THE ROLE OF THE MACROPHAGE MIGRATION INHIBITORY FACTOR IN STROKE

by

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ABSTRACT

The macrophage migration inhibitory factor (MIF) is a 12kDa cytokine with pro-inflammatory properties. Initially characterized as a lymphocyte-secreted factor which inhibits macrophage migration in vitro, MIF has emerged as a multi-faceted cytokine involved in many processes, including cellular responses to ischemia/reperfusion injury in the heart. The main objective of this thesis was to determine whether human MIF expression is induced following cerebral ischemia, the underlying mechanism by which MIF expression is regulated under stroke conditions and its role therein. Previous studies have shown that MIF expression and release from cells is induced during hypoxia. However, the underlying mechanism is not clear. To examine whether the induction of MIF gene expression was mediated by its transcriptional upregulation, the human MIF gene promoter was cloned and a luciferase assay was used to determine the presence of a hypoxia responsive region in the human MIF promoter. The presence of a functional HIF-1α binding site was demonstrated using an electrophoretic mobility shift assay (EMSA). The results showed that upregulation of MIF gene expression under stroke is mediated by the effect of hypoxia on an HRE in MIF gene promoter. MIF has been shown to protect cells from ischemia and oxidative stress-induced cell death. To determine whether MIF has a similar protective effect on neurons, rat primary cortical neurons were cultured and subjected to either oxygen-glucose deprivation or treatment with hydrogen peroxide. MIF significantly reduced both OGD and H₂O₂-induced cell death. The expression of MIF in human brain has not been characterized. To determine whether the expression of MIF in human brain is altered following ischemia, brain sections from 10 stroke patients were immunostained with an antibody against MIF. Blood vessel endothelial cells in the peri-infarct region of ischemic brain displayed strong MIF immunoreactivity. Normal brain endothelium showed no MIF immunoreactivity. To understand the consequence of increased MIF expression by endothelial cells following stroke, the adhesion of human monocytes to human brain endothelial cells exposed to MIF was evaluated in vitro. MIF suppressed the monocyte adhesion to endothelial cells. The findings presented here are the first to suggest a role for MIF in cerebral ischemia.
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LIST OF ABBREVIATIONS

ABC.....................................................ATP binding cassette
AMI ....................................................acute myocardial infarction
AMPK ..................................................AMP-activated protein kinase
ATP ....................................................adenosine triphosphate
BBB....................................................blood brain barrier
BCL-xL ..............................................B-cell lymphoma-x long
bHLH...................................................basic helix-loop-helix
BRE ...................................................TFIIB recognition element
CCR ..................................................CC chemokine receptor
CBP ...................................................CREB binding protein
cDNA ................................................complementary DNA
CMV...................................................cytomegalovirus
CREB ................................................cyclic-AMP responsive element binding
CXCR ................................................CXC chemokine receptor
DNA ..................................................deoxyribonucleic acid
DCE...................................................downstream core element
DPE ...................................................downstream core promoter element
EMSA................................................electrophoretic mobility shift assay
EPO ...................................................erythropoietin
ERK...................................................extracellular-regulated kinase
FIH ....................................................factor inhibiting HIF-1α
GLUT ..................................................glucose transporter
GRE...................................................glucocorticoid response element
HAOEC ................................. human aortic endothelial cell
HBEC ................................. human brain endothelial cell
HEK ......................................... human embryonic kidney
HIF .......................................... hypoxia inducible factor
HMIF ....................................... human macrophage migration inhibitory factor
HPH .......................................... HIF prolyl hydroxylase
HRE .......................................... hypoxia response element
HUMMR .................................... hypoxia-upregulated mitochondrial movement regulator
HUVEC .................................... human umbilical vein endothelial cell
ICAM ....................................... intercellular adhesion molecule
IFN .......................................... interferon
IGFBP-1 ................................. insulin-like growth factor binding protein 1
IL ............................................. interleukin
I/R ........................................... ischemia-reperfusion
JMJD1A/JMJD2B ......................... jumonji family proteins
JNK .......................................... c-Jun N-terminal kinase
LPS ......................................... lipopolysaccharide
MAPK ....................................... mitogen-activated protein kinase
MCAO ....................................... middle cerebral artery occlusion
MCP-1 ....................................... monocyte chemoattractant protein 1
MIF .......................................... macrophage migration inhibitory factor
MIP-1β ...................................... macrophage inflammatory protein-1β
MKK ......................................... MAP kinase kinase
mRNA ...................................... messenger RNA
MTE ......................................... motif ten element
MTT ........................................................... methylthiazolyldiphenyl-tetrazolium bromide
NADPH ........................................................ nicotinamide adenine dinucleotide phosphate
NF-κB ........................................................ nuclear factor–kappa B
NLS ............................................................ nuclear localization signal
NOS ............................................................. nitric oxide synthase
ODD .............................................................. oxygen-dependent degradation domain
OGD ............................................................. oxygen-glucose deprivation
PAS ............................................................... Per, ARNT, Sim
PIC ............................................................... pre-initiation complex
PKCδ ............................................................ protein kinase c δ
PMN .............................................................. polymorphonuclear leukocytes
RANTES ........................................................... regulated on activation, normal T-cell expressed and secreted
RNA ............................................................... ribonucleic acid
ROS .............................................................. reactive oxygen species
rt-PA .............................................................. recombinant tissue plasminogen activator
SDF-1α ........................................................... stromal cell derived factor-1α
siRNA ............................................................. silencing RNA
Sp1 ............................................................... specificity protein 1
TAF .............................................................. TBP associated factor
TBP .............................................................. TATA-binding protein
TF ............................................................... transcription factor
TIA ............................................................... transient ischemic attack
TNF .............................................................. tumor necrosis factor
TrKB .............................................................. tyrosine kinase receptor B
TSS.....................................................transcription start site
VCAM..................................................vascular cell adhesion molecule
VEGF ..................................................vascular endothelial growth factor
VHL.....................................................von Hippel-Lindau tumor suppressor
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CHAPTER 1: INTRODUCTION

1.1 STROKE

Stroke is a neurological disorder resulting from the attenuation of blood flow to a region of the brain supplied by a cerebral artery. Without a blood supply, neurons lose their source of nutrients and oxygen. As a result, brain tissue experiencing ischemia is fatally compromised. Clinically, stroke is defined as the sudden onset of neurological dysfunction which either persists for more than 1 hour and presents evidence of a lesion via imaging, or continues for more than 24 hours (Liberato et al. 2005). Events precipitating neurological dysfunction lasting less than 1 hour and without evidence of a lesion through imaging are classified as transient ischemic attacks (TIAs) (Albers et al. 2002).

1.1.1 Classification of Stroke

There are two main types of stroke: ischemic and hemorrhagic. Ischemic stroke occurs when a vessel is occluded, leading to tissue ischemia and infarction. Hemorrhagic stroke occurs when a blood vessel ruptures, leading to blood accumulation and tissue damage. There are four main mechanisms of stroke: atherosclerotic, lacunar, cardioembolic and cryptogenic (Liberato et al. 2005). Atherosclerotic infarctions stem from the formation and build up of atherosclerotic plaques in arteries. Two major consequences of atherosclerotic lesion development contribute to stroke. First, plaques may develop to a size which restricts downstream blood flow. Areas of the brain supplied by the terminal branches of an occluded artery can be affected by the occlusion, and the extent of damage of these areas is subsequently dependent on the availability of collateral blood flow from neighbouring vessels. Second, fragments (emboli) may be released
from plaques, and become lodged in distal vessels too narrow to allow continued passage of the embolus, thus occluding blood flow. Lacunar infarcts are usually small in size, and are caused by disease or blockade of small vessels which penetrate the deeper regions of the brain. Cardioembolic infarcts are those which occur as a result of thrombi originating in the atria or ventricles of the heart. Lastly, cryptogenic infarcts are those which cannot be classified based on patient history and imaging into one of the three stroke types described above.

### 1.1.2 Risk Factors

There are several risk factors associated with the incidence of stroke. These include hypertension, atrial fibrillation, presence of a patent foramen ovale, diabetes, high serum levels of low density lipoprotein, and carotid artery disease (Amarenco & Labreuche 2009, Lloyd-Jones et al. 2009, Lechat et al. 1988). Behavioural risk factors include cigarette smoking, excessive alcohol consumption and obesity (Lloyd-Jones et al. 2009).

Uncontrollable risk factors include age, sex, and race (Carandang et al. 2006, White et al. 2005). The annual prevalence of stroke in the U.S.A is estimated at 2,500,000 for males and 3,900,000 for females over the age of 20 (Lloyd-Jones et al. 2009). Furthermore, the ability to perform post-stroke tasks such as eating, getting dressed or getting up from bed tends to be greater in males (Petrea et al. 2009). In addition, mortality due to stroke tends to be less in males (Petrea et al. 2009, Lloyd-Jones et al. 2009).
1.1.3  Pathology

1.1.3.1 Macroscopic

Swelling and softening of brain regions affected by stroke are the most pronounced macroscopic changes in the infarcted brain (Petito 2005). These occur due to changes in the permeability of the blood brain barrier (BBB) and resulting cerebral edema.

1.1.3.2 Microscopic

Haematoxylin and eosin staining of human brain tissue containing an infarct reveals shrunken cells with nuclei containing condensed chromatin, referred to as pyknotic nuclei (Petito 2005). In acute infarcts, neurons undergo eosinophilic neuronal necrosis (ischemic necrosis) characterized by shrunken eosinophilic cell bodies and pyknotic nuclei. When reperfusion is delayed or absent, neuronal necrosis is represented by “ghost” neurons, which are neurons that are unable to take up haematoxylin, and in addition, only weakly stain with eosin. This leaves neurons with an unstained nucleus and faint trace of a cell body. Another major visible change detectable in the infarcted region of the human brain is the presence of polymorphonuclear leukocytes (PMNs) and macrophages. PMNs are the first cells that infiltrate the brain following an infarct. These cells typically cross the blood brain barrier 18 to 24 hours following infarction and are present up to 48-72h later. Monocytes cross the blood barrier approximately 48h post-infarction and infiltration may proceed for 5 more weeks.

The penumbra is an area surrounding the infarct core which is considered salvageable. Within this region, neurons are compromised, but death is not imminent. Whereas the neurons of the infarct core become rapidly necrotic, the delayed death experienced by neurons in the
penumbra still allows for gene regulation and protein synthesis to occur (reviewed by (Weinstein et al. 2004). Given that these processes occur under hypoxia, the penumbra represents a unique yet fragile environment which provides an opportunity for investigating suitable targets in order to prevent delayed neuronal death.

1.1.4 Animal Models of Stroke

1.1.4.1 Middle Cerebral Artery Occlusion (MCAO)

The MCAO model of stroke is probably the most commonly used method of inducing focal ischemia in rodents. Typically, this model is used in rats, although it has been adapted for use in mice (Welsh et al. 1987, Yang et al. 1994). The MCAO model is highly reproducible in rats although its reproducibility and effectiveness as well as variability among different strains of mice have been debated (Belayev et al. 1999, Connolly et al. 1996b). The MCAO model was initially performed in rats by exposing the middle cerebral artery through a craniotomy and ligating it with a suture (Robinson 1979, Robinson et al. 1977). Techniques were then developed to occlude the middle cerebral artery by advancing a suture into the internal carotid artery until it reaches the anterior cerebral artery, blocking blood flow into the middle cerebral artery in the process (Longa et al. 1989).

1.1.4.2 Hypoxia-Ischemia

The hypoxia-ischemia model is a more recent animal model of infarct used in mice. In this model, one of the carotid arteries is ligated and the mouse is subsequently exposed to hypoxia (8% oxygen) (Vannucci et al. 2001, Zhang et al. 2004). The combination of ischemia
and hypoxia produces an acute unilateral lesion in the ipsilateral hemisphere of the brain. It offers an advantage over the MCAO model in that it is slightly less invasive, faster, and easier to perform.

1.1.5 Stroke Treatment

The treatment of stroke is severely limited. The lack of effective treatment options and modest success of primary prevention strategies on reducing stroke incidence are largely responsible for the high mortality rates associated with stroke. Indeed, stroke mortality approaches 50% one year after the event (Donnan et al. 2008). Available treatments for stroke include the administration of recombinant tissue plasminogen activator (rt-PA), aspirin, and anticoagulants. Administration of rt-PA within 3h after stroke results in short-term (24h) and longer term (3 months) neurological improvement in up to 14% of patients (1995). In the same study, however, rt-PA treated patients were significantly more likely to experience intracerebral haemorrhage within 36h after stroke onset. Furthermore, rt-PA administration had no effect on mortality assessed at 3 months after stroke. Aspirin given to patients after stroke was reported to result in a significant decrease in recurrent ischemic strokes and a slight but non-significant decrease in mortality after 6 months (1997). Heparin, an anticoagulant, was found to have no significant effect on neurological symptoms or mortality following stroke (1997).

1.1.6 Role of the Blood Brain Barrier

The blood brain barrier is composed of the endothelial cells that line the cerebral blood vessels. These endothelial cells form a strict barrier to certain substances by means of inter-endothelial tight junctions and absence of a vesicular transport system, while selectively
mediating transport of necessary substances into the brain, and allowing passive diffusion of gases, such as oxygen. While gases like oxygen can diffuse across the blood brain barrier, brain endothelial cells are equipped with transporters for amino acids, sugars (e.g. glucose) and other hydrophilic substances (Begley & Brightman 2003).

The blood brain barrier plays two important roles in the context of stroke pathology. First, blood brain barrier permeability is compromised by reperfusion following ischemia. In an animal model of ischemia, both water and salts accumulated in the brain following permanent and temporary ischemia, causing cerebral edema (Ito et al. 1979). Interestingly, the time within which reperfusion was established seemed to determine the extent of edema. Cerebral edema was greater in animals in which reperfusion was established more than 3 hours after ischemia compared to animals in which reperfusion was established less than 2 hours after ischemia (Ito et al. 1979). Using an MCAO model of focal ischemia, it was found that reperfusion leads to increased blood brain barrier permeability, accompanied by greater edema and increase in infarct volume (Yang & Betz 1994). The evidence surrounding blood brain barrier disruption due to reperfusion has created controversy regarding the use of thrombolytic agents to treat stroke patients. In fact, it was reported that t-PA promotes blood brain barrier permeability and may underlie vasogenic edema following ischemia (Yepes et al. 2003). Considering also the effect of reperfusion time on edema formation, this may explain why the clinical efficacy of t-PA is limited to administration within short periods of time following stroke, and furthermore, is associated with increased haemorrhage/edema in some stroke patients.

