Intracerebral matrix metalloproteinase 9 in fatal diabetic ketoacidosis

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ABSTRACT

There is increasing awareness that in addition to the metabolic crisis of diabetic ketoacidosis (DKA) caused by severe insulin deficiency, the immune inflammatory response is likely an active multicomponent participant in both the acute and chronic insults of this medical crisis, with strong evidence of activation for both the cytokine and complement system. Recent studies report that the matrix metalloproteinase enzymes and their inhibitors are systemically activated in young Type 1 diabetes mellitus (T1D) patients during DKA and speculate on their involvement in blood-brain barrier (BBB) disruption. Based on our previous studies, we address the question if matrix metalloproteinase 9 (MMP9) is expressed in the brain in the fatal brain edema (BE) of DKA. Our data show significant expression of MMP9 on the cells present in brain intravascular areas. The presence of MMP9 in intravascular cells and that of MMP$^+$ cells seen passing the BBB indicates a possible role in tight junction protein disruption of the BBB, possibly leading to neurological complications including BE. We have also shown that MMP9 is expressed on neurons in the hippocampal areas of both BE/DKA cases investigated, while expression of tissue inhibitor of metalloproteinases 1 (TIMP1) was reduced in the same areas. We can speculate that intraneuronal MMP9 can be a sign of neurodegeneration. Further studies are necessary to determine the role of MMP9 in the pathogenesis of the neurologic catastrophe of the brain edema of DKA. Inhibition of MMP9 expression might be helpful in preserving neuronal function and BBB integrity during DKA.

1. Introduction

Varying degrees of metabolic dysregulation and the associated oxidative stress (Lee et al., 2002) initiate and perpetuate an inflammatory response that can progress from a chronic low-grade inflammatory milieu (Heier et al., 2015) to the metabolic and immunologic crisis of DKA (Hoffman et al., 2003a). Similarly, acute and more severe dysregulation results in perturbation with an increased morbidity (Peeters et al., 2017). Evidence for the early pathogenesis of the dysregulated metabolic stress of DKA comes from in-vitro studies of ketone bodies and hyperglycemia activating both rodent (Isales et al., 1999) and human cerebral capillary endothelial cells (Hoffman et al., 2002), resulting in an increased expression of proinflammatory cytokines and chemokines such as CXCL1 and CXCL8 and promoting neutrophil adhesiveness (Omatsu et al., 2014). Among the numerous perturbations in systemic metabolic/immunological variables that have been suggested to increase the stress of type 1 diabetes mellitus/DKA include: 1) deoxy carbonyls (Hoffman et al., 2003a; Turk et al., 2006); 2) advanced glycation end products (AGE)/RAGE axis (Skrha Jr et al., 2012; Peeters et al., 2018); 3) AGE and oxidized low density lipoproteins (LDL) (Lopes-Virella et al., 2012); 4) inflammatory cytokines (Hoffman et al., 2003b; Karavanaki et al., 2011; Close et al., 2013); 5) complement activation (Jerath et al., 2005; Speidl et al., 2011) and MMP9 (Woo et al., 2016a, 2016b; Garro et al., 2017).

Matrix metalloproteinases (MMPs) are potent zinc-dependent endopeptidases produced by numerous cell types including leukocytes, microglia, astrocytes and neurons (Fedorova et al., 2018). MMP9 is one of the few MMPs enzymes that are constitutively expressed. It has a diverse spectrum of activity: 1) degrades tight junction proteins and all components of the extracellular matrix proteins including laminin,

collagen and fibronectin (Feng et al., 2011); 2) facilitates leukocyte migration and invasion (Lavini-Ramos et al., 2017); 3) integration of immunoregulatory pathways (Lavini-Ramos et al., 2017); 4) maintenance of tissue barriers by processing a range of non-matrix proteins, including cytokines and chemokines (Van Lint and Libert, 2007; Nissinen and Kahari, 2014; Smigiel and Parks, 2017); and 5) staging serum and tissue in metastatic conditions (Wieczorek et al., 2015). They are also involved in the pathogenesis of acute complications such as acute coronary syndromes (Suzuki et al., 2006). Although MMPs have broad substrate specificity, they are tightly regulated in inflammation and play a significant role in biological processes of autoimmune diseases (Ram et al., 2006).

