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Regulation of Leukocyte-Derived Matrix Metalloproteinases and Azurophilic Enzymes in Human Diabetic Ketoacidosis

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

REGULATION OF LEUKOCYTE-DERIVED MATRIX METALLOPROTEINASES AND AZUROPHILIC ENZYMES IN HUMAN DIABETIC KETOACIDOSIS

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by

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ABSTRACT

Diabetic Ketoacidosis (DKA) is associated with pediatric cerebrovascular-related complications. DKA-associated inflammation instigates leukocyte adherence to the brain microvascular endothelium. As adhered leukocytes release enzymes that compromise vascular integrity, we questioned a role for leukocyte-derived matrix metalloproteinases (MMPs) and azurophilic enzymes (elastase, proteinase-3, myeloperoxidase). Our aims were to measure leukocyte-derived enzymes in DKA plasma, determine associations with DKA severity and investigate their effect on the cerebrovascular endothelium.

Plasma was obtained from children with type-1 diabetes, either in acute DKA or insulin-controlled. DKA was associated with altered plasma levels of ↓MMP-2, ↑MMP-8, ↑MMP-9 and ↑TIMP-4, which are largely leukocyte in origin. DKA was also associated with elevated plasma leukocyte elastase, proteinase-3 and myeloperoxidase. MMP-8, MMP-9 and proteinase-3 were positively correlated with DKA severity. Azurophilic enzymes decreased ZO-1 and degraded β-catenin in cerebrovascular endothelium.

In summary, DKA is associated with dynamic regulation of leukocyte proteolytic enzymes that can impair blood brain barrier integrity.

Key words: Human, Pediatric, Plasma, Diabetic ketoacidosis, Matrix metalloproteinase, Tissue inhibitor of metalloproteinase, Leukocyte, Elastase, Proteinase-3, Myeloperoxidase, β-catenin, ZO-1, Cerebrovascular endothelial cells.

CO-AUTHORSHIP STATEMENT

Dr. G Cepinskas and Dr. DD Fraser provided valuable guidance in designing the experimental approach and interpreting the data collected in this study. The primary human brain microvascular endothelial cells were provided by Dr. M Bani (NRC, Ottawa).

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LIST OF ABBREVIATIONS

ADC	Apparent diffusion coefficient
AJ	Adherens junctions
ANOVA	Analysis of Variance
AP-1	Activating protein-1
BBB	Blood-Brain Barrier
BCA	Bicinchoninic acid
bFGF	Basic fibroblast growth factor
BUN	Blood urea nitrogen
CD	Cluster of differentiation
CT	Computerized tomography
CTLA	Cytotoxic T-lymphocyte-associated protein
CTSG	Cathepsin G
CVEC	Cerebrovascular endothelial cell
CXCL	C-X-C motif ligand
DKA	Diabetic ketoacidosis

DKA-CE	Diabetic ketoacidosis-cerebral edema
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ET-1	Endothelin-1
ETV-4	E26 transformation-specific translocation variant 4
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
GM-CSF	Granulocyte monocyte- colony stimulating factor
HbA_{1c}	Hemoglobin A_{1c}
hBMEC	Human brain microvascular endothelial cells
HIF	Hypoxia-inducible factor
HLE	Human leukocyte elastase
ICAM	Intercellular adhesion molecule
IFN	Interferon
IgG	Immunoglobulin

IL	Interleukin
JAM	Junctional adhesion molecule
LFA	Lymphocyte function-associated antigen
MAC	Macrophage-associated protein
MCP	Monocyte chemoattractant protein
MMP	Matrix metalloproteinase
MPO	Myeloperoxidase
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MT	Membrane-type
NF-κB	Nuclear factor- κB
NSP	Polymorphonuclear neutrophil serine protease
OD	Optical Density
PAGE	Polyacrylamide gel electrophoresis
PECAM	Platelet endothelial cell adhesion molecule
PMN	Polymorphonuclear neutrophil
PR-3	Proteinase-3

SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
T1DM	Type 1 diabetes mellitus
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteinase
TJ	Tight junction
TNF	Tumor necrosis factor
uPA	Urokinase plasminogen activator
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor
VL	Vasculife media
VNTR	Variable number tandem repeat
WBC	White blood cell
ZO	Zonula occludens protein

CHAPTER 1: INTRODUCTION

1.1 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is a serious affliction that persists throughout life (4). Globally, the incidence of T1DM varies greatly, anywhere from 0.5-30.3/100 000 people per year with significant ethnic diversity (5). T1DM occurs predominantly in children younger than 15-18 years of age and in the past few decades, its incidence has been increasing (5, 6). Among juveniles with diabetes mellitus, T1DM accounts for over 90% of the cases (7). Cases of T1DM account for 5-10% of all diabetics (4). Approximately 14.9 billion dollars is expended on T1DM annually, thus treatment and management of T1DM is of great significance to patients, healthcare professionals and researchers globally (8).

T1DM is a condition caused by the destruction of the β-cells of the islets of Langerhans in the pancreas. These cells are responsible for all insulin production in the body and their destruction leads to an insulin deficiency. A lack of insulin results in an inability to regulate blood glucose effectively, leading to chronic hyperglycemia. T1DM is generally classified into 2 categories: Type 1A is the result of an autoimmune response destroying the β-cells whereas Type 1B is idiopathic destruction of the β-cells (4). Type 1A diabetes mellitus has a predominantly genetic origin where polymorphisms in genes residing in the human leukocyte antigen (HLA) loci, the *insulin*-variable number tandem repeat (VNTR) and cytotoxic T-lymphocyte-associated protein (CTLA)-4 genes can confer susceptibility to T1DM (9, 10). The other facet of T1DM susceptibility involves environmental triggers that initiate the immune-mediated destruction of the β-cells in those already predisposed to it. Examples of triggers include viruses such as rubella,

environmental toxins such as N-nitroso compounds and food components such as cow's milk proteins (11, 12).

Symptoms of T1DM include polydipsia, polyphagia, weight loss, blurred vision, polyuria with glycosuria and fatigue. The symptoms of T1DM start to present themselves after approximately 90% of the β-cells have been destroyed (7). Therefore, by the time it is detected by clinicians, patients are already suffering from a severe insulin deficiency. Complications that can arise from uncontrolled blood glucose in T1DM are hypokalemia, hypophosphatemia, hypoglycemia, peripheral venous thrombosis, mucormycosis, rhabdomylosis, acute pancreatitis, acute renal failure, sepsis and aspiration pneumonia (13). Longer term complications include retinopathy, nephropathy, neuropathy, cardiovascular disease, cognitive impairment and increased susceptibility to certain infections (14). The most severe acute complication of T1DM is diabetic ketoacidosis (DKA).

1.2 Diabetic Ketoacidosis

DKA involves the breakdown and metabolism of fatty acids and amino acids for energy. It is caused by a severe deficiency in insulin together with altered levels of counterregulatory hormones such as catecholamines, glucagon, cortisol and growth hormones. Increased glycogenolysis and gluconeogenesis along with impaired glucose uptake by peripheral tissues results in a hyperglycemic and hyperosmolar state (15, 16). Increased serum glucose levels, beyond that which renal excretion is able to rectify, causes osmotic diuresis and depletion of electrolytes, leading to potentially fatal dehydration (15). Occurring concurrently is the metabolism of fatty acids in the liver to the ketone bodies, β-hydroxybutyrate and acetoacetate, which accumulate in peripheral

circulation, overwhelming the bicarbonate buffering system and contributing to metabolic acidosis. Clinically, DKA manifests with dehydration, Kussmaul respiration, nausea, vomiting, acute abdominal pain and reduced or loss of consciousness (15). Children who develop DKA possess a higher risk for intracranial vascular complications such as cerebral edema (17), hemorrhage (18) and stroke (19).

The biochemical criteria for DKA involves hyperglycemia (blood glucose > 11 mmol/L), venous pH < 7.3 or bicarbonate < 15 mmol/L and ketonemia or ketonuria (15). DKA is classified according to the severity of acidosis: Mild DKA is distinguished by a pH < 7.3, moderate DKA is distinguished by pH < 7.2 and severe DKA is distinguished by pH < 7.1 (20).

DKA often occurs in “first time” diabetics, in undiagnosed and therefore, untreated T1DM cases. Other common events that precipitate DKA are the accidental or deliberate stoppage of insulin treatment (i.e., weight control in adolescents). Risk factors for DKA are extremely variable, including children with poor metabolic control, peripubescent females, children from troubled households and children with psychiatric disorders (21).

1.3 DKA Cerebral Edema

DKA, though a previously highly lethal condition, following the advent of insulin, has become much more manageable with a mortality rate below 2% (16). However, DKA still retains significant mortality due to related complications, one of which is cerebral edema (DKA-CE). DKA-CE is a serious and highly lethal condition, despite advances in management techniques. In North America, the incidence of DKA-CE among DKA cases is 0.5-0.9% with a devastating mortality rate of 21-24% (17, 22, 23). Some studies have

even reported a mortality rate of up to 90% (24). DKA-CE is the leading cause of death in pediatric cases of T1DM and leaves 30-40% of survivors with significant neurological damage (25). It has been surmised that DKA-CE mortality accounts for 80% of all diabetes-related deaths among children under 12 year of age (24). Ninety-five percent of DKA-CE patients are under 20 years of age and 33% are under 5 years of age (25). As such, DKA-CE is considered mainly a pediatric pathology. The reason for the apparent predisposition in children to this complication is not yet clear.

Cases of DKA-CE usually occur within 2-24 hours after the onset of DKA treatment. Patients present with a sudden headache and reduced level of consciousness followed by rapid neurological degeneration and eventual herniation of the brain stem (13, 26). Thus, many researchers suspect DKA-CE to be a side effect of the interventions traditionally administered for DKA. However, it should be noted that cases of DKA-CE have been reported before the initiation of treatment (17, 22). Therefore, the etiology of DKA is more complex than just an adverse response to treatment. DKA-CE can manifest itself in two ways: from admission, the patient's condition will steadily deteriorate into a comatose state or, more frequently, the patient will appear to recover and then suddenly worsen (24, 27).

Much discussion has centered over the distinction between subclinical and symptomatic cerebral edema. In a study done on 6 children between the ages of 11 and 14 years old with DKA who were asymptomatic for DKA-CE, it was discovered that all 6 showed evidence of cerebral swelling via computerized tomography (CT) scans taken at the onset of treatment and at hospital discharge (28). Additionally, another study using brain encephalography detected the presence of significant cerebral swelling in 81% of

patients between the ages of 13-42 years old undergoing treatment for DKA, despite the absence of symptomatic cerebral edema (29). Taken together, these studies suggest that DKA-CE is far more common in pediatric cases of DKA than previously suspected. Conversely, another study evaluated ventricle size via CT following DKA treatment in patients 4-15 years old and found that subclinical DKA-CE did not occur frequently (30). These controversial findings may be explained by DKA-CE possessing a spectrum of severities from mild subclinical DKA-CE to severe symptomatic DKA-CE (31).

Cerebral edema can be classified into 2 main categories: vasogenic edema and cytotoxic edema. Vasogenic edema is the flow of water into the extracellular space due to destabilization of the Blood-Brain Barrier (BBB). Cytotoxic edema is the flow of water into the intracellular space resulting in cell swelling (32). Though both cytotoxic and vasogenic edema are observed in pathological conditions, it has been suggested that the primary form of edema present in DKA, prior to treatment is that of the vasogenic type. Diffusion-weighted magnetic resonance imaging (MRI) of pediatric cases of DKA showed elevated brain apparent diffusion coefficient (ADC), indicating vasogenic edema (33, 34).

The exact cause of DKA-CE is still largely unknown at this point, though theories that have been proposed include increases in BBB permeability, increases in hydrostatic pressure due to aggressive fluid resuscitation concomitantly with decreased osmotic pressure, loss of cerebral autoregulation, osmotic disequilibrium from accumulation of intracellular osmoles in the brain, hypoxia and ischemia and intracranial acidosis (35).

A suggested mechanism of DKA-CE and perhaps the most convincing is the initiation of a pro-inflammatory cytokine cascade that results in BBB dysfunction. Observed consistently in DKA is an inflammatory cascade similar to that of systemic inflammatory response syndrome (36). This inflammatory cascade may trigger transient opening of the BBB. DKA has been shown to initiate a widespread inflammatory cascade involving the cytokines, C-X-C motif ligand (CXCL)-1, CXCL-8 and interferon (IFN)-α2 (37). The same study also showed increased adherence of polymorphonuclear neutrophils (PMN) to the endothelium, providing convincing evidence of leukocyte involvement. Also reported to be upregulated in patients suffering from DKA are interleukin (IL)-1β, IL-6, IL-2 and tumor necrosis factor (TNF)-α, whose increase correlates with the timeframe of the development of subclinical DKA-CE (38). An immunological response during DKA is further supported by the detection of elevated levels of components of the complement pathway (C3a, Bb and C5b-9) (39) as well as C-reactive protein (CRP) (40). In juvenile mice, DKA increased circulating levels of numerous endothelial-specific molecules such as E-selectin, intracellular adhesion molecule (ICAM)-1 and vascular cell adhesion protein (VCAM)-1, indicating an onset of systemic inflammation (41). Thus, the onset of DKA involves a significant activation of the inflammatory cascade. It is well established that cytokines and the inflammation cascade lead to activation of the BBB endothelium as well as leukocytes and subsequent BBB disruption, resulting in increased water flux into the brain, hence the prevalence of cerebral edema in DKA (42-45).

Furthermore, it is believed that acidosis *per se* plays an important role in DKA-CE. The ketone bodies, acetoacetate and β-hydroxybutrate are known to be pro-inflammatory agents that activate the endothelial cells in the BBB by increasing endothelin (ET)-1 and

vascular endothelial growth factor (VEGF) secretion (46). Both VEGF and ET-1 are known as vascular permeability inducing factors. In another study, acetoacetate was shown to increase expression of ICAM-1, a receptor that mediates leukocyte adhesion to the endothelium (47). Thus, ketone bodies are able to trigger inflammatory processes at the BBB endothelium and lead to the release of permeability inducing factors.

Hyperglycemia, present during precipitation of DKA can initiate an inflammatory response as well. Hyperglycemic crises were associated with increased levels of circulating CRP, free fatty acids, CXCL-8, IL-6, IL-1β and TNF-α (48). Hyperglycemic conditions can also facilitate the activation of human T-lymphocytes, thus providing a mechanism by which hyperglycemia is able to trigger inflammation (49). Therefore, not only DKA, but also hyperglycemia *per se* is able to trigger an inflammatory response in the body by facilitating the production and release of circulating cytokines. The activation of the BBB endothelium by many of these cytokines leads to an increase in permeability of the BBB.

Some studies have also addressed the role of hypoxia and ischemia in the development of DKA-CE. Factors like increased blood urea nitrogen (BUN) levels (17), and lower pCO_2 levels (22) have been frequently associated with the development of DKA-CE. Studies have also shown that cerebral blood flow is decreased in rats with untreated DKA and that cerebral blood flow was directly tied to pCO_2 (50). DKA commonly results in dehydration which could lead to, in combination with a low pCO_2, increased vasoconstriction and consequently cerebral ischemia, hypoxia and increased capillary permeability (24, 51, 52). Increased capillary permeability would result in increased fluid flux across the BBB into the brain resulting in cerebral edema.