Several factors have been implicated in contributing to increased blood brain barrier permeability following ischemia including the c-reactive protein (Kuhlmann et al. 2009), the fractalkine receptor CX3CR1 (Denes et al. 2008), NADPH oxidase (Kahles et al. 2007), vascular endothelial growth factor (Zhang et al. 2000), P-selectin (Kisucka et al. 2009), RANTES (Terao et al. 2008), and p38 MAPK (Nito et al. 2008). In animal studies, both
albumin and superoxide dismutase were found to decrease blood brain barrier permeability following ischemia (Belayev et al. 2005, Armstead et al. 1992). Second, the blood brain barrier mediates the entry of immune cells into the brain. Regulation of immune cell infiltration has been implicated in the severity of the stroke lesion.

1.1.7 Role of Infiltrating Cells

Activation of the inflammatory response is a major consequence of stroke. Leukocytes, including neutrophils, lymphocytes and monocytes leave the systemic circulation and infiltrate the brain following focal ischemia. Brain-resident microglia become activated following ischemia and contribute to infarct development.

Early studies showed that labelled leukocytes infiltrated the human brain and remained activated in the peripheral blood for as long as 2 weeks following infarction (Pozzilli et al. 1985, Violi et al. 1988). Interestingly, leukocyte infiltration was observed to be associated with stroke severity. Additional studies found that infiltration of leukocytes persisted for as long as 5 weeks post-infarct (Wang et al. 1993). Interferon beta (IFN-β), an anti-inflammatory cytokine, administered daily for a week during reperfusion following MCAO in rats has shown to reduce the number of leukocytes, and more specifically, the numbers of monocytes and neutrophils in the ischemic hemisphere. Animals receiving IFN-β also displayed significantly smaller lesions than non-treated animals. However, another study found that INF-β does not have a protective effect, although this study did not extensively characterize or quantify the inflammatory response in these animals.
Polymorphonuclear leukocytes (PMNs) are the first cells which infiltrate the brain following stroke. PMNs appear near the infarct core within the first few hours following ischemia (Hallenbeck et al. 1986). Using antisera to neutrophils, investigators initially reported that a reduction in circulating neutrophils led to a significant decrease in infarct size in rabbits (Bednar et al. 1991). However, administration of an antibody to a surface protein important for neutrophil adhesion to the endothelium (CD18) did not have an effect on infarct size following ischemia in cats and rabbits (Clark et al. 1991, Takeshima et al. 1992). More recently, CD18 knockout mice undergoing transient MCAO were found to have less neutrophil accumulation and reduced infarct volumes than wildtype mice (Prestigiacomo et al. 1999, Soriano et al. 1999). Interestingly, wildtype and CD18 knockout animals undergoing permanent ischemia displayed similar numbers of neutrophils in the ischemic hemisphere and similar infarct volumes. In a separate report, animals treated with a CD18 antagonist in conjunction with thrombolysis following an embolic model of stroke had significantly less neutrophil accumulation and smaller infarcts than animals receiving thrombolytic therapy alone (Zhang et al. 2003).

Other factors have been shown to mediate neutrophil infiltration following cerebral ischemia, leading to improvement of stroke pathology. Depletion of neutrophils prior to MCAO in mice results in significant decreases in infarct volume and mortality, improvement in neurological deficit scores and cerebral blood flow (Connolly et al. 1996a). In the same study, it was found that the intercellular adhesion molecule-1 (ICAM-1), an endothelial adhesion molecule that mediates neutrophil adhesion and infiltration, may underlie the protection afforded by decreased neutrophil infiltration which supplements previous observations that inhibition of ICAM-1 protects against ischemic injury (Zhang et al. 1994). Protein kinase C delta (PKCδ) has also been found to underlie neutrophil-mediated stroke injury following reperfusion. Knockout
of PKCδ leads to decreased neutrophil infiltration and infarct size following transient focal ischemia (Chou et al. 2004). In addition, PKCδ knockout resulted in decreased neutrophil activity, including superoxide and proteolytic enzyme release, mechanisms suggested to contribute to brain injury following ischemia, specifically following reperfusion (Chou et al. 2004).

P-selectin is an important endothelial cell adhesion molecule that mediates neutrophil rolling on the luminal surface of the endothelium prior to firm adhesion. Inhibition of P-selectin presentation on the surface of endothelial cells results in reduced infiltration of neutrophils and reduced infarct volumes in mice undergoing transient MCAO (Atkinson et al. 2006, Connolly et al. 1997).

1.1.7.2 Lymphocytes

Lymphocytes (B- and T-lymphocytes) are another type of inflammatory cell which infiltrate the brain following acute cerebral ischemia. Mice induced to harbour T-lymphocytes secreting the anti-inflammatory cytokine IL-10 at increased levels were found to have reduced infarct sizes and improved behavioural outcomes following stroke. This was attributed to an inhibition of infiltrating macrophages and neutrophils during reperfusion (Frenkel et al. 2003).

Knockout of the proinflammatory interleukin-1 type 1 receptor (IL-1R1) results in increased T cell infiltration and reduced infarct volumes following experimental hypoxia/ischemia (Lazovic et al. 2005). A specific subset of regulatory T cells were found to protect the brain from ischemic injury by exerting anti-inflammatory effects through IL-10 and were associated with decreased expression of the proinflammatory cytokines TNF-α and interferon-γ (IFN-γ) (Liesz et al. 2009).
A definite protective role for T-lymphocytes in infarct development has not been established as mice lacking T-lymphocyte subsets (CD4+ or CD8+) were found to exhibit smaller infarct volumes than wildtype animals while mice lacking B-lymphocytes exhibited similar infarct volumes as wildtype animals (Yilmaz et al. 2006). In addition, a separate study showed that mice lacking both T- and B-lymphocytes display smaller infarct lesions (Hurn et al. 2007). Furthermore, the γδT-lymphocyte subset is a source of interleukin-17 (IL-17) following cerebral ischemia. Knockout of IL-17 results in reduced infarct volume, reduced production of the proinflammatory cytokines TNF-α and IL-1β by macrophages and other infiltrating inflammatory cells while having no effect on inflammatory cell infiltration (Shichita et al. 2009).

Though the reports above suggest a role for TNF-α in mediating inflammatory responses following cerebral ischemia, it should be noted that TNF-α was reported to decrease infarct development in cerebral ischemia (Bruce et al. 1996).

1.1.7.3 Microglia

Microglia become activated and subsequently produce neurotoxic substances following cerebral ischemia (Giulian & Vaca 1993). Treatment of animals with either minocycline or doxycycline before or after cerebral ischemia reduces the activation of microglia and is neuroprotective (Yrjanheikki et al. 1998). Minocycline treatment also prevents activated microglia from accumulating just outside the infarct centre and prevents microglial production of IL-1β (Yrjanheikki et al. 1999). Activated microglia appear with increasing numbers in the area surrounding the infarct for up to 7 days while microglia in the ischemic core die within 3.5 hours after an ischemic insult (Ito et al. 2001). Microglia exposed to oxygen and glucose deprived (OGD) neurons were able to elicit neuronal death in neurons cultured under normal conditions.
through TNF-α-induced activation of the apoptotic cascade in an in vitro model simulating the penumbra (Kaushal & Schlichter 2008).

However, microglia exposed to OGD and applied to slice cultures undergoing OGD, were found to be neuroprotective when applied before or within 2h of OGD (Neumann et al. 2006). Furthermore, by selectively killing off proliferating microglia, it was shown that stroke injury is more severe, and that microglia may exert neuroprotective effects following ischemia by releasing trophic factors (Lalancette-Hebert et al. 2007). Microglia were also shown to be neuroprotective in a study that shows microglia engulf live PMNs following ischemia, and by doing so, reduce PMN-mediated tissue damage (Neumann et al. 2008).

1.1.7.4 Monocytes/Macrophages

Monocyte-derived macrophages are apparent in the brain following ischemia. Although there has been some debate as to whether the appearance of macrophages following cerebral ischemia is due to blood-borne monocytes or brain resident microglia, it is becoming increasingly evident that both lineages contribute to the population of macrophages following cerebral ischemia. Advances in techniques have allowed researchers to distinguish more easily between blood-borne macrophages and resident microglia in the brain. Monocytes infiltrate the peri-infarct area and become phagocytic, with cell numbers achieving levels similar to those of resident activated microglia 7 days after ischemia (Tanaka et al. 2003). Monocyte numbers also increase in the infarct core to a maximum 14d after ischemia (Tanaka et al. 2003). In a separate study, monocytes were found to infiltrate the brain, although infiltration was found to involve much fewer cells (Denes et al. 2007).

Knockout of the receptor for the monocyte chemoattractant protein-1 (MCP-1), a chemokine that regulates monocyte transendothelial migration and brain infiltration, results in
significantly fewer infiltrating monocytes and reduction of infarct volume following transient focal ischemia (Dimitrijevic et al. 2007). Knockout of MCP-1 also results in a reduction of infarct volume and a decrease in infiltrating monocytes (Hughes et al. 2002). Knockout of CD39, an ADP/ATP diphosphohydrolase, results in increased monocyte infiltration and monocyte expression of the adhesion receptor CD18, and is associated with greater stroke lesions following focal ischemia (Hyman et al. 2009).

1.1.8 HIF-1α Upregulation as a Consequence of Hypoxia Following Cerebral Ischemia

HIF-1α is upregulated in the ischemic penumbra shortly after MCAO in rats. By 4 hours, HIF-1α mRNA levels, and by 20h, HIF-1α protein levels are significantly increased in the penumbra (Bergeron et al. 1999). HIF-1α protein levels are increased in the infarct core within 1h of focal ischemia (Baranova et al. 2007). Neuron-specific knockout of HIF-1α results in neuroprotection following transient global ischemia in mice (Helton et al. 2005). Conversely, neuron-specific knockout of HIF-1α was shown to result in larger infarcts, poorer neurological outcome and probability of survival following MCAO in mice (Baranova et al. 2007). Knockdown of HIF-1α in an in vitro model of stroke has also been found to result in more significant neuronal death (Guo et al. 2009). Although several HIF-1 target genes have been discovered, only a handful of studies have examined the role of these genes in the context of cerebral ischemia. The proapoptotic protein Noxa was found to be upregulated by HIF-1α under hypoxic conditions and during ischemia while facilitating brain infarction following transient MCAO in rats (Kim et al. 2004). HIF-1α regulates IL-20 expression, a cytokine which is upregulated in glia following transient focal ischemia, and inhibition of which, leads to reduced stroke-induced injury in rats (Chen & Chang 2009). The inducible nitric oxide synthase gene
iNOS) is also regulated by HIF-1α (Melillo et al. 1995). Inhibition of iNOS in vivo results in reduced infarct volume following MCAO in rats, while knockout of the iNOS gene in mice results in delayed neuroprotection following MCAO (Iadecola et al. 1997, Iadecola et al. 1995). The receptor for advanced glycation end products (RAGE) is a target of HIF-1α gene regulation, is upregulated following hypoxia-ischemia, and may be neuroprotective, since knockout of the RAGE gene leads to more severe infarcts than in wildtype mice (Pichiule et al. 2007). The role of the vascular endothelial growth factor (VEGF) has been the focus of much research since the discovery that its expression is regulated by HIF-1α (Forsythe et al. 1996). VEGF has been implicated in the development of the infarct, angiogenesis in the penumbra, and edema following stroke (van Bruggen et al. 1999, Zhang et al. 2000, Sun et al. 2003).

1.2 HYPOXIA AND THE HYPOXIA-INDUCIBLE FACTOR (HIF-1α)

1.2.1 Definition of Hypoxia

The term “hypoxia” originates from the Greek prefix “hypo-”, meaning under or low, and the word “oxi”, referring to oxygen. In medicine, hypoxia refers to specific conditions under which tissues experience abnormally low oxygen levels. Since blood supplies oxygen to the tissues of the body, hypoxia can be traced back to the occlusion of blood supply either locally or distally.

1.2.2 Discovery of HIF-1α and HIF-1β

Erythropoietin (EPO) is a hormone synthesized chiefly in the kidneys and, to a much lesser extent, in the liver in adult mammals in response to hypoxemia (Fried 1972, Jacobson et
al. 1957, Fried et al. 1981). EPO is involved at several steps during the differentiation of red
blood cells and its production is tightly linked to oxygen levels (reviewed in Jelkmann (1992)).
Diminished oxygen carrying capacity of the blood due to anemia or haemorrhage stimulates
EPO production (Baer et al. 1987). It was soon discovered that factors binding to DNA elements
in the 3’ flanking region of the EPO gene were responsible for increased transcription of a
reporter gene under hypoxic conditions in the EPO-producing human hepatoma cell line, Hep3B
(Semenza et al. 1991). This suggested that the sensitivity of EPO to oxygen was mediated by a
factor promoting transcription of the EPO gene. Initially, the presence of the suspected factor
was detected by using electric mobility shift assays (EMSAs) to show DNA-protein complexes
arising from hypoxia-exposed cell lines. The mystery factor was given the name “hypoxia-
inducible factor 1” (HIF-1) and was initially found to recognize and bind the DNA sequence: 5’
CTACGTGCT 3’ (Semenza & Wang 1992). Shortly thereafter, HIF-1 was found to be inducible
by hypoxia in many mammalian cell lines including the Chinese hamster ovary (CHO), human
embryonic kidney 293 (HEK293), and HeLa cell lines (Wang & Semenza 1993). HIF-1 was
soon found to be a heterodimer, that is, composed of two different subunits. The two subunits
were identified and named HIF-1α and HIF-1β (Wang & Semenza 1995). Both HIF-1α and
HIF-1β were found to be basic helix-loop-helix-PAS (bHLH-PAS) proteins, capable of binding
DNA and protein dimerization (Wang et al. 1995). PAS proteins are named for the Drosophila
genomes Per, ARNT and Sim and share a common domain, the PAS domain.

1.2.3 Mechanisms Regulating HIF-1α Expression

HIF-1α has an apparent mass of 120kDa and, structurally, it bears resemblance to other
transcription factors designated bHLH proteins, possessing both a DNA-binding region (b) and a
region (HLH) for interacting and forming dimers with HIF-1β (Wang & Semenza 1995, Wang et
HIF-1α heterodimerizes with HIF-1β in the absence of DNA (Jiang et al. 1996). HIF-1α contains a domain for transcriptional activation at its carboxy terminus, which plays an important role during hypoxia (Jiang et al. 1996). HIF-1β contains a nuclear localization signal (NLS), located near the N-terminus of the protein and required for translocation into the nucleus (Eguchi et al. 1997). HIF-1α was also found to contain a NLS near its carboxy terminus (Kallio et al. 1998).

