after cold-injury can also lead to (1) desquamation, (2) degeneration and necrosis of the endothelial lining, (3) thrombosis formation, and (4) basement membrane disruption (Vorbrodt et al 1993). There is evidence for apoptosis after cold-injury including DNA fragmentation and shrunken nuclei (Murakami et al 1999). Originally apoptotic cells were widespread in the entire lesion, however by 3 days post-injury they were only detected in the marginal zone surrounding the lesion (Murakami et al 1999). Other pathological observations of cryogenic lesions include phospholipid degradation and lipid peroxidation (Chan et al 1983), pericapillary swelling of astrocytes mediated by activation of ornithine decarboxylase (Trout et al 1995), presence of oxygen free radicals (Ikeda et al 1989), and nitric oxide synthase localization (Gotoh et al 1998, and Nag et al 2001).

1.6.3. Factors Implicated in the Pathogenesis and Repair

Thus far, there have been various factors implicated in the pathogenesis and repair following cortical cold-injury, such as VEGF (Nag et al 1997) and nitric oxide (Nag et al 2001). Angiopoietins are a recently-discovered family of glycoproteins which are indispensable for peripheral vascular development and integrity (Patan 1998, and Suri et al 1996). The main goal of this thesis is to characterize the expression of Angiopoietins in the normal cerebrovascular system and following cortical cold-injury model of vasogenic cerebral edema.
CHAPTER 2

RATIONALE AND HYPOTHESES
Chapter 2 – Rationale and Hypotheses

2.1. Rationale

As indicated in the literature review, Ang-1 promotes association of the vascular endothelium with perivascular cells in the peripheral vasculature, thus increasing vascular permeability and integrity (Patan 1998, Suri et al 1996, and Thurston et al 1999). Furthermore, Ang-1 can alter phosphorylation and association of tight junctional proteins (Gamble et al 2000). Recently, it has been shown that Ang-1, but not Ang-2, is expressed in normal brain cortical vessels (Hashimoto et al 2001). Knowing that blood-brain barrier integrity is dependent on the complex inter-endothelial tight junctions and association of peri-endothelial supporting cells (Nagy et al 1984, Pardridge 1998, and Schulze and Firth 1992), it is conceivable that Ang-1 may play a role in maintaining blood-brain barrier integrity. If this hypothesis were true, it would be expected that Ang-1 expression would decrease during blood-brain barrier breakdown, or there would be an increased expression of the antagonizing Ang-2. Furthermore, as Ang-1 and Ang-2 are both involved in developmental and pathological angiogenesis, it is conceivable that they play a role in cerebral angiogenesis during the repair and remodeling of injured cerebral vessels. Therefore, assuming Angiopoietins are expressed in cerebrovasculature and considering their significant effect on endothelial permeability, Angiopoietins may modulate blood-brain barrier integrity in steady and pathological states.
2.2. Hypotheses

I. Angiopoietin-1 contributes to the integrity of the blood-brain barrier; therefore, in cryo-induced vasogenic cerebral edema there will be loss of Angiopoietin-1 expression in cortical vessels.

II. Angiopoietin-2 antagonizes the stabilizing action of Angiopoietin-1, thus selectively modulating blood-brain barrier integrity, and facilitating endothelial cell activation in response to VEGF and other angiogenic growth factors; therefore, cold-injury will produce increased expression of Angiopoietin-2 post-trauma associated with the angiogenic response.

2.3. Specific Aims

The rat cortical cold-injury model will be utilized to study the spatial and temporal expression of Ang-1 and Ang-2 at the gene and protein levels during the 6 hours to 6 days period post-trauma. A semi-quantitative estimate of Ang-1, Ang-2, and Tie-2 mRNA expression in the normal rat brain cortex and following cortical cold-injury will be determined by RT-PCR. A semi-quantitative estimate of Ang-1 and Ang-2 proteins in normal rat brain cortex and after cortical cold-injury will be determined by western blotting. Tissue localization of Ang-1, Ang-2, and Tie-2 proteins in normal rat brain and post-injury will be detected by immunohistochemistry. Immunostaining results of cortical vessels containing an intact blood-brain barrier will be compared to vessels of the choroid plexus, ependyma, and posterior pituitary which lack blood-brain barrier characteristics. Ang-1 and Ang-2 immunoreactivity will be correlated with blood-brain barrier breakdown to protein. The latter will be determined by serum protein immunostaining.
CHAPTER 3

METHODOLOGY
Chapter 3 – Methodology

3.1. Rat Cortical Cold-Injury Model

Male Wistar rats (180 to 200 g, Charles River Laboratories, Wilmington, MA, USA) were anaesthetized using methoxyflurane (Metophane, Janssen Pharmaceutica, Titusville, NJ, USA) by inhalation. A 2.3 mm dental drill bit (Horico, Berlin, Germany) was used to produce a concave hole midway between the lambdoid and coronal sutures on the parietal bone. The cold probe consisted of a 20 ml syringe with a blunt copper wire tip, filled with liquid nitrogen. The cold probe was applied over the concave hole for 45 seconds (Figure 4a). The incision was sutured, and the analgesic buprenorphine hydrochloride (Buprenex, 0.033 ml per 100 g body weight, Reckitt & Colman Pharmaceuticals Inc., Hull, England) was injected intraperitoneally. This protocol was in accordance with the guidelines set by the Canadian Council on Animal Care and was further approved by the local animal care committee.

For RT-PCR, a time-course of 6 hours, 2, 4, and 6 days post-injury was studied, and three samples were taken from the normal brain cortex of each control rat (n=5). For western blotting, a time-course of 2 and 4 days post-injury was analyzed using normal brain cortex as control (n=5). For immunohistochemistry, a time-course of 6 hours, 2, 3, 4, and 6 days post-injury was analyzed using normal brain cortex as control (n=3).

3.2. Sample Preparation and Treatment

For RT-PCR and western blotting, rats were decapitated and cortical tissue samples weighing 20 mg on average from lesion, peri-lesion, and contralateral to lesion area were flash-frozen in liquid nitrogen and stored at −80 °C (Figure 4b). The entire pituitary gland was flash-frozen without separating the anterior and posterior lobes.
Rat Cortical Cold-Injury Model

Figure 4 (a) Surgical Steps.
Steps in production of the cold-injury include making an incision overlying the left parietal bone (i), drilling a concave hole midway between the coronal (cor) and lambdoid (lam) sutures (ii), and placement of a syringe filled with liquid nitrogen and having a blunt copper tip at the craniotomy site (iii).

Figure 4 (b) Tissue Sampling.
(i) A severe 4-day lesion is shown. Note the area of necrosis within the lesion surrounded by hemorrhage. (ii) For RT-PCR and western blotting, samples weighing 20 mg on average were obtained from the lesion area (L), peri-lesion area (P), and contralateral to the lesion area (C).
For immunohistochemistry, rats were perfused using 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.3) at a pressure of 110 mmHg, by a cannula in the ascending aorta. Brains were removed and sliced into 3 mm coronal slabs. Slices were fixed for 16 hours at room temperature in the same fixative, and paraffin-processed on the Histomatic Tissue Processor (Model 166 MP, Fisher Scientific Ltd., Nepean, ON, Canada) using the following schedule for the different solutions: (1) 80% alcohol, 30 minutes; (2) 95% alcohol, twice, 20 minutes each; (3) 100% alcohol, twice, 40 minutes each; (4) 100% alcohol, 50 minutes; (5) xylene, twice, 50 minutes each; and (6) paraffin wax at 60 °C, twice, 30 minutes each. Slices were then embedded in paraffin blocks using the TBS 88 Tissue Embedding System (Medite, Burgdorf, Germany).