Several studies have attributed DKA-CE occurrence to rapid osmolar changes resulting from intravenous infusion during treatment (53-55). In a hyperosmolar environment, such as that commonly seen in hyperglycemia, cells in the brain generate intracellular osmoles in order to maintain their volume and osmolarity. Hyperglycemic rabbits showed an intracellular increase in various ionic solutes, sorbitol, lactate, urea, amino acids (56) and other idiogenic osmoles since identified as taurine and myoinositol (57-59). These changes were accompanied by equalization of brain and cerebrospinal fluid osmolality. When the hyperglycemia was corrected, the rabbits rapidly developed an osmotic gradient from brain to plasma, showed an increase in unidentified osmoles and other solutes in brain cells, an increased cerebral water content and most significantly, precipitation of cerebral edema (56). During treatment for DKA with fluids and insulin, plasma osmolarity decreases rapidly and if the intracellular osmoles do not dissipate accordingly, the result is that brain cells become relatively hyperosmolar to the serum, thus favouring inward fluid flux and consequentially, the formation of cytotoxic DKA-CE.

Another proposed theory behind the pathophysiology of DKA-CE is the involvement of aquaporin channels, likely the result of fluid administration. Aquaporin channels are transmembrane channels whose function it is to transport water from extracellular to intracellular compartments. They are expressed in many epithelial and endothelial cells throughout the body as well as glial cells of the BBB. Aquaporins are highly expressed in the endfeet of astrocytes in contact with the abluminal surface of the cerebral endothelial cells (60). Aquaporin activity is pathologically associated with the formation of cerebral edema after injury in rats, both vasogenic and cytotoxic(61). They

have been shown to mediate cerebral edema in other pathological conditions such as traumatic brain injury and cerebral ischemia (61, 62). Acidotic conditions were shown to worsen cerebral edema in rats through the actions of aquaporin-1 (63).

1.4 Blood-Brain Barrier

The BBB is present in all mammals with a complex nervous system and plays a crucial role in keeping the brain isolated from peripheral circulation. The microvasculature in the brain provides a massive combined area for blood-brain gas, nutrient and waste exchange. A large functional surface area is critical due to the brain's role as the most metabolically active organ in the body. With an average vessel diameter of < 8 μm, the microvessel functional area for blood-brain exchange in humans is approximately 100-200 cm^2/g in brain tissue, or approximately 12-18 m^2 total in an average adult (64). The BBB does not protect the areas of the brain involved in autonomic nervous control and endocrine function; it is present in every region of the brain except the circumventricular areas (65).

The BBB is not a single protective brain partition as its name might imply, rather it refers to protective and exclusive characteristics that is inherent to brain vasculature itself, specifically the microvasculature. The BBB is composed of multiple cell types such as endothelial cells, astroglia and pericytes (66). The spatial arrangement of the BBB is very distinct. The luminal side of the barrier is composed of the cerebrovascular endothelial cells (CVEC) which form the vessel wall (Figure 1-1). The CVEC are arranged continuously, associating tightly together to form the tubular structure of the vessel. On the abluminal side of the vessel, underlying the CVEC is basal lamina which sheaths the capillary. Discontinuously associated with this surface are pericytes and

perivascular macrophages (45, 66). Further into the parenchyma are astrocytes, which project long endfeet onto the abluminal surface of the CVEC, and microglia (Figure 1-1). Astrocytes are the most abundant cell type interacting with CVEC and covers a majority of the microvascular abluminal surface (67). In terms of function, the term BBB refers to the actions and effects of the CVEC and to a lesser extent, pericytes and astrocytes (45, 66).

The main function of the blood-brain barrier is twofold. The first is to protect the brain and sensitive neural tissue from neurotoxic compounds, soluble factors, microorganisms, proteins and other cells that might be present in peripheral circulation. The second is to maintain homeostatic conditions in the neuronal extracellular environment by regulating the passage of blood-borne compounds into the brain parenchyma. In order to fulfill these roles, the BBB must be selectively permeable; it needs to be able to restrict entry of blood-borne macromolecular compounds with extreme prejudice, but at the same time, allow essential nutrients and compounds that the brain needs to function to access the brain parenchyma. The BBB accomplishes this through physical barriers and expression of selective transporters (64). Transporters specific for many important substances have been identified in the BBB, including ones specific for glucose compounds, amino acids, cholines, purines and monocarboxylic acids (68). The BBB functions to isolate cerebral circulation from peripheral circulation under physiological conditions. There are many macromolecules present in peripheral circulation that would be harmful to the brain. Proteins and factors such as albumin, prothrombin and plasminogen, if allowed to access the brain, are potent signalling

activators and can stimulate unwanted cell processes, resulting in damage and cell death (69-71).

The BBB must also exclude peripheral neurotransmitters from the brain. Since both the central and peripheral nervous signalling uses the same neurotransmitters, it is imperative that the two systems remain independent of each other. Likewise, this also prevents excitotoxicity due to peripheral neurotransmitter imbalances spreading to the brain and disrupting signalling (72). The BBB also excludes immune cells from the cerebral environment. Activated immune cell infiltration into the brain can damage delicate neural tissue by releasing destructive factors and enzymes and turning on unwanted signalling pathways. As such, under normal physiological conditions, the brain is normally excluded from peripheral immune surveillance (72).

Another function of the BBB is ion regulation. The BBB possesses a multitude of ion channels and transporters that preserve the ionic composition of the brain and keeps the extracellular space optimal for neuronal signalling. It is essential to keep the brain ionic content separate from peripheral ionic concentrations. For example, K^+, an important ion in nervous signalling is held at ~2.5 mM in the brain, in spite of ionic fluctuations occurring in the periphery (64).

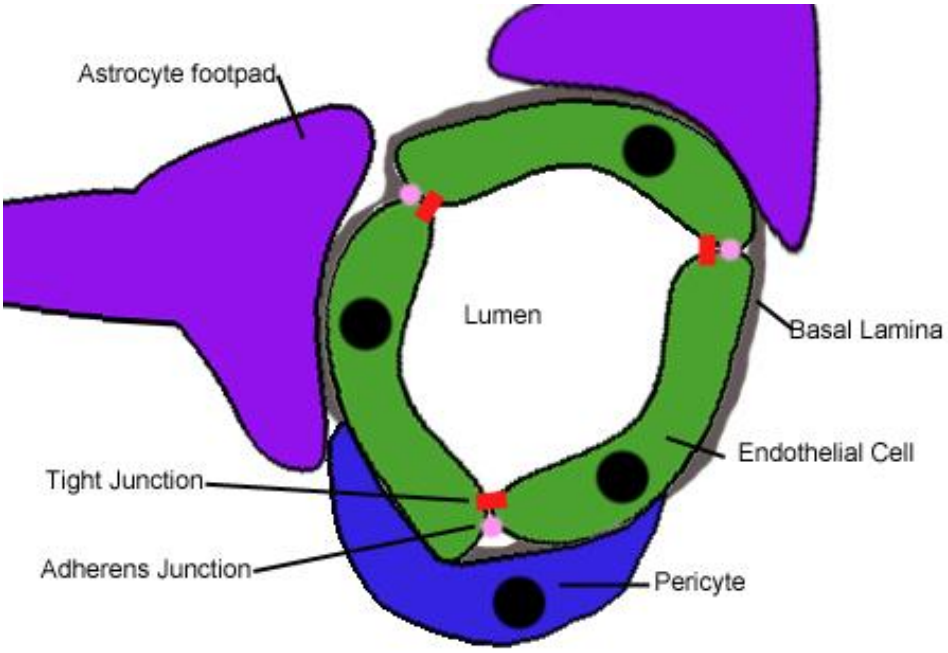

FIGURE 1-1. Schematic Diagram of the Blood-Brain Barrier. The BBB is found at the level of cerebral microvessels. Composing the wall of the vessel itself are continuous CVEC, tightly sealed against each other. Pericytes are located discontinuously on the abluminal side of the endothelial cells within the endothelial basal lamina. Perivascular macrophages also share this location. Extending cellular processes to make contact with the vessel from the neuropil are astrocytes. TJs and AJs function to seal the gaps between the adjacent endothelial cells.

1.5 Cerebrovascular Endothelial Cells

The component of the BBB that is most responsible for its exclusionary properties are the CVEC. CVEC differ from endothelial cells elsewhere in the body by their relative lack of fenestrae. Fenestrae are pores that go through endothelial cells which allow for rapid exchange of material between blood vessels and the extracellular space without the use of transporters or channels and so in CVEC, this lack of fenestrae act to greatly reduce paracellular movement of substances, lending to the BBB's permeability characteristics (45, 66).

CVEC possess tight junctions (TJ) which are complexes of transmembrane proteins whose primary purpose is to hold adjacent endothelial cells together and accordingly, they span the intercellular gap between adjacent endothelial cells. The TJs are also heavily involved in signal transduction in the cells. TJs are composed of a chain of many, almost continuous, but discrete points of intercellular contact, which are mediated by protein complexes. These are arranged in a belt-like formation around the apical end of the cell (45, 73). These points of tight contact between adjacent cells essentially occlude the paracellular passage. TJs act to increase electrical resistance across the endothelial layer as well as hindering paracellular transport of soluble compounds (45). TJs lend the BBB the functional properties of a single continuous cell membrane (64). This belt imparts polarity upon the cell by physically partitioning the apical and basal ends, allowing directional transport via asymmetrical expression of transporter proteins. TJs in the brain differ from those elsewhere in the body by being far tighter and more complex (64).

Tight junctions are composed of high levels of intercellular spanning transmembrane proteins, occludin and the claudins and the intracellular component, zonula occludens proteins (ZO) (Figure 1-2) (64). Occludin expression is higher in BBB TJs than in any other peripheral TJ, indicating a central role in the BBB (74). Of the over 20 isoforms of claudin, only claudin-1 and -5 have been shown to be a component of BBB TJs (65). Both occludin and claudins are directly associated with the cytoplasmic scaffolding and signalling proteins ZO-1, -2, -3 and cingulin (64, 75). The TJ-ZO complex is also connected to the actin cytoskeleton. Many studies have associated disruption of occludin, claudin and ZO-1 with impaired BBB function (76-78). The final proteins involved in the TJs are junctional adhesion molecules (JAM). JAMs engage in heterogeneous and homogenous binding across the intercellular gap. Only JAM-A and -C are involved in the BBB TJs (79). Accordingly, the JAM family decreases BBB permeability and has been associated with an increased transendothelial electric resistance, inhibition of leukocyte extravasation and occludin localization (80).

Another important feature of the CVEC in the BBB is the presence of adherens junctions (AJ). AJs are composed of transmembrane proteins called cadherins, anchored to the actin cytoskeleton in the cytosol via the catenins (α, β and γ) (Figure 1-2). In the intercellular space, vascular endothelial (VE)-cadherins homogenously bind to their adjacent cell counterpart, anchoring the 2 adjacent cells together (81). AJs play a role in cell-cell adhesion, signalling and transcriptional regulation (82). Perturbances to AJs have been shown to facilitate disruption of the endothelium (83, 84).

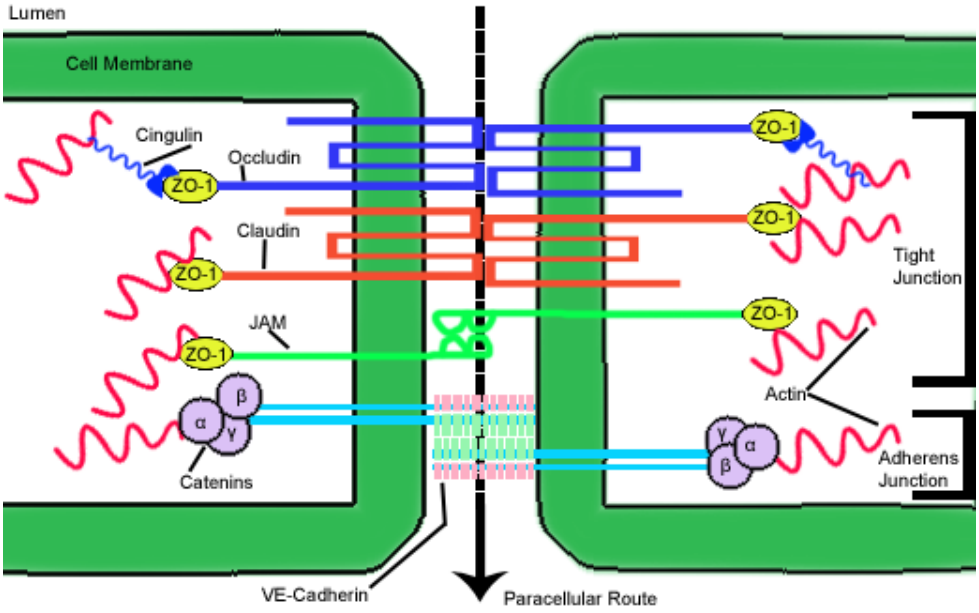

FIGURE 1-2. Schematic Diagram of the CVEC Junctional Complexes. Tight junctions are composed of three main proteins: occludin, claudin and junctional adhesion molecules (JAM). Intracellularly, these proteins are connected to the cytoskeleton by ZO-1. The junctional complex also consists of adherens junctions, which are composed of VE-cadherin, α-catenin and β-catenin. Together, they are responsible for physically sealing adjacent endothelial cells together in order to prevent paracellular movement of luminal solutes as well as playing a role in signal transduction. (1)

1.6 Matrix Metalloproteinases

Matrix metalloproteinases are a family of more than 20 $zinc^{2+}$ dependent endopeptidases, found both secreted into the extracellular environment and membrane bound. In humans, there are 23, a number which includes the collagenases (MMP-1, -8, -13, -18), the stromelysins (MMP-3, -10, -11), the matrilysins (MMP-7, -26) and the gelatinases (MMP-2, -9), as well as the membrane-type MMPs anchored to the cell surface by a C-terminal transmembrane domain (MMP-14 [MT1-MMP], -15 [MT2-MMP], -16 [MT3-MMP] and -24 [MT5-MMP]) or a glycophosphatidylinositol (GPI) anchor (MMP-17 [MT4-MMP] and -25 [MT6-MMP]) (85-87).

All MMPs are synthesized as preproenzymes with an endoplasmic reticulum-targeting signal peptide and secreted as inactive zymogens or active enzymes (88). In terms of primary structure, MMPs are composed of several domain motifs, depending on the specific MMP. The catalytic domain is approximately 170 amino acids long and contains a zinc-binding motif inside the catalytic cleft (Figure 1-3). The zinc ion binds to a water molecule and is activated by a glutamate residue also located in the catalytic domain, in order to hydrolyze the target peptide bond (89). The propeptide domain, approximately 80 amino acids, is located at the N-terminus and is conserved among the MMP family (Figure 1-3). It contains a cysteine residue, also known as the cysteine switch that is able to bind to the catalytic site in order to suppress the proteolytic activity of the site in the propeptide. Some MMPs have a furin cleavage site between the pro-domain and the catalytic domain. These enzymes are secreted in their active forms (90). Thus, the propeptide domain's function is to maintain the inactivity of the MMP's catalytic domain. Most MMPs contain a C-terminal hemopexin-like domain as well

(Figure 1-3), which functions mainly as a recognition site for specific substrates, though most MMPs retain their proteolytic activity without it (91). The hemopexin domain is however, absolutely necessary for the degradation of triple helical collagen (92). Similarly, the gelatinases have a series of three fibronectin type II inserts in the catalytic domain which function to recruit and bind gelatin (93). The hemopexin domain in some MMPs is also involved in mediating activation via other MMPs (94).