MMP9, MMP2 and TIMP1 expression were reported in T1D (Maxwell et al., 2001; Jacqueminet et al., 2006; Symeonidis et al., 2013). Woo et al. (2016a) reported an increase of MMP9 in DKA and after its treatment along with other leukocyte azurophilic enzymes, such as proteinase 3 (PR-3), whose level correlated with DKA severity and was shown to be involved in tight junction protein disruption in vitro. Recent publications by Woo et al. (2016b) and by Garro et al. (2017) reported a positive correlation between DKA severity and the prevalence of MMP8 and MMP9, and hypothesized that these enzymes are playing a role in the disruption of the blood-brain barrier by extracellular matrix cleavage. Activation of MMP9 before DKA treatment likely involves: hyperglycemia, ketones, methylglyoxal and endothelin 1 (ET-1) (Isales et al., 1999; Kim et al., 2012; Koyama et al., 2017); hypercortisolemia and free fatty acids (Boden and Song, 2008), in addition to the bidirectional modulation by inflammatory cytokines and chemokines (Strazielle et al., 2003), whose cleavage by MMP9 can result in a gain of function of some substrates (Van Lint and Libert, 2007). Also, of potential importance is that insulin has been reported to activate MMP9 (Fedorova et al., 2018). This possibility suggests a similar pattern of activation for inflammatory cytokines, like in systemic inflammatory response (SIR) and for complement activation during DKA treatment (Hoffman et al., 2003b; Jerath et al., 2005).

We investigated the intracerebral expression of MMP9 and TIMP1, along with the previously reported membrane attack complex (MAC) or C5b-9 (Hoffman et al., 2006) using immunohistochemistry in five brain regions (basal ganglia, choroid plexus, hippocampus, frontal cortex and cerebellum) of two adolescent cases with fatal DKA/brain edema (Hoffman et al., 2006, 2007). Our results show that the neuronal expression of MMP9 in the hippocampal areas of both BE/DKA cases was associated with decreased expression of TIMP1. In addition, significant expression of MMP9 was found on the cells present in brain intravascular areas. The presence of MMP9 in intravascular cells indicates a possible role in BBB tight junction protein disruption, possibly leading to neurological complications including BE.

2. Materials and methods

2.1. Case 1

An adolescent female had a four-year history of poorly controlled T1D, which resulted in recurrent hospitalizations for DKA. The admission was preceded by a 12-h history of abdominal pain and several episodes of emesis. There was no history of fever or enteritis. On physical examination, the patient was orientated but drowsy. There was no evidence of infection. The Tanner stage of puberty was B3 and P3. Admission laboratory tests consisted of a pH 7.10; pCO2 15 mmHg; pO2 106 mmHg; glucose 810 mg/dL; Na 132 mEq/L; K 5.7 mEq/L; Cl 93 mEq/L; HCO_3 5 mEq/L; and BUN 30 mg/dL. The patient was treated in a Pediatric Intensive Care Unit lead for the correction of hyperglycemia and metabolic acidosis. Twelve hours following treatment, the child developed a mild headache. Seven hours later, she developed sudden onset of labored respirations and within 20 min had a cardio-respiratory arrest. An emergency CT scan of the head showed sulcal effacement and cerebral and pontine edema with evidence of

herniation. Efforts at resuscitation were unsuccessful and she was pronounced dead after the cardio-respiratory arrest.