3.3. Reverse Transcriptase – Polymerase Chain Reaction

The RNeasy Kit (QIAGEN, Mississauga, ON, Canada) is optimized to extract RNA molecules longer than 200 nucleotides, thereby excluding small RNAs such as 5.8S RNA, 5S RNA, and tRNA. RNA extraction was performed according to the manufacture’s protocol. Samples weighing 20 mg on average were homogenized in 350 μl RLT buffer containing 1% β-mercaptoethanol. The homogenate was centrifuged for 3 minutes at 13,000 rpm, and thoroughly mixed with an equal volume of 70% ethanol by pipetting. The mixture was added onto the RNeasy mini spin column, and centrifuged for 15 seconds at 10,000 rpm. Then the following solutions were pipetted onto the spin column, followed by centrifugation: (1) 700 μl of RW1 buffer, and centrifuged for 15 seconds at 10,000 rpm to wash; (2) 500 μl of Buffer RPE, and centrifuged for 15 seconds at 10,000 rpm to wash; and (3) 500 μl Buffer RPE, and centrifuged for 2 minutes at 13,000 rpm to dry the RNeasy membrane. RNeasy column was transferred into a new 1.5-ml collection tube and 30 μl of RNase-free water was directly
pipetted onto the RNeasy membrane. The column was centrifuged for 1 minute at 10,000 rpm to elute the RNA. The absorbance of the RNA was measured at 260 and 280 nm using SpectraMax-250 spectrophotometer and the SOFTmax-Pro computer software (Global Medical Instrumentation Inc., Clearwater, MN, USA). RNA concentration and purity were estimated as follows: [RNA] = OD_{260} \times \text{Dilution Factor} \times 40, \text{Purity} = \frac{\text{OD}_{260}}{\text{OD}_{280}}.

Reverse-transcription was performed in 40 μl of reaction volume containing 2 μg of sample RNA, 0.450 μg random primers (GibcoBRL, Burlington, ON, Canada), 200 μM deoxynucleotide triphosphate (dNTP) mixture, 400 units of Moloney Murine Leukemia Virus reverse transcriptase (GibcoBRL, Burlington, ON, Canada), 1mM DTT, and 22640 units RNAguard RNase inhibitor (Amersham Pharmacia Biotechnology, Piscataway, NJ, USA).

PCR amplification was performed in 50 μl total volume containing 2-10 μl reverse transcription product, 200 μM dNTP mixture, 50 pmol of each primer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and 2.5 units of Taq polymerase (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The sequence of primers were: Ang-1 [sense 5'-CACGAC AGACCAGTACAACACAAACG-3' and antisense 5'-GACGACTGTTGTTGGTAGCTCT-3']; Ang-2, partially degenerate primers [sense 5'-GT(GT)GA(CT)TT(CT) CAGAG(ACGT)AC(ACGT)TGG-3' and antisense 5'-CGA(AG)TAGCC(GT)GA(ACGT) CC(CT)TTCCA-3']; Tie-2 [sense 5'-CAGGACCTTCACAACAGCTTCTATCGGACT-3' and antisense 5'-CTGTCGAAGAATGTCACTAAGGGTCCAAGC-3']; GAPDH [sense 5'-CTCTAAGGCTGTGGCAAGGTCATGACTAAGGGTCCAAGC-3' and antisense 5'-CTGTA-3']. Amplification cycles were 30 for Ang-1, Ang-2, and Tie-2, and 25 for GAPDH.

The sizes of the PCR products were 570 bp, 450 bp, 322 bp, and 343 bp for Ang-1, Ang-2, Tie-2, and GAPDH respectively. The amplified cDNA segments were run on 2% agarose gels.
containing 4% ethidium bromide. Signals were visualized using a gel scanning system (Bio-Rad Laboratories, Hercules, CA, USA).

3.4. Western Blotting

Pre-weighed tissue samples were lysed in 6 times the corresponding volume of RIPA buffer (1.4 M NaCl, 13 mM Na₂HPO₄, 2.3 mM NaH₂PO₄, pH 7.4) containing various protease inhibitors (1% Nonidet P-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 10 μl/ml PMSF, 30 μl/ml Aprotinin, 10 μl/ml Sodium Orthovanadate). Using a Pellet Pestle Motor (Kontes, Vineland, NJ, USA), the samples were homogenized on ice. Homogenates were incubated on ice for 30 minutes, and centrifuged at 12,500 rpm for 10 minutes at 4 °C. For each sample, the supernatant was transferred to another tube and centrifuged again at 12,500 rpm for 10 minutes.

Protein concentration was estimated using the modified Lowry’s method (Harrington 1990) and the detergent-compatible protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The samples were standardized against two identical bovine serum protein concentration gradients. Protein content was determined by measuring absorbance at 750 nm using the SpectraMax-250 photospectrometer and the SOFTmax-Pro computer software (Global Medical Instrumentation Inc., Clearwater, MN, USA).

Samples were run on 6% Tris-Glycine polyacrylamide gels (Invitrogen Life Technologies, Burlington, ON, Canada), under denaturing conditions of β-mercaptoethanol (X-Cell II Mini Cell, Helixx Technologies Inc., Toronto, ON, Canada). MultiMark Multi-Colored standard marker (Invitrogen Life Technologies, Burlington, ON, Canada) was used to estimate corresponding band sizes. 75 μg of protein was run on each lane using a protein denaturing buffer (running buffer: 0.025 M Tris base, 0.2 Glycine, 0.1% SDS, pH 8.3) and a voltage difference of 120 V.
Samples were transferred from the gel (transfer buffer: 0.04 M Tris base, 0.19 M Glycine, 20% methanol) onto a nitrocellulose membrane (Invitrogen Life Technologies, Burlington, ON, Canada), using a current of 60 mA for 16 hours (mini-PROTEAN II Cell, Bio-Rad Laboratories, Hercules, CA, USA). Transfer from the gel was confirmed with Coomassie Blue staining (BioShop, Burlington, ON, Canada). Transfer onto the membrane was confirmed by Ponceau S staining (Sigma, St. Louis, MO, USA).

The nitrocellulose membranes were blocked in Tris-buffered saline (TBS) buffer (0.08 M Tris, 0.1 M NaCl, 0.1% Tween-20) containing 7% skim milk for Ang-1, and 5% skim milk (Nestle, Willowdale, ON, Canada) for Ang-2, for 2 hours at room temperature.

For Ang-1 staining, nitrocellulose blots were incubated in Ang-1 polyclonal goat antibody (N-18, 1:300; Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in 2% skim milk TBS buffer for 6 hours at 4 °C. For Ang-2 staining, nitrocellulose blots were incubated in carboxy-terminal Ang-2 polyclonal goat antibody (C-19, 1:200; Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in 2% skim milk TBS buffer for 2 hours at room temperature. Alternatively, nitrocellulose blots were incubated in the amino-terminal Ang-2 polyclonal goat antibody (F-18, 1:200; Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in 2% skim milk TBS buffer for 2 hours at 4 °C. Ang-2 (C-19) is against the carboxy-terminus, whereas Ang-2 (F-18) is against the amino-terminus of Ang-2 protein.

Following treatment with primary antibody, nitrocellulose blots were washed three times in TBS buffer for 10 minutes each at room temperature. Blots were then stained with HRP-conjugated human/mouse pre-adsorbed donkey anti-goat immunoglobulin-G (IgG) secondary antibody (1:2000; Santa Cruz, Santa Cruz Biotechnology, CA, USA) diluted in 2% skim milk TBS buffer for 1 hour at room temperature.

Blots were washed twice in TBS buffer and once in TBS buffer without Tween-20 for 10 minutes each at room temperature. The ECL chemiluminescent detection kit for western
blotting was used according to the manufacturer's protocol (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The reaction was exposed onto emulsion x-ray films (Kodak, Rochester, NY, USA) for a duration of 30 seconds to 10 minutes, depending on the intensity of the reaction.

Nitrocellulose blots were washed in stripping buffer (10% SDS, 62.5 mM Tris-HCl, 100 mM β-mercaptoethanol) twice for 30 minutes each at 70 °C. Blots were then washed in TBS, three times for 10 minutes each at room temperature. Blocking was performed in TBS buffer containing 5% skim for 2 hours at room temperature. Nitrocellulose blots were incubated in mouse β-actin monoclonal antibody (1:5000; Sigma, St. Louis, MO, USA) diluted in 2% skim milk TBS buffer for 16 hours at 4 °C. Blots were then stained with HRP-conjugated goat anti-mouse IgG secondary antibodies (1:2000; Sigma, St. Louis, MO, USA), diluted in 2% skim milk TBS buffer for 1 hour at room temperature. Developing was performed with the ECL kit, as described above.

Total plasma protein was extracted by mixing rat blood thoroughly with 15% tripotassium ethylenediamine tetra-acetate (K₂EDTA) anti-coagulating solution (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA), followed by centrifugation at 3,000 rpm for 10 minutes.