Since the MMPs possess extremely potent proteolytic activity, their activity and expression is regulated at many levels. A large portion of MMP regulation takes place at the transcriptional level. MMP transcription is under the control of a number of factors such as activating protein (AP)-1, E26 transformation-specific translocation variant (ETV)-4, nuclear factor (NF)-κB (95) and hypoxia-inducible factor (HIF)-1α (96). A feature common to most MMPs is that they are inducible. MMP gene expression can be enhanced or suppressed by many factors. Examples of inducers of MMP gene expression are cytokines, growth factors, phorbol esters, actin disrupters, physical stress and oxidative stress (94, 97). Factors such as interleukins, interferons, epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) can all increase expression of MMPs through activation of the AP-1 or the ETV-4 elements (95). MMP gene expression can also be downregulated by transforming growth factor (TGF)-β, retinoic acids and glucocorticoids (94).

Once secreted, MMPs are activated via cleavage of the propeptide domain, removing the inhibiting cysteine residue from the catalytic cleft (Figure 1-4) (94). This cleavage can be mediated enzymatically by other MMPs or proteases such as plasmin (98). Activation can also be mediated non-enzymatically, which involves disruption of

the inhibitory interaction between the Zn^{2+} residue and the cysteine residue. Thus, MMPs may be activated by S-nitrosylation or oxidation without cleavage of the propeptide domain (99, 100). Reactive oxygen species, mercurial compounds and denaturants may also nonenzymatically activate MMPs (101, 102). Nevertheless, *in vivo*, a majority of proMMP activation is mediated by tissue or plasma proteases. Numerous studies have suggested that the urokinase plasminogen activator (uPA)/plasmin system is a critical physiological activator of proMMPs, including proMMP-2, -3, -9, -12 and -13 (103-105).

Catalytic MMP activation can also be mediated by other MMPs. For example, MT1-MMP, MT2-MMP and MT5-MMP have been shown to activate proMMP-2 (106-108). As well, MMP-2, MMP-3 and MMP-13 are able to cleave and activate proMMP-9 (109-111). ProMMP-2 can be activated by MT1-MMP, but this activation is tightly regulated by the complexing of tissue inhibitor of metalloproteinase (TIMP)-2 and MMP-2 in cells (112). It is thought that the TIMP-2 subunit is required for the localization of the whole complex and thus, MMP-2 localization to the cell membrane where it can then be proteolytically activated by MT1-MMP (113). Thus, MMPs play a significant role in the modulation of each other and allow for even tighter regulation of activity.

Once they are activated however, the proteolytic activity of MMPs is modulated either by nonspecific protease inhibitors, α2-macroglobulin or by a family of endogenous regulators called tissue inhibitor of metalloproteinases. There are 4 known members of the TIMP family: TIMP-1, -2, -3 and -4, ranging from 21-30 kDa in size. TIMPs noncovalently bind to and inhibit MMPs in a 1:1 stoichiometric ratio (114). The mechanism by which TIMPs inhibit the catalytic activity of MMPs is not well known. Studies have reported on the interaction between TIMP-1 and MMP-3: TIMP-1 possesses

a cysteine residue that is capable of chelating the catalytic Zn^{2+}, thus disrupting its ability to carry out hydrolytic attack on other species. Additionally, TIMP-1 possibly obstructs any potential substrate binding in MMP-3 (115).

The TIMPs do not show strong specificity for any particular MMPs; each TIMP can inhibit every MMP physiologically (116). However, differences between the TIMPs do exist. TIMP-1 and TIMP-2 have been observed to exhibit a preference for MMP-9 and MMP-2 respectively (117). TIMP-1 shows a lower affinity for the membrane-type MMPs (118). Additionally, TIMP-2 and TIMP-3 show a lower affinity for MMP-3 and MMP-7 than TIMP-1 (119). TIMP-3 displays a wider range of MMP inhibition than the other TIMPs. The expression of TIMP-1 and TIMP-3 are thought to be highly inducible, whereas TIMP-2 expression is mostly constitutive (120).

The major targets of the MMPs are the proteins that compose the extracellular matrix and basal lamina such as fibronectin, gelatin, collagen and proteoglycans; indeed, virtually all protein components of the extracellular matrix (ECM) can be degraded by the MMPs (88). They serve to modulate tissue architecture through turnover of the ECM and intercellular junctions.

MMPs not only serve to degrade the ECM but also play a role in the generation of substrate cleavage fragments that have a biological function, and modify, activate or deactivate signaling molecules during inflammation (121). MMP-1, -3 and -9 have shown the most potential to regulate chemokine signaling (122). CXCL-8, a major inflammatory chemokine has been shown to be potentiated via MMP-8 and-9 processing (123, 124). Another well-known example is the effect of MMP-7 on the generation on CXCL-1

gradient. MMP-7 is able to release the chemokine into the ECM thereby generating a gradient to facilitate PMN infiltration. MMPs have also been shown to possess certain anti-inflammatory actions. For example, the cytokines CXCL-8 and CXCL-1 have been shown to be degraded and inactivated by MMP-9. Many MMPs can inactivate CXCL-12 as well (125). Furthermore, in addition to inactivating pro-inflammatory cytokines, MMPs can also further process them into potent antagonists (126). Examples of this are the processing of CX3CL-1 and MCP-2 by MMP-2, and MCP-1 by MMP-8 (127). Thus, MMPs represent a bidirectional level of regulation of inflammatory signals.

Despite their important physiological roles, MMPs, through their ability to promote ECM breakdown and remodeling, have also been implicated in numerous pathologies. MMPs are also known to be an important contributor to pathological processes like arthritis, cancer and cardiovascular diseases (128-130). In particular, the gelatinases, MMP-2 and -9, have been shown to play a critical role in processes such as tumour invasion, angiogenesis and neovascularisation.

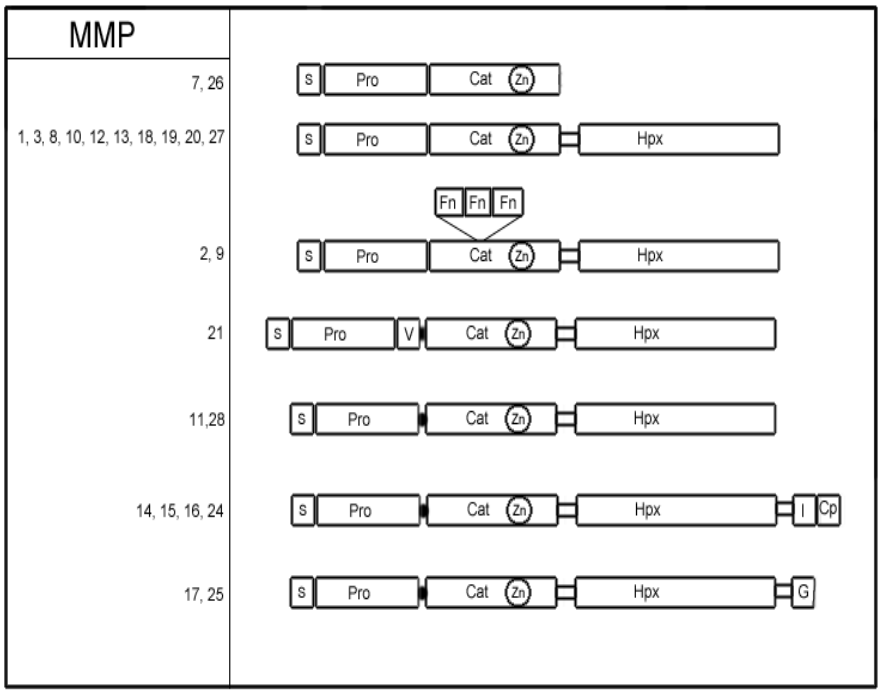

FIGURE 1-3. Schematic Diagram of the Common Different Functional Domains of the MMP Family.

S, signal peptide; Pro, propeptide domain; Cat, Catalytic domain; Zn, Zinc residue; Fn, Fibronectin domain; Hpx, Hemopexin domain; I, Type I transmembrane domain; Cp, Cytoplasmic region; G, GPI anchor; ■, Furin cleavage site; V, Vitronectin insert.

Classically, MMPs are composed of 3 main regions, the propeptide domain which inactivates the enzyme, the catalytic domain which facilitates target cleavage and the hemopexin/vitronectin domain which facilitates substrate recognition. Other domains include the signal peptide which locates the preproenzyme to the endoplasmic reticulum, Type 1 transmembrane domain and GPI anchor which serve to attach MT-MMPs and the furin cleavage site which facilitates activation via furin. (2)

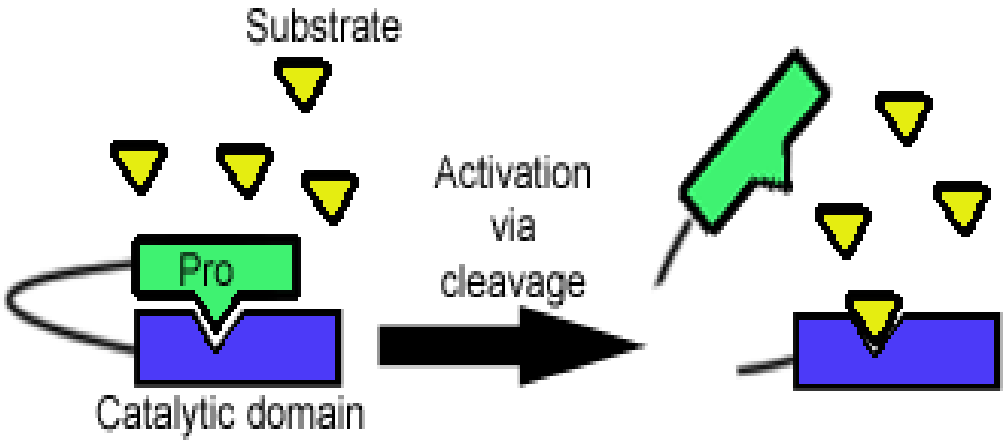

FIGURE 1-4. Schematic Diagram of the Classic Activation of MMPs. The catalytic activity of the proenzyme is inhibited by the presence of propeptide domain which binds to and obstructs the catalytic cleft. Cleavage of the propeptide domain results in dissociation of the inhibitory complex and allows the MMP catalytic cleft to access and digest substrate.

1.7 Polymorphonuclear Neutrophils

Polymorphonuclear neutrophils are the most abundant subtype of granulocyte white blood cell present in mammalian circulation. They are a key component of the innate immune system whose main role is to defend the body against microbes and pathogens during inflammation and are considered the "first line of defense". PMN are constantly circulating throughout the body, moving through vessels in a nonadherent state and constantly probing the vascular wall. However, during inflammation, they transition into an adherent state. The endothelial cells of the vascular wall, upon inflammation, become activated and express many different membrane proteins such as E- and P-selectin as well as ICAMs (131). The PMN themselves constitutively express L-selectin and P-selectin glycoprotein ligand (PSGL)-1 which can bind to all 3 types of selectin (132-134). Coupled with vessel dilation due to inflammation and flow stress, PMN are allowed to contact the vessel wall (135). Following inflammatory cytokine-mediated activation of endothelial cells, both expression of E-selectin and ICAM-1 are upregulated. L-selectin and PSGL-1, expressed on PMN, physically interact with E- and P-selectin, expressed by the endothelial cell, transiently recruiting the PMN to the endothelium. The PMN then "rolls" along the endothelium, jumping from selectin molecule to selectin molecule. Exposure to chemokines quickly triggers PMN to become more firmly adherent to the endothelium, a state change mediated by leukocyte integrins, leukocyte-function-associated antigen (LFA) and macrophage-associated protein (MAC)-1 on the PMN interacting with ICAM-1 on the endothelial cell (134). The presence of chemokines during inflammation serves to activate integrins, thus increasing their binding strength immensely. At this stage, the rate at which PMN roll across the

endothelium is greatly diminished. Binding of leukocyte integrins to endothelial immunoglobulin (IgG)-superfamily molecules mediates leukocyte arrest (134, 136). Once its rolling has been arrested and the PMN firmly adheres to the endothelium, it is able to respond to inflammatory chemoattractants and undergoes chemotaxis. The PMN can then transmigrate through the endothelial layer into inflamed tissue, a process actively facilitated by endothelial platelet-endothelial cell adhesion molecule (PECAM)-1 and JAMs (Figure 1-5).

PMN are able to coordinate the body's immune system response as they can generate chemotactic signalling molecules, TNF and other cytokines such as IL-1β, monocyte chemoattractant protein (MCP) -1 and CXCL-1 to activate and direct immune cells (3, 137, 138). They can even direct macrophage differentiation into either a pro- or anti-inflammatory phenotype (138).

PMN, however, are more recognized for their direct antimicrobial action. The cadre of microbicidal processes that PMN possess include their phagocytic function, their ability to release granules filled with proteolytic enzymes and their ability to produce reactive oxygen species (139, 140). The hallmark of all granulocytes and indeed, PMN, are the granules which form intracellularly during differentiation in the bone marrow (141). These granules can be divided into 3 main categories based on the proteins contained within them: primary/azurophilic granules, secondary/specific granules and tertiary/gelatinase granules. Azurophilic granules primarily contain myeloperoxidase (MPO) which facilitates peroxidation of cell membranes, 3 serine proteases, PMN elastase (HLE), proteinase-3 (PR-3) and cathepsin G (CTSG) as well as defensins and lysozyme. Specific granules contain lactoferrin, an enzyme that sequesters away iron and

copper, MMP-8 and lysozyme. Tertiary granules contain MMP-9 and MMP-25 (139). PMN also possess secretory vesicles which contain plasma proteins, complement receptor and Fc receptors (142). The type of granule produced coincides with the differentiation stage of the PMN such that azurophilic granules are indicative of relatively young PMN while gelatinase granules are that of mature PMN (143). These granules are released in a process called degranulation which occurs upon activation, during and after the transmigration of PMN through the endothelial layer into tissue. Different granules are released at different times during PMN extravasation. Secretory vesicles are exocytosed upon contact with the endothelium and so act to facilitate a further immune response (139, 144). In general, the order of granule subtype released depends upon the ease of mobility. Secretory vesicles are most easily mobilized and are therefore, released first, onto the PMN membrane within the bloodstream and at the endothelial surface. Tertiary granules are the second easiest mobilized granule and are released as PMN are moving through the endothelial layer whereas primary and secondary granules are the most difficult to mobilize and are thus, released directly at the site of inflammation (3). The release of these highly destructive enzymes is crucial for antimicrobial activity. However, unwanted or improper release due to severe inflammation can cause serious damage to tissue. Pathologically, PMN play a deleterious role in inflammatory conditions such as ischemia-reperfusion injury and sepsis (145-147).