2.2. Case 2

An adolescent female had an eight-year history of poorly controlled T1D, which had resulted in recurrent hospitalizations for DKA. The admission was preceded by an 18-hour history of capillary blood glucoses of over 300 mg/dL; ketonuria; a four-hour history of headache; and several episodes of emesis. There was no history of fever or enteritis. On physical examination, the patient was slightly confused and lethargic. The Tanner stage of puberty was B5 and P4. There was no evidence of infection. Admission laboratory tests consisted of: pH 7.16; pCO2 17 mmHg; pO2 100 mmHg; blood glucose 581 mg/dL; Na 130 mEq/L; K 4.8 mEq/L; Cl 89 mEq/L; HCO_3 6 mEq/L; and BUN 28 mg/dL. Treatment was in a Pediatric Intensive Care Unit and correction of the hyperglycemia metabolic acidosis was performed. Ten hours following initiation of treatment, she became unresponsive and was treated with mannitol and hyperventilation and placed on mechanical ventilation. An emergency CT scan of the head showed diffuse cerebral edema. She was pronounced dead approximately 10 h after the cardiorespiratory arrest.

Control normal brain tissue arrays were obtained from Pro Sci Incorporated (Poway, CA) and Chemicon (Temecula, CA).

2.2.1. Immunohistochemistry

The paraffin sections were processed as described previously (Hoffman et al., 2006). After xylene deparaffinization and epitope retrieval using a Target Retrieval Solution (DAKO, Carpinteria, CA), sections were treated with 3% H_2O_2 to remove endogenous peroxidase. Sections were preincubated for 30 min with normal goat serum then with a rabbit monoclonal antibody (mAb) against human MMP9 (CST, Danvers, MA) or a rabbit polyclonal antibody against human TIMP1 (Millipore Sigma, Burlington, MA) over-night at 4 °C in a humid chamber. MMP9 (D6O3H) XP® Rabbit mAb recognizes the full-length, proenzyme (92 kDa) and the cleaved, active enzyme (84 kDa) of MMP9. In addition, we were unable to perform additional analysis by Western blot due to the lack of tissue to further confirm the specificity of the MMP9 antibody. Similar steps were performed for RGC-32 using a rabbit IgG anti-RGC-32 generated by us (Fosbrink et al., 2005) and for C5b-9 neoantigens with the monoclonal antibody against C5b-9 (Quidel, San Diego, CA) as described (Hoffman et al., 2006). Then sections were washed 3 times for 3 min at room temperature with Tris buffer saline pH 7.4 (TBS), and then incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). After washing, the specific deposits were developed using NovaRed (Vector Laboratories, Burlingame, CA). The nuclei were counterstained with Mayer's hematoxylin (Sigma Chemical Co, St. Louis, MO). Controls for the specificity of each immunohistochemical reaction were performed by replacing the primary antibody with PBS, mouse, or rabbit IgG. Immunostaining was independently evaluated by two investigators in a blinded fashion.

2.2.2. Statistical analysis

Comparisons between groups were performed using unpaired two-tailed Student's *t*-test. *p* values < .05 were considered significant. Statistical analysis was performed using Graph Pad software, version 7.