3.5. Immunohistochemistry

Paraffin-embedded 6 μ coronal sections were deparaffinized using the following solutions for the specified times: (1) xylene, twice, 5 minutes each; (2) 100% ethanol, twice, 3 minutes each; (3) 95% ethanol, 3 minutes; (4) 70% ethanol, 2 minutes; (5) 50% ethanol, 2 minutes; and (6) distilled water, 5 minutes, at room temperature.
Sections were partially digested in 0.5% pepsin in 0.01 M HCl for 30 minutes at 37 °C. Then they were washed in distilled water three times, for 2 minutes each. Endogenous peroxidase activity was blocked by 0.3% methanolic peroxide for 20 minutes. Sections were then washed twice in phosphate-buffered saline (PBS) buffer (1.4 M NaCl, 13 mM Na₂HPO₄, 2.3 mM NaH₂PO₄, pH 7.4), 3 minutes each, and blocked in normal serum diluted 1:20 in PBS for 15 minutes. Normal rabbit serum (Vector Laboratories, Burlingame, CA, USA) was used for the Ang-1 and Ang-2 antibodies, and normal goat serum (Dimension Laboratories Inc., Mississauga, ON, Canada) was used for the Tie-2 antibody.

For Ang-1 and Ang-2 staining, slides were incubated in polyclonal goat Ang-1 (N-18, 1:110; Santa Cruz, Santa Cruz Biotechnology, CA, USA) and Ang-2 (C-19, 1:100; F-18, 1:100; Santa Cruz, Santa Cruz Biotechnology, CA, USA) in diluting buffer (DAKO Diagnostics Inc., Mississauga, ON, Canada) for 16 hours at 4 °C. Ang-2 (C-19) is against the carboxy-terminus, whereas Ang-2 (F-18) is against the amino-terminus of Ang-2 protein. For Tie-2 staining, slides were incubated in polyclonal rabbit Tie-2 (C-20, 1:575; Santa Cruz, Santa Cruz Biotechnology, CA, USA) in diluting buffer for 2 hours at room temperature. Sections were washed three times in PBS, for 3 minutes each. Ang-1 and Ang-2 slides were then incubated in biotinylated Rabbit anti-Goat IgG (1:175; Vector Laboratories, Burlingame, CA, USA) for 30 minutes at room temperature. Tie-2 slides were incubated in biotinylated goat-anti-rabbit IgG (1:400; Sigma, St. Louis, MO, USA) for 30 minutes at room temperature.

Slides were washed three times in PBS, for 3 minutes each, and were incubated with HRP-conjugated streptavidin (1:300; DAKO Diagnostics Inc., Mississauga, ON, Canada) for 30 minutes. Sections were then washed twice with Tris-Saline (0.05 M Tris, 0.14 M NaCl, pH 8.0) for 3 minutes each, and incubated in a substrate consisting of 0.25% 3',3'-Diaminobenzidine (Sigma, St. Louis, MO, USA) in Tris-Saline and 0.45% Hydrogen Peroxide for 3 minutes at room temperature. Slides were rinsed with tap water for 3 minutes.
Sections were counter-stained with Harris’ Hematoxylin for 30 seconds. Slides were then dehydrated in 100% alcohol (twice, 1 minute each), and xylene (twice, 5 minutes each). Sections were mounted with Permount (Fisher Scientific Ltd., Nepean, ON, Canada).

Negative controls included omission of primary antibody, and neutralization of the primary antibody with blocking peptides, and using non-immune serum. Positive controls used were the lung for Ang-1, the prostate for Ang-2, and the ovary for both Ang-1 and Ang-2. Glial fibrillary acidic protein immunostaining (GFAP, rabbit polyclonal antibody, 1:2000; DAKO Diagnostics Inc., Mississauga, ON, Canada) of brain cortex was used as experimental positive control.

3.6. Densitometry, Quantitative Morphometry, and Statistical Analyses

Densitometry was performed using the MCID Image Analyzer 5+ (Imaging Research Inc., St. Catharines, ON, Canada). Density was estimated as [Optical Density] X [Area] for each time-period post-injury. In order to compare density of signals on the same gel, all values were normalized to their corresponding GAPDH signals. In order to compare RT-PCR results on two different gels, the signal density of 15 normal samples was averaged and assigned the arbitrary value of 1. All other signals were calculated as ratio of normal density.

Quantitative morphometry was performed by counting vessels, macrophages, and polymorphonuclear leukocytes (PMN’s) in three high power fields of 0.283 mm² on a 40X objective using a Leitz Orthoplan (Wetzlar, Germany) microscope.

Statistical analysis was performed using SYSTAT 9 (SPSS Science Inc., Chicago, IL, USA). The analysis of variance (ANOVA) with Tukey’s post hoc test was used to determine the significance between the means of control and test groups. Differences were considered...
significant at a value of \( p < 0.05 \). Pearson’s correlation test was used to correlate changes in Ang-1 and Ang-2 mRNA expression during 6 hours to 6 days post-injury.

3.7. Methodological Constraints

A drawback of the model is that freeze-injuries are rare in the clinical setting. However, the histological changes resemble those of cerebral contusions. A second drawback is the slight variability in the response of different rats to the cold-injury. The variability of the lesions in rats are kept to a minimum by maintaining the following factors constant: (1) the size of the burr hole; (2) the duration of cold-probe exposure; and (3) the pressure of cold-probe application.

RT-PCR is only semi-quantitative, and it is difficult to estimate the actual number of mRNA species. Moreover, primers have different binding affinities for their targets, and as a result it is very difficult to compare results obtained with different primers. The integrity of the PCR amplification result is dependent on the specificity of the primer.

Detection of desired antigen using western blotting technique is dependent in part on the specificity and sensitivity of the antibody. The advantage of western blotting is the ability to approximate the target’s molecular weight, and to eliminate obvious non-specific bands of unexpected molecular weight. On the other hand, western blotting may also result in a false-positive having a molecular weight similar to the protein of interest. In addition, western blotting does not allow for cellular localization of the target protein.

Integrity of immunohistochemistry, similar to western blotting, is limited to the specificity and sensitivity of the antibody used. Furthermore, the preservation of epitopes by fixation of proteins in the tissue will determine the binding-affinity of the antibody for its epitopes. If fixation is insufficient or excessive, the epitope could become destroyed or masked.
For both western blotting and immunohistochemistry, the choice of antibody is critical. The two types of antibodies are monoclonal and polyclonal. Although monoclonal antibodies offer high specificity against a single epitope, in turn if that single epitope is destroyed or unavailable due to experimental manipulation, binding will not occur. On the other hand, polyclonal antibodies offer specificity against multiple epitope binding sites on the desired target, and therefore are more likely to bind. Unfortunately, this broad specificity also means non-specific binding to similar epitopes on unwanted targets. In western blotting, this may increase spurious bands, and in immunohistochemistry it may produce background staining or false-positive staining. Western blotting and immunohistochemistry techniques when augmented can be much more powerful techniques for the detection of protein expression and identification of protein localization respectively.
CHAPTER 4

RESULTS AND ANALYSES
Chapter 4 – Results and Analyses

4.1. Histology of the Cold-Lesion

The histology of the lesions was comparable to previous findings in our laboratory (Nag 1996, Nag et al 1997, and Nag et al 2001) (Figure 5).

4.2. Reverse Transcriptase – Polymerase Chain Reaction

Baseline Ang-1, Ang-2, and Tie-2 expression was detected in control cerebral cortex (Figure 6a) and in the normal pituitary (Figure 6b).

There was no significant statistical change in Ang-1 mRNA 6 hours after cerebral trauma within the lesion area, whereas Ang-2 mRNA increased by 7.0 ± 2.4 fold (p < 0.005). Thereafter, Ang-1 mRNA showed a progressive increase, whereas Ang-2 mRNA displayed a progressive decrease within the lesion area. Ang-1 mRNA expression was increased to 3.5 ± 0.8 fold (p < 0.001) relative to control levels, whereas there was no statistically significant difference in Ang-2 mRNA expression by 6 days compared to control (Figures 6, a and c). Pearson’s correlation coefficient (r) for Ang-1 and Ang-2 mRNA expression during 6 hours to 6 days post-injury within the lesion is −0.871, which implies a significant inverse relationship (r can range from -1 to +1, the former signifying a perfect inverse relationship, and the latter a perfect proportional relationship). Tie-2 mRNA expression remained constant throughout the time-course (Figure 6a).
HEE stain x 250

(d) At 6 days post-injury, the lesion site is occupied by neovessels separated by few mononuclear cells. Intrinsic axotoblasts show mural thickening.

(e) At 4 days post-injury, endothelial proliferation and macrophages are at their maximum. Furthermore, neovessels start to appear.