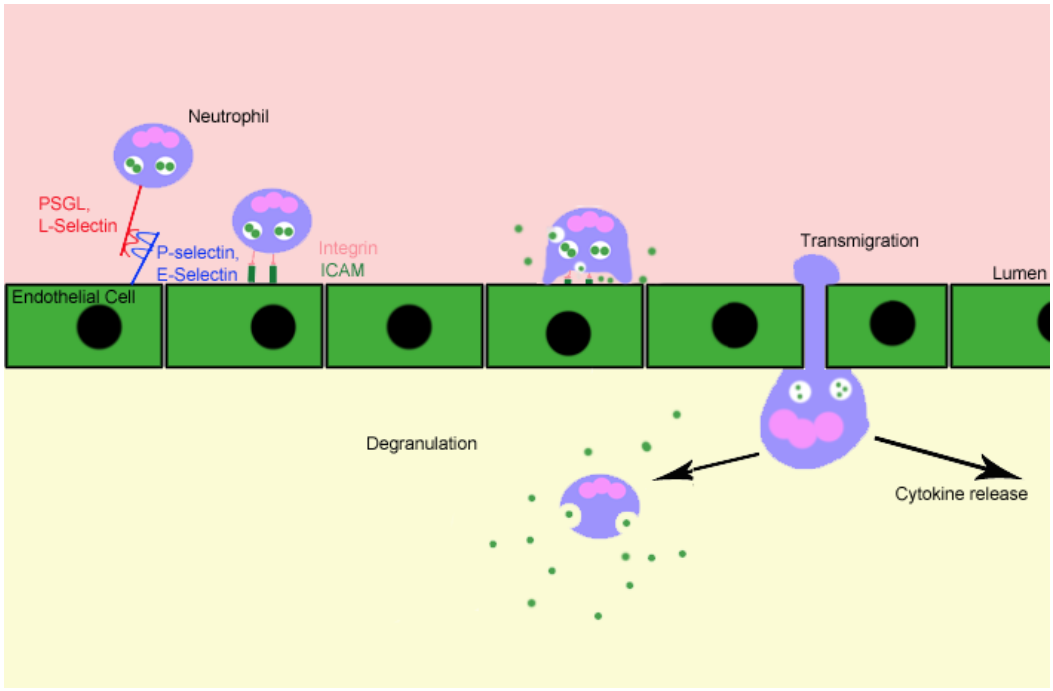

FIGURE 1-5. Diagram of PMN Activation and Recruitment to the Vascular Endothelium. During inflammation, endothelial cells express P- and E-selectin on the cell surface. These proteins interact with ligands on the PMN resulting in margination and rolling of PMN on the endothelial surface. Adhesion molecules of IgG-superfamily (e.g., ICAM-1) expressed on vascular endothelium interact with PMN adhesion molecules (e.g., integrins) resulting in PMN capture and firm adhesion. Subsequently, PMN migrate across the endothelial barrier (a process mediated by PECAM-1 and JAMs) in response to chemotactic stimulus. Once inside the infected/inflamed tissue, PMN release their vesicles and microbicidal enzymes in a process called degranulation. (3)

1.8 PMN Serine Proteases

The PMN serine proteases (NSP), as previously mentioned, include HLE, PR-3 and CTSG. The major sources of these serine proteases are PMN, but also include monocytes (148, 149) and other granulocytes such as eosinophils (150). Similar to MMPs, NSPs are expressed as proenzymes (151-153) and require processing into active forms. However, unlike MMPs which are secreted as proenzymes, NSPs are generally processed into their active form prior to packaging into their respective granules and are therefore, active upon release (154).

These homologous proteases depend upon a catalytic triad formed from aspartate, histidine and serine (154). As its name implies, serine proteases contain a catalytic serine residue. The catalytic unit utilizes the hydroxyl group on the residue and free H_2O to hydrolyze the target peptide bond (155). They are all neutral proteases with their optimal pH range being around 7.5-8.5 (154, 156).

Physiologically, they are irreversibly inhibited by the endogenous protease inhibitor family, the serpins, such as α1-proteinase inhibitor and α1-antichymotrypsin, or to a lesser extent, α2-macroglobulin (154). Interestingly, α1-proteinase inhibitor displays specificity for HLE but nevertheless, inhibits all serine proteases (157). A conserved loop domain on α1-proteinase inhibitor and other serpins, binds to the protease catalytic site. When the loop is cleaved, the inhibitor undergoes a rapid conformational change, trapping the NSP and the inhibitor in an inactive state (158). These inhibitors are extremely prominent in plasma, in far excess of circulating NSPs (157). However, it is thought that during inflammation, the release of these proteases is significant enough to

overwhelm the local inhibitory capacity and exert its actions for a brief period of time (159, 160).

Intracellularly, NSPs are stored in phagolysosomes and act to digest phagocytised pathogens. They are, however, more recognized for their extracellular role in ECM destruction and peptide cleavage. NSPs are able to cleave a variety of ECM components including elastin, fibronectin, laminin, collagens and proteoglycans (161-163). They are also able to cleave a wide range of plasma proteins such as complement and factor VII (164, 165). Due to their extremely broad range of overlapping substrates, it is believed that they are non-specific proteases.

NSPs also play a role in innate immunity (166) and regulation of the inflammatory response (167). NSPs have been shown to augment the inflammatory cytokine response. HLE can induce release of CXCL-8 (168-171), IL-33 and GM-CSF (170). PR-3 has been shown to be able to induce the release of CXCL-8 (171), active IL-18 which can promote PMN activation (172, 173), TNF converting enzyme and IL-1β converting enzyme (174). The NSPs can not only induce cytokine release from other cells but are also able to modify the biological function of cytokines. For example, both TNF-α and IL-6 are cleaved and inactivated by all three of the NSPs (175, 176). PR-3 was also shown to be able to generate more active forms of CXCL-8 whereas HLE was shown to inactivate it (177, 178). HLE has also been shown to cleave IL-2, a potent T-cell activator, into inhibitory fragments (179). NSPs may also function to facilitate PMN transmigration through the endothelium. NSPs could, potentially, be used to degrade various proteins of the ECM and impeding intercellular junctions (180-182). Interestingly though, it has been shown in some studies that inhibition of the various NSPs does not

inhibit neutrophil migration (183), indicating an incomplete understanding of the mechanism by which PMN penetrate the endothelium.

Pathologic regulation of NSPs has been observed in many diseases such as ischemia-reperfusion injury, arthritis and emphysema. As powerful proteases, much of the focus has been on their proteolytic activity. NSPs have been shown to contribute to lung injury due to its ability to degrade both endothelial VE-cadherin, thereby promoting microvascular permeability and E-cadherin which contributes to alveolar flooding (184). HLE has been shown to be associated with fatal sepsis (147) and is thought to function by disrupting endothelial integrity. This junctional disruption could be mediated by NSPs (182, 185).

1.9 Rationale

Pediatric DKA has been linked to numerous intracranial cerebrovascular pathologies such as vasogenic edema and hemorrhagic stroke (19, 186-189). Although the exact mechanisms of these crises are not known, a common element to all cerebrovascular complications is endothelial dysfunction. A prominent theory is that DKA results in the breakdown or destabilization of the brain endothelium, increasing endothelial permeability and allowing solutes and water to cross into the extracellular space. BBB disruption was observed in conjunction with DKA in examination of brain tissue from 2 deceased human patients. In the same study, DKA was associated with decreased expression of numerous tight junction proteins such as occludin, claudin-5, ZO-1 and JAM-A in all areas of the brain, indicating a dysfunction in the formation or composition of the TJs at the BBB (190). Functionally, increased albumin leakage into the brain from peripheral circulation was also observed in the study, providing direct evidence of compromised BBB function.

Pathological MMP activity is widely implicated in BBB disruption (98, 191-193). Increased MMP expression and activity have been seen in severe sepsis, traumatic brain injury, cerebral ischemia, bacterial meningitis, stroke and migraine and have been linked to increased permeabilization of the BBB (96, 192, 194-199). MMPs can contribute to the disruption of the BBB by directly degrading TJs between the CVEC, leading to increased water and solute movement across the BBB (200). Increased MMP-2 and -9 activity and expression was associated with degradation of claudin-5 and occludin in cerebral vessels following ischemic reperfusion injury in rats, indicating decomposition of the TJs (201).

MMPs can also contribute to BBB or any endothelial disruption by releasing permeability-inducing factors that are normally sequestered away in the ECM such as vascular endothelial growth factor (VEGF), thus facilitating an increase in biological activity (202, 203). It has also been demonstrated that MMPs are able to proteolytically activate latent factors such as TGF-β (204), which has been shown to significantly increase endothelial permeability (205).

MMPs are known to play a role in cytokine processing and generation of biologically active fragments (127). Processing of the cytokines, CXCL-8, CXCL-5 and CXCL-6 by both MMP-8 and -9 has been shown to potentiate their chemotactic effects and biological function (206). MMP-mediated cytokine processing acts to facilitate leukocyte activation and migration, thus facilitating endothelial disruption indirectly (127).

Previous work in our lab has shown that DKA is associated with a pro-adhesive phenotype of endothelial cells (41). DKA also results in increased circulating pro-inflammatory cytokines such as CXCL-8, CXCL-1 and IFN-α2, which results in increased activation and adhesion of PMN (37). PMN possess granules full of destructive enzymes which are released upon activation. PMN-derived MMP-9 can facilitate the pathological breakdown of the BBB during ischemic-reperfusion injury (191). The PMN serine proteases, elastase and cathepsin G were shown to be able to induce a disruption of the BBB and increase vascular permeability in rats (207). Additionally, PMN elastase and PR-3 were shown to be able to induce apoptosis of endothelial cells (208, 209). Activated PMN and their associated proteases have been associated with widespread endothelial damage (207, 210, 211). MPO generates extremely powerful oxidizing species such as

hypochlorous acid from H_2O_2 and has been shown to severely disrupt BBB function *in vitro* and *in vivo* (212). It also plays a role in generation of endothelial intracellular oxidative species and drives apoptosis in some cell types (213, 214).

Similar to the MMPs, the PMN protease are known to play a role in the processing of cytokines, rendering them either active or inactive. For example, elastase and PR-3 are known to be able to cleave immunoglobulin, IL-2 receptor (215), TNF-α (175) and TNF-α converting enzyme (174). All 3 serine protease also process CXCL-8 and CXCL-1 (177).

Previous experiments done in our lab have shown leukocyte adherence to the endothelium under DKA conditions indicating significant leukocyte involvement. We believe that the intracranial vascular complications often seen in DKA are the result of the destructive enzymes released by adherent leukocytes during the DKA inflammatory response. Therefore, is it believed that disturbance in the BBB and other endothelial barriers are exacerbated by the presence of DKA through perturbances in circulating leukocyte-derived MMPs as well as azurophilic enzymes.

1.10 Hypothesis

I hypothesize that children with DKA will present with increased plasma leukocyte-derived proteases and enzymes compared to insulin-controlled T1DM. In addition, enzyme levels will correlate with DKA severity and result in brain microvascular junctional disruption.

1.11 Objectives

I. To examine plasma MMPs/TIMPs in pediatric DKA patients and to correlate these with DKA severity.

II. To (1) examine the systemic PMN azurophilic enzyme profile of pediatric DKA patients and to correlate these with DKA severity and (2) to examine the potential for PMN azurophilic enzymes to disrupt brain microvascular intercellular junctions.

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CHAPTER 2: DYNAMIC REGULATION OF PLASMA MATRIX METALLOPROTEINASES IN HUMAN DIABETIC KETOACIDOSIS

A version of this chapter has been submitted for review.

2.1 Introduction

Type 1 diabetes mellitus (T1DM) is a chronic affliction that occurs predominantly in children, and is expected to double over the next decade (1). The most frequent complication of T1DM is diabetic ketoacidosis (DKA), a state of insulin deficiency that leads to metabolic acidosis, hyperglycemia and ketonemia (2). DKA is associated with intracranial cerebrovascular-related complications such as stroke (3), hemorrhage (4) and vasogenic edema (5).

DKA is associated with systemic inflammation (6-9). We recently reported that DKA elicits significant elevations in the chemokines CXCL-1 (GROα) and CXCL-8 (IL-8), resulting in leukocyte adhesion to human-derived cerebral microvascular endothelium (10). Adhered leukocytes can release substances that mediate endothelial damage and vascular destabilization (i.e., matrix metalloproteinases [MMP]) (11).

MMPs are a family of endogenous proteases that include collagenases (MMP-1,-8,-13), stromelysins (MMP-3,-10,-11), matrilysins (MMP-7,-26), gelatinases (MMP-2,-9) and membrane-type MMPs (MMP-14, -15, -16, -17,-24, -25) (12). Leukocytes are a major source of circulating MMPs during inflammation (13). Alterations of MMPs and their endogenous tissue inhibitors (TIMPs) are observed in inflammation-related pathologies (14-16) and MMPs have potential to compromise cerebrovascular endothelial barrier function (17-19).

We hypothesized that DKA is associated with elevated leukocyte-derived MMPs. Our aims, using blood from acute pediatric DKA patients, were (1) to measure plasma

levels of MMPs/TIMPs, (2) to correlate the MMP/TIMP levels with DKA severity, and (3) to determine if MMPs are leukocyte-derived.

2.2 Methods

This study was approved by the Health Sciences Research Ethics Board at Western University. Patients were recruited at our regional tertiary care centre; the Children's Hospital, London Health Sciences Centre (London, ON).

2.2.1 Human Subjects

Consent was obtained from the legal guardians of all pediatric patients admitted with DKA, and both legal guardian consent and patient assent were obtained for T1DM insulin-controlled patients. Biochemical diagnostic criteria for DKA included hyperglycemia > 11 mmol/L, bicarbonate < 15mmol/L and ketonurea (20). DKA is classed according to severity of acidosis as mild DKA (venous pH < 7.3), moderate DKA (pH < 7.2) or severe DKA (pH < 7.1) (21, 22). The majority of DKA cases used in this study were severe. Clinic patients with insulin-controlled T1DM (HbA_{1c} < 10% and no DKA for $\geq$ 3 months) served as controls (CON).

2.2.2 Blood Collection and Processing

Blood for research purposes was obtained on hospital presentation at the time of clinically-indicated blood draws. Blood was drawn into citrate-containing tubes (Vacutainers®, BD Biosciences, Mississauga, ON) by certified nursing personnel, placed on ice, and immediately transferred to the Translational Research Centre facility for processing by standard operating procedures (www.translationalresearch.ca, London, ON) (23, 24). Briefly, blood was centrifuged at 1500 g for 15 min (4^oC), and the upper

plasma layer collected into 250 µl aliquots. Next, the buffy coat was removed and also aliquoted. Both plasma and buffy coat aliquots were immediately frozen at −80 °C until usage. When needed for experiments, plasma was thawed and maintained briefly on ice. Freeze-thaw cycles were avoided.

2.2.3 MMP/TIMP Antibody Microarray

The plasma concentrations of MMP-1, MMP-3, MMP-8, MMP-10, MMP-13, TIMP-1, TIMP-2 and TIMP-4 were measured using a multiplex enzyme-linked immunosorbent assay (ELISA)-based array, the Quantibody® Human MMP array (RayBioTech, Inc.; Norcross, GA) which utilizes a biotin conjugated detection antibody and a streptavidin-labeled Cy3 equivalent dye that produces a fluorescent signal intensity proportional to concentration. Undiluted plasma was loaded into each well and assayed according to the manufacturer's instructions. The completed arrays were then shipped back to the manufacturer for data extraction and analysis.