3. Results

3.1. Immunohistochemical localization of MMP9 in DKA and control brains

Hippocampal neurons exhibit extensive MMP9 specific deposits (Fig. 1A and B). We did not found similar MMP9 deposits in midbrain, substantia nigra or Purkinje neurons (Table 1). MMP9 deposits were
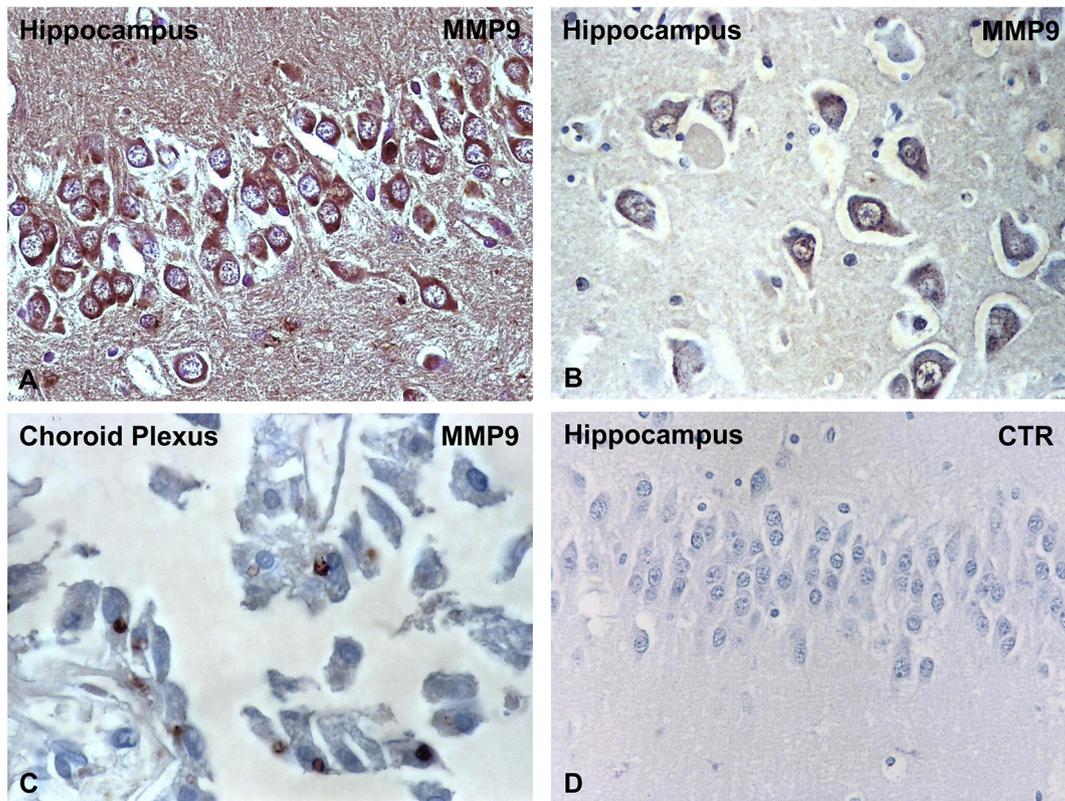
Fig. 1. Immunohistochemical localization of MMP9 in DKA brains.
MMP9 was localized by indirect immunoperoxidase in DKA brains as described in Material and Methods section. We found that hippocampal neurons exhibit extensive MMP9 intracytoplasmatic staining (A, B). In addition, immunostaining for MMP9 was found in choroid plexus epithelial cells (C), while controls of immunoperoxidase reaction were negative (D). Original magnification: x400 (in A-C) and x200 (D).

Table 1
Expression of MMP9 and TIMP1 in DKA and normal brains.

	Region	Intravascular		Neurons	
		MMP9	TIMP1	MMP9	TIMP1
DKA brain #1	Basal ganglia	−	+	−	+
	Hippocampus	+ +	+	+ +	+
	Choroid plexus	−	+	−	−
	Frontal cortex	+	+	−	+
	Cerebellum	−	+	−	+ +
DKA brain #2	Basal ganglia	+ +	+	−	+ +
	Hippocampus	+ + +	+ +	+ + +	+ +
	Choroid plexus	+	+ +	−	−
	Frontal cortex	+ +	+	−	+ +
	Cerebellum	+ +	+ +	−	+ +
Normal brain	Frontal cortex	−	+ +	−	+ +
	Occipital cortex	−	ND	−	ND
	Parietal cortex	−	+ +	−	+ +
	Temporal cortex	−	ND	−	ND
	Cerebellum	−	+ +	−	+ +
	Pons	−	ND	−	ND

−, negative; +, slightly positive; + +, positive; + + +, highly positive; ND, not determined.