(f) At 2 days post-injury, PMN's, endothelial proliferation is present at the margin of the lesion.

(g) At 2 days post-injury, there is infiltration of macrophages and some remaining vessels at the lesion site.

(h) At 6 hours post-injury, there is coagulative necrosis, loss of neurons, infiltration of polymorphonuclear leukocytes (PMN's), and only few lymphocytes.

Figure 5: Histology of the Cold-Lesion.
(a) Ang-1, Ang-2, and Tie-2 mRNA Expression in Normal and Cold-Injured Rat Brain Cortex

<table>
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<tr>
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(b) Ang-1, Ang-2, and Tie-2 mRNA Expression in the Normal Pituitary

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Figure 6 (a and b)  Ang-1, Ang-2, and Tie-2 mRNA Expression in Normal and Cold-Injured Rat Brain Cortex, and in Normal Pituitary.

(a) A representative RT-PCR analysis shows expression of Ang-1 (570 bp), Ang-2 (450 bp), and Tie-2 (322 bp) at 6 hours, 2, 4, and 6 days post-injury in comparison to normal control rats. Basal expression of Ang-1, Ang-2, and Tie-2 is observed in the cerebral cortex of a control rat. For each time-period n=5. Progressive increase in Ang-1 mRNA signal intensity is observed over the 6 day period post-injury, compared to control levels. The Ang-2 mRNA signal shows a large increase in intensity as early as 6 hours post-injury compared to control levels, and then shows a progressive decrease. For each rat, samples were taken from the lesion area (L), contralateral to lesion area (C), and peri-lesion area (P).

(b) RT-PCR analysis shows basal expression of Ang-1 (570 bp), Ang-2 (450 bp), and Tie-2 (322 bp) in normal rat pituitary glands (i to v).
Figure 6 (c) Quantitation and Statistical Analysis.

(i) Ang-1 mRNA Expression after Cortical Cold-Injury

(ii) Ang-2 mRNA Expression after Cortical Cold-Injury
Figure 6 (c) Quantitation and Statistical Analysis.

Graphical representations of Ang-1 (i) and Ang-2 (ii) mRNA after cortical cold-injury are shown. Ang-1 mRNA at 6 hours and 2 days post-injury does not show a statistically significant change from control levels. However, Ang-1 mRNA is increased $2.8 \pm 0.7 \ (p < 0.01)$ fold by 4 days, and $3.5 \pm 0.8 \ (p < 0.001)$ fold by 6 days post-injury, compared to control brain cortex. Ang-2 mRNA shows a $7.0 \pm 2.4$ fold upregulation as early as 6 hours post-injury, and a $6.1 \pm 3.3$ fold increase by 2 days post-injury. At 4 and 6 days post-injury, Ang-2 mRNA levels are not significantly different from control levels. These observations indicate that Ang-2 mRNA increases in the early phase post-injury, whereas Ang-1 mRNA shows a delayed increase. Moreover, during the period of 6 hours to 6 days post-injury Ang-1 mRNA shows a progressive increase, while there is a progressive decrease in Ang-2 mRNA. A significant inverse relationship between Ang-1 and Ang-2 mRNA levels 6 hours to 6 days post-injury is implied by their Pearson’s correlation coefficient of $-0.871 \ (* p < 0.05, ** p < 0.01, *** p < 0.005, **** p < 0.001$ in comparison with normal control cortex using ANOVA with Tukey’s post hoc test; $n=15$ for controls, and $n=5$ for each test group; bars represent mean relative normalized optical density multiplied by area ± standard error).
4.3. Western Blotting

Ang-1 protein was very abundant in normal rat brain lysates (Figure 7a). The signal corresponded to approximately 75 kDa. At 2 and 4 days post-injury, the Ang-1 signal in the lesion was decreased or undetectable compared to the contralateral cortex. In the distal peri-lesional parenchyma, there was also a decreased Ang-1 signal, but to a lesser extent than in the lesion. The Ang-1 signal in the contralateral hemisphere was comparable to control rat brain samples. Pure Ang-1 protein was not used as a positive control, since the available pure recombinant protein has a modified amino-terminus and thus will not be recognized by the amino-terminus-specific Ang-1 antibody used in this experiment. Ang-2 signals followed the same pattern as Ang-1 within all sampling areas, and also migrated at 75 kDa (Figure 7a). Ang-2 western blotting was repeated using an antibody against the carboxy-terminus, as supposed to the amino-terminus, and similar downregulation within the lesion and peri-lesion samples were observed (Figure 7a). Pure recombinant Ang-2 was used as a positive control, and it co-migrated with the Ang-2 signals using both the carboxy- and amino-terminus antibodies (Figure 7a).

Since the apparent concordant downregulation of Ang-1 and Ang-2 proteins in lesion samples do not corroborate with their corresponding RNA results obtained by RT-PCR, the lack of Angiopoietin expression within the lesion samples may be due to an artifact. Several possibilities for the lack of signals within the lesion were considered including blockage of the antigen binding site and proteolytic breakdown. Since neither the carboxy-terminus nor the amino-terminus Ang-2 antibodies were able to detect any signal within the lesion, the possible proteolytic breakdown of Ang-2 was examined. Several experiments involving pure recombinant Ang-2 protein, total plasma protein, and lesion samples were performed (Figure 7b). The available pure recombinant Ang-1 was not used in these experiments, since it
**Figure 7 (a) Ang-1 and Ang-2 Western Blotting at 4 Days Post-Injury.**

Representative western blotting assay of Ang-1 and Ang-2 protein expression in normal control cortex (N), and a 4-day lesion (L), contralateral to lesion cortex (C), and peri-lesion cortex (P) are shown. Brain cortex of a control rat shows a 75 kDa band for both Ang-1 and Ang-2. Following cortical cold-injury, there is a significant decrease in Ang-1 and Ang-2 signals in the lesion area, and to a lesser degree in the peri-lesion area compared to control levels. The intensity of Ang-1 and Ang-2 signals in the cortex contralateral to the lesion area is comparable to the cortex of a control rat. β-actin intensity is comparable in all samples, confirming equal loading. Each lane contains 75 µg of total protein.
(i) Addition of Recombinant Ang-2 Protein to Lesion Sample

\[
\begin{align*}
\text{Ang-2 (ng)}: & \quad \text{none} & \quad 30 & \quad 30 \\
+ \hspace{1cm} \text{Lesion (\mu g)}: & \quad 75 & \quad \text{none} & \quad 225 \\
\end{align*}
\]

(ii) Effect of Plasma Protein Concentration on Recombinant Ang-2 Protein Signal

\[
\begin{align*}
\text{Ang-2 (ng)}: & \quad 30 & \quad 30 & \quad 30 & \quad 30 & \quad 60 \\
+ \quad \text{PP (\mu g)}: & \quad \text{none} & \quad 75 & \quad 150 & \quad 300 & \quad 600 & \quad 600 \\
\end{align*}
\]

(iii) Effect of Lesion Sample on Excess Recombinant Ang-2 Protein

\[
\begin{align*}
\text{Ang-2 (ng)}: & \quad 300 & \quad 300 \\
+ \hspace{1cm} \text{Lesion (\mu g)}: & \quad \text{none} & \quad 75 \\
\end{align*}
\]

Figure 7 (b)  Inhibition Studies.
(i) Addition of 225 \( \mu \)g of the lesion sample to 30 ng of recombinant Ang-2 protein decreased its signal intensity. A novel 30 kDa band is observed in the mixture.
(ii) In order to test the possible inhibitory effect of plasma protein present in the lesion samples on Ang-2, increasing concentrations of plasma protein were mixed with 30 ng of recombinant Ang-2. Ang-2 signal intensities showed a progressive decrease in response to increasing concentrations of plasma protein.
(iii) To test whether a component of plasma protein present in the lesion may mediate proteolytic breakdown of Ang-2, excess Ang-2 was mixed with 75 \( \mu \)g of a 4-day lesion sample. There was a significant increase in the levels of the 30 kDa and 50 kDa bands present in recombinant Ang-2 sample following the addition of the lesion sample.
contains a modified amino-terminus and is thus unable to bind our amino-terminus-specific Ang-1 antibody. First, 30 ng pure recombinant Ang-2 protein was mixed with 225 µg of a 4-day lesion sample. The signal for the pure recombinant Ang-2 was decreased following the addition of a 4-day lesion sample. Moreover, a new signal at 30 kDa was observed in the mixture (Figure 7b, i). This result suggested that a component in the lesion sample may be causing the proteolytic breakdown of Ang-2. To test this hypothesis, 75-600 µg of purified total plasma protein was added to 30 ng pure recombinant Ang-2 protein. There was a concentration-dependant decrease in the Ang-2 signal relative to the total plasma protein added (Figure 7b, ii). To further investigate the possible proteolytic breakdown of Ang-2 by a component in the lesion, an excess amount of 300 ng pure recombinant Ang-2 protein was added to 75 µg of lesion. There was an increase in the intensity of two smaller bands at 30 kDa and 50 kDa by approximately 3.8 and 3.6 fold respectively (Figure 7b, iii).