2.2.4 MMP Gelatin Zymography

MMP-2 and MMP-9 were assayed using gelatin zymography, as we reported previously (16). Plasma samples were diluted 1:1 in PBS. Protein in the diluted plasma samples was then quantified using the bicinchoninic acid assay (Thermo Fisher Scientific, Waltham, MA). Equal amounts of protein (75µg) were mixed with non-reducing sample buffer and loaded in each well. Samples were run on a 10% sodium dodecyl sulphate polyacrylamide electrophoretic gel impregnated with 0.1% gelatin (m/v) under non-reducing conditions at 140V until the dye front left the gel. Enzymes were re-natured in 50mL of 2.7% Triton X-100 (v/v) in distilled water with gentle shaking for 1 hour at room temperature. After washing gels for 5 minutes in 50mL of distilled water on

a rotary mixer, the gels were developed in 50mL of developing buffer (50mM Tris-base pH 7.4, 200mM NaCl, 5mM $CaCl_2 \cdot 2H_2O$) containing 1 cOmplete EDTA-free Protease Inhibitor Cocktail Tablet (Roche Applied Science, Indianapolis, IN) for 20 hours at 37°C (5% CO_2). The gels were then stained with 0.5% Commassie Brilliant Blue R-250 (Roche Diagnostics, Mississauga, ON) for 1 hour and then de-stained with a solution consisting of 30% MeOH (v/v) and 10% acetic acid (v/v) until bands of proteolysis were clear. Gels were scanned using a GS-690 densitometer (Bio-Rad Laboratories, Hercules, CA). Proteolytic bands were quantified as a ratio of the optical density of the experimental band to a known amount of human recombinant active MMP-2 standard (EMD Millipore, Etobicoke, ON) run on the same gel. Quantification of bands was performed using image quantification software (FroggaBio, Toronto, ON). Negative control zymograms were incubated in the presence of 20 mM ethylenediaminetetraacetic acid (Bioshop, Burlington, ON).

Since the zymography technique measures protein as gelatinolytic activity as opposed to concentration, it was necessary to estimate the concentration of MMP-9 from proteolytic bands in a separate experiment. In order to quantify the MMP-9 present in a zymogram, identical volumes (1.0 µL) of three representative age- and sex-matched sample pairs were loaded and run on a zymogram as previously described. A dose-response curve of a known amount of recombinant human active MMP-9 standard (EMD Millipore) was loaded and run on the same gel. The optical density of the proteolytic bands were measured as previously described and the standard dilutions were used to generate a standard curve to quantify the mass of MMP-9 present in a given proteolytic band. The plasma concentration (ng/mL) of MMP-9 in each experimental condition was

estimated by adjusting the average mass of MMP-9 in the zymogram for the volume of plasma initially loaded in the gel and the dilution factor.

2.2.5 Quantitative Real-Time PCR

Quantitative real-time polymerase chain reaction (qPCR) was used to quantify the expression of the genes found to be significantly altered in zymography and the ELISA array. RNA was isolated using TRIzol® LS Reagent (Life Technologies, Grand Island, NY) from buffy coat extracted from CON and DKA patients then subjected to Turbo DNA-free (Life Technologies) digest according to the manufacturer's suggested protocol. The RNA integrity number (RIN) of the samples was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON) at the London Regional Genomics Center, samples had an RIN $\geq$ 6.7. 2µg of RNA from each sample was then reverse transcribed using iScript™ Advanced Reverse Transcriptase (Bio-Rad). TaqMan® Gene Expression Assays (Life Technologies) were used with the SensiFAST™ Probe No-ROX kit (Bioline, London, UK) for qPCR. The assay IDs were MMP-2 (Hs01548727_m1), MMP-8 (Hs01029058_m1), MMP-9 (Hs00957562_m1) and TIMP-4(Hs00162784_m1). Target genes were normalized to β-actin (Hs01060665_g1) and GAPDH (Hs02758991_G1). The reaction was carried out with CFX96 Real-Time PCR Detection System-IVD (Bio-Rad).The PCR protocol was as follows: 2 minute initial denaturation at 95°C, 70 cycles of 10 second denaturation at 95°C, 30 second annealing and extension at 60°C.

2.2.6 Statistical Analysis

All calculations were done using Graphpad Prism software. Data was assessed with the Mann-Whitney U test for nonparametric data with Bonferroni’s correction for

multiple comparisons when applicable. Correlation analysis utilized Spearman's rank correlation coefficient. All data is presented as mean ± standard error (SEM). Statistical significance utilized a P value of < 0.05.

2.3 Results

Study patients

Plasma was obtained from T1DM patients either in an acute DKA or in an insulin-controlled state (CON). The two groups were age- and sex-matched (n=16/group; Table 2-1). Patients with DKA had significantly higher HbA_{1c} values, compared to those with insulin-controlled T1DM ($P<0.001$), indicating elevated blood glucose over the previous three months. DKA patients all had elevated blood glucose (27.4±8.3 mmol/L) and moderate to severe metabolic acidosis (pH 7.00±0.03; PCO_2 20.8±2.3 mmHg; HCO_3^- 5.9±0.8 mmol/L; lactate 2.9±0.7 mmol/L).

ELISA array measurements of plasma MMPs/TIMPs

In order to generate an MMP/TIMP profile, the levels of MMP and TIMP species were measured in plasma from both DKA and CON groups. Out of all the plasma MMP and TIMP species measured on the array, only MMP-8 and TIMP-4 were found to be significantly increased in DKA (17.1-fold increase and 2.1-fold increase, respectively), as compared to CON (Table 2-1; $P < 0.001$; n=16 per group). These results demonstrate selective elevation of the collagenase MMP-8, and to a lesser extent, TIMP-4.

TABLE 2-1. Clinical Data and Plasma MMP/TIMP Levels in Type-1 Diabetes Patients.

Either insulin-controlled (CON) or with acute diabetic ketoacidosis (DKA) (n= 16/group)

	CON	DKA	P value
Clinical Data			
Mean age (years)	11.4 ± 1.0	11.7 ± 0.8	0.830
Male: Female ratio	8:8	8:8	1.000
HbA_{1c}	8.3 ± 0.3	11.7 ± 0.5	**< 0.001**
MMP/TIMP Microarray			
MMP-1	1.22 ± 0.72	2.24 ± 1.18	0.865
MMP-3	6.04 ± 1.34	9.45 ± 1.72	0.086
MMP-8[a]	39.96 ± 12.90	684.25 ± 307.59	**< 0.001**
MMP-10	0.28 ± 0.04	0.60 ± 0.16	0.181
MMP-13	0.03 ± 0.01	0.04 ± 0.02	0.201
TIMP-1	5.46 ± 1.75	10.70 ± 2.28	0.318
TIMP-2	4.42 ± 0.37	4.71 ± 0.47	0.925
TIMP-4	1.08 ± 0.12	2.26 ± 0.22	**< 0.001**

a; Concentrations of MMP-8 are reported in pg/mL (mean ± SEM). All the rest are all reported in ng/mL (mean ± SEM). To control for repeated measures in MMP/TIMP analyses, a P value<0.005 was considered significant. Boldface indicates significant P value.

Gelatin zymography for MMP-2 and MMP-9

To assess MMP-2 and MMP-9 levels in DKA, we assayed gelatinolytic activity of both CON and DKA plasma via zymography. A representative zymogram of 3 different CON/DKA sample pairs is shown in Figure 2-1A. MMP-9 was significantly increased 1.7 fold in DKA plasma compared to CON plasma (Figure 2-1B; ~100 kDa MW; $P < 0.05$). Conversely, MMP-2 was significantly decreased 2.8 fold in DKA plasma compared to CON plasma (Figure 2-1C; ~70 kDa MW; $P < 0.001$). In addition, DKA plasma had a faint ~135 kDa band on gelatin zymography that may reflect a well-described complex of MMP-9 with neutrophil-gelatinase-associated lipocalin (25). Overall, these results suggest distinct opposing regulation of the MMP gelatinases by DKA in children, and a potential neutrophilic source of MMP-9.

The plasma concentration of MMP-9 was estimated from zymography (data not shown) using 3 age-/sex-matched sample pairs (CON ~670 ng/mL vs. DKA ~1,509 ng/mL).

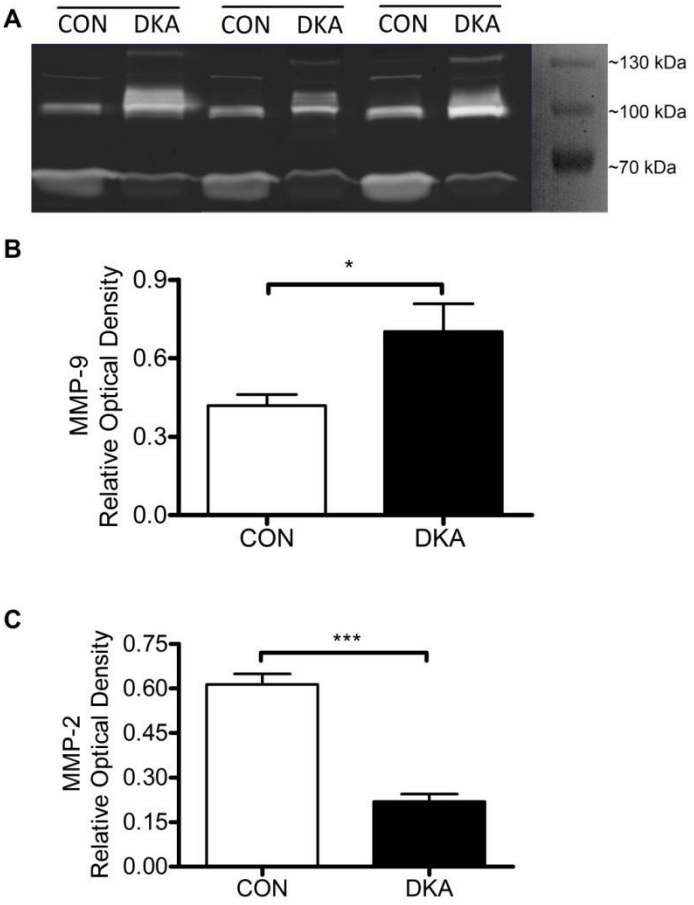

FIGURE 2-1. Gelatin Zymography of DKA Plasma. Gelatin zymography was used to measure both MMP-2 and MMP-9. **(A)** A representative zymogram of 3 plasma DKA/CON experimental pairs. The right-most lane contains a molecular weight ladder. MMP-9 is represented at ~100 kDa, likely representing the latent form. MMP-2 is represented by the bands at ~70 kDa, also representing the latent form. **(B)** MMP-9 levels in DKA plasma were significantly increased relative to CON plasma. Results are presented as relative optical density. **(C)** MMP-2 levels in DKA plasma were significantly decreased from CON plasma. Results are presented as relative optical density. *P<0.05; ***P<0.001; n= 16/group.

MMP-8 and MMP-9 correlated with DKA severity

In order to determine correlations between altered MMP/TIMP levels in DKA plasma and a clinically relevant parameter, correlation analysis was applied to data points graphed as MMP concentrations versus blood pH (acidotic blood pH reflects greater DKA severity). Both MMP-8 and MMP-9 were inversely correlated with blood pH (Figure 2-2A; r_s= -0.71; $P < 0.01$ and Figure 2-2B, r_s= -0.60, $P < 0.05$, respectively). In contrast, no correlations could be determined for either MMP-2 or TIMP-4 (r_s= 0.26, P= 0.34 and r_s= 0.07, P= 0.80, data not graphically shown). These results suggest that MMP-8 and MMP-9 plasma levels are correlated with DKA severity.

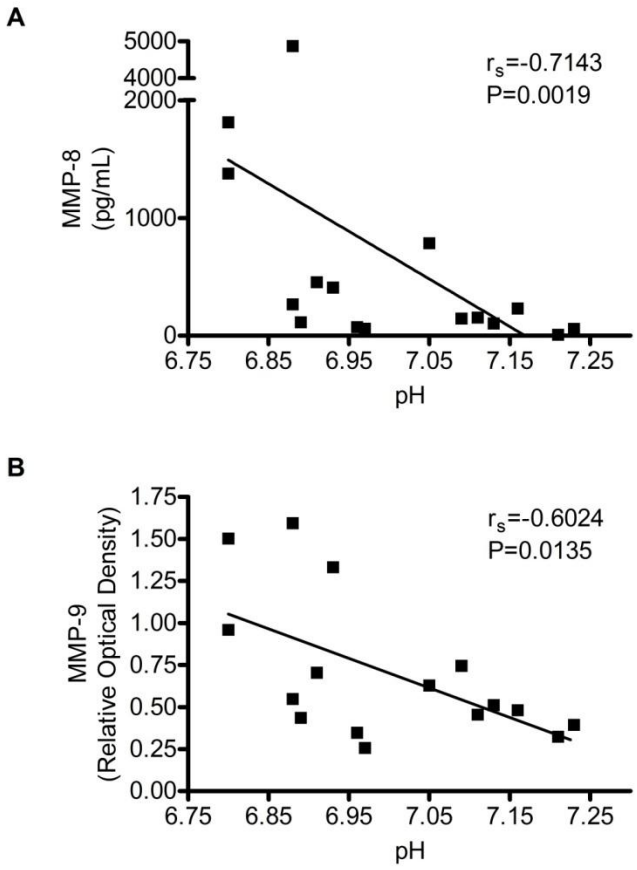

FIGURE 2-2.Plasma MMP Correlation with pH in DKA. MMPs correlate with DKA severity. **(A)** MMP-8 plasma concentrations were inversely correlated with blood pH in DKA patients. A best-fit line was added to aid in visual interpretation of the graphs (r_s= -0.714, P= 0.0019, n= 16). **(B)** MMP-9 plasma concentrations were inversely correlated with blood pH in DKA patients. A best-fit line was added to aid in visual interpretation of the graphs (r_s= -0.6024, P= 0.0135; n= 16).

Leukocyte mRNA expression of MMPs and TIMPs

To determine whether the observed changes in MMP-2, MMP-8, MMP-9 and TIMP-4 were leukocyte derived, we assayed the respective gene mRNA levels with qPCR. MMP-8, MMP-9 and TIMP-4 gene expression was significantly increased in leukocytes from DKA patients, as compared to CON patients (Figure 2-3; 45.0, 6.3 and 31.8 fold-change respectively; $P < 0.0025$). In contrast, MMP-2 showed no significant changes. These results suggest that elevated plasma MMP-8, MMP-9 and TIMP-4 are, at least in part, leukocyte derived.

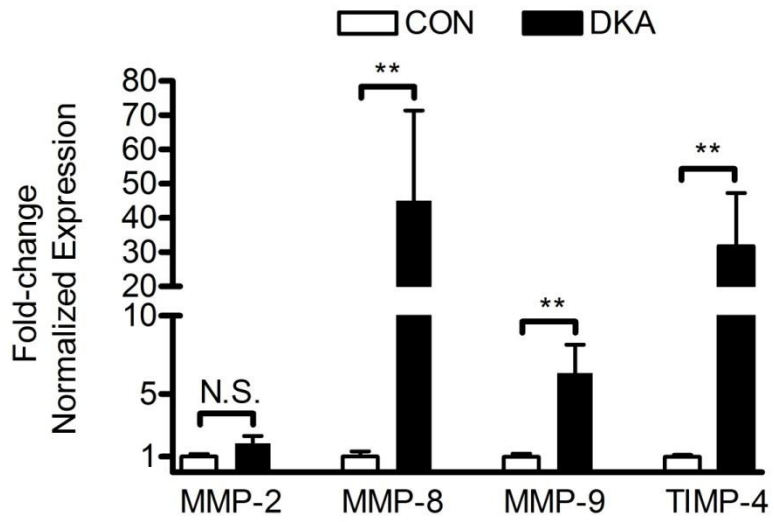

FIGURE 2-3. Leukocyte MMP Gene Expression in DKA. Elevated MMPs/TIMP-4 in DKA plasma are leukocyte-derived. Gene expression of MMP-8, MMP-9 and TIMP-4 were significantly elevated in DKA, as compared to CON patients. Target gene expression is normalized to β-actin and GAPDH and presented as fold-change from CON. N.S.: not significant; **P < 0.005 (controlled for repeated measures); n= 5/group for MMP-2, n= 7/group for all other genes. Mean CT values for CON and DKA samples were MMP-2 (37.73; 37.35), MMP-8 (29.91; 35.09), MMP-9(25.83; 28.11), TIMP-4 (39.40; 43.08), respectively.