The mechanisms underlying the regulation of HIF-1α under normoxic conditions are strict and complex. It was initially discovered that HIF-1α is constitutively expressed and continuously degraded through the ubiquitin-proteasome pathway during normoxia, leading to the observed short half-life of HIF-1α levels in vitro (Salceda & Caro 1997). Since this initial observation, knowledge of the mechanisms underlying HIF-1α regulation has grown and it is now known that several enzymes mediate the process of HIF-1α degradation. While it had been shown that HIF-1α is degraded by the ubiquitin-proteasome system, it still remained unknown why HIF-1α protein levels were unstable, with a half-life of 5-10 minutes. It was soon discovered that HIF-1α contains an oxygen-dependent degradation domain (ODD) (Huang et al. 1998). Deletion of this domain and expression of the resulting truncated protein led to an increase in the stability of HIF-1α and strong expression of HIF-1α under normoxia. In addition, this domain resulted in rapid degradation of a protein to which it was fused.

The importance of the ODD in regulating HIF-1α levels was unravelled by several reports. The von Hippel-Lindau protein (VHL) was the first protein identified to be required for targeted and continuous degradation of HIF-1α under normoxia (Maxwell et al. 1999). This study also demonstrated that iron is essential for VHL binding of HIF-1α. Two years later, it was found that the ODD of HIF-1α is hydroxylated by specific prolyl-hydroxylases (HPHs) and acetylated by the acetyl-transferase ARD1 (Epstein et al. 2001, Jeong et al. 2002). Hydroxylation and acetylation of the ODD of HIF-1α is required for VHL binding. HPHs
belong to the class of 2-oxoglutarate dependent oxygenases which use dioxygen (O\textsubscript{2}) and H\textsubscript{2}O to hydroxylate substrates and require iron for their enzymatic activity (Schofield & Zhang 1999), which explains the inability of VHL to bind HIF-1\textalpha in the absence of iron in previous reports. As such, the HPHs serve as molecular oxygen sensing enzymes and are one of the hypoxia sensors that upregulate HIF-1\textalpha levels under hypoxia. VHL binding to HIF-1\textalpha further promotes binding of factors required for subsequent ubiquitination and degradation of HIF-1\textalpha via the proteasome (reviewed in Sharp (2004)), such as FIH1.

The factor inhibiting HIF-1\textalpha (FIH1) is an enzyme similar to the HPHs. It is a 2-oxoglutarate dependent oxygenase, which requires iron to hydroxylate HIF-1\textalpha (Lando et al. 2002, Mahon et al. 2001). Unlike HPHs, however, FIH1 hydroxylation of HIF-1\textalpha prevents binding of HIF-1\textalpha to the CREB-binding protein, p300/CBP (Lando et al. 2002). Binding of HIF-1\textalpha to p300/CBP is essential for HIF-1\textalpha transcription activity (Sang et al. 2002). Thus, FIH1 is an oxygen sensor, and contributes to HIF-1\textalpha inactivity under normal oxygen conditions by inhibiting HIF-1\textalpha transcriptional activity.

1.2.4 Genes Regulated by HIF-1\textalpha

HIF-1\textalpha regulates the expression of a variety of proteins involved in a myriad of processes through regulation of genes at the transcriptional level during hypoxia. HIF-1\textalpha has been found to be a major regulator of angiogenesis (VEGF, Leptin, Angiopoietin-2 and -4 (Forsythe et al. 1996, Ambrosini et al. 2002, Yamakawa et al. 2003)), insulin growth factor signalling (IGFBP-1 (Tazuke et al. 1998)), iron transport and uptake (Transferrin (Tacchini et al. 1999)), metabolism and pH regulation (Carbonic anhydrase 9 (Wykoff et al. 2000)), neutrophil survival (MIP-1\beta, NF-\kappaB (Walmsley et al. 2005)), vasodilation (eNOS (Coulet et al. 2003)), DNA methylation (JMJD1A and JMJD2B (Beyer et al. 2008)) inflammation (IL-20 (Chen & Chang 2009)),
apoptosis (BCL-xL (Chen et al. 2009)) and mitochondrial transport along axons of neurons (HUMMR (Li et al. 2009)). In addition, HIF-1α regulates the expression of the neurotrophin receptor TrKB and the adenosine receptor A2B (Martens et al. 2007, Kong et al. 2006). HIF-1α also regulates the expression of the BACE1 gene, leading to increased severity of Alzheimer disease pathology and behavioural deficits in a mouse model (Sun et al. 2006, Zhang et al. 2007b).

1.2.5 The Role of Hypoxia in Development

HIF-1α is required for proper development of the cardiovascular system (Licht et al. 2006, Krishnan et al. 2008, Yin et al. 2002), components of the immune system (Kojima et al. 2002), chondrogenesis and joint development (Provot et al. 2007), early human embryonic development (Caniggia et al. 2000), development of the lung (Saini et al. 2008), and skeletal development (Wang et al. 2007).

1.3 EUKARYOTIC GENE TRANSCRIPTION

1.3.1 Definition of a Gene Promoter

A gene promoter is a sequence of DNA that is absolutely required for basal transcription of the proximal downstream gene.
1.3.2 The Core Promoter

The core promoter is a short sequence of the promoter which is essential for initiation of transcription. Current knowledge holds that there are two distinct types of core promoter regions: the focused and the dispersed (Juven-Gershon et al. 2008, 1995). Focused core promoters contain either a single or several transcription initiation sites within a very narrow region of the promoter (several nucleotides). Dispersed core promoters are comprised of multiple transcription initiation sites typically spread over 50 to 100 nucleotides (Juven-Gershon et al. 2008). Dispersed core promoters comprise the majority of core promoters in humans and other vertebrates (Juven-Gershon et al. 2008, 1995).

1.3.2.1 Core Promoter Elements

The core promoter and the elements necessary for transcription initiation are still poorly understood. Although several elements have been found to bind proteins involved in the initiation of transcription of focused core promoters, including the initiator element (Inr), TATA box, BRE, MTE, DPE and DCE, the presence of any or all of these elements is not necessary for transcription at most core promoters. The initiator binds TFIID, the TATA box binds the TATA-box binding protein TBP (a subunit of TFIID), the BRE TFIIB (upstream or downstream of the TATA box), the DPE TAF6 and TAF9 (subunits of TFIID) (subunits of TFIID), and the DCE TAF1 (a subunit of TFIID) (reviewed in (Juven-Gershon et al. 2008, 1995). While these elements have been shown to play a role in certain focused core promoters, a distinct requirement for specific promoter elements in dispersed core promoters does not seem to be necessary. Since the majority of human genes are driven by dispersed core promoters, the
promoter regions of human genes are highly irregular, which highlights the necessity for individual examination and functional characterization of the promoter regions of human genes.

1.3.3 Brief Summary of the Mechanism of Transcription

A consensus for the general components that are necessary for constituting a functional promoter region of a gene has not been established due to variations in transcriptional machinery among gene promoters. Such is the case even for the basic mechanisms of transcription initiation leading to recruitment of RNA Polymerase II. Indeed, it seems that the only common underlying mechanisms of transcription initiation of mRNA are the steps following recruitment of RNA Polymerase II to the transcription initiation site. Since a thorough review of the discoveries and description of the transcriptional process would be well beyond the scope of this thesis, what follows is a brief description of the aspects of transcription initiation which have been elucidated and is adapted from two recent and thorough reviews by Thomas and Chiang (2006) and Sikorski and Buratowski (2009). Initiation of transcription is dependent upon the formation of a functional complex of proteins, often referred to as the pre-initiation complex (PIC). The PIC responds to and assembles at elements constituting the core promoter region (described above). However, there is no consensus for a unified PIC. In fact, different models of PIC formation exist.

The major non-RNA polymerase II constituents of the PIC are the general transcription factors (GTFs) TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIF. There are two views on the formation of the PIC complex with respect to the order of binding of the aforementioned transcription factors. The first one describes the binding of TFII species by ordered binding. In this proposed pathway, binding of the core promoter is first accomplished by TFIID, followed by IIA, IIB, IIF, IIE and IIH. The second view on PIC assembly is based on observations that RNA
polymerase II can be purified in a complex containing TFIIB, IIE, IIF and IIH. From these observations, it has been suggested that TFIID binds the core promoter, followed by binding of a RNA polymerase II-GTF complex.

In TATA box-containing promoters, the TATA box is bound by the TATA binding protein (TBP) subunit of the basal transcription factor TFIID. TFIID was found to be a protein complex consisting of several factors. In addition to TBP, there are approximately 14 TBP-associated factors (TAFs) that comprise TFIID. Certain functions of the remaining GTFs include stabilizing bound TFIID and TBP to DNA (IIB), stabilizing RNA polymerase II binding to the PIC (IIF), promoting the enzymatic functions of TFIIH (IIE), and unwinding the DNA for exposure of the template strand for transcription (IIH).

A significant amount of human gene promoters do not contain functional TATA boxes. These genes tend to be non-housekeeping genes. TATA-less promoters present an interesting challenge to the model of PIC formation outlined above. One major alternate factor driving TBP-TFIID binding to the core promoter is the specificity protein 1 (Sp1) transcription factor. Studies have shown that Sp1 binds to TAF7 of TFIID and can help form the PIC by recruitment of TFIID, thereby promoting PIC formation and transcription initiation in TATA-less gene promoters.

1.3.4 The Human MIF Promoter

1.3.4.1 Promoter Characteristics

The human MIF promoter is TATA-less and it appears that basal transcription of the human MIF gene relies on two transcription factors: Sp1 and the cyclic-AMP responsive element binding (CREB) transcription factor (Roger et al. 2007). The transcription start site (TSS) of the
human MIF gene promoter has been identified (Paralkar & Wistow 1994). Formation of the PIC has been suggested to occur via Sp1-dependent recruitment of TFIID (Dorris & Struhl 2000, He & Weintraub 1998). More recently, MIF has been shown to be transcriptionally activated by the glucocorticoid dexamethasone, and a functional glucocorticoid response element (GRE) was identified approximately 800bp upstream of the TSS (Leng et al. 2009). The human MIF promoter was also shown to be hypoxia inducible, although the experiments in this study are not entirely convincing and don’t fully demonstrate the requirement for and interaction between HIF-1α and the human MIF promoter (Baugh et al. 2006).

1.3.4.2 MIF Promoter Polymorphisms

There have been several studies showing that polymorphisms in the human MIF gene promoter are associated with higher incidence of certain diseases, including systemic lupus erythematosus and prostate cancer and increased severity of disease, including cystic fibrosis and rheumatoid arthritis (Plant et al. 2005, Meyer-Siegler et al. 2007, Baugh et al. 2002, Sanchez et al. 2006). The polymorphism in question is a tetranucleotide repeat, CATT, whose terminal thymidine nucleotide resides at -794 of the human MIF promoter (Baugh et al. 2002). The number of repeats ranges from 5 to 8, and exist in any combination as heterozygous or homozygous pairs of alleles with the current exception of 8,8 homozygotes (Baugh et al. 2002). The most common CATT repeat allele frequency varies according to different studies, however it appears that the combination of 5,5 and 5,6 CATT repeats are most common in the normal population (Plant et al. 2005, Sanchez et al. 2006, Baugh et al. 2002). It has been reported that promoter activity varies according to the number of CATT repeats, where the presence of 5 CATT repeats displays the lowest promoter activity and longer repeats display higher promoter activity (Baugh et al. 2002). In addition to low promoter activity, the 5,5 CATT promoter
polymorphism is associated with less MIF secretion than longer CATT repeats (Miller et al. 2008, Qi et al. 2009).

1.4. MACROPHAGE MIGRATION INHIBITORY FACTOR (MIF)

1.4.1 Structure

MIF was initially described as a soluble factor released from lymphocytes, which had the ability to inhibit the migration of macrophages over a glass surface (Bloom & Bennett 1966). It was shown that treatment of MIF with neuraminidase results in reduction of MIF activity, suggesting that MIF is a glycoprotein (Remold & David 1971). The macrophage-migration inhibitory activity of MIF was then found to correspond to a protein in the molecular weight range of 12.4 to 25kDa (Papageorgiou et al. 1972). Though early studies identified lymphocytes as the central producers of MIF, later studies demonstrated that macrophages themselves are necessary for the production of MIF in lymphocyte-macrophage migration experiments (Nelson & Leu 1975). In addition to inhibiting migration of macrophages over a glass surface, MIF was found to inhibit the detachment of macrophages from glass surfaces under shear conditions (Weiss & Glaves 1975).

The human MIF gene is located on chromosome 22q11.2 (Budarf et al. 1997). The human MIF mRNA-coding region was cloned by expressing a cDNA library from a stimulated T-cell line in COS-1 cells and assaying for MIF-induced inhibition of monocytes (Weiser et al. 1989). Sequence analysis showed that the MIF gene contains 345 nucleotides encoding a 115-amino acid protein with a molecular weight of 12,650 Da. The sequence of MIF was found to lack conserved motifs associated with known secreted proteins, which left the mechanism of MIF secretion, until recently, unknown. The secondary structure of human MIF is primarily
composed of 6 β-sheets, and contains 2 alpha helices (Bernhagen et al. 1994, Sun et al. 1996). Crystal structure analysis of MIF suggests that MIF monomers can assemble to form a trimer with a barrel-like structure containing a positively charged central channel (Sun et al. 1996). Human MIF does not appear to be glycosylated (Bernhagen et al. 1994).

1.4.2 Characterization and Expression

B and T cells express and release MIF, which inhibits macrophage migration (Yoshida et al. 1973). In addition, T cells secrete a soluble factor which inhibits B cell production of MIF (Cohen & Yoshida 1977). MIF is expressed by unstimulated murine macrophages and human monocytes and is released by macrophages upon stimulation with LPS (Calandra et al. 1994). Human umbilical vein endothelial cells have also been found to express and release MIF upon exposure to LPS (Nishihira et al. 1998). MIF is expressed in the mouse liver, spleen, kidney and brain (Calandra et al. 1994). Human MIF is expressed in the heart, brain, lung, liver and kidney (Paralkar & Wistow 1994). Murine macrophages secrete MIF in response to TNF-α and IFN-γ, but not IL-1β or IL-6 (Calandra et al. 1994). MIF is detectable in the rat brain by immunohistochemistry. MIF was found to be expressed in neurons and astrocytes in one study, but not in another. However, strong expression was seen in epithelial cells of the choroid plexus in both studies (Bacher et al. 1998, Ogata et al. 1998). To date, the expression of MIF by cells of the human central nervous system has not been investigated.
1.4.3 Major Actions of MIF

MIF is an important factor involved in the regulation of inflammation by glucocorticoids. MIF can stimulate TNF-α release from murine macrophages (Calandra et al. 1994). MIF secretion is induced by the glucocorticoid dexamethasone and is stored by cells of the anterior pituitary (Bernhagen et al. 1993). Incubation of human monocytes with MIF relieves the glucocorticoid-mediated inhibition of TNF-α. Furthermore, MIF inhibits IL-1β expression and glucocorticoid-mediated protection of LPS-induced endotoxemia in mice (Bernhagen et al. 1993, Calandra et al. 1995). ISO-1, a synthetic MIF inhibitor, protects against endotoxemia by inhibiting MIF-induced TNF-α production (Al-Abed et al. 2005). Metabolic derivatives of acetaminophen are able to inhibit the counter-glucocorticoid actions of MIF, notably, MIF-induced TNF-α production (Senter et al. 2002).