4.4. Immunohistochemistry

Marked Ang-1 immunostaining was observed in all vascular endothelium of normal brain cortex (Figure 8a). Only mild Ang-2 immunoreactivity was present in occasional vessels of normal cortex (Figures 8b). Quantitative morphometry showed that Ang-1 immunoreactivity was present in 99.3 ± 0.2 % of cortical vessels, whereas Ang-2 immunoreactivity was found in 0.5 ± 0.2 % of cortical vessels. Both Ang-1 and Ang-2 immunostaining were observed in the cilia of ependymal epithelium lining brain ventricles. The cilia of ependyma lining the roof of the fourth ventricle showed particularly high Ang-2 immunoreactivity (data not shown). In the choroid plexus, vascular endothelium showed immunoreactivity for Ang-1, but not for Ang-2 (Figures 8, c and d). Choroid plexus epithelium showed granular Ang-1 and Ang-2
Figure 8.
Figure 8. Ang-1 and Ang-2 Immunoreactivity in Normal Brain.
(a) Marked endothelial Ang-1 immunoreactivity is present in all vessels of the parietal cortex (arrows). X 250
(b) Majority of normal neocortical vessels lack immunoreactivity for Ang-2 (arrow), except for occasional vessels which show mild endothelial immunoreactivity (arrowhead). X 250
(c and d) Choroid plexus epithelial cells show granular immunoreactivity for both Ang-1 and Ang-2 (arrowheads). Vascular endothelium of choroid plexus shows Ang-1, but not Ang-2, immunoreactivity (arrows). X 600
(e and f) Cilia (arrows) and luminal membrane (arrowheads) of ependymal epithelium lining brain ventricles show immunoreactivity for both Ang-1 and Ang-2. X 400
immunostaining, in addition to diffuse Ang-2 immunostaining (Figures 8, c and d).

Vascular endothelium of the posterior pituitary showed Ang-1 immunoreactivity, and occasional Ang-2 immunoreactivity (Figures 9, a and b). Granular Ang-1 and Ang-2 immunostaining was also observed in the anterior pituitary (Figures 9, c and d). Furthermore, vascular endothelium within the area postrema showed high Ang-1 immunoreactivity compared to the adjacent brain stem (data not shown). Both antibodies used to obtain Ang-2 immunostaining gave similar results.

Immediately post-trauma at 6 hours, vascular Ang-1 immunostaining was decreased within the lesion and peri-lesion, being predominantly present in remnants of intrinsic vessels. There was mild Ang-2 immunoreactivity in disrupted vascular endothelium at this time. PMN’s infiltrated into the lesion area and were positive for both Ang-1 and Ang-2 immunoreactivity (Figure 10, a and b). Early in the remodeling phase at 2 days post-trauma, there was moderate Ang-1 immunoreactivity within vessels of lesion and peri-lesion, whereas vascular Ang-2 immunostaining was only mildly detectable. PMN’s were now markedly positive for Ang-1 and Ang-2 immunostaining. Furthermore, few macrophages were observed at this time, many of which showed Ang-1 and Ang-2 immunoreactivity (Figure 10, c and d). At 3 days post-trauma, moderate Ang-1 immunoreactivity persisted in vascular endothelium of lesion area, whereas vascular Ang-2 immunoreactivity was significantly more prevalent compared to 2 days post-injury. The increasing number of macrophages and PMN’s showed Ang-1 and Ang-2 immunoreactivity (Figure 10, e and f). During the peak of vascular remodeling at 4 days post-trauma, vascular endothelium within the lesion was markedly positive for both Ang-1 and Ang-2 immunoreactivity. Also, marked Ang-1 and Ang-2 immunostaining was observed within macrophages and PMN’s (Figure 10, g and h). During the later remodeling phase at 6 days post-trauma, Ang-1 and Ang-2 were still markedly positive within lesion vessels.
Figure 9. Ang-1 and Ang-2 Immunoreactivity in the Posterior and Anterior Pituitary.

(a) Vascular endothelium of posterior pituitary shows Ang-1 immunoreactivity (arrows).
(b) Within the posterior pituitary, mild Ang-2 immunoreactivity is observed only in occasional vascular endothelium (arrow), and is absent in most vessels (arrowhead).
(c and d) Anterior pituitary cells near capillaries show granular immunostaining for Ang-1 and Ang-2 (arrows). X 600
Figure 10.
Figure 10. Time-course of Ang-1 and Ang-2 Immunoreactivity after Cortical Cold-Injury.

(a and b) 6 hours post-injury, endothelial Ang-1 and to a lesser extent Ang-2 immunoreactivity is present in lesion vessels (arrows). Ang-1 and Ang-2 immunoreactivity is also present in PMNs (arrowheads).

(c and d) 2 days post-injury, serial sections of the same vessel show endothelial immunoreactivity for Ang-1 and to a lesser extent Ang-2 (arrows). Ang-1 and Ang-2 immunoreactivity is present in macrophages and PMNs (arrowheads).

(e and f) 3 days post-injury, serial sections of the same vessel show endothelial immunoreactivity for both Ang-1 and Ang-2 (arrows). Ang-1 and Ang-2 immunostaining is also present in macrophages and PMNs (arrowheads).

(g and h) 4 days post-injury, Ang-1 and Ang-2 immunoreactivity is present in endothelium of vessels (arrows), macrophages and PMNs (arrowheads).

(i and j) 6 days post-injury, Ang-1 and Ang-2 immunoreactivity is observed in vessels (arrows), macrophages and PMNs (arrowheads). X 250
comparable to 4 days post-injury. Macrophages and PMN's also showed marked Ang-1 and Ang-2 immunoreactivity (Figure 10, i and j). Vascular Tie-2 immunostaining did not differ between lesion and non-lesion cortex (data not shown).

Quantitative morphometry at 2 days post-injury showed that Ang-1 immunoreactivity was present in 51.8 ± 2.3 % of lesion vessels, which is a significant decrease (p < 0.001) compared to 99.3 ± 0.2 % observed in normal cerebral vessels. At 4 days post-injury, percentage of Ang-1 immunoreactive vessels showed an increase to 87.0 ± 5.2 %, which is not significantly different from control levels, but is significantly increased compared 2 days post-injury (p < 0.001). Vascular Ang-2 immunoreactivity at 2 days post-injury was observed in 18.7 ± 3.1 % of lesion vessels, which is a significant increase compared to 0.5 ± 0.2 % in normal cerebral vessels (p < 0.01). At 4 days post-injury, vascular Ang-2 immunoreactivity had increased to 88.9 ± 3.9 % of lesion vessels, and this is significantly higher compared to both normal and 2 days post-injury (p < 0.001) (Figure 11).

At 2 days post-injury, Ang-1 immunoreactivity was present in 77.6 ± 1.0 % of PMN’s and 45.5 ± 10.1 % of macrophages. At 4 days post-injury, Ang-1 immunoreactivity was present in immunoreactivity was present in 76.8 ± 10.4 % of PMN’s and 75.7 ± 7.3 % of macrophages. Ang-2 immunoreactivity at 2 days was observed in 83.6 ± 1.3 % of PMN’s and 30.3 ± 3.1 % of macrophages. At 4 days post-injury, Ang-2 immunoreactivity was present in 76.3 ± 9.8 % of PMN’s and 83.0 ± 4.9 % of macrophages within the 4-day lesions.

Serum protein immunoreactivity was not observed in normal cortical vessels as described previously (Nag 1984 a, and Nag 1996). Immediately post-trauma at 6 days, there was serum protein immunoreactivity within lesion and peri-lesion. From 2 to 4 days post-injury, serum protein immunoreactivity shows a progressive decline (Figure 12). By 6 days post-injury, serum protein immunoreactivity is significantly diminished within lesion and peri-lesion
Figure 11. Quantitative Morphometry of Vascular Ang-1 and Ang-2 Immunoreactivity in Normal and Cold-Injured Brain Cortex.