2.4 Discussion

In this study, we report that DKA induced a markedly altered plasma MMP/TIMP profile in acute pediatric DKA patients, and that both MMP-8 and MMP-9 concentrations correlated with DKA severity. DKA-induced MMPs were, at least in part, leukocyte derived. MMP-8 and MMP-9, at concentrations similar to those measured in DKA plasma, have been reported to disrupt brain endothelial cell tight junctions. To our knowledge, this study is the first to employ human DKA tissues in relation to pathological changes in systemic proteases.

Our current data, taken together with our previously reported results (10), show that DKA is an inflammatory state associated with highly elevated levels of systemic cytokines (IL-6 and IFN-α2), chemokines (CXCL-1 and CXCL-8) and MMPs (i.e., MMP-8 and MMP-9). MMPs have diverse actions that that may involve exacerbation of existing inflammatory cascades (26), as well as direct actions on the microvasculature (27). This latter action of MMPs is particularly intriguing given that DKA induced leukocyte adherence to the brain microvasculature (10) and that the DKA-induced MMPs appear to be leukocyte-derived.

MMP-8 was significantly elevated in DKA plasma and plasma MMP-8 concentrations correlated with DKA severity. MMP-8 mRNA was elevated in leukocytes from DKA patients, a finding that is consistent with the almost exclusive production of MMP-8 by neutrophils (28, 29). The primary substrates for MMP-8 are the fibrillary collagens, I, II, III, as well as other extracellular matrix components (13). Vascular leukocyte trafficking and blood brain barrier disruption are mediated by MMP-8, and may involve cleavage of the endothelial cell tight junction proteins (30, 31). Our results

raise the possibility that the DKA-induced elevations in MMP-8 may contribute to cerebrovascular endothelial perturbations.

MMP-9 was significantly increased in DKA plasma and plasma MMP-9 activity on zymogram correlated with DKA severity. The 100 kDa MMP-9 band suggests that the plasma MMP-9 is primarily a latent form (32, 33), but it is available for immediate activation. Neutrophils are a likely source of MMP-9 in DKA plasma given that increased MMP-9 mRNA was found in our leukocyte preparation, that a ~135 kDa band was observed on zymography that likely reflects a well-described complex of MMP-9 with neutrophil-gelatinase-associated lipocalin (25), and that early rises in plasma MMP-9 have been attributed to neutrophil degranulation of stored MMP-9 (34). Delayed sources of plasma MMP-9 may include de novo synthesis via monocytes, lymphocytes, dendritic cells and endothelial cells (35). CXCL-8, known to be elevated in DKA, stimulates expression of MMP-9 (36, 37). MMP-9 can proteolytically degrade virtually all components of the extracellular matrix, and MMP-9 has the ability to disrupt components of junctional complexes in the brain microvasculature (27, 38).

Our data suggests that plasma MMP-2 (~70 kDa) is decreased in DKA, possibly due to the hyperglycemic conditions that are characteristic of DKA (39). Decreased MMP-2 facilitates increased extracellular matrix deposition in vascular structures as a primary function of MMP-2 is extracellular matrix turnover (40). Indeed, decreased plasma levels of MMP-2 during DKA may contribute to chronic thickening of basal lamina and pathologic remodeling of the extracellular matrix over multiple DKA episodes.

Plasma TIMP-4 was also mildly elevated in DKA and was at least partially leukocyte-derived. Increases in TIMP-4 are likely due to increased monocytic expression. While TIMP-4 is considered a broad MMP inhibitor, it also displays some specificity against MMP-2 (41) and has the ability to prevent cell surface activation of MMP-2 (42). Depressed levels of MMP-2, in conjunction with elevated plasma TIMP-4, could facilitate extracellular matrix deposition.

The actions of elevated plasma MMP-8 and MMP-9 in DKA are largely unknown. The main role attributed to MMPs during inflammation is its proteolytic ability on cellular junctions, however, MMPs are now known to play a role in cytokine processing and generation of biologically active or inactive fragments (26). Processing of the DKA-relevant chemokine CXCL-8 (10), by both MMP-8 and MMP-9, has been shown to potentiate its chemotactic effects and biological function (43), MMP-mediated cytokine processing acts to facilitate leukocyte migration, thus facilitating endothelial disruption indirectly (26).

Higher concentrations of plasma MMP-8 and MMP-9 were associated with greater DKA severity (lower pH). Acidic pH directly stimulates the induction and release of cellular MMPs (44-47), and a lower pH is associated with markedly more MMP-8 and MMP-9 catalytic activity (44, 48-50). Thus, DKA patients, by virtue of their blood acidic pH, are particularly susceptible to the deleterious actions of MMP-8 and MMP-9. DKA correction with intravenous fluid and exogenous insulin administration normalizes the blood pH. While MMP-8 activity would decrease with increasing pH to normal values (48, 50), MMP-9 still maintains much of its catalytic activity (44, 49). In contrast, MMP-

2 is noted to have decreased activity in acidic pH conditions (51), perhaps resulting in further reduced MMP-2 functional capacity.

MMPs have been shown to increase permeability of human brain microvascular endothelium (i.e., vasogenic edema) (18, 19), likely by degrading tight junction proteins, including occludin, claudin-5 and ZO-1 (17). Active MMP-9 has been most thoroughly investigated, and exerts actions at concentrations (100-250 ng/ml) that are compatible with those measured for latent MMP-9 in DKA plasma (1,509 ng/ml) (17, 18). The sequential addition of MMPs (i.e., MMP-8) has been demonstrated to boost substrate degradation (52).

Our study has several limitations worthy of discussion. First, our data showed a leukocyte origin of the MMP-8 and MMP-9, but we cannot rule out other cellular contributions (i.e., endothelial cells). Future studies should try to isolate leukocyte subtypes for analyses from fresh blood samples. Second, the measured concentrations of MMP-8 and MMP-9 might not represent the exact concentrations encountered at the brain endothelial cell layer. The MMP concentrations adjacent to leukocytes adhered to the brain microvascular endothelium are almost certainly higher, but we have no way of measuring concentrations in such localized domains.

In summary, our data show for the first time that DKA is a disease state associated with dynamic regulation of plasma MMPs. In particular, leukocyte-derived MMPs (e.g., MMP-8 and MMP-9) correlated with disease severity and they have the potential to degrade key components of the cerebrovascular tight junctions, perhaps explaining the susceptibility of children with DKA to intracranial complications. Our

data, provide a plausible mechanism for DKA-induced cerebrovascular perturbations, and represents a possible link between DKA mediated inflammation and DKA cerebrovascular crises.

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CHAPTER 3: ELEVATED LEUKOCYTE AZUROPHILIC ENZYMES IN HUMAN DIABETIC KETOACIDOSIS PLASMA DEGRADE CEREBROVASCULAR ENDOTHELIAL JUNCTIONAL PROTEINS

3.1 Introduction

Type 1 diabetes mellitus (T1DM) is a chronic affliction that occurs primarily in children, whose diagnosis has been occurring at an increasingly younger age (1, 2). A serious complication of T1DM is diabetic ketoacidosis (DKA), which results from severe insulin deficiency. DKA refers to the triad of metabolic acidosis, hyperglycemia and ketonemia (3). Intracranial cerebrovascular complications such as stroke (4), hemorrhage (5, 6) and cerebral edema (7, 8) have all been shown to be associated with DKA in children. Previous work in our lab has shown that DKA is associated with a systemic inflammatory response that stimulates polymorphonuclear neutrophil (PMN) adherence to the brain microvascular endothelium via CXCL-1/CXCL-8 (9, 10).

PMN antimicrobial/proteolytic enzymes are normally stored safely in azurophilic granules (11). Upon inflammatory stimulation, PMN undergo degranulation and granules containing azurophilic enzymes (e.g., leukocyte elastase [HLE], proteinase-3 [PR-3], cathepsin G [CTSG] and myeloperoxidase [MPO]) are released by activated circulating or adherent PMNs. Elevated azurophilic enzymes produced by adherent PMNs may contribute to degradation of the vascular element and potentially contribute to destabilization of the BBB (12, 13).

We hypothesized that DKA is associated with elevated PMN azurophilic enzymes and that some or all of these enzymes may lead to BBB dysfunction. Thus, our aims using human DKA blood were: (1) to measure plasma levels of PMN azurophilic enzymes, (2) to determine any relationships between enzyme levels and DKA severity and (3) to determine the consequential effects of enzyme levels on cerebrovascular endothelial junctional proteins *in vitro*.

3.2 Methods

This study was approved by the Health Sciences Research Ethics Board at Western University. Patients were recruited at our regional tertiary care centre; the Children's Hospital, London Health Sciences Centre (London, ON).

3.2.1 Human Subjects

Consent was obtained from the legal guardians of all pediatric patients admitted with DKA, and both legal guardian consent and patient assent were obtained for type-1 diabetes control patients. Biochemical diagnostic criteria for DKA include hyperglycemia > 11 mmol/L, bicarbonate < 15 mmol/L and ketonurea (14). DKA is classed according to severity of acidosis as mild DKA (venous pH < 7.3), moderate DKA (pH < 7.2) or severe DKA (pH < 7.1) (15, 16). A majority of DKA cases used in this study were severe. Clinic patients with insulin controlled type-1 diabetes (HbA_{1c} < 10% and no DKA for at least 3 months) served as controls (CON).

3.2.2 Blood Collection and Processing

Blood for research purposes was obtained on hospital presentation at the time of clinically-indicated blood draws. Blood was drawn into citrate-containing tubes (Vacutainers®, BD Biosciences, Mississauga, ON) by certified nursing personnel, placed on ice, and immediately transferred to the Translational Research Centre facility for processing by standard operating procedures (www.translationalresearch.ca, London, ON) (17, 18). Briefly, blood was centrifuged at 1500 g for 15 min ($4^{o}C$), and the upper plasma layer was collected in 250 µl aliquots and frozen at −80 °C. Thawed plasma was maintained on ice for short periods prior to use in experiments and freeze-thaw cycles were avoided.

3.2.3 Polymorphonuclear Neutrophil Enzyme Measurement

The plasma concentrations of several major PMN-associated enzymes were measured via enzyme-linked immunosorbent assay (ELISA) in patients. We measured the plasma concentration of the serine proteases human leukocyte elastase (HLE; 1:100 dilution; Abcam, Cambridge, UK), proteinase-3 (PR-3; 1:30 dilution; Cloud-Clone Corp., Houston, TX) and cathepsin G (CTSG; undiluted; MyBioSource, Inc., San Diego, CA). The plasma concentration of myeloperoxidase (MPO; 1:1 dilution; Abcam) as a marker of PMN activation was also assessed. Concentrations were adjusted for dilution factor.

3.2.4 Cell Culture

Primary human brain endothelial primary cells (hBMEC) were kindly provided by Dr. Mahmud Bani (NRC, Ottawa, ON) and used as a model of brain microvascular endothelium in vitro. hBMEC were cultured in EBM-2 Endothelial Growth Basal Medium (Clonetics®; Lonza) supplemented with Clonetics® EGM®-2 MV SingleQuots kit (Lonza), 1% GA-1000 (Lonza) on gelatin (0.5% w/v in water; Sigma-Aldrich) coated plates. The cells were maintained at 37°C in a humidified atmosphere with 5% CO2 and reseeded when the cell monolayer became sub-confluent. hBMEC at passage 2-15 were used in all experiments. For treatments, hBMEC were seeded into 24-well plates (8.0×10^5 cells/well) and grown until confluence.

3.2.5 *In vitro* Experimental Approach

hBMEC monolayers were treated with human neutrophil PR-3 (Athens Research & Technology, GA), HLE (Abcam) and MPO (Abcam) diluted in serum-free VascuLife® basal medium (VL; Lifeline Cell Technology). Growth media was aspirated and wells were treated with basal medium only (-), HLE (2 µg/mL) (19), PR-3 (5 µg/mL) (20, 21)

or MPO (35 ng/mL) (22) by themselves or all in combination (Total) for 1 hour at 37°C (5% CO_2). MPO-treated wells (MPO and Total) were supplemented with 80 µM H_2O_2 to provide a substrate for free radical generation.

3.2.6 Immunoblotting

Degradation of zonula occludens-1 (ZO-1) and β-catenin were assessed using immunoblotting. Following protease treatment, wells were washed 3 times with ice-cold phosphate buffered saline (PBS) then lysed in 200µL of hot SDS-electrophoresis sample buffer. Samples were then stored at -20°C. Prior to running, samples were boiled for 5 minutes then electrophoresed with 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred overnight to a BioTrace polyvinylidene diflouride (PVDF) membrane (Pall Corporation, Port Washington, NY). Following transfer, the membranes were blocked in 5% skim milk in 0.1% Tween tris-buffered saline (TBST) for 1 hour at room temperature. Membranes were then incubated with primary antibody, polyclonal rabbit anti-ZO-1 (mid) 1:2000 (Invitrogen, Carlsbad, CA) or polyclonal rabbit anti-β-catenin 1:4000 (Abcam, Cambridge, UK) in 2% skim milk in tris-buffered saline (TBS) for 2 hours at room temperature. The membrane was then washed 3 times for 5 min each with TBST then incubated with secondary horseradish peroxidase-conjugated antibody, goat anti-rabbit (Invitrogen) in 2% skim milk in TBS for 1 hour at room temperature. Finally, membranes were washed for 5 min twice and then 10 min with TBS. For a loading control, each membrane was also probed with monoclonal anti-lamin-β1 (Abcam). Bound target antibodies were visualized using enhanced chemiluminescence detection (2.5mM Luminol, 0.4mM p-coumaric acid, 0.02% H_2O_2 in 100mM Tris buffer; Sigma-Aldrich). Images of the immunoblots were captured using a MicroChemi imaging system

(Froggabio) and band quantification was done with GelQuant Pro Software (Froggabio). Target bands were quantified as an optical density (O.D.) ratio to its corresponding lamin-β1 band.

3.2.7 Quantification of Monolayer Detachment

To determine whether there was significant hBMEC detachment following PMN enzyme treatment, we quantified the number of cells present in the culture media following treatment. hBMEC were grown on gelatin-coated 24-well plates until the monolayer reached confluency. Cells were treated for 1 hour at 37°C with PMN enzymes. Subsequently, the culture media from wells were collected into microtubes. Samples were centrifuged at 20,000 g for 10 minutes, supernatant discarded and pellets were resuspended in cold 0.4% Trypan Blue in PBS. Cells were resuspended in a much smaller volume (10X concentrated) relative to the original sample volume to allow for a more accurate cell counting. The number of cells in each sample was counted using a Haemocytometer (Hausser Scientific, Horsham, PA) and expressed as the number of cells detached per well.

3.2.8 Statistical Analysis

Data was screened for normality and assessed with either the Mann-Whitney U test (nonparametric data) or the Student's t-test (parametric data). For multiple comparisons, the Kruskal-Wallace test with post-hoc Tukey test (nonparametric data) or a One-Way Analysis of Variance (ANOVA) test with post-hoc Holm-Sidak test (parametric data) was used. Correlation analysis utilized the Spearman's rank correlation coefficient. All data is presented as mean ± SEM, statistical significance ($P < 0.05$).