MIF exhibits enzymatic activity in vitro, although demonstration of relevant in vivo targets has not been shown. MIF displays dopachrome, phenylpyruvate, and p-hydroxyphenylpyruvate tautomerase activity, which is dependent on both the N-terminal Proline residue adjacent to the methionine start codon, as well as the C-terminus region of the MIF trimer (Bendrat et al. 1997, Rosengren et al. 1997, Swope et al. 1998). MIF also catalyzes the conversion of the toxic quinones dopaminechrome, epinephrinechrome and norepinephrinechrome to neuromelanin (Matsunaga et al. 1999).

MIF can also act as a thiol protein oxidoreductase in vitro on the substrate insulin (Kleemann et al. 1998). The oxidoreductase activity of MIF is dependent on the C-X-X-C motif C-A-L-C comprising amino acids 57 through 60 of the MIF protein since mutation of the cysteine residues abrogates oxidoreductase activity. MIF can form a complex with the reductase PAG, which mutually inhibits their individual enzymatic activities (Jung et al. 2001).
Expression of MIF can be induced by glucose in insulin-producing islet cells of the pancreas. Inhibition of MIF during glucose stimulation of islet cells results in decreased insulin production (Waeber et al. 1997). MIF is involved in glycolysis in muscle and liver since it mediates expression of the 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2) enzyme, a regulator of fructose-2,6-bisphosphate, a key activator of the glycolytic pathway. In addition, inhibition of MIF in vivo reduces glycolysis stimulated by TNF-α administration (Benigni et al. 2000).

MIF inhibits the transcriptional activity of the tumor suppressor p53, and by doing so, inhibits downstream events associated with p53 activity, including cell growth arrest, apoptosis and cellular senescence (Hudson et al. 1999, Jung et al. 2008). Inhibition of p53 activity by MIF has been suggested to occur by direct interaction, although it seems to also depend on cyclooxygenase-2 (cox-2), p21, E2F and Rb family of proteins (Petrenko et al. 2003, Taranto et al. 2009, Mitchell et al. 2002). The pro-proliferative properties of MIF have been linked to activation of Rho-GTPase, downstream activation of the MAP/ERK kinase pathway and subsequent transcriptional activation of cyclin D1 expression (Swant et al. 2005, Mitchell et al. 1999). Interestingly, MIF interacts with Jab intracellularly and inhibits Jab-mediated degradation of the cell cycle progression inhibitor p27 and subsequent proliferation when Jab is overexpressed in cells. However, overexpression of MIF alone does not inhibit proliferation (Kleemann et al. 2000).

1.4.4. MIF Cell Surface Receptors

Despite mounting evidence of diverse functions of MIF in experiments involving extracellular application of this cytokine, the identification of a surface receptor for MIF remained elusive for quite some time. In one particular study, a cDNA library obtained from
stimulated monocytes was transfected into a non-MIF binding cell line. Sequencing of clones conferring MIF-binding activity revealed that the invariant chain of the major histocompatibility complex II (MHC II), CD74, was responsible for the observed activity. Further experiments confirmed that MIF binds to CD74, and CD74 is necessary for MIF-induced activation of ERK-1/2 and subsequent cell proliferation (Leng et al. 2003).

The requirement of additional components of a functional MIF receptor has been reported. Evidence exists for the necessary recruitment and interaction of the membrane protein CD44 with CD74 necessary for eliciting ERK-1/2 signalling and activation of downstream events such as inhibition of p53-mediated apoptosis (Shi et al. 2006). MIF also binds the chemokine receptors CXCR2 and CXCR4, which seems to underlie interactions between endothelial cells and leukocytes (Bernhagen et al. 2007). A receptor complex formation comprised of CD74 with either CXCR4 or CXCR2 has been demonstrated (Bernhagen et al. 2007, Schwartz et al. 2009).

1.4.5 Secretion

Although the secretion of MIF from cells in response to various stimuli is a well-observed phenomenon, the mechanisms underlying MIF secretion have only recently been studied. MIF is constitutively expressed and stored intracellularly, with the potential to be released upon exposure to appropriate stimuli, for example, LPS, after which de novo MIF synthesis replenishes depleted intracellular stores (Bacher et al. 1997). Both the ATP-binding cassette (ABC) transporters and the Golgi-associated protein p115 have been shown to be important in regulating MIF secretion (Flieger et al. 2003, Merk et al. 2009). These studies show that inhibition of either factor leads to significant reduction in MIF secretion from cells. However, additional factors which regulate MIF secretion must be involved since, for example,
inhibition of p115 does not completely abolish MIF secretion. Furthermore, the exact mechanism by which MIF is secreted from cells is still very poorly understood. Since MIF does not enter either the ER or the Golgi network, is found preformed in the cytoplasm, and seems to co-localize with p115 near the Golgi, as well as in the cytoplasm, further investigations into the degree of association of MIF with the Golgi apparatus and the mechanisms underlying secretion therein are required.

### 1.4.6 Role of MIF in Ischemia/Reperfusion (I/R)

Interest with respect to the role of MIF in ischemia-related events has developed since early studies demonstrated that MIF expression is upregulated following acute myocardial infarction (AMI) in rats. Six hours following AMI in rats, MIF protein levels are elevated in the serum, while MIF mRNA levels are increased in myocytes, suggesting an early release of MIF from myocytes in response to AMI, followed by de novo synthesis of MIF (Yu et al. 2003). In the same study, macrophages were shown to accumulate in the infarct area after AMI and stain positive for MIF.

It has been reported that MIF protein levels are elevated in the serum following intestinal I/R, which is associated with an increase in TNF-α, and decreased survival of animals with no bearing on neutrophil influx into infarcted tissue (Amaral et al. 2007).

MIF has been shown to play a prominent role in I/R injury of the mouse heart. During heart I/R in mice, MIF expression and release by cardiomyocytes is increased, leading to increased AMPK phosphorylation, cell surface glucose transporter-4 (GLUT4) expression, glucose uptake and protection mediated by the CD74-CD44 MIF signalling complex (Miller et al. 2008). Importantly, I/R injury results in MIF release into the bloodstream and a human MIF promoter polymorphism associated with decreased MIF promoter activity was shown to decrease
MIF secretion and AMPK phosphorylation during hypoxia (Miller et al. 2008). MIF is also involved in another signalling pathway during I/R in the heart. MIF suppresses activation of the JNK pathway during I/R through suppression of the upstream JNK kinase, MKK4, leading to reduced JNK-induced apoptosis (Qi et al. 2009).

1.5 OBJECTIVE AND SPECIFIC AIMS

1.5.1 Objective and Hypotheses

Increasing evidence suggests a prominent role for MIF in the regulation of cell survival following cardiac ischemia-reperfusion injury. These results are intriguing and they present the possibility that MIF plays a role during ischemia in other organs, namely the brain, which is of chief importance. A major consequence of tissue ischemia is hypoxia and subsequent activation of the HIF-1α transcription factor. Recent evidence suggests that the MIF promoter contains putative hypoxia response elements which bind to HIF-1α. Therefore, the main objective of this study is to examine the hypotheses that: 1) the MIF gene is a direct downstream target of hypoxia signalling, 2) MIF expression is increased in the human brain following stroke, and 3) MIF contributes to cell survival following ischemia-induced cell death of neurons.

1.5.2 Specific Aims

This thesis tries to establish a role for MIF during stroke through the following specific aims:

1. To confirm and characterize the transcriptional activation of the human MIF promoter during hypoxia by the HIF-1α transcription factor.
2. To determine the pattern of expression of MIF in normal and infarcted human brain tissue

3. To examine the effect of MIF on oxidative stress and in vitro ischemia-like induced cell death
CHAPTER 2: MATERIALS AND METHODS

2.1 CLONING OF HUMAN MIF PROMOTER FRAGMENTS

The 5’ flanking region of the human MIF gene was amplified by PCR using 5’-CTAGCTAGCGCCTAGACATGCAGTCT forward and 5’-CCCAAGCTTAGCTACGTGCCTGACTTC reverse primers for the human MIF promoter. The resulting DNA fragments spanned -2634 to +35 bp of the human MIF gene’s 5’ flanking region (+1 is denoted as the transcription start site). The corresponding DNA fragments were cloned into the promoter-less vector pGL3-Basic (Promega, Madison, WI, USA) at the NheI and HindIII sites, thereby generating plasmids containing a part of the human MIF promoter regions upstream of the luciferase reporter gene, pHMIF-Luc. Primers were designed to include restriction enzymes sites so that the resulting PCR-amplified fragment could be easily cloned into the multi-cloning sites of pGL3-Basic. Software analysis for putative transcription factor binding sites was performed using Genomatix MatInspector online software (www.genomatix.de, Ann Arbor, MI, USA). Deletion constructs were created by PCR-amplification using pHMIF-Luc as template. The following primer pairs were used to create pHMIF-HIF, forward, 5’-CCGCTCGAGGCCTCCCAGCATCCTATCCT and reverse, 5’-CCCAAGCTTAGCTACGTGCCTGACTTC, pHMIF-HIFB, forward, 5’-CCGCTCGAGCTAGCCGCCAAGTGGAGAACAG and reverse 5’-CCCAAGCTTAGCTACGTGCCTGACTTC, pHMIF-HIFC, forward 5’-CCCAAGCTTAGCTACGTGCCTGACTTC and reverse, 5’-CCGCTCGAGCTAGCCGCCAAGTGGAGAACAG and reverse 5’-CCCAAGCTTAGCTACGTGCCTGACTTC, pHMIF-HIFD, forward, 5’-CCGCTCGAGCTAGCCGCCAAGTGGAGAACAG and reverse, 5’-
CCCAAGCTTACTGTGGTCCCGCCTTTTGTGA. All deletion constructs were cloned into the XhoI and HindIII sites of the pGL3 Basic vector. pHMIF-HIF spans -553 to +8, pHMIF-HIFB -194 to +35, pHMIF-HIFC -553 to +8 and pHMIF-HIFD -194 to +8 of the human MIF promoter sequence. Confirmation of positive clones was first assessed by restriction enzyme digest followed by 1% agarose gel analysis. Samples containing appropriate size inserts were sequenced to confirm for correct insertion of target DNA.

2.2 HEK 293 CELL CULTURE, LUCIFERASE ASSAY, INCLUDING PLASMIDS AND SIRNA

Human embryonic kidney (HEK293) cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 1 mM of sodium pyruvate, 2 mM of L-glutamine and 50 units of Penicillin and 50 µg of Streptomycin (all from GIBCO BRL, Burlington, Ontario). Cells were maintained at 37 °C in an incubator containing 5% CO2. Cells were seeded onto 24-well plates one day prior to transfection and grown to approximately 70% confluence by the day of transfection. Cells were transfected with 0.5 µg of plasmid DNA per well using 1 µl Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The Renilla (sea pansy) luciferase vector pCMV-Rluc was cotransfected to normalize for transfection efficiency. Cells were maintained either under normoxic (21% O2) or hypoxic (2% O2) conditions following transfection. Cells were harvested at 48 hours after transfection and lysed with 50 µl 1X passive lysis buffer (Promega) per well. Firefly luciferase activity and Renilla luciferase activity were measured sequentially using the Dual-luciferase reporter assay system (Promega). The firefly luciferase activity was normalized according to Renilla luciferase activity and expressed as relative luciferase units (RLU) to reflect the promoter activity. pHIF-1α is a CMV-driven expression vector expressing hemaglutinin (HA)-tagged H1F-1α, and pEpoE-Luc is a luciferase
reporter plasmid containing a functional HIF-1 binding site from the human erythropoietin 3’ enhancer region (Huang et al. 1998). HIF-1α siRNA (human HIF-1α siGENOME SMARTpool) was purchased from Dharmacon Research (Lafayette, CO) and used as previously described (Sun et al. 2006). siRNA treatment of HEK293 cells was performed by using a Lipofectamine 2000 based delivery (Invitrogen) and following the manufacturer’s directions. DSCR1 siRNA served as the control siRNA.

2.3 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The following pairs of oligos were annealed for EMSA: HIF-1(2), forward, 5’-TGTTGC CACGAGGTCCA and reverse 5’-TGGGACCCTCGTGGCACCA, and HIF-1(4), forward, 5’- GTCAGGACGTAGCTCAGC and reverse, 5’-GCTGAGCTACGTGCCTGAC. The sequence of the HIF-1 consensus probe and wildtype competitor oligos was: forward, 5’-GCATGCAGAGCGTGACGCACGTGTGGCAG and reverse, 5’-CTGCCACACGTGCGTCACGTCCCGCATGC. The sequence of the HIF-1 mutant competitor oligos was: forward, 5’-GCATGCAGAGAATTGACGCAATTGCGCAG and reverse 5’-CTGCCACACGTGCCGTCACTCGTCCCGCATGC. Oligos were annealed at 65°C for 10 minutes in annealing buffer (0.1M Tris-HCL, pH 7.5, 0.5M NaCl, 0.05M EDTA). Binding of oligos to whole cell lysates from hypoxia-treated HEK293 cells was performed using the EMSA buffer kit for the Li-Cor Odyssey following the manufacturer’s directions (LI-COR Biosciences, Lincoln, NE, USA). 0.5pmol of labelled probe was used in each reaction. Competition was performed by adding 200 or 400 fold molar excess of competitor. After completion of the binding reaction, samples were run on a 6.25% tris-glycine-edta gel for 70 minutes in the dark. The gel was then scanned at 700nm using the Li-Cor Odyssey (LI-COR Biosciences).
2.4 PRIMARY CORTICAL NEURON CULTURE

For the culture of primary neurons, E18-E19 embryos were isolated from timed pregnant Sprague-Dawley rats. The study complied with all institutional policies and was approved by the ethics committee of the Animal Care Centre of The University of British Columbia. The heads of the embryos were separated from the bodies and were placed in a separate sterile dish where the skulls were opened and the brains removed. The freshly isolated brains were placed immediately in ice-cold dissection buffer containing 1X Hank’s Balanced Salt Solution without Ca+2 and Mg+2 (GIBCO), 20mg/mL glucose, 5mg/mL sucrose and 3.56mg/mL Hepes (Sigma, St. Louis, MO). The cortex of each hemisphere was removed and placed in fresh ice-cold dissection buffer. The dissection buffer was removed and the cortices were incubated with a 0.25% solution of trypsin at 37ºC for 30 minutes. The cortices were then washed with DMEM containing 10% FBS to inhibit and remove traces of trypsin. The tissue was then suspended in plating media containing 0.5mM Glutamax-I, 2% B-27 (GIBCO), and 25µM glutamic acid (Sigma) in Neurobasal Media (GIBCO). The suspension was centrifuged at 2,000 rpm for 1 minute. Plating media was removed and the tissue was resuspended in fresh plating media. The tissue was passed through a 10mL pipette several times to facilitate dissociation of the tissue after which it was further triturated by placing a 100µL pipette tip onto a 10mL pipette. Once dissociation was observed to be complete, cell viability was assessed by trypan blue staining. The day before plating of the isolated neurons, 96-well plates were coated with a 10ug/mL solution of poly-D-lysine (PDL) (Sigma) and incubated in the dark overnight at room temperature. On the day of plating, PDL was removed from the plates, wells were washed with sterile ddH₂O to remove traces of PDL solution, after which the ddH₂O was removed and plates
were left to dry. Cells were seeded onto 96-well plates at a density of 5 x 10^4 cells per well and cultured in a 37° C incubator containing 5% CO2 and 21% O2. This was counted as day in vitro 1 (DIV1). The next day, and every 3rd day thereafter, half of the culture media was replaced with feeding media containing 0.5mM Glutamax-I and 2% B-27 in Neurobasal Media.