Nearly all vessels in the normal brain cortex show endothelial immunoreactivity for Ang-1 (99.3 ± 0.2 %), whereas Ang-2 immunoreactivity is found only in occasional vessels (0.5 ± 0.2 %). At 2 days post-injury, Ang-1 immunoreactivity is observed in 51.8 ± 2.3 % of vessels, and Ang-2 is present in 18.7 ± 3.1 % of vessels within the lesion. At 4 days post-injury, Ang-1 immunoreactivity is observed in 87.0 ± 5.2 % of vessels, and Ang-2 is detected in 88.9 ± 3.9 % of vessels within the lesion (* \(p < 0.001\) with respect to vascular Ang-1 immunoreactivity in normal brain cortex; † \(p < 0.01\) and †† \(p < 0.001\) with respect to vascular Ang-2 immunoreactivity in normal brain cortex; for both control and test groups, n=3; bars represent mean percentage of immunoreactive vessels in three high power fields of 0.283 mm² ± standard error).
Figure 12. Serum Protein Immunoreactivity after Cortical Cold-Injury.

(a) A representative lesion shows extravasation of serum proteins at 2 days post-injury. Note the spread of serum proteins from the lesion site into the adjacent white matter. X 40

(b) Higher magnification shows a peri-lesion vessel with blood-brain barrier breakdown to serum protein at 2 days post-injury. X 900
compared to earlier time-periods.

On adjacent sections of a representative 4-day lesion (Figure 13, a, c, and e), vessels which show serum protein extravasation also show Ang-2, but not Ang-1, immunostaining. However, vessels which lack serum protein extravasation show Ang-1, and not Ang-2, immunostaining. Serial sections of a representative 4-day perilesion area (Figure 13, b, d, f) show that vessel which are markedly positive for serum protein immunostaining display decreased Ang-1 and marked Ang-2 immunostaining. However, vessels which are not showing serum protein immunoreactivity show marked Ang-1, but no Ang-2, immunostaining.
Figure 13. Co-localization of Ang-1, Ang-2, and Serum Protein Immunoreactivity on Adjacent Sections, 4 Days Post-Injury.

(a, c, and e) In serial sections of a 4 day-lesion, vessels which show serum protein extravasation also show Ang-2, but not Ang-1, immunostaining (arrows). However, vessels not showing serum protein immunostaining show Ang-1, but not Ang-2, immunostaining (arrowheads).

(b, d, and f) In serial sections of a peri-lesional area (4 day lesion), vessels which show marked serum protein extravasation also show marked Ang-2 and only mild Ang-1 immunostaining (arrows). However, vessels lacking serum protein extravasation show marked Ang-1, and not Ang-2, immunostaining (arrowheads). X 250
CHAPTER 5

DISCUSSION
Chapter 5 – Discussion

Expression of Ang-1 and Ang-2 mRNA and protein was detected in normal brain and after cortical cold-injury. Increased Ang-2 relative to Ang-1 mRNA was observed in the early phase post-injury during the known period of blood-brain barrier breakdown. Two to 6 days post-injury, the progressive increase in Ang-1 and decrease in Ang-2 coincided with cerebrovascular angiogenesis, maturation of neovessels, and restoration of the blood-brain barrier.

5.1. Angiopoietin Expression in Normal Brain

5.1.1. Areas with Vessels Having Blood-Brain Barrier Characteristics

RT-PCR showed basal Ang-1 and Ang-2 mRNA expression in normal rat cortex. Other studies using RT-PCR confirm our findings, showing Ang-1 and Ang-2 mRNA expression in normal human (Hashimoto et al 2001) and rat (Mandriota et al 2000) brain cortex. The Ang-1 signal was much more prominent than the Ang-2 signal in this study. Since both reactions were performed for the same number of cycles, and assuming the sensitivities of the probes for their targets is similar, Ang-1 mRNA expression seems to exceeds Ang-2 mRNA expression in normal brain cortex. This observation is consistent with in situ hybridization experiments showing Ang-1 exceeds Ang-2 mRNA expression in normal brain cortex (Beck et al 2000, Hashimoto et al 2001, Mandriota et al 2000, and Stratmann et al 1998).

By in situ hybridization, Ang-1 was expressed either exclusively in vessels (Hashimoto et al 2001) or in neurons (Beck et al 2000), while Ang-2 was absent in normal human brain. However, Stratmann and colleagues (2001) reported both vascular and neuronal Ang-1, and also neuronal Ang-2 in normal human brain by this technique. These inconsistent results may be due to technical differences such as probe sensitivity and tissue fixation. In this study, RT-
PCR was preferentially chosen over in situ hybridization, since the former is associated with less technical variability.

Western blotting results corroborate with the RT-PCR results, showing basal expression of both Ang-1 and Ang-2 proteins in normal brain cortex. Ang-2 protein expression was verified by two different polyclonal antibodies against its amino- and carboxy-terminus. Recently, western blotting assay of Angiopoietins using antibodies distinct from those used in this study has shown baseline expression of both Ang-1 and Ang-2 proteins in normal human brain cortex (Hashimoto et al 2001), thus confirming our findings.

Cellular localization of Ang-1 and Ang-2 protein in normal rat brain cortex was performed by immunohistochemistry. All cortical vessels showed Ang-1 immunoreactivity, while Ang-2 immunoreactivity was only detectable in occasional vessels of normal brain cortex. These findings do not agree with a recent study showing Ang-1 immunoreactivity in neurons and only occasional vascular endothelial cells, and lack of Ang-2 immunoreactivity in normal human brain cortex (Audero et al 2001). The discrepancies could be due to technical differences such as sensitivity of the antibodies, tissue fixation, and antigen retrieval.

In this study, significant Ang-2 protein was detected by western blotting in normal brain cortex, however Ang-2 immunoreactivity was not prevalent. Therefore, there is an apparent discrepancy in Ang-2 results by western blotting and immunohistochemistry in normal brain cortex. Tissues for western blotting were homogenized and treated with reducing reagents, whereas those for immunohistochemistry were perfusion-fixed. As a result, the integrity and accessibility of a particular antigen by the same antibody differs in these methods. This may explain the discrepancy observed in Ang-2 protein results.

Presence of Angiopoietins in microvasculature of normal brain could have important implications. Since Ang-1 increases tight junctional proteins and alters their phosphorylation states (Gamble et al 2000), constitutive Ang-1 expression in normal cerebral endothelium may
maintain inter-endothelial tight junctions thus maintaining blood-brain barrier integrity. Furthermore, since prolonged exposure to Ang-2 causes Tie-2 phosphorylation and activation (Teichert-Kuliszewska et al. 2001), basal Ang-2 expression in normal brain vessels may have similar agonistic effects as Ang-1.

5.1.2. Areas with Vessels Lacking Blood-Brain Barrier Characteristics

Certain areas of the brain contain vessels which resemble peripheral vessels rather than cerebrocortical vessels, lacking an intact blood-brain barrier under normal physiological states (Baldwin 2000, Ganong 2000, and Michel and Curry 1999). They are collectively referred to as the circumventricular organs because of their vicinity to brain ventricles (Ganong 2000).

Ang-1 but not Ang-2 immunoreactivity was observed in endothelium of all choroid plexus vessels. Also, granular Ang-1 and Ang-2 immunoreactivity was observed in the choroid plexus epithelium. The cilia and luminal membrane of ependymal cells lining brain ventricles also showed immunoreactivity for both Ang-1 and Ang-2. Therefore, Ang-1 and Ang-2 may regulate the permeability of endothelium and epithelium in the choroid plexuses and ependyma under normal conditions.

Various cytokines and growth factors are expressed by the pituitary gland (Ray and Melmed 1997, and Tsagarakis et al. 1998), and it is also a target for many cytokines and growth factors (Renner et al. 1996). These cytokines may induce pituitary hormone synthesis and release, pituitary cell proliferation and differentiation, or effect other organs including other endocrine glands (Tsagarakis et al. 1998). Basal Ang-1, Ang-2, and Tie-2 mRNA expression was observed in the pituitary by RT-PCR. Ang-1 and to a lesser extent Ang-2 immunoreactivity was present in the vascular endothelium of the posterior pituitary. Granular Ang-1 and Ang-2 immunoreactivity was observed within anterior pituitary cells adjacent to capillaries suggesting that they may be released into the circulation.
To our knowledge, this is the first report of Angiopoietin expression within the choroid plexus, ependyma, and the pituitary gland.