also present on choroid plexus (Fig. 1C). Controls of the immunoperoxidase reaction were negative (Fig. 1D). MMP9 deposits were absent on neurons or choroid plexus of the control normal brains (Table 1). The number of neurons with MMP9 deposits in DKA patients and control brains was quantified by two independent observers. For each brain, the number of positive neurons was determined by counting 200 cells. We found a statistically significant increase of MMP9 expression in neurons in DKA brains as compared to control brains ($p = .002$) (Fig. 2A). In addition, we found that many brain blood

vessels had positive intravascular cells for MMP9 (Fig. 3A–D). The presence of MMP9 in intravascular cells and that of MMP9$^+$ cells seen passing the BBB (Fig. 3C–D, arrows) indicates a possible role in tight junction protein disruption of the BBB. Some MMP9$^+$ cells are attached to the blood vessel wall (Fig. 3D, arrowhead). Some of the intravascular cells had morphology compatible with neutrophils and lymphocytes (Fig. 3B). Since the anti-MMP9 antibody used recognized both the latent and active form, we were unable to delineate the functional role of the molecule. Controls of immunoperoxidase reaction were negative (Fig. 3E). There were no positive MMP9 intravascular cells in control normal brains (Table 1). These data suggest an increased and selective neuronal vulnerability of hippocampus in DKA/BE.

3.2. Immunohistochemical localization of TIMP1 in DKA and control brains

The main function of TIMPs is their inhibitory effect on MMPs. TIMPs irreversibly inactivate MMPs by direct binding to their catalytic zinc cofactor and resultant inhibition of proteinase function (Smigiel and Parks, 2017). It has been also shown that pro-MMP9 binds TIMP1 (Smigiel and Parks, 2017). In Case #2, TIMP1 was present intravascularly and in the neurons in all five regions studied (Fig. 4A) but not in the choroid plexus (Table 1). Intravascular expression of TIMP1 was present in all five brain regions investigated for Case#1, with low degree of expression (Fig. 4B, C). Low expression of TIMP1 was also found in the neurons of all regions studied (Table 1). Some of the intravascular cells which were TIMP1 positive had morphology typical for neutrophils while others for lymphocytes (Fig. 4C). Controls of immunoperoxidase reaction were negative (Fig. 4D). Concerning expression in hippocampus, the number of positive TIMP1 neurons was determined by counting 200 cells in DKA and control brains. We found significantly higher levels of TIMP1 expression in neurons in controls as
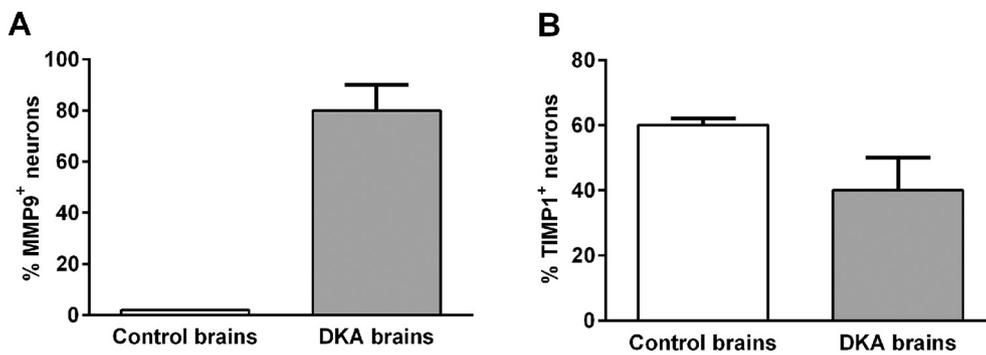
Fig. 2. Quantitative analysis of the expression of MMP and TIMP1 in control and DKA brains.

The percentage of MMP9+ (A) and TIMP1+ (B) hippocampal neurons was determined by counting 200 cells from 3 controls and 2 DKA brains. We did not find MMP9+ neurons in control brains (A) and an arbitrary value of 1 was given. There was a statistically significant increase of MMP9+ neurons in DKA brains as compared to control brains ($p = .002$). In addition, we found significantly higher levels of TIMP1 expression in neurons in the control brains as compared to DKA brains ($p = .03$) (B). Data are shown as mean ± SEM.