2.5 OXYGEN GLUCOSE DEPRIVATION (OGD), HYDROGEN PEROXIDE (H2O2)
TREATMENT AND MTT ASSAY

Fourteen day-old cultures of rat neurons were subjected to treatment with H2O2 or underwent OGD. For H2O2 treatment, cells were treated with 75µM H2O2 (Sigma) for 24 hours. For cells undergoing OGD, culture media were replaced with either fresh Neurobasal media (control cells) or glucose-free Neurobasal media (OGD treatment). Cells undergoing OGD were treated with human MIF or vehicle and placed under hypoxic or normoxic conditions for 12 hours. After 12 hours, media were replaced with Neurobasal media with or without MIF and cells were incubated under normoxic conditions for an additional 24 hours. Cell viability after H2O2 or OGD treatment of neurons was assessed using the MTT assay. MTT (Sigma) was added to each well to a final concentration of 0.8mg/mL. Cells were incubated with MTT for 2.5 hours. Formazan crystals were dissolved in DMSO (Sigma) and absorbance was measured at 540nm. Cell viability was calculated by dividing the absorbance of treated or untreated cells by the average absorbance of non-treated (control cells) and expressed as a percentage.

2.6 ISOLATION OF HUMAN MONOCYTES

Human monocytes were isolated from fresh blood of human volunteers using the RosetteSep Human Monocyte Enrichment Cocktail (Stemcell Technologies, Vancouver, BC)
according to the manufacturer’s instructions. Briefly, EDTA was added to whole blood to a final concentration of 1mM. 50μL/mL RosetteSep was added to the EDTA-blood mixture and incubated for 20 minutes. The sample was then diluted with PBS containing 2% FBS and 1mM EDTA. Samples were layered on top of Histopaque (Sigma) and centrifuged for 20 minutes at 1200x g. The monocyte-enriched cell layer was removed and washed twice with PBS containing 2% FBS and 1mM EDTA. Residual red blood cells were lysed and the sample was washed a final time. The final pellet was suspended in RPMI containing 10% human AB serum. Monocytes were stained with trypan blue, and the number of viable cells was counted under a phase contrast microscope.

2.7 ISOLATION AND CULTURE OF HUMAN BRAIN (HBEC) AND HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS (HUVEC)

Isolation of HBEC was performed as previously described (Dorovini-Zis et al. 1991). The study has complied with all institutional policies and was approved by the ethics committees of the University of British Columbia and the Vancouver General Hospital. HBMEC were plated onto fibronectin-coated 96-well plates and cultured in medium 199 (M199, GIBCO) supplemented with 10% horse plasma-derived serum (Cocalico Biologicals, Reamstown, PA) 2mM L-glutamine, 100U/mL penicillin, 100μg/mL streptomycin, 0.25μg/mL amphotericin B (all from Gibco), 20μg/mL endothelial cell growth supplement and 100μg/mL heparin (Sigma) at 37°C in a humidified 5% CO₂/95% air incubator. Culture media was replaced every 2 days and confluence was reached after 8-10 days.

Isolation of HUVEC was performed as previously described (Jaffe et al. 1973). Briefly, umbilical cords from normal pregnancies were washed with PBS and incubated with collagenase (1mg/mL) for 15 minutes at 37°C. Umbilical cords were flushed with M199, and endothelial
cells collected in M199 containing 20% horse serum. HUVEC cultures were maintained as described above for HBEC cultures.

2.8 MONOCYTE ADHESION ASSAY

Human endothelial cells were grown to confluence on fibronectin-coated 96-well plates. TNF-α (100 units/mL, Sigma) was used to stimulate endothelial cells for 24h to upregulate endothelial cell adhesion molecules and served as a positive control. Prior to incubation of endothelial cells with MIF, the monolayers were washed 2 times with medium M199 containing 10% human AB serum (Sigma). Monolayers were incubated with MIF (50ng/mL or 100ng/mL) for 2 hours at 37°C in an incubator containing 5% CO₂ and 21% O₂, during which time human monocytes were isolated (see above). At the end of the incubation period, freshly isolated monocytes were added to each well at a concentration of 5 × 10⁵ cells/mL and incubated for 1hr at 37°C. The monolayers were then washed gently three times in M199 and once in PBS to remove non-adherent monocytes. Monolayers with adherent monocytes were fixed in 4% paraformaldehyde for 20 minutes at room temperature. The monolayers were then washed twice with Tris buffer containing tween, followed by an endogenous peroxidase block for 25 minutes at room temperature using a solution containing 0.75% H₂O₂ in 100% methanol. Monolayers were washed three times with Tris buffer containing tween and incubated for 1 hour at room temperature with the primary antibody, mouse anti-human leukocyte common antigen (LCA/CD45, 1:100, DAKO, Glostrup, Denmark) in order to stain the monocytes. The cultures were then washed and incubated with the secondary antibody, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for 90 minutes at room temperature (1:200, Jackson Immunoresearch, West Grove, PA). Monolayers were then washed in Tris buffer, incubated
with the chromogenic substrate for HRP, 3,3’-diaminobenzidine (DAB), for 20 minutes at 4°C washed with ddH₂O and counterstained with haematoxylin. Monolayers were then washed with ddH₂O, and upon removal of ddH₂O, were allowed to dry. Adherent monocytes were enumerated by counting the number of adherent monocytes per millimeter square (mm²) of the monolayer in five fields per well with a 20X objective lens using an ocular grid under a Nikon labophot light microscope. The average number of adherent monocytes per field per well was calculated and expressed as the number of monocytes per mm² of endothelial monolayer. Controls included HBEC incubated in culture media in the absence of MIF.

2.9 IMMUNOHISTOCHEMISTRY

Tissue from recent ischemic infarcts was obtained from 10 patients at post mortem examination. The study was approved by the ethics committee of the University of British Columbia. Sections were fixed in 10% buffered formalin and embedded in paraffin. Three micron thick sections were stained with the indirect immunoperoxidase technique for the demonstration of MIF. Antigen retrieval was performed by incubating sections in citrate buffer (18mM citric acid, 18mM sodium citrate in ddH₂O, pH 6.0) at 95-100°C for 30 minutes. Sections were then cooled to room temperature and rinsed with Tris-Tween buffer. Endogenous peroxidase was blocked with 1% H₂O₂ in 100% Methanol for 30 minutes followed by three washes in Tris-Tween buffer. Sections were then blocked with 4% normal donkey serum (NDS) in Tris-Tween buffer for 1 hour at room temperature. Following blocking with NDS, sections were incubated with 1° antibody (goat anti-human MIF, 1:100, R&D Systems, MN, USA) overnight at 4°C. At the end of the incubation period, sections were washed three times with Tris-Tween buffer and incubated with 2° antibody (donkey anti-goat, 1:500, Abcam Inc., MA, USA) for 2 hours at room temperature. Sections were washed twice in Tris-Tween buffer
followed by one wash in 0.1M acetate buffer (pH 5.2, 0.05% Tween). Sections were incubated with the chromogen AEC for 4 minutes, washed with ddH2O, and counter stained with haematoxylin for 4 min. Crystal mount (Electron Microscopy Sciences, PA, USA) was added to the sections, which were then allowed to dry overnight. Sections were mounted with Entellan (Electron Microscopy Sciences) diluted in xylene, coverslipped, and allowed to dry overnight. In some of the cases, monocytes within the infarct were immunostained for the monocytic marker CD68 (mouse anti-human IgG, clone KP1, Dako, Denmark, at 1:60 dilution) by the same indirect immunoperoxidase technique. The number of blood vessels immunoreactive for MIF was quantified by counting the number of vessels with positive staining endothelial cells in 5 to 10 fields within the peri-infarct zone under a 20X objective lens. The average number of positive vessels per field per case was calculated and the average of 10 cases was calculated and expressed as the average number of positive vessels per field per case. Negative controls included omission of primary antibodies and sections of age-matched normal brains. Paraffin sections of tonsils or lymph nodes served as positive controls.

2.10 CLONING, EXPRESSION AND PURIFICATION OF HUMAN MIF

The sequence for the human MIF (HMIF) coding sequence was PCR-amplified using the forward primer 5’CCGGAATTCGCCACCATGCGATGTTCATCGT 3’ and either 1 of 2 reverse primers, 5’ TGCTCTAGAGGCGAAGGTGGTTGTTTGGGAGTTTGGT 3’ or 5’ TGCTCTAGATTAGGCGAAGGTGGTTGTTTGGGAGTTTGGT 3’ and cloned into pCDNA4(A) (Invitrogen, Carlsbad CA, USA) at the XbaI and EcoRI sites to generate either pHMIFmychis or pHMIFstop, respectively. Confirmation of positive clones was first assessed by restriction enzyme digest followed by 1% agarose gel analysis. Samples containing appropriate size inserts were sequenced to confirm for correct insertion of target DNA. pHMIFmychis contains the full
coding sequence for MIF with a mychis tag while pHMIFstop does not encode a tagged protein. pEGFP-N3-MIF was used as template (a plasmid bearing the human MIF coding sequence which was a gift from Dr. Lijuan Wang).

To over-express human MIF protein, pHMIFmychis and pHMIFstop were transfected into HEK293 cells at approximately 50-60% confluence using a calcium-phosphate dependent transfection method. 48 hours after transfection, cells were washed in cold 1X PBS and harvested. To obtain mychis-tagged human MIF under conditions that preserve biological activity, Nickel-NTA magnetic agarose beads were used and the procedure for purification of 6X His-tagged proteins under native conditions was followed according to the manufacturer’s protocol (Qiagen, Mississauga, Ontario). After protein purification, the concentration of MIF was assayed using a colorimetric protein quantitation assay (Bio-Rad Laboratories, CA, USA).

2.11 SDS-PAGE GEL ELECTROPHORESIS AND WESTERN BLOTTING

To confirm expression of HMIF in transfected HEK293 cells, 48 hours after transfection, cells were harvested and lysed in RIPA-DOC lysis buffer containing 0.15M NaCl, 0.05M Tris-HCl pH 7.2, 0.1% SDS, 1% sodium deoxycholate, and 1% triton x-100. Lysates were sonicated and cellular debris was pelleted by centrifugation at 14,000xg for 10 minutes. Protein concentration was determined as described above. SDS-PAGE gel electrophoresis was performed using 10% tris-tricine gels. For coomasie-blue detection of total protein following electrophoresis, coomasie blue was incubated with the gel, followed by washes with ddH₂O, and destaining using a solution containing 40% methanol and 7% glacial acetic acid for 1.5 hours at room temperature. Gels were then rinsed with ddH₂O, with a final rinse left overnight. For western blotting, proteins were transferred from the gel onto a PVDF membrane (Immobilon-FL,
Millipore, MA, USA). Membranes were probed with either anti-myc (9E10, 1:10) or rabbit anti-MIF (FL-115, 1:200, Santa Cruz Biotechnology, CA, USA) antibodies in milk containing PBS with 0.1% Tween-20 and 0.01% sodium azide, overnight at 4°C. The membranes were then washed and incubated with goat anti-mouse 800CW or goat anti-rabbit 680CW (LI-COR Biosciences). Membranes were then scanned at 700 and 800nm wavelengths using an Odyssey Infrared Imaging System (LI-COR Biosciences).

2.12 DATA COLLECTION AND STATISTICS

Data were collected in the following ways: using the TD Luminometer software for luciferase assay (Turner Biosystems, Sunnyvale, CA), the Fluoroskan Ascent software for analysis of MTT assays (Thermo Fisher Scientific, Waltham, MA), or by visual inspection for analysis of adhesion assays. Data were analyzed, organized into graphical form, and statistics generated by using GraphPad Prism 4 software (GraphPad Software, La Jolla, CA).
CHAPTER 3: RESULTS

3.1 THE HUMAN MIF PROMOTER IS HYPOXIA INDUCIBLE

A portion of the 5’ untranscribed (UTR) region comprising nucleotides -2634 to +35 of the human MIF promoter was cloned into the promoter-less vector, pGL3 Basic, upstream of the luciferase reporter gene (Fig.1). A series of MIF promoter deletion plasmids were constructed (Fig. 2). pHMIF-Luc, the plasmid containing the longest promoter sequence of the human MIF gene from -2634 to +35, was initially assayed for promoter activity. pHMIF-Luc exhibited significantly higher promoter activity than the promoter-less control plasmid, pGL3 Basic following transfection into HEK293 cells (Fig.3, 14.5 ± 0.8 fold increase compared to control, P<0.0001). Previous studies had shown that hypoxia induced the expression of MIF mRNA and MIF protein release in human glioblastoma cells, and was able to enhance the promoter activity of the mouse MIF promoter (Bacher, Schrader et al. 2003). The human MIF promoter was found to exhibit hypoxia-dependent activity and this was suggested to be based upon a hypoxia response element (HRE) at +27 of the promoter (Baugh, Gantier et al. 2006). To confirm the hypoxia-inducible nature of the human MIF promoter and the identification of the HRE responsible, pHMIF-Luc was initially transfected into HEK293 cells. Transfected cells were then incubated under 2% (hypoxia) or 21% O₂ (normoxia). The luciferase assay revealed a significantly higher activity of the human MIF promoter fragment and positive control, pEpoE-Luc under hypoxic conditions, confirming the results from the previous study (Fig.4, 4.9 ± 0.1 and 7.2± 0.09 fold increases under hypoxia, respectively, relative to pGL3 promoter and compared to normoxia, P<0.0001). To determine whether HIF-1α activation was likely contributing to increased MIF promoter activity during hypoxia, the HIF-1α expression plasmid,
pHIF-1α was overexpressed and its effect on MIF promoter activity under normoxia was evaluated (Fig.5). pHMIF-Luc and pEpoE-Luc promoter activities were significantly upregulated compared to control (4.0 ± 0.04 and 4.9 ± 0.04 fold increases, respectively, relative to pGL3 Basic and compared to control, P<0.0001). To further demonstrate that participation of HIF-1α in the observed effect on MIF promoter activity during hypoxia treatment, siRNA was used to successfully knockdown expression of HIF-1α during hypoxia (Fig.6). Inhibition of HIF-1α accumulation during hypoxia led to significantly blunted promoter activity of pHMIF-Luc and, importantly, pEpoE-Luc (19.5 ± 0.4% of control and 29.6 ± 0.5% of control, respectively P<0.0001). HIF-1α siRNA treatment did not have an effect on the activity of the control promoter, pGL3 promoter (94.6 ± 1.0% of control, P=0.07).