5.2. Angiopoietin-1 and Angiopoietin-2 during Blood-Brain Barrier Breakdown

The time-course of blood-brain barrier breakdown was determined by serum protein immunohistochemistry. Similar to previous observations (Nag 1996), serum protein extravasation was maximal at 6 hours and 2 days post-injury, and decreased thereafter. Ang-1 mRNA was not significantly altered at 6 hours and 2 days post-injury, however immunohistochemistry showed that endothelial Ang-1 is reduced by 51.8 ± 2.3 %. Since the observed PMN’s also showed Ang-1 immunoreactivity, they could be a potential source of the observed Ang-1 mRNA. The relative decrease in the Ang-1 to Ang-2 mRNA at 6 hours and 2 days post-injury coincides with maximum blood-brain barrier breakdown.

Ang-1 is known to promote association of the vascular endothelium with perivascular cells in the peripheral vasculature (Patan 1998, Suri et al 1996, and Thurston et al 1999), and alter phosphorylation and association of tight junctional proteins in vitro (Gamble et al 2000). Moreover, association of cerebral endothelium with perivascular cells (Arthur et al 1987, Janzer and Raff 1987, and Stewart and Wiley 1981), and the integrity of inter-endothelial circumferential tight junctions are important in maintaining blood-brain barrier integrity to protein in normal cerebral vessels (Brightman et al 1973, Brightman and Reese 1969, Reese and Karnovsky 1967, and Staddon et al 1995). Therefore, the relative decrease in Ang-1 to Ang-2 mRNA in the early phase post cold-injury may be important in breakdown of the blood-brain barrier to protein.
Leaky vessels, as determined by serum protein immunostaining, contained Ang-2 but not Ang-1 immunoreactivity within the lesion and peri-lesional area. However, non-leaky vessels contained Ang-1 but not Ang-2. Assuming Ang-2 interferes with the maturation-inducing effect of Ang-1 in the cerebral cortex similar to its action in the periphery (Maisonpierre et al 1997), the observed changes in Ang-1 and Ang-2 may contribute to loosening of interendothelial tight junctions and dissociation of peri-endothelial supporting cells from vascular endothelium. This vascular destabilization will induce loss of blood-brain barrier integrity. Since Ang-1 induces protein-leakage resistance in peripheral vessels (Thurston et al 1999), decreased Ang-1 concurrently with the breakdown of blood-brain barrier suggests that Ang-1 may also play a role in maintaining blood-brain barrier integrity of normal cerebrovasculature.

VEGF has been implicated in the blood-brain barrier breakdown occurring after cortical cold-injury (Nag et al 1997, and Papavassiliou et al 1997). VEGF induces vascular permeability by targeting inter-endothelial tight junctions (Antonetti et al 1999, and Kevil et al 1998). Since Ang-1 modulates phosphorylation of tight junctional proteins (Gamble et al 2000), a decrease in Ang-1 or an increase in Ang-2 in the early phase post-injury may allow for the permeability-inducing actions of VEGF. This hypothesis is supported by the observation that overexpression of Ang-1 inhibits the permeability-inducing effect of VEGF (Thurston et al 1999). Also, mouse cornea neovascularization assays have shown that VEGF-induced neovascularure is not associated with peri-endothelial cells, whereas abundant peri-endothelial cells are observed in neovascularure that is co-induced with both Ang-1 and VEGF (Asahara et al 1998).

The relative increase in Ang-1 mRNA and protein immunoreactivity compared to Ang-2 at 4 and 6 days post-injury coincides with blood-brain barrier restoration. Assuming that Ang-1 has a similar function in cerebral vessels compared to peripheral vessels, the increase in Ang-1 in later phases post-injury may promote association of newly-formed vessels with peri-
endothelial pericytes and astrocytes, and therefore lead to restoration of blood-brain barrier. Furthermore, since Ang-2 activates Tie-2 at high concentrations (Teichert-Kuliszewska et al 2001) and after prolonged exposure (Kim et al 2000 d), Ang-2 in later phases post-injury may have an agonistic effect on Tie-2, and hence may promote blood-brain barrier restoration.

5.3. Angiopoietin-1 and Angiopoietin-2 during Cerebral Angiogenesis

Ang-1 and Ang-2 expression within the lesion seem to be inversely related over the 6 day period post-injury within the lesion, having a Pearson’s correlation coefficient of –0.871. The relative decrease in Ang-1 to Ang-2 at 6 hours post-injury may have a role in the initial steps of vascular remodeling and angiogenesis, namely breakdown of the pre-existing endothelial junctions. Also, since Ang-1 promotes endothelial cell survival (Fujikawa et al 1999, Hayes et al 1999, Jones et al 1999, Kim et al 2000 a, Kwak et al 1999, 2000, and Papapetropoulos et al 1999, 2000), and Ang-2 has been observed just prior to onset of vascular apoptosis (Cohen et al 2001, and Zagzag et al 2000), the significant decrease in Ang-1 relative to Ang-2 mRNA at 6 hours post-injury compared to normal levels may induce regression of the intrinsic lesion vessels. The progressive relative increase in Ang-1 compared to Ang-2 mRNA during 2 hours to 6 days post-injury may play a role in later stages of angiogenesis and vascular remodeling after cortical cold-injury, namely maturation of the newly-formed vessels by association of cerebral endothelium with peri-endothelial cells such as pericytes and astrocytes.

The abrupt upregulation of Ang-2 mRNA during non-neoplastic cerebral angiogenesis has been previously described by RT-PCR, northern blotting, and in situ hybridization in human arteriovenous malformations (Hashimoto et al 2001), by RNase protection assay following systemic hypoxia in rat brain (Mandriota et al 2000), and by in situ hybridization after middle cerebral artery occlusion in the rat brain (Beck et al 2000). Ang-1 mRNA in these models have
been similar or only slightly increased compared to control brains. This difference could be due to the particular phase post-injury during which Ang-1 mRNA was assessed. In this study, Ang-1 mRNA was not significantly altered at 6 hours and 2 days post-injury. In situ hybridization documented Ang-1 and Ang-2 mRNA upregulation in cerebral vessels during non-neoplastic (Beck et al 2000, and Hashimoto et al) and neoplastic cerebral angiogenesis (Audero et al 2001, and Stratmann et al 1998). This upregulation was confirmed by northern blotting in glioblastoma multiforme (Stratmann et al 1998). The progressive decrease in Ang-2 mRNA after it’s early surge at 6 hours can be due to feedback inhibition, since recombinant Ang-2 protein has been demonstrated to decrease Ang-2 mRNA expression in BME (Mandriota and Pepper 1998).

The apparent concordant downregulation of Ang-1 and Ang-2 protein levels detected by western blotting in lesion and peri-lesion samples at 2 and 4 days post-injury do not reflect the significant upregulation of their corresponding mRNA. To assay whether the observation was due to an artifact of a component within the lesion samples, pure recombinant Ang-2 protein was mixed with a 4-day lesion sample. The mixture showed a reduction in Ang-2 signal intensity, which may be due to inhibition of antibody-epitope interaction by a component within the lesion samples. Also, a new 30 kDa band appeared as a result of this mixing, which may be due to proteolysis of Ang-2 by a component within the lesion samples. Serum protein components were likely candidates for this inhibitory or proteolytic effect, since the lesion samples are enriched with the extravasated serum protein. This hypothesis was tested by mixing increasing concentrations of purified total plasma protein with recombinant Ang-2 protein. Ang-2 signal intensity decreased with increasing concentrations of plasma protein suggesting that a component within the plasma protein led to the blockage or degradation of Ang-2 epitopes. In order to visualize the potential breakdown products, a 10-times excess amount of recombinant Ang-2 was mixed with a lesion sample. The intensity of two bands at
30 kDa and 50 kDa were significantly increased, and thus may be potential breakdown products of recombinant Ang-2. Proteolytic breakdown of Ang-1 was proposed to explain the decreased Ang-1 protein expression in human arteriovenous malformations (Hashimoto et al 2001). These authors did not observe a significant change in Ang-1 mRNA by RT-PCR, however Ang-1 protein expression was decreased by 34% ($p = 0.01$) (Hashimoto et al 2001). Interestingly, brain arteriovenous malformations are associated with extravasation of serum proteins due to blood-brain barrier breakdown, and formation of vasogenic cerebral edema (Young et al 1996). Evidence in this study suggests that a component of serum protein may interfere with Angiopoietins.