3.3 Results

Study patients

Plasma was obtained from type-1 diabetes patients either in acute DKA or an insulin-controlled state (CON). The two groups were age- and sex-matched (Table 3-1; n= 16 patients/group). Patients with DKA had significantly higher HbA_{1C} values, compared to those with controlled type-1 diabetes ($P < 0.001$), indicating elevated blood glucose over the previous three months. DKA patients all had elevated blood glucose (27.4 ± 8.3 mmol/L) and metabolic acidosis on blood gas measurements (pH 7.00 ± 0.03; PCO_2 20.8 ± 2.3 mmHg; HCO_3^- 5.9 ± 0.8 mmol/L; lactate 2.9 ± 0.7 mmol/L).

DKA patients had an elevated complete blood leukocyte count (Table 3-2; n= 16) relative to age-specific normal ranges. Specifically, all DKA patients had neutrophilia and monocytosis (n= 14; Two patients did not have a differential WBC count done).

TABLE 3-1. Human Clinical and Biochemical Data for Type-1 Diabetes Patients

Insulin Controlled (CON) or with Acute Diabetic Ketoacidosis (DKA). (n= 16/group)

	CON	**DKA**	**P value**
Mean age (years)	11.4 ± 1.0	11.7 ± 0.8	0.83
Male: female ratio	8:8	8:8	1.00
HbA_{1c} (%)	8.3 ± 0.3	11.7 ± 0.5	**< 0.001**
pH	N/A	7.00 ± 0.03	
HCO_3^- (mmol/L)	N/A	5.9 ± 0.8	
pCO_2 (mmHg)	N/A	20.8 ± 2.3	
Lactate (mmol/L)	N/A	2.9 ± 0.7	
Blood glucose (mmol/L)	N/A	27.4 ± 8.3	

Data presented as mean ± SEM.

a; Boldface indicates significant P value ($P < 0.05$). N/A; data not gathered

TABLE 3-2. Differential White Blood Cell Count for DKA Patients

Cell type	n= 16	Healthy Reference Range	Status
Leukocytes, total ($x10^9$/L)	22.99 ± 2.16	4.0-10	Elevated
Neutrophils ($x10^9$/L)	18.43 ± 1.96	4.0-5.3	Elevated
Lymphocytes ($x10^9$/L)	2.04 ± 0.17	1.4-4.0	Normal
Monocytes ($x10^9$/L)	1.54 ± 0.19	0.2-0.8	Elevated
Eosinophils ($x10^9$/L)	0.02 ± 0.01	0.0-0.8	Normal
Basophils ($x10^9$/L)	0.11 ± 0.01	0.0-0.1	Normal

Data presented as mean ± SEM.

Polymorphonuclear neutrophil enzyme measurements

In order to assess PMN degranulation, plasma levels of the major azurophilic granule enzymes were investigated. The concentration of HLE, PR-3, CTSG and MPO was measured in DKA and CON plasma. It was found that HLE was significantly increased in DKA plasma (85.99 ng/mL) compared to CON plasma (31.61 ng/mL; $P < 0.001$; Figure 3-1A). PR-3 was significantly increased in DKA plasma (27.04 ng/mL) compared to CON plasma (8.87 ng/mL; $P < 0.001$; Figure 3-1B). CTSG was assessed but found to be not significantly changed between CON and DKA plasma ($P=0.81$; Figure 3-1C). MPO, a major component of the PMN oxygen-dependent antimicrobial pathway, was significantly increased in DKA plasma (5.49 ng/mL) compared to CON plasma (3.25 ng/mL) ($P < 0.001$; Figure 3-1D).

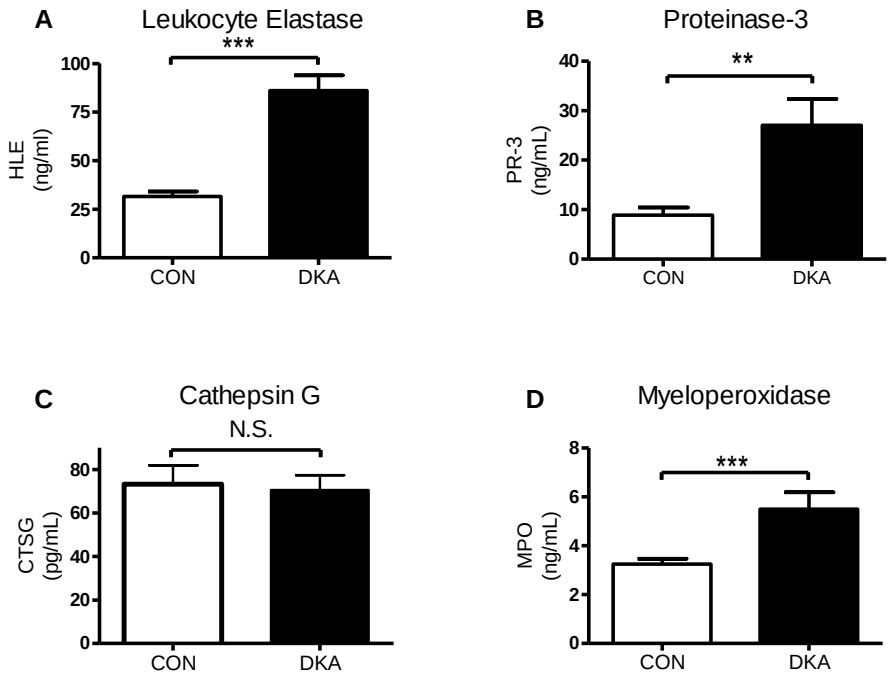

FIGURE 3-1. Plasma Leukocyte Azurophilic Enzymes in DKA. DKA plasma was associated with increased circulating PMN enzymes. Circulating HLE increased in DKA plasma compared to CON plasma **(A)**. The average concentrations in CON and DKA plasma were 31.6 ng/mL and 85.99 ng/mL, respectively. PR-3 was also increased significantly in DKA plasma compared to CON plasma **(B)**. The average concentrations for CON and DKA were 8.87 ng/mL and 27.04 ng/mL, respectively. CTSG was not significantly changed between CON and DKA groups **(C)**. Finally, MPO increased in DKA plasma compared to CON **(D)**. Observed concentrations were 5.49 ng/mL and 3.25 ng/mL, respectively. N.S. not significant; **P < 0.01; ***P < 0.001; n= 14 per group (**A**); n= 15 per group **(B)**; n= 16 per group (**C**, **D**).

PR-3 was inversely correlated with blood pH

In order to determine the relationship between level of significantly altered plasma PMN azurophilic enzymes in DKA samples and disease severity, correlation analysis was applied to data points graphed as concentration versus blood pH which was used as a surrogate of DKA severity. Plasma PR-3 (Figure 3-2B) concentration in DKA was found to be significantly inversely correlated with blood pH ($r_s = 0.73$, $P < 0.01$). In contrast, no correlation could be detected for HLE (Figure 3-2A) or MPO (Figure 3-2C) ($r_s = -0.24$, $P = 0.41$ and $r_s = 0.06$, $P = 0.82$, respectively).

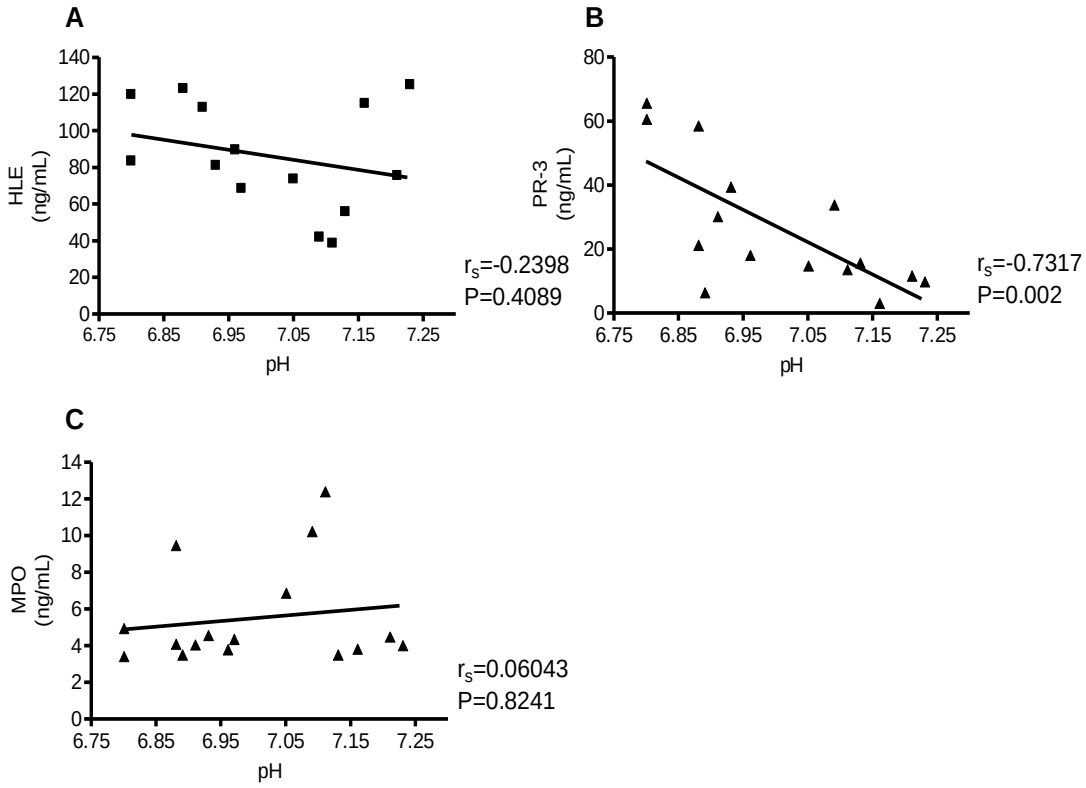

FIGURE 3-2. Plasma PMN Azurophilic Enzyme Correlation with pH in DKA. PR-3 was significantly correlated with disease severity. Correlation analysis was applied to the concentration of the 3 significantly increased PMN azurophilic enzymes, HLE, PR-3 and MPO in DKA plasma with blood pH. Only PR-3 concentration displayed significant inverse correlation with blood pH (r_s= -0.7317, P= 0.002, n= 15) **(B)**. Both HLE (r_s= -0.24, P= 0.41, n= 14) **(A)** and MPO (r_s= 0.06, P= 0.82, n= 16) **(C)** were not significantly correlated with blood pH. A line of best fit was added to the graphs to aid in interpretation of the data points.

PMN enzymes disrupt ZO-1 and β-catenin in vitro

Elevated PMN enzymes were applied to hBMEC monolayers in order to assess their effect on protein junctions. Specifically, we investigated degradation of both ZO-1 and β-catenin, critical structural elements of TJs and AJs, respectively. PR-3 and total enzyme mixture both significantly decreased ZO-1 expression (Figure 3-3A, B; $P < 0.05$, $P < 0.01$, respectively). No changes were seen in regards to ZO-1 degradation products. PR-3 and total enzyme mixture also both significantly increased the appearance of smaller molecular weight β-catenin degradation products (Figure 3-3C, D; $P < 0.01$ and $P < 0.001$, respectively). No significant changes were seen with regard to full length β-catenin. H_2O_2 by itself as a control did not have an effect on either ZO-1 or β-catenin, full length or degradation (Data not shown). The changes in protein levels were not due to cell detachment from the monolayer as there were no changes in the number of detached cells in treatment media following treatment ($P= 0.60$; $n= 3$; Data not shown).

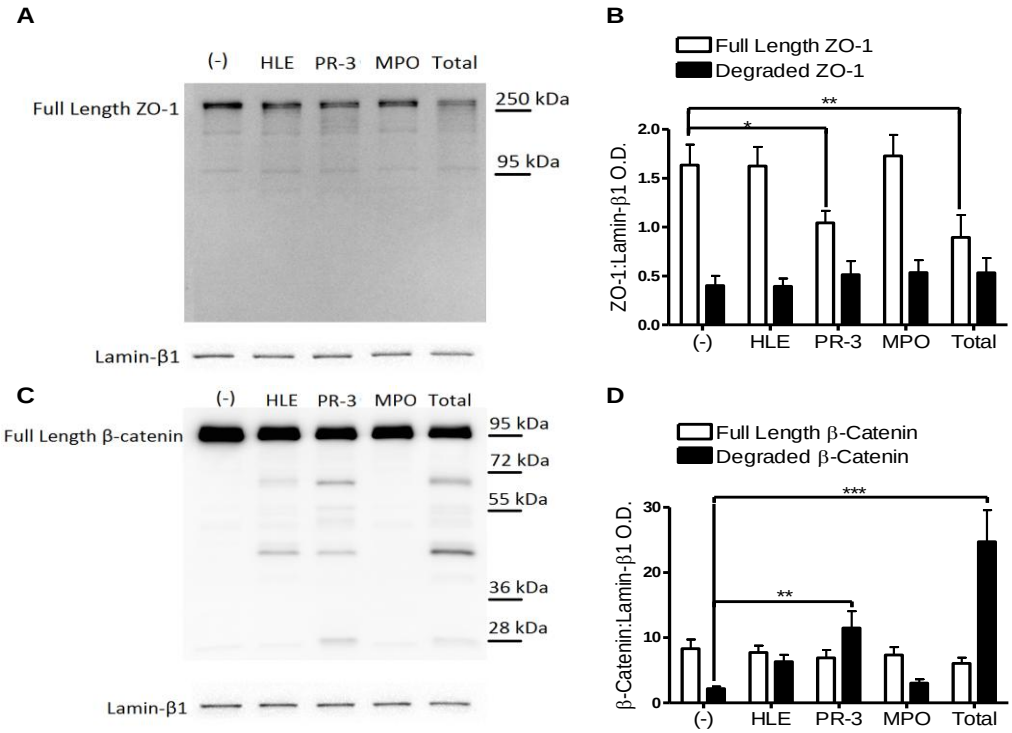

FIGURE 3-3. Enzyme-Mediated Degradation of ZO-1 and β-Catenin. An enzyme cocktail mixture consisting of HLE, PR-3 and MPO was able to degrade β-catenin. hBMEC monolayers were treated with a combination of significantly elevated PMN azurophilic enzymes; (-), basal media only; HLE, 2 µg/mL HLE; PR-3, 5 µg/mL PR-3; MPO, 35 ng/mL MPO + 80 µM H_2O_2; Total, 2 µg/mL HLE + 5 µg/mL PR-3 + 35 ng/mL MPO + 80 µM H_2O_2. Representative blots for ZO-1 and β-catenin are shown in (**A)** and **(C)**, respectively. Full length ZO-1 expression **(B)** was significantly decreased by PR-3 treatment and total enzyme treatment whereas full length β-catenin **(D)** was not significantly altered. No significant ZO-1 degradation products were observed **(B)**. Total enzyme mixture and PR-3 treatment resulted in an increase of a range of smaller molecular weight β-catenin degradation bands **(D)**. *P < 0.05, **P < 0.01, ***P < 0.001; n= 7-8.

3.4 Discussion

In this present study, we report that DKA is associated with elevated neutrophils and induces increased PMN degranulation. While HLE, PR-3 and MPO were all elevated in DKA plasma, only PR-3 was positively correlated with DKA severity. PR-3 alone, or in combination with the other PMN enzymes, resulted in decreased expression of the tight junction protein ZO-1 and degradation of the adherens junction protein β-catenin *in vitro*. To our knowledge, this study is the first to employ human DKA tissues in relation to pathological changes in systemic PMN azurophilic enzymes.