Sequence analysis of the 2.6kb fragment of the human MIF promoter revealed four putative HREs. Since the previous study examined a significantly shorter region (1.2kb) of the human MIF promoter, it remained possible that upstream HREs could also contribute to regulation of the human MIF promoter. To identify which of the four HREs conferred hypoxia responsiveness to pHMIF-Luc, a series of deletion plasmids containing truncated lengths of the 2.6kb MIF promoter fragment were generated (Fig 2, and Fig.7). Deletion of the region upstream of -553 (pHMIF-HIF) resulted in a fragment which still responded strongly to hypoxia (2.9 ± 0.03 fold increase over control, P<0.0001). Further deletion of the 5’ region (pHMIF-HIFB) did not affect the promoter’s response to hypoxia (2.9 ± 0.02 fold increase over control, P<0.0001). This suggested that the 3’ most HRE is likely the sole functional HRE involved in hypoxia-induced increases in the human MIF promoter, at least within the first 2.6kb. A deletion plasmid bearing nucleotides -553 to +8, thereby effectively removing the suspect HRE, whose core sequence begins at +27, was created (pHMIF-HIFC). This HRE-less construct was unable to respond to hypoxia (0.84 ± 0.01 fold over control, P<0.01). Truncation of the 5’ end of pHMIF-HIFC (generating pHMIF-HIFD) resulted in retention of non-responsiveness to hypoxia
(0.69 ± 0.02 fold over control, P<0.001), confirming that the HRE located at +27 of the human MIF promoter was indeed a functional HRE as previously suggested. Since the effects of hypoxia are broad, a slight possibility still remained that the region observed to behave in an HRE-like manner did not contain a true HIF-1α binding element.

To demonstrate that the region between +8 and +35 contained a HIF-1α binding element containing a core binding sequence (5’ACGT) beginning at +27 of the human MIF promoter, an electric mobility shift assay (EMSA) was performed (Fig.8). The free, labelled HIF-1 probe is shown in lane 1. Upon incubation of HIF-1 probe with whole cell lysate obtained from hypoxia-treated HEK293 cells, a notable shifted band was observed, indicating a protein-DNA complex had formed by the specific interaction between proteins binding to the consensus labelled HIF-1α binding sequence (lane 2). Addition of an excess of unlabelled, HIF-1α wildtype competitor oligos to the binding reaction resulted in a disappearance of the shifted band, indicating that the shifted band was specifically representing binding of protein to the HIF-1α consensus sequence (lanes 3 and 4). To further confirm specific binding of protein to the HIF-1α recognition sequence, an excess of unlabelled, HIF-1α mutant competitor oligos were added instead of wildtype oligos (lanes 5 and 6). The shifted band, as seen in lane 2, re-appeared, indicating that specific recognition of the HIF-1α consensus sequence was necessary for a protein-DNA complex to occur. The ability of the +27 HRE (HIF-1(4)) to compete for occupation of the HIF-1α binding site with the HIF-1α probe was assessed (lanes 9 and 10). Like the wildtype HIF-1α competitor oligos, HIF-1(4) successfully served to compete for binding of protein to the HIF-1α probe, confirming that HIF-1(4) is indeed a HIF-1α binding element. Notably, the software-predicted site at -1002 (HIF-1(2)), within a region not necessary for hypoxia-induced activity of the human MIF promoter, was unable to successfully compete away binding of the HIF-1α probe, resulting in protein-DNA complex formation (lanes 7 and 8).
3.2 MIF PROTECTS NEURONS AGAINST OXIDATIVE STRESS AND OXYGEN-GLUCOSE DEPRIVATION

MIF has been found to play a protective role in ischemia-reperfusion injury in the intestine and heart, while the function of MIF in cerebral ischemia has not been studied. To understand the role of MIF under ischemia-like conditions, we tested the hypothesis that MIF plays a protective role in cerebral ischemia similar to its reported role in intestinal and cardiac ischemia.

In order to be able to assess the effect of MIF on the survival of cultured neurons in response to oxidative stress and OGD, it was necessary to produce MIF using a method which allows for reasonable expression levels of MIF, while, ideally, retaining both the native state of the protein necessary for its biological activity, and expressing the protein in the most naturally relevant system available. To achieve as many of these desired properties as possible, human MIF cDNA was cloned into the pcDNA4.mycHis plasmid in which a myc-his tag was fused with MIF at its C-terminus, and the MIF protein was expressed in HEK293 cells (Fig.9A, B). Retrieval and purification of ectopic expression of MIF in HEK293 cells was performed using Nickel-NTA agarose magnetic beads (Fig.9C). This approach yielded relatively concentrated samples of a protein represented by a single band corresponding to the expected size of HMIFmychis following SDS-PAGE gel electrophoresis (MIF P, 7.5ug compared to the equivalent amount of unpurified protein sample, MIF NP, 7.5ug). Western blotting using an antibody to the mychis tag (9E10) confirmed that the purified protein was indeed HMIFmychis (Fig.9D).

Oxidative stress contributes to cell death induced by ischemia, particularly following reperfusion (Niizuma, Endo et al. 2009). To determine the effect of MIF on oxidative stress-induced death of neurons, primary cultures of cortical neurons isolated from rat embryos were
treated with hydrogen peroxide (Fig.10). Hydrogen peroxide treatment of cortical neurons resulted in a significant decrease in neuronal viability, as assessed by the MTT assay (46.8 ± 3.0% cell viability versus control, 100 ± 3.7%, P<0.0001). Interestingly, when cortical neurons exposed to hydrogen peroxide were incubated with MIF, cell death was significantly prevented (93.9 ± 7.7% cell viability versus vehicle, 46.8 ± 3.0%, P<0.0001).

To assess the effect of MIF on neuronal survival under ischemia-like conditions, cultured cortical neurons were subjected to the in vitro model of OGD to mimic ischemia (Fig.11). Although hypoxia rather than complete oxygen deprivation (anoxia) was employed, the effects are comparable, requiring only slightly longer incubation times (Arumugam, Tang et al. 2007). Subjecting cultured cortical neurons to OGD for 12h resulted in significant cell death (52.1 ± 2.8% of cell viability versus control, 100 ± 2.8%, P<0.0001). Treatment of cortical neurons with MIF during OGD and for 12h during “in vitro reperfusion” significantly protected neurons against OGD-induced death (70.1 ± 2.6% cell viability versus vehicle, 52.1 ± 2.8%, P<0.001).

Taken together, these results provide evidence for a protective role of MIF on neurons under in vitro ischemia-reperfusion-like conditions, which complements MIF’s previously described role as a protective agent during ischemia-reperfusion in the heart and intestine.

3.3 MIF EXPRESSION IS INCREASED IN THE HUMAN BRAIN FOLLOWING STROKE

The regional and cellular expression of MIF in the human brain has not been studied to any extent by which definite conclusions can be drawn regarding the expression profile of MIF in human brain. However, it has been shown that MIF is expressed at low levels in only a few dispersed cells in the frontal cortex (Bacher, Schrader et al. 2003). In order to determine whether the increase in MIF expression following focal ischemia in the rat is a phenomenon
which also occurs in and is relevant to stroke in humans, the expression of MIF in human brains following stroke was examined using immunohistochemistry. Expression of MIF was not detected in the normal brain (Fig.12A). In contrast, MIF expression was readily observed in sections taken from acute cerebral infarcts. MIF was primarily localized in the endothelium of brain microvessels in the peri-infarct region (penumbra) and in the subarachnoid space overlying the infarcted area (Fig.12B). Endothelial cells showed intense cytoplasmic staining for MIF (Figs.) often in association with a reactive phenotype indicated by enlarged, vesicular nuclei. Quantification of the average number of positively-stained vessels per 10 fields per stroke case within the peri-infarct area versus normal brains is shown in Fig.12C. MIF was also strongly expressed by foamy macrophages that infiltrated the necrotic tissue (Fig.13). These results indicate that MIF is not normally expressed in normal human brain, in agreement with previous observations. In addition, these findings indicate that MIF is expressed de novo by cerebral endothelial cells and blood-born infiltrating monocytes following focal brain ischemia.

3.4 MIF INHIBITS MONOCYTE ADHESION TO ENDOTHELIAL CELLS

Previous studies have demonstrated that infiltration of the infarcted brain tissue by blood-born monocytes occurs following stroke. After successful migration across the blood-brain barrier and invasion of the necrotic tissue, monocytes become phagocytic and while they facilitate removal of the debris of necrotic neurons, glial cells and myelin, they may also exacerbate damage in the peri-infarct area by phagocytosing vulnerable, albeit salvageable neurons. Thus, monocyte infiltration into the brain following stroke is an event, manipulation of which may offer therapeutic potential. In light of the novel finding that MIF is strongly expressed in endothelial cells following stroke in humans, it was necessary to investigate the functional significance of this observation. Considering the large and historical role of MIF as a
mediator of macrophage migration, and recent mechanistic insights into its effects on monocyte adhesion to aortic endothelial cells (Bernhagen, Krohn et al. 2007), the effect of MIF on monocyte adhesion to human large vessel endothelial cells and human brain endothelial cells was investigated. To this end, human MIF was expressed, purified and incubated with either human brain (HBEC) or human umbilical vein endothelial cells (HUVEC, Fig.14). Primary human monocyte adhesion to endothelial cells was quantified in the presence and absence of MIF (Fig.15). Representative results of at least 3 independent experiments are shown. It should be noted that the experiments were performed using at least two sets of primary endothelial cell cultures and primary monocytes from different subjects, indicating that the effect observed was probably not subject-specific. TNF-α treatment served as a positive control. Addition of MIF to HUVEC at a concentration of 50ng/ml or 100ng/ml prior to, and during monocyte incubation, resulted in a significant decrease in monocyte adhesion (210 ± 25 cells/mm², P<0.0001 and 191 ± 43 cells/mm², P=0.001, respectively, compared to control, 586 ± 17 cells/mm²). A similar trend was observed using HBEC, although significance was approached, but not achieved (320 ± 12 cells/mm² compared to control, 400 ± 40 cells/mm², P=0.125). These results suggest that MIF inhibits monocyte adhesion to human endothelial cells in vitro. Furthermore, these results suggest that stroke-induced MIF expression by endothelial cells inhibits monocyte adhesion to the endothelium, thereby providing evidence for a novel mechanism regulating monocyte trafficking to the brain following stroke.
CHAPTER 4: DISCUSSION

4.1  INDUCTION OF THE HUMAN MIF PROMOTER BY HYPOXIA

The expression of the human MIF gene is controlled by a TATA-less promoter. TATA-less promoters lack characteristic and unifying recognition elements and are likely very highly and individually regulated by a unique assortment of transcription factors. Basal transcription of the human MIF promoter region has been shown to be dependent on the transcription factors Sp1 and CREB (Roger et al. 2007). Regulation of basal MIF transcription by the ubiquitous transcription factors Sp1 and CREB may underlie the constitutive nature of MIF expression in a variety of cell types. While the search for and demonstration of additional transcription factors which regulate the activity of the human MIF promoter was not undertaken for quite some time, a recent report suggested that the human MIF promoter was hypoxia inducible (Baugh et al. 2006). The study also suggested that the site conferring hypoxia-responsiveness to the MIF promoter lied at +25. The results presented in this thesis complement these observations and provide definitive evidence for a HIF-1α binding site with a core consensus sequence beginning at +27. Furthermore, the experiments reported herein suggest that the sequence of nucleotides from +21 to +39 is sufficient for the binding of HIF-1α. This was supported by a recent report that the presence of a HIF-1α binding site with a core consensus sequence beginning at +25 (Elsby et al. 2009).

Understanding the factors that regulate MIF expression is important, especially considering that MIF has been reported to be involved in many disease processes including endotoxemia, rheumatoid arthritis, cancer, and atherosclerosis. In addition, MIF has been shown to be associated with neuritic plaques in Alzheimer’s disease, an event which could potentially
be brought upon by HIF-1α regulated MIF expression through hypoxia as a result of vascular events, such as stroke (Sun et al. 2006, Bacher et al. 2010). Equally important is to understand the factors that stimulate the secretion of MIF. One of the first mechanisms to be identified was the LPS-induced release of MIF from cells of the anterior pituitary gland (Bernhagen et al. 1993). Glucocorticoids such as dexamethasone can also stimulate MIF secretion. Glucose was found to stimulate secretion of MIF from insulin-producing islet cells of the pancreas while the proinflammatory cytokines TNF-α and IFN-γ contribute to MIF release from murine macrophages (Waeber et al. 1997, Calandra et al. 1994). In addition to the findings that activation of the human MIF promoter is regulated by hypoxia through HIF-1α, MIF has been found to be significantly upregulated at the transcriptional level (mRNA) in HUVEC within 16h of hypoxia, in a human lung fibroblast and HeLa cell line within 24h of exposure to hypoxia and in a glioblastoma cell line beginning after 12h of exposure to hypoxia (Bacher et al. 2003, Flamant et al. 2009, Baugh et al. 2006). The results from these studies also indicate that hypoxia serves as a mediator of MIF secretion. Hypoxia leads to MIF secretion, detectable by 12h in the culture media of a human glioblastoma cell line, which suggests that hypoxia contributes to both transcriptional upregulation and secretion of MIF. The timing of MIF upregulation and release has not been, in any way, established by the aforementioned studies. However, if these studies are any indication, they suggest that MIF is released first upon exposure to hypoxia with a concomittant upregulation of intracellular MIF through HIF-1α, perhaps to restore intracellular pools and if hypoxia persists, facilitate a cycle of release and replenishment. However, the mechanism by which hypoxia elicits MIF secretion requires further investigation. In a recent report, it was shown that MIF mRNA expression by peripheral blood mononuclear cells (monocytes, t cells and b cells) is significantly upregulated following stroke in human patients. In addition, stroke patients exhibited increased levels of MIF protein in the plasma. Both intra- and extracellular MIF levels were positively associated with increasing severity of stroke.
Moreover, MIF expression was maximal within 3 days following stroke, and returned to baseline levels by 7 days following stroke (Wang et al. 2009). While the role of circulating inflammatory cells expressing MIF following stroke needs to be examined, these results indicate that MIF is intrinsically linked to the inflammatory response following stroke. In addition, these results further suggest a strong link between intracellular MIF upregulation and secretion.