The low Ang-2 immunoreactivity at 2 days post-injury does not correlate with the early surge in Ang-2 mRNA observed with RT-PCR. There are several possible explanations for this observation: (1) proteolytic breakdown of Ang-2 occurs in response to the high serum protein extravasation during the early phase post-injury; (2) there is a delay in production of Ang-2 protein from mRNA, involving translation and post-translational modifications; and (3) the main source of Ang-2 mRNA during the early phase post-injury could be PMN’s which show marked Ang-2 immunoreactivity.

The increase in Ang-1 and Ang-2 mRNA and immunoreactivity between 3 and 6 days post-injury compared to controls correlates with proliferation of endothelial cells and formation of neovessels, and therefore may be related to the cerebrovascular angiogenesis and vascular remodeling after cold-injury. An increase in Ang-1 and Ang-2 immunoreactivity during cerebrovascular angiogenesis has also been reported in human glioblastoma multiforme (Audero et al 2001).

Interestingly, VEGF immunoreactivity also increases following a cortical cold-injury (Nag et al 1997, and Papavassiliou et al 1997). Angiopoietins and VEGF interact during pathological angiogenesis (Peters 1998), and they are both indispensable during this process.
(Siemeister et al 1999). Furthermore, concurrent upregulation of Angiopoietins and VEGF occur during both neoplastic (Ding et al 2001), and non-neoplastic (Mandriota et al 2000) cerebral angiogenesis. Therefore, the concurrent increase in VEGF and Angiopoietins may facilitate cerebrovascular angiogenesis after cortical cold-injury.

5.4. Angiopoietin-1 and Angiopoietin-2 Immunoreactivity in Inflammatory Cells

This is the first report of Ang-1 and Ang-2 immunoreactivity within PMN’s and macrophages during cerebrovascular angiogenesis. Ang-1 and Ang-2 immunoreactivity have been previously observed in macrophage-like mesenchymal cells during the angiogenesis occurring in human pyogenic granuloma (Yuan et al 2000 b).

Ang-1 and Ang-2 immunoreactivity in polymorphonuclear leukocytes and macrophages following brain trauma could have various implications. This immunoreactivity could either be due to (1) synthesis of Ang-1 and Ang-2 within the inflammatory cells, (2) uptake of Ang-1 and Ang-2 from the neuropil, or due to (3) a false-positive reaction related to the high myeloperoxidase content of inflammatory cells. Non-radioactive in situ hybridization can be used to characterize Ang-1 and Ang-2 mRNA expression after cortical cold-injury in order to determine whether the inflammatory cells synthesize these proteins or take them from the surrounding tissue.

Assuming PMN’s and macrophages indeed express Ang-1 and Ang-2 proteins, they may have a role in the various pathological processes after cold-injury. Macrophages express an alternatively-spliced Ang-2 with 443 amino acids during their maturation (Kim et al 2000 e). A significant increase in the frequency of Ang-2 immunoreactive macrophages was observed at 4 days compared to 2 days post-injury, whereas the frequencies of Ang-1 immunoreactive macrophages and Ang-1 and Ang-2 immunoreactive PMN’s were not significantly altered.
between 2 and 4 days post-injury. Interestingly, the rates of infiltration and maturation of macrophages significantly increased between 2 and 4 days post-injury. Macrophages are known to secrete other angiogenic factors (Knighten et al 1983), and thus may promote angiogenesis (Polverini et al 1977, and Sunderkotter et al 1994). Since Angiopoietins have previously been shown to alter phosphorylation of inter-endothelial tight junctional proteins (Gamble et al 2000), expression of Angiopoietins by macrophages may be involved in tight junctional remodeling following cold-injury. Macrophage-mediated tight junctional modulation is critical in the process of diapedesis (Edens and Parkos 2000) and this process may be partly mediated by Ang-2. This hypothesis is supported by the observation that Ang-1 can inhibit TNF-α-stimulated leukocyte transmigration, and thus may act as an anti-inflammatory agent in HUVECs (Gamble et al 2000). The increased Ang-2 to Ang-1 ratio may induce endocytosis in macrophages. This potential Ang-2-mediated induction of vesicular transport may also be involved in other areas of dominant Ang-2 expression accompanied with high pinocytic activity such as within lesion and peri-lesion vessels, and in the ependyma and pericytes.
CHAPTER 6

CONCLUSIONS AND PERSPECTIVES
Chapter 6 – Conclusions and Perspectives

Ang-1 and Ang-2 mRNA and protein expression were observed in the normal rat brain cortex. Nearly all cortical vessels showed immunoreactivity for Ang-1, whereas Ang-2 immunoreactivity was only present in occasional vessels. Since Ang-1 enhances inter-endothelial tight junctions, and promotes association of endothelial cells with peri-endothelial cells in non-cerebral vessels, constitutive expression of Ang-1 in the vascular endothelium of normal brain cortex suggests a role in maintaining blood-brain barrier integrity. Ang-2 was more prevalent in areas lacking a blood-brain barrier such as the choroid plexus, ependyma, and the pituitary gland.

Interestingly, at 6 hours and 2 days after cortical cold-injury Ang-1 mRNA was comparable to control levels, whereas Ang-2 mRNA was significantly increased. At 2 days post-injury, vascular Ang-1 immunoreactivity was less prevalent than in control cortex, whereas vascular Ang-2 immunoreactivity was more abundant. The increase in Ang-2 occurred during the period of maximal blood-brain barrier breakdown post-injury. By 4 days post-injury, comparable levels of Ang-1 and Ang-2 immunoreactivity were observed within lesion vessels, suggesting a role in cerebrovascular angiogenesis and remodeling. During 2 to 6 days post-injury, Ang-1 mRNA showed a progressive increase, whereas Ang-2 mRNA showed a progressive decrease compared to 6 hours post-injury. The delayed increase in Ang-1 may be important in restoration of the blood-brain barrier integrity. Immunostaining for Ang-1, Ang-2, and serum protein on adjacent sections showed that permeable lesion and peri-lesion vessels display diminished Ang-1 and increased Ang-2 immunoreactivity, whereas vessels with an intact blood-brain barrier to protein show marked Ang-1 but not Ang-2 immunoreactivity. Therefore, Ang-1 may have a role in promoting blood-brain barrier integrity. In addition, Ang-2 may be a factor involved in blood-brain barrier breakdown by antagonizing Ang-1.
and Ang-2 immunoreactivity was also present in PMN’s and macrophages within the lesion, and may facilitate inflammatory processes such as infiltration and maturation, or regulate angiogenesis and cerebrovascular remodeling. Expression of Ang-1 and Ang-2 in PMN’s and macrophages may also facilitate blood-brain barrier breakdown and restoration.

It would be interesting to examine Ang-1, Ang-2, and Tie-2 mRNA expression by in situ hybridization in normal brain and after cortical cold-injury, in order to verify our current results and those obtained in other models. Even more intriguing, functional studies can be pursued to examine the role of Angiopoietins in normal and injured brains. For example, Ang-1 can be infused after cortical cold-injury in order to see if vascular permeability decreases. If overexpression of Ang-1 induces leakage-resistance in the injured cerebral capillaries as it does in normal peripheral capillaries (Thurston et al 1999), it could have great potential in treating diseases in which the blood-brain barrier to protein is compromised. Infusion of recombinant VEGF into adult rat brain cortex induced cerebral angiogenesis, however neovessels lacked blood-brain barrier markers (Rosenstein et al 1998). If Ang-1 promotes association of vascular endothelium with peri-endothelial cells in the cerebral vasculature as it does in the peripheral vasculature, co-infusion of Ang-1 with VEGF into cerebral cortex may lead to cerebral angiogenesis producing vessels with blood-brain barrier characteristics. One aspect of angiogenic therapy involves modulation of growth factors in order to control blood vessel growth (Plate 1999, and Waltenberger 1997). For example, anti-angiogenesis therapy may be used to decrease the vascularity of brain tumors (Plate 1996), and pro-angiogenesis therapy could be used to reduce infarction in stroke (Hayashi et al 1998). Evidence in this thesis implies that the fine balance between Ang-1 and Ang-2 may be essential in regulating cerebrovascular angiogenesis and blood-brain barrier integrity. A better understanding of the function of Angiopoietins in the brain may provide potential therapy against cerebrovascular diseases involving blood-brain barrier breakdown and cerebral angiogenesis.
CHAPTER 7

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Chapter 7 – References


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