In this study, the differential white blood cell count of DKA patients showed monocytosis and neutrophilia, consistent with previous reports of increased circulation of leukocytes during DKA (23, 24). Also, we show that PMN in acute DKA patients undergo degranulation with the release of azurophilic granule-specific enzymes, HLE, PR-3 and MPO. Ubiquitous PMN activation in DKA patients is in direct agreement with our previous work that showed increased PMN-endothelial adherence due to the cytokines CXCL-1 and CXCL-8 (10) as well as DKA-induced regulation of leukocyte-derived MMPs (Chapter 2).

The increased circulating enzymes, HLE, PR-3 and MPO, are relevant to intracerebral complications as there is evidence linking them to endothelial perturbation (25-28). HLE can disrupt junctional proteins such as E-cadherin (19, 25, 29) and β-catenin (30) and break down components of the extracellular matrix (31, 32). HLE has also been shown to facilitate PMN transmigration, thereby contributing to endothelial disruption indirectly (33). HLE can also activate MMP-9 (34, 35), thus potentiating the destructive effects of the MMPs that we have shown to be elevated in DKA plasma

(Chapter 2). The presence of extracellular PMN enzymes may also affect endothelial stability indirectly; HLE has also been shown to induce CXCL-8 expression (36, 37) which has been shown to facilitate PMN adherence to the endothelium in DKA (10).

PR-3 is also involved in PMN transmigration (38), and MMP activation (35). PR-3 has been shown to potentiate the effects of CXCL-8 by cleavage into a more potent form (39). Only PR-3 was positively correlated with DKA severity. This latter finding may be due to PMN activation by acidic extracellular pH as it has been shown that a sub fraction of PMN PR-3 localizes not to azurophilic granules but to secretory vesicles (40, 41). Secretory vesicles are the first vesicles to be released by PMN following stimulation and tend to be released in a dose-dependent manner (42). These vesicles are released faster than azurophilic granules and so it is conceivable that only PR-3, at this stage in DKA progression, is released in a pH-dependent manner

Through its ability to generate hypohalous acid, MPO can facilitate endothelial dysfunction, both *in vitro* and *in vivo* (22, 28, 43), as well as degrade the endothelial extracellular matrix (26). Hypochlorous acid generated by MPO can activate MMP-9 (44), and MMP-9 was elevated in DKA (Chapter 2). Apart from its oxidative compound-generating ability, MPO facilitates leukocyte activation and adhesion to the endothelium during inflammation (45, 46).

In support of the notion that PMN azurophilic enzymes contribute to vascular dysfunction in DKA, a total enzyme cocktail containing significantly increased azurophilic enzymes, HLE, PR-3 and MPO decreased ZO-1 expression in brain microvascular endothelial cells *in vitro*. ZO-1 is an integral component of the tight

junction as it anchors both occludin and claudin to the actin cytoskeleton and is important for junctional regulation (47). Reduced expression of ZO-1 facilitates endothelial permeability (48-50).

Our total enzyme cocktail also degraded β-catenin in human brain endothelial cells. β-catenin is one of the terminal cytoplasmic proteins in the adherens junction. Its role is to anchor the intercellular component VE-cadherin to the cytoskeleton and it is responsible for junctional regulation (51). β-catenin loss is associated with vascular instability and hemorrhage (52). However, we did not observe a significant decrease in full length β-catenin concurrently with appearance of the degradation products, indicating increased protein turnover as opposed to only protein breakdown. Also, since we did not observe any enzyme-mediated degradation products with ZO-1, this implies that β-catenin and ZO-1 are differentially processed. These changes most likely occurred through extracellular-intracellular signalling as it has been shown that disruption of both the membrane-bound proteins occludin and VE-cadherin result in disruption of their cytosolic counterparts (53, 54).

The concentrations of PMN enzymes used in our *in vitro* experiments were greater than what we measured in plasma. Physiologically, PMN degranulation occurs at the endothelial surface, or inside the intercellular compartment. In actuality, the concentrations at the endothelium of PMN-derived molecules are almost certainly greater than those that we measured in plasma. In fact, HLE (55, 56), lactoferrin and defensins (57) and cytokines (58) have been suggested to reach local concentrations of up to 2 mg/ml, following PMN degranulation. The levels of enzymes we measured in plasma are most likely “spill over” from more localized domains and would be diluted as compared

to the effective concentrations at the level of the endothelium. Therefore, we used enzyme concentrations used in other publications (19-22)

As neutral proteases, both HLE and PR-3 may display slightly reduced catalytic activity at acidic pH but as pH is normalized during DKA treatment, HLE and PR-3 activity would increase (59, 60). Our observation that PR-3 release is correlated with a lower pH raises the possibility that increased PR-3 release under acidotic conditions may compensate for decreased catalytic activity. MPO is known to be more active under acidic conditions as well, thereby potentiating its ability to generate oxidative compounds in DKA patients prior to treatment (61).

This study has several limitations. First, we assumed that HLE and PR-3 were of PMN origin due to their release concurrent with PMN-specific MPO. Other notable plasma sources of HLE and PR-3, however minor, include endothelial cells (62) and monocytes (63). Future studies should examine specific leukocyte components isolated from DKA patients. Second, the release and maximal effects of the individual enzymes, HLE, PR-3 and MPO during DKA and its treatment are currently unknown and require further examination.

We have shown previously that pediatric DKA is associated with inflammation and PMN adherence to brain microvascular endothelial cells. We now demonstrate increased circulating PMN azurophilic enzymes (HLE, PR-3 and MPO) that reduced the expression of the tight junction protein, ZO-1 and degraded the adherens junction protein, β-catenin. These findings support the notion of DKA as an inflammatory condition and in

conjunction with our other work (10), implicates PMN activation and degranulation in DKA-associated BBB disruption.

3.5 References

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CHAPTER 4: DISCUSSION

4.1 Discussion

DKA is associated with intracerebral vascular complications in children, such as stroke (1), hemorrhage (2) and cerebral edema (3). The mechanisms of these complications are not well known.

DKA is an inflammatory condition involving increases in circulating cytokines such as CXCL-1 (GROα), CXCL-8 (IL-8), IL-6 and IFN-α2 (4). These cytokines are known to cause activation of leukocytes, including PMN (5). Thus, I hypothesized that during pediatric DKA, inflammation leads to widespread activation of leukocytes which then release destructive enzymes. This enzyme surge could be responsible for facilitating cerebrovascular damage.

In this thesis, we observed alterations in the plasma level of a number of MMPs and TIMP-4 as well as PMN azurophilic enzymes. DKA plasma was associated with decreased MMP-2 and increased MMP-8, MMP-9 and TIMP-4. DKA plasma was also associated with increased PMN azurophilic enzymes, HLE, PR-3 and MPO. Our findings are consistent with a widespread inflammatory condition occurring in DKA patients. HLE, PR-3 and MPO are major components of PMN azurophilic granules, which are only released upon degranulation following PMN activation (6). In fact, MPO is exclusively a component of the PMN azurophilic granules and as such is widely utilized as a marker for PMN activation. MMP-8 is found in the PMN secondary granules (7) and is released upon activation as well, albeit earlier in inflammation than the other PMN enzymes (8). Interestingly, active transcription of MMP-8 is thought to occur only in immature PMN, indicating mobilization from bone marrow of immature PMN during DKA: a hallmark of an overwhelming inflammatory response (6). MMP-9 is, among

other sources, associated with the PMN tertiary granule. Further support for notion of PMN-associated MMP-9 comes from the detection of lipocalin-associated MMP-9 complex in DKA plasma, characteristic of PMN-associated MMP-9.

The plasma levels of MMP-8, MMP-9 and PR-3 were positively correlated with DKA severity. The aforementioned correlations are in agreement with studies that have shown that acidic pH directly stimulates release of MMPs from cells (9-11) and leads to enhanced PMN activation (12). The severity of acidosis during DKA has previously been shown to be determinant of the magnitude of consciousness impairment. Cerebral function in DKA patients with cerebral edema is closely tied to pH; a lower pH is correlated with increased level of cognitive impairment (13). Our data supports the notion that the relationship between pH and severity of DKA neurological impairment may involve pathological regulation of MMP-8 and MMP-9 as well as PR-3.

We have shown that DKA plasma is associated with increased levels of MMP-8, MMP-9, HLE, PR-3 and MPO. The presence of these enzymes at the level of the endothelium could potentially lead to breakdown of the intercellular junctions and facilitate fluid movement into the brain (e.g., vasogenic edema). Indeed, using human brain microvascular endothelial cells, we have shown a potential role for PR-3, HLE and MPO in disruption of both AJs and TJs. Numerous studies have implicated the MMPs and PMN azurophilic enzymes in facilitating breakdown of the BBB (14-17).

Our data raises the possibility of a 2-stage insult to the brain microvasculature. We have shown that DKA stimulates the release of MMP-8, MMP-9, HLE, PR-3 and MPO. Following DKA-induced release of these enzymes, MMP-8 and MPO can be

expected to display higher catalytic activity during the initial stages of DKA when blood pH is low (18, 19). During treatment and the ensuing normalization of pH, the catalytic activity of MMP-9 (20), HLE and PR-3 (21) can be expected to increase, potentially leading to a second wave of protease activity.

These studies have provided insight into the circulating protease profile occurring in pediatric DKA patients. Future studies could examine the effects on these enzymes *in vivo* by assessing markers of BBB disruption in DKA plasma. Solubilized junctional proteins in circulation like occludin, claudin-5 and cadherin might indicate cleavage and subsequent release from the brain endothelium. Unfortunately, due to the ethical ramifications of patient studies, it is difficult to probe more into the physiological functions. The effects of these enzymes on BBB structure and function could be assessed *in vitro*. Isolated PMN could be applied to human cerebromicrovascular endothelial monolayers under DKA conditions (e.g., acidosis, hyperglycemia, and DKA-specific cytokine mixture) in order to assess their direct effect on monolayer integrity. The effect of PMN on the BBB could be assessed in a juvenile mouse model of DKA as well (22).

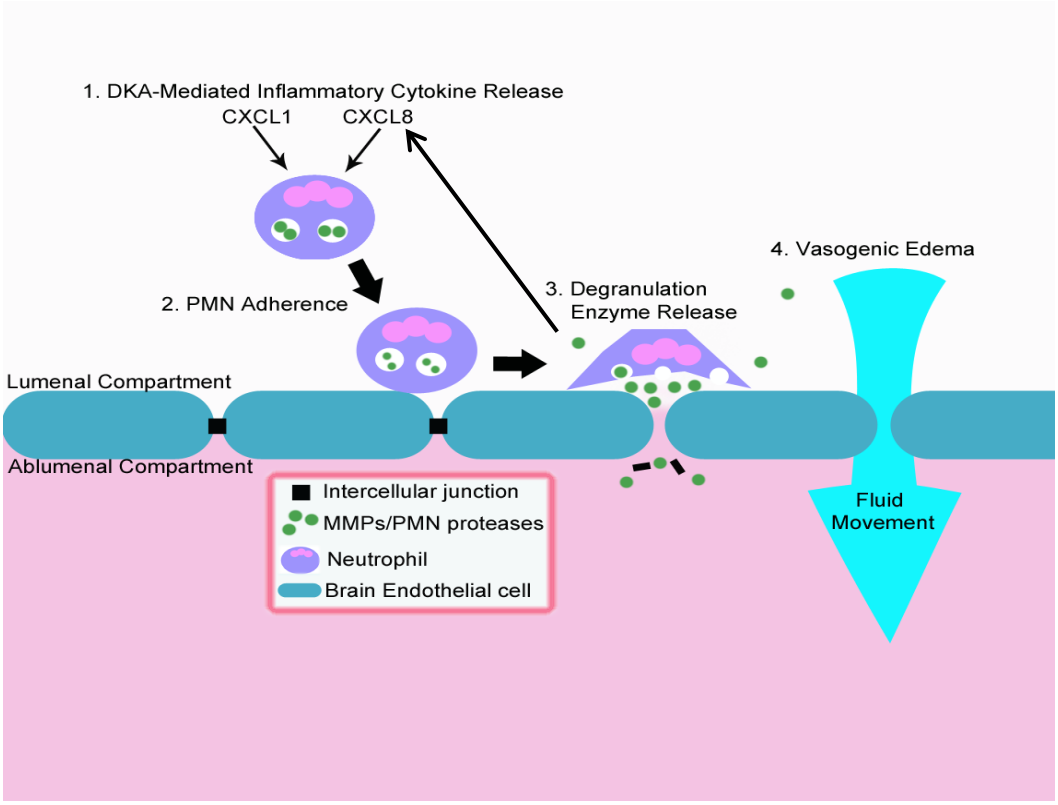

FIGURE 4-1. Proposed Mechanism of PMN-Mediated DKA Endothelial Perturbations. DKA is a well-known inflammatory condition. Inflammatory cytokines in circulation serve to activate endothelial cells and PMN, recruiting them to the endothelial surface. PMN degranulate upon activation and the released proteases destroy the junctions between adjacent cells. Released proteases may also potentiate existing DKA-mediated cytokines, thereby facilitating a positive feedback mechanism. This results in the loss of vascular integrity, leading to fluid and solutes moving across the BBB, thus facilitating DKA-mediated cerebrovascular dysfunction.

4.2 References

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APPENDIX A

Western

Office of Research Ethics

The University of Western Ontario
Room 4180 Support Services Building, London, ON, Canada N6A 5C1
Telephone: (519) 661-3036 Fax: (519) 850-2466 Email: ethics@uwo.ca
Website: www.uwo.ca/research/ethics

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.D. Fraser
Review Number: 16986E **Review Level:** Expedited
Review Date: March 24, 2010 **Approved Local # of Participants:** 250
Protocol Title: Translational Research Centre: Repository of control biological specimens from healthy volunteers for future research purposes.
Department and Institution: Paediatrics, London Health Sciences Centre
Sponsor: CHILDREN'S HEALTH FOUNDATION
Ethics Approval Date: May 04, 2010 **Expiry Date:** March 31, 2015
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent. Email poster.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

[redacted]

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
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cc: ORE File
LHRI

Office of Research Ethics

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Western

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.D. Fraser
Review Number: 16993 **Review Level:** Full Board
Review Date: March 23, 2010 **Approved Local # of Participants:** 50
Protocol Title: Translational Research Centre: Repository of biological specimens from patients with critical illness and/or traumatic conditions for future research purposes.
Department and Institution: Paediatrics, London Health Sciences Centre
Sponsor: Childrens's Health Foundation
Ethics Approval Date: May 20, 2010 **Expiry Date:** April 30, 2015
Documents Reviewed and Approved: UWO Protocol, Letter of information & consent form dated April 26/10, 24 hours Post-Collection Letter of information & consent form dated May 10/10, Assent for Ages 13-17 dated May 10/10 & Assent for Ages 7 to 12 dated April 26/10

Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

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Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
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Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

[redacted]

Chair of HSREB: Dr. Joseph Gilbert
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Western Research

Research Ethics

Use of Human Participants - Revision Ethics Approval Notice

Principal Investigator: Dr. Douglas Fraser
File Number:6078
Review Level: Delegated
Protocol Title: Translational Research Centre: Tissue Collection and Database - 15996E
Department & Institution: Schulich School of Medicine and Dentistry\Paediatrics,Children's Hospital of Western Ontario
Sponsor:
Ethics Approval Date: April 02, 2014 **Expiry Date:**March 31, 2019
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Revised Study End Date	The study end date has been extended to March 31, 2019.	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

[redacted]

Ethics Officer to Contact for Further Information

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This is an official document. Please retain the original in your files.

VITA

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