4.2 MIF IS NEUROPROTECTIVE AGAINST OXIDATIVE STRESS AND OXYGEN-GLUCOSE DEPRIVATION

Oxygen radicals produced following cerebral ischemia impart oxidative stress in the penumbra and may be one of the insults which lead to neuronal apoptosis following ischemia (Niizuma et al. 2009). Specifically, H₂O₂ levels have been shown to be increased in the striatum of rats undergoing global ischemia (Hyslop et al. 1995). Since the levels of the superoxide anion, O₂⁻, are increased following ischemia/stroke, elevated H₂O₂ levels may be a reflection of the conversion of O₂⁻ to H₂O₂ via superoxide dismutase (SOD). Oxidative stress, induced by reactive oxygen species (ROS) such as H₂O₂, may lead to cell death through initiation of p53-dependent apoptosis. The results presented in this thesis provide evidence that MIF acts as a protective agent against oxidative stress-induced neuronal apoptosis. There is evidence to suggest that MIF is secreted in response to H₂O₂ in cardiomyocytes (Takahashi et al. 2001, Fukuzawa et al. 2002). Since the presence of endogenous MIF was not determined in the rat neuronal cultures, it is not possible to know the extent to which endogenous MIF played a role in the response of neurons to oxidative stress. However, other investigators have demonstrated that MIF is expressed in cultured neurons from newborn rats, and MIF is upregulated at the mRNA and protein level upon exposure to H₂O₂ (Harrison & Sumners 2009). Unfortunately, the study failed to examine whether H₂O₂ also elicited MIF secretion. Further experiments are needed to
determine whether the observed protection of exogenously applied MIF serves to supplement an existing MIF-mediated protective mechanism, by examining whether blocking or inhibiting endogenous MIF during oxidative stress results in decreased cell survival. Although it has been reported that MIF mRNA is expressed in human brain, there are no data demonstrating its cellular localization (Paralkar & Wistow 1994). Therefore it is unknown whether MIF is expressed in neurons or glial cells in the human brain. Thus, it would be important to determine the type of cells in the brain that express MIF and investigate whether H2O2-induced upregulation of MIF is a property shared by these cells. The protective nature of MIF on cultured neurons undergoing oxidative stress reported here is in agreement with the previously published role of MIF in inhibiting thiol starvation- and diamide-induced apoptosis in a HeLa cell and HL-60 promyeloblast cell line (Nguyen et al. 2003).

Given the possibility that MIF may have been expressed endogenously in the neuronal cultures used in the experiments presented here, there are two putative routes of MIF action to consider: one concerned with intracellular MIF and another one mediated by the binding of MIF to a receptor. However, since MIF release and secretion are seemingly interconnected events, the effects of intracellular and secreted MIF on cell viability may parallel and complement each other in situations of oxidative stress or ischemia. Intracellular interactions of MIF will be discussed first.

There are several studies that highlight the involvement of MIF in the regulation of p53-mediated apoptosis. After exogenously applied MIF was initially found to inhibit p53-mediated apoptosis in fibroblasts and macrophages (Hudson et al. 1999), subsequent studies have begun to unravel the underlying mechanisms. MIF suppression of p53 activity and apoptosis may depend on the cyclooxygenase cox-2 (Mitchell et al. 2002). Interestingly, MIF was shown to directly bind p53, which depended primarily on the DNA binding domain of p53 and cysteine 81 of MIF, leading to inhibition of p53-dependent transcription and apoptosis (Jung et al. 2008a). MIF itself
interacts with and appears to be regulated by the tumor suppressor NM23-H1. NM23-H1 negatively regulates MIF, and, by doing so, promotes p53-dependent apoptosis (Jung et al. 2008b).

MIF may also inhibit apoptosis by interacting with factors that are activated downstream of p53 transcriptional activation. The protein Bim is one such factor, which plays a major role in apoptosis (Putcha et al. 2001). The JNK signalling pathway has been shown to be required for Bim activation leading to Bax-mediated apoptosis in mouse granule neurons by JNK phosphorylation of the Ser65 residue of Bim (Harris & Johnson 2001, Lei & Davis 2003, Putcha et al. 2003). MIF has been shown to interact with the proapoptotic Bim protein, and in doing so, partially inhibits Bax-mediated cytochrome c release from mitochondria resulting in a reduction of Bim-mediated and diamide-induced apoptosis (Liu et al. 2008).

While these studies have examined the intracellular components which may be involved in ROS-generated and MIF-mediated p53 inhibition, the extracellular receptors or components by which MIF induces inhibition of p53-mediated apoptosis have not thus far been identified. Thus, it will be necessary to determine the extracellular and/or intracellular pathways underlying the protection of cultured neurons undergoing oxidative stress by MIF.

The results presented in this thesis suggest that MIF may protect neurons during cerebral ischemia since it was demonstrated that MIF protects cultured neurons undergoing OGD-induced cell death. Previous reports have demonstrated the protective nature of MIF in both intestinal and myocardial infarction (Amaral et al. 2007, Miller et al. 2008, Qi et al. 2009). Insight into the mechanisms underlying the role of MIF in I/R injury prevention has recently been established. Exogenous application of MIF induces AMPK phosphorylation in cardiomyocytes, resulting in increased glucose uptake via glucose transporter-4 (GLUT4) translocation to the cell surface. The CD74-CD44 receptor complex was found to be necessary for MIF-mediated AMPK phosphorylation. While AMPK activation in cerebral ischemia has
been shown to be harmful rather than beneficial to the survival of neurons (McCullough et al. 2005, Li et al. 2007), the contribution of GLUT4 in glucose uptake in the brain has, thus far, only been shown to function in the granule cell layer of the cerebellum (Bakirtzi et al. 2009).

Since GLUT1 and GLUT3 are the major glucose transporters in the brain (reviewed in Simpson et al. 2007), AMPK activation in cerebral ischemia must recruit signalling pathways leading to responses other than the MIF-AMPK-GLUT4 protective pathway reported in myocardial infarction.

The recognition of MIF by CD74 may also play an integral part in the JNK signalling pathway and JNK-mediated apoptosis. JNK activation plays a role in apoptosis during cerebral hypoxia-ischemia (Kuan et al. 2003). Knockout of JNK3 in mice leads to decreased brain damage following cerebral hypoxia-ischemia, which seems to be a result of decreased Bim activation. Furthermore, comparison of cell death in primary hippocampal cultures from wildtype and JNK3-knockout mice exposed to OGD showed that JNK activity was associated with increased cell death. In addition to protecting mouse hearts from I/R injury through extracellular signalling via CD74, MIF has recently been shown to protect mouse hearts from I/R injury by inhibiting the JNK signalling pathway (Qi et al. 2009). In this study, the binding of MIF to CD74 during I/R was found to negatively regulate MKK4 dependent phosphorylation and activation of JNK. Knockout of MIF resulted in increased MKK4 and JNK activation, leading to more severe tissue injury.

Other than the CD74-CD44 signalling complex, the only other cell surface MIF receptors that have been identified are the chemokine receptors CXCR2 and CXCR4 (Bernhagen et al. 2007). Interestingly, CXCR4 is upregulated by hypoxia and H₂O₂ (Schioppa et al. 2003, Staller et al. 2003, Saccani et al. 2000). In addition, CXCR4 is upregulated within the infarct and penumbra following focal ischemia in the rat (Schonemeier et al. 2008). The chemokine SDF-1α (CXCL12) also binds to CXCR4, and protects cells against apoptosis during myocardial
infarction and hypoxia-induced cell death (Hu et al. 2007, Zhang et al. 2007a). Whether or not MIF can exert a similar effect during cerebral ischemia is worth investigating. However, any hypothesis concerning the potential role of a MIF-CXCR4 axis participating in neuronal survival is complicated by the fact that there are numerous and conflicting reports regarding the role of CXCR4 in regulating both survival and death of neurons (reviewed in (Tran & Miller 2003).

4.3 MIF IS EXPRESSED IN ENDOTHELIAL CELLS FOLLOWING STROKE AND INHIBITS MONOCYTE ADHESION TO HUMAN ENDOTHELIAL CELLS

One of the first described properties of MIF was its ability to inhibit the migration of macrophages in vitro. Since then, there have been many functions ascribed to MIF, as discussed in section 1.4.3. A handful of studies have examined the effects of MIF on adhesion of leukocytes to endothelial cells. Early studies demonstrated that MIF promotes leukocyte adhesion and infiltration into aortic and kidney tissues during inflammation, an effect suggested to be a result of MIF-induced endothelial expression of cell adhesion molecules such as ICAM-1 and VCAM-1 (Lan et al. 1997, Lin et al. 2000). MIF was found to promote the adhesion of monocytes to human aortic endothelial cells (HAOEC) when endothelial cell cultures had been pretreated with MIF (Schober et al. 2004). The mechanisms underlying MIF-mediated adhesion of monocytes to HAOECs were recently described. Monocyte adhesion to HAOECs requires functional CXCR2 receptors on monocytes and MIF-induced ICAM-1 expression on endothelial cells. Interestingly, MIF treatment of HAOECs induces MIF expression on the surface of endothelial cells, where it interacts with CXCR2 on the surface of monocytes to promote adhesion (Bernhagen et al. 2007). The CCR2 receptor has also been implicated in MIF-mediated monocyte adhesion to the endothelium. Treatment of lung microvascular endothelial cells with MIF induces expression of the ligand for CCR2, monocyte chemoattractant protein MCP-
1(CCL2). Knockout of CCL2 reduces MIF-mediated monocyte adhesion in vivo (Gregory *et al.* 2006).

The results presented here demonstrate that MIF treatment of HUVEC and to some degree HBEC, results in slightly decreased monocyte adhesion, which is in stark contradiction to the aforementioned studies. There are a couple of possibilities which may have contributed to the observation that MIF decreases rather than increases monocyte adhesion to HUVEC and HBEC. First, the studies described above examined monocyte adhesion to either HAOECs or lung endothelial cells. Thus, there is a significant difference with respect to the cells used for the adhesion assays between this thesis and previously published studies. Second, the migration of monocytes beneath the endothelial monolayer may have contributed to the decrease of the number of monocytes still adherent to endothelial cells following a one hour adhesion assay. In fact, the immunoperoxidase staining of the monocytes revealed cells with different morphology. In addition to the monocytes which appeared flattened with a fairly uniform shape, some monocytes appeared much more shrunken. This may reflect monocytes which have begun to migrate beneath the monolayer, leaving only a portion of their cell body visibly stained. These smaller-looking monocytes were not counted and thus are not represented in the adhesion assay counts, which may underlie the non-canonical results. Furthermore, the apparent increase in the adhesion of monocytes to HBEC may be a result of reduced trans-endothelial migration in HBEC culture, since HBEC have tighter intercellular junctions than HUVEC. To be sure, further experiments are needed where migration of monocytes is quantitated using a double chamber chemotaxis system which allows monocytes to migrate across the endothelial cell monolayers and quantified.

The studies presented in this thesis are the first to document the presence of MIF in the human brain following stroke and to specifically identify the cellular expression of MIF in acute cerebral infarcts. Endothelial cells in the peri-infarct area showed strong MIF expression, while
there was a complete absence of MIF immunoreactivity in control tissue. Since HIF-1α is increased in the penumbra following focal ischemia (Bergeron et al. 1999) and MIF transcription is regulated by HIF-1α, it is possible that the MIF-expressing microvascular endothelial cells are the cells that upregulate MIF in response to hypoxia in the penumbra. While the expression of MIF by HBEC has not been studied until now, the expression, upregulation, and secretion of MIF by HUVEC following LPS stimulation has been previously demonstrated (Nishihira et al. 1998). Hypoxia regulates transcription of a variety of genes in HUVEC, including MIF (Flamant et al. 2009). This may likely be the result of HIF-1α activity, since HIF-1α is expressed in HUVEC (Li et al. 2000). Therefore, it is quite possible that the observed expression of MIF by HBEC following stroke in humans is the result of hypoxia-induced HIF-1α activation and upregulation of MIF. Of course, it will be necessary to demonstrate whether: 1) HIF-1α is upregulated upon exposure of HBEC to hypoxia and 2) MIF is expressed at the endothelial cell surface where it modulates monocyte adhesion/migration by binding to its receptors, CCR2 or CXCR2, on monocytes. MIF is secreted in response to hypoxia by cells other than endothelial cells (discussed in section 4.1). In addition, MIF treatment of HAOECs results in MIF presentation at the cell surface. Thus, MIF may be secreted in response to hypoxia during ischemia, followed by autocrine/paracrine stimulation of MIF presentation at the cell surface in neighbouring endothelial cells. In fact, MIF was found to be released into the circulation during cardiac I/R (Miller et al. 2008).

The consequences of MIF expression/upregulation by endothelial cells following stroke require further investigation. Since MIF has been reported to enhance the adhesion of monocytes to extracerebral endothelial cells, it may affect inflammatory cell infiltration following cerebral ischemia. In view of recent published evidence that supports a role for MIF as a promoter of adhesion and migration, the effects of MIF on monocyte adhesion to HBEC presented in this thesis will have to be further investigated and supplemented with in vitro
transendothelial migration studies, so that a stronger conclusion can be drawn regarding the role of MIF in regulating inflammatory cell adhesion/migration to HBEC. Post-ischemic inflammation plays a critical role in regulating neuronal survival (see section 1.1.7). During ischemia, MIF may modulate post-ischemic inflammation by regulating infiltration of the area of damage by T cells and monocytes, since it has been shown that MIF regulates adhesion of these cells to HAOECs through binding to the CXCR4 and CXCR2 receptors, respectively. Therefore, MIF may serve as a novel target for modulating post-ischemic inflammation. Post-ischemic inflammation occurs in the penumbra, in addition to the infarct core. While neurons of the infarct core become necrotic rapidly, neurons of the penumbra undergo a delayed cell death and are most likely to be salvaged by therapeutic intervention. Considering also that post-ischemic inflammation occurs for weeks following infarction, modulating inflammatory processes provides an opportunity to target neurons undergoing delayed death, therefore offering a longer therapeutic time window than current conventional treatment. Considering MIF as a putative target for modulating post-ischemic inflammation, it would be worthwhile to investigate the effect of blocking MIF in vivo following focal ischemia in an animal model.
CHAPTER 5: CONCLUSIONS

5.1 SUMMARY AND SIGNIFICANCE

Stroke is a leading cause of death in North America. The irreversible and rapid necrosis of neurons in the ischemic core severely limits any means of therapeutic intervention to a strict time window. However, neurons of the peri-infarct region (penumbra), where neuronal death is a more protracted affair involving apoptosis, may be amenable to treatment.

Current evidence suggests that MIF is a key regulator of cell death due to I/R injury in the heart and that MIF is upregulated in the brain of rats and in blood mononuclear cells of human stroke patients. The first aim of the study was to examine the mechanisms underlying increased expression of MIF during cerebral ischemia. Tissue hypoxia is a direct result of ischemia. A previous report from our laboratory identified a functional association between HIF-1α activation during hypoxia, β-secretase 1 transcriptional upregulation and Alzheimer’s disease pathogenesis. In light of this novel observation and based on previous studies demonstrating that MIF is hypoxia inducible, the hypoxia-inducibility of the human MIF promoter as a result of direct interaction with HIF-1α was examined. The studies presented herein are the first to clearly identify a HIF-1α responsive element in the human MIF promoter, which interacts with HIF-1α during hypoxia. These results demonstrated HIF-1α driven MIF upregulation following stroke. The second aim of this thesis was to examine the effect of MIF on neurons undergoing ischemic challenges in vitro. To this end, cultured neurons were subjected to OGD, an in vitro model of ischemia, which induces apoptosis, and H₂O₂, an oxygen radical, which imparts oxidative stress and induces apoptosis. The results presented here
strongly suggest that MIF protects neurons against apoptosis, which is consistent with in vivo and in vitro data from previous reports using non-neuronal cell lines.

Since MIF has been previously shown to be expressed in the rat brain following focal ischemia, the third aim of this study was to determine whether such an event also occurs in the human brain. Furthermore, by examining the expression of MIF in human cerebral ischemia, insight would be gained into the largely unknown expression profile of MIF in normal and ischemic brain. The results reported here indicate that MIF is expressed in endothelial cells following stroke in human subjects, whereas MIF is absent in endothelial cells of normal brain. In addition, infiltrating CD68+ macrophages/microglial cells in the core of human infarcts were found to express MIF. These novel results suggest that MIF is upregulated in human brain endothelial cells following ischemia, and implicate a role for hypoxia-driven MIF expression in these cells following stroke.

The death of neurons following cerebral ischemia invokes a major inflammatory response. Both brain-resident microglia and blood-borne macrophages phagocytose dead cellular debris left behind from necrotic neurons in the infarct core. In addition, neutrophils and blood mononuclear cells infiltrate the brain following ischemia. While a definitive conclusion cannot be made as to whether infiltration of inflammatory cells following stroke is beneficial or detrimental, it is apparent that modulating infiltration plays an important role in the salvation of the penumbra. The fourth aim of this thesis was to study whether MIF affects adhesion of monocytes, a type of blood mononuclear cell, to human umbilical vein and, more importantly, brain endothelial cells. While previous reports have shown that MIF promotes adhesion of cells to the endothelium, the results presented here show that MIF inhibits adhesion of monocytes. Considering the substantial amount of evidence supporting a role for MIF in promoting adhesion, it is quite possible that the results here are reflective of monocyte transmigration during the adhesion assay, leading to a reduction in observed adherent monocytes.
Together, these results: 1) suggest that HIF-1α activation as a consequence of ischemia-induced tissue hypoxia may underlie MIF upregulation at the transcriptional and 2) support a dual role for MIF in cerebral ischemia, including neuroprotection from apoptosis and regulation of inflammatory cell recruitment to the infarct.

5.2 FUTURE DIRECTIONS

While this thesis provides novel findings, which suggest a role for MIF during cerebral ischemia, it has merely done just that. More importantly, the results of this thesis provoke several interesting questions, which should be addressed if the consequences of MIF upregulation following ischemia are to be understood and, ultimately, serve as a platform for treating stroke. First, while the results suggest that MIF may protect rat neurons from apoptosis in vitro, it will be necessary to determine the underlying mechanisms proffering such an effect. Since neurons do not express Class II MHC molecules, it is unlikely that they would express CD74, a receptor that has been implicated in mediating the protective effect of MIF during I/R in the heart. A likely candidate is the CXCR4 chemokine receptor, which has been shown to be expressed in rat embryonic cortical primary neurons and is upregulated in response to both hypoxia and H2O2 treatment. Following identification of a MIF-receptor complex, the downstream signalling mechanisms involved in inhibiting apoptosis should be examined. It will also be necessary to examine the contribution of intracellular MIF to the protection of neurons from oxidative stress and OGD in vitro.

While MIF was demonstrated to exhibit neuroprotective effects in vitro, it would be ideal to demonstrate a similar effect in vivo. In the future, our lab will be investigating the effect of MIF on stroke pathology by inducing focal ischemia in MIF knockout mice and comparing stroke severity to wildtype mice. If the results presented here are any indication, it is expected
that MIF knockout mice will display more severe infarct volumes than wildtype mice. As mentioned previously, MIF has been shown to have an effect on adhesion of inflammatory cells to endothelial cells. The work presented in this thesis shows that MIF is expressed by endothelial cells and macrophages/microglia in the infarct core. Since the function of MIF in these cells is unknown, the effect of MIF knockout on these cell types will also have to be established. Thus, any in vivo work involving mice will have to consider and determine the contribution of at least three actions of MIF in the context of ischemia: a neuroprotective effect, an effect on regulating inflammatory cell infiltration, and an effect on infarct-associated microglia/macrophages.

Since the main objective of examining the role of MIF in stroke is to understand whether MIF may have any therapeutic potential, it will eventually be necessary to modulate MIF. Whether enhanced MIF expression in brain, via exogenous delivery, can enhance neuronal survival in the penumbra will need to be investigated. In addition, the effect of modulating MIF, either by inhibiting or enhancing its expression in endothelial cells as a means of controlling post-ischemic inflammation, should also be examined.
Fig. 1. The 2.6kb Human MIF Promoter Sequence. PCR was used to amplify the region from -2634 to +35bp of the human MIF promoter and the resulting DNA fragment was cloned into the promoterless vector, pGL3 Basic. The transcription initiation site is designated as +1. Underlined sequences are putative HIF-1α binding sites, as predicted by software analysis. The red box denotes the fourth putative HIF-1α binding site (designated HIF-1(4)), which was not retrieved by software analysis.
Fig.2. Restriction Enzyme Digestion Analysis of Cloned MIF Promoter Constructs. Positive clones were digested with the appropriate pair of restriction enzymes so that incorporation of the cloned sequences of the human MIF promoter into pGL3 Basic could be confirmed by gel electrophoresis prior to sequencing of the DNA.
Fig.3. Promoter Activity of pHMIF-Luc. HEK293 cells were transfected with either pGL3 Basic (negative control), pGL3 promoter (positive control), pHMIF-Luc, or pEpoE-Luc. pHMIF-Luc contains -2634 to +35bp of the human MIF promoter sequence. pEpoE-Luc contains a functional HIF-1 responsive region of the human erythropoietin gene promoter. A luciferase assay was used to measure promoter activity of the constructs 48h after transfection. *P<0.0001 by student’s t-test, n=4 per treatment.
Fig.4. The Activity of the Human MIF Promoter is Increased During Hypoxia. HEK293 cells were transfected with either pGL3 promoter, pHMIF-Luc, or pEpoE-Luc. Cells were then incubated under either normoxia (21% O₂) or hypoxia (2% O₂) for 48h. After 48h, transfected cells were harvested and a luciferase assay was performed to reflect the promoter activity of the transfected cells. *P<0.0001, relative to pGL3 Promoter, by student’s t-test, n=4 per treatment.
Fig. 5. The Activity of the Human MIF Promoter is Increased in Response to HIF-1α.
HEK293 cells were transfected with either pGL3 promoter (n=4), pHMIF-Luc (n=4) or pEpoE-Luc (n=3) in conjunction with either pHIF-1α or empty vector. pEpoE-Luc served as positive control. Cells were incubated under normoxia for 48h. A luciferase assay was performed to assess promoter activity. *P<0.0001 by student’s t-test.
Fig.6. Inhibition of HIF-1α Reduces the Response of the Human MIF Promoter to Hypoxia. 

A) HEK293 cells were transfected with pHIF-1α and treated with either HIF-1α specific or control siRNA for 44h. A western blot was then performed to detect HIF-1α protein levels. B) HEK293 cells were transfected with either pGL3 Basic, pHMIF-Luc or pEpoE-Luc and either HIF-1α or control siRNA. Cells were then incubated under hypoxia for 44h. Cells were then harvested and a luciferase assay was performed to reflect promoter activity. *, P<0.0001 by student’s t-test, n=4 per treatment.
Fig. 7. Deletion of Putative HREs in the Human MIF Promoter Inhibits the Response to Hypoxia. A) Diagram showing the deletion constructs made and their start and end positions with respect to the human MIF promoter. B) HEK293 cells were transfected with either pHMIF-Luc or one of a series of deletion constructs. Cells were then exposed to hypoxia for 48h. Cells were then harvested and a luciferase assay performed to represent promoter activity. *P<0.0001, #P<0.001, ^P<0.01, by student’s t-test, n=4 per treatment.
Fig.8. Identification of an HRE Within the Human MIF Promoter. An EMSA was performed to determine the interaction of short oligonucleotide sequences of the human MIF promoter with HIF-1α. Whole cell extract of protein was obtained from HEK293 cells treated with CoCl₂ to induce accumulation of HIF-1α. The free probe was loaded in lane 1 (left). Addition of protein from a whole cell preparation resulted in a shifted band, representing a DNA-protein complex (lane 2). Various competing, unlabelled oligonucleotides were added (lanes 3 through 10). Addition of a molar excess of the wildtype oligonucleotide (Wt-HIF-1) competed for binding, resulting in the failure of the probe to form a DNA-protein complex. Addition of an oligonucleotide bearing a mutated HIF-1α binding sequence failed to compete. HIF-1(2) and HIF-1(4) represent the putative HIF-1 binding sites in the human MIF promoter (Fig. 1).
HEK293 cells were transiently transfected with the plasmid pHMIFmychis, which contains the human MIF cDNA sequence cloned into the mammalian protein expression vector, pCDNA4(A). Following 48h transfection, a western blot was performed to detect the expression of transfected MIF in the cell lysate with (pHMIFmychis) or without a myc-his tag (pHMIFstop) by blotting with anti-MIF antibody (A) or anti-myc (B). HMIFmychis protein was purified from the cell lysate by using Ni-NTA agarose beads. C) Protein gel stained with coomasie blue showing untreated whole cell lysate (MIF NP, 100µg), 7.5µg untreated whole cell lysate (MIF NP 7.5) or 7.5µg of the purified whole cell lysate (MIF PUR). D) The same samples in C) were run in a western blot using anti-myc antibody to detect HMIFmychis.
Fig. 10. MIF Protects Cultured Neurons Against Oxidative Stress-Induced Cell Death.
Cultured primary rat cortical neurons were treated with 75µM H₂O₂ for 24 hours (H₂O₂ + Vehicle; H₂O₂ + MIF). Treatment of cells with either vehicle solution or MIF alone served as controls, respectively. Cell viability was assessed by using an MTT assay. Values represent the average absorbance as a percentage of the average control absorbance values. *P<0.0001, by student’s t-test, n=12 per treatment.
Fig.11. MIF Protects Cultured Neurons Against Oxygen-Glucose Deprivation-Induced Cell Death. Cortical neurons cultured from rat embryos were subjected to oxygen-glucose deprivation by culturing cells in glucose-free media and incubating them under hypoxic conditions (OGD-Vehicle, OGD-MIF, n=8). Cells which served as controls were either treated with vehicle or MIF and cultured in normal media containing glucose under normoxic conditions (Vehicle, n=13 MIF, n=11). Following 12h of OGD, cell viability was assessed by using an MTT assay. Values represent the average absorbance as a percentage of the average control absorbance values. *P<0.001, by student's t-test.
Fig. 12. MIF is Expressed in Endothelial Cells Following Stroke in Human Brain. Ten cases of stroke in human brain were evaluated for MIF expression by immunohistochemistry using an antibody to MIF. A) Brain tissue from a normal subject stained for MIF. B) Brain tissue from a stroke patient stained for MIF showing a blood vessel whose endothelial cells exhibit positive staining for MIF (arrows). C) The number of blood vessels immunoreactive for MIF was quantified by counting the number of vessels with positive staining endothelial cells in 5 to 10 fields within the peri-infarct zone under a 20X objective lens. The average number of positive vessels per field per case was calculated and the average of 10 cases was calculated and expressed as the average number of positive vessels per field per case.
Fig.13. MIF Positive Cells in the Infarct Region are Likely Macrophages. Brain tissue from a human stroke patient was analyzed by immunohistochemistry using an antibody to either MIF (A) or a monocytic marker, CD68 (B). The morphology of the cells which stained positive for MIF in the infarct display similar morphology to cells which stained positive for CD68 in the infarct. The morphology of these cells is consistent with that of macrophages.
Fig. 14. MIF Inhibits the Adhesion of Monocytes to Human Umbilical Vein Endothelial Cell Monolayers. Confluent monolayers of HUVEC were incubated with freshly isolated primary human monocytes for 1 hour. Prior to addition of monocytes, monolayers were treated with vehicle (A), 50ng/mL MIF (C) or 100ng/mL MIF (D) for 2 hours or 100U/mL TNF-α (B) for 24 hours. Following incubation with monocytes, monolayers were washed, fixed and stained with an antibody to the leukocyte common antigen (LCA) to visualize monocytes (brown). Cells were counterstained with haematoxylin to visualize endothelial cells (blue).
Fig. 15. Quantification of Adhesion Assay Results. Adherent monocytes were enumerated by counting the number of adherent monocytes per millimeter square (mm²) of the endothelial monolayer in five fields per well with a 20X objective lens. The average number of adherent monocytes per field per well was calculated and expressed as the number of monocytes per mm² of A) HUVEC and B) HBEC endothelial monolayer.

*P<0.0001, **P=0.001, ^P<0.01, #P<0.05, by student’s t-test, n=3 per treatment, except n=5 for MIF (50ng/mL) in A).
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APPENDIX-CERTIFICATE OF ETHICS APPROVAL

Application Number: A04-1466

Investigator or Course Director: Weihong Song

Department: Psychiatry

Animals:

- Mice APP and PS transgenic mice 50
- Invertebrates C. Elegans 100
- Rats Spraugue Dawley 100
- Mice B6SJLF1, 129P3, B6EiC3Sn-a/A-Ts65Dn and Control 100

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  Funding Title: Regulation of the BACE Expression in the Alzheimer's Disease Pathogenesis

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Regulation of BACE by Presenilins and the pathogenesis of Alzheimer's Disease.

- Funding Agency: Canadian Institutes of Health Research (CIHR)
  Funding Title: Regulation of the BACE Gene Expression in the Alzheimer's Disease

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.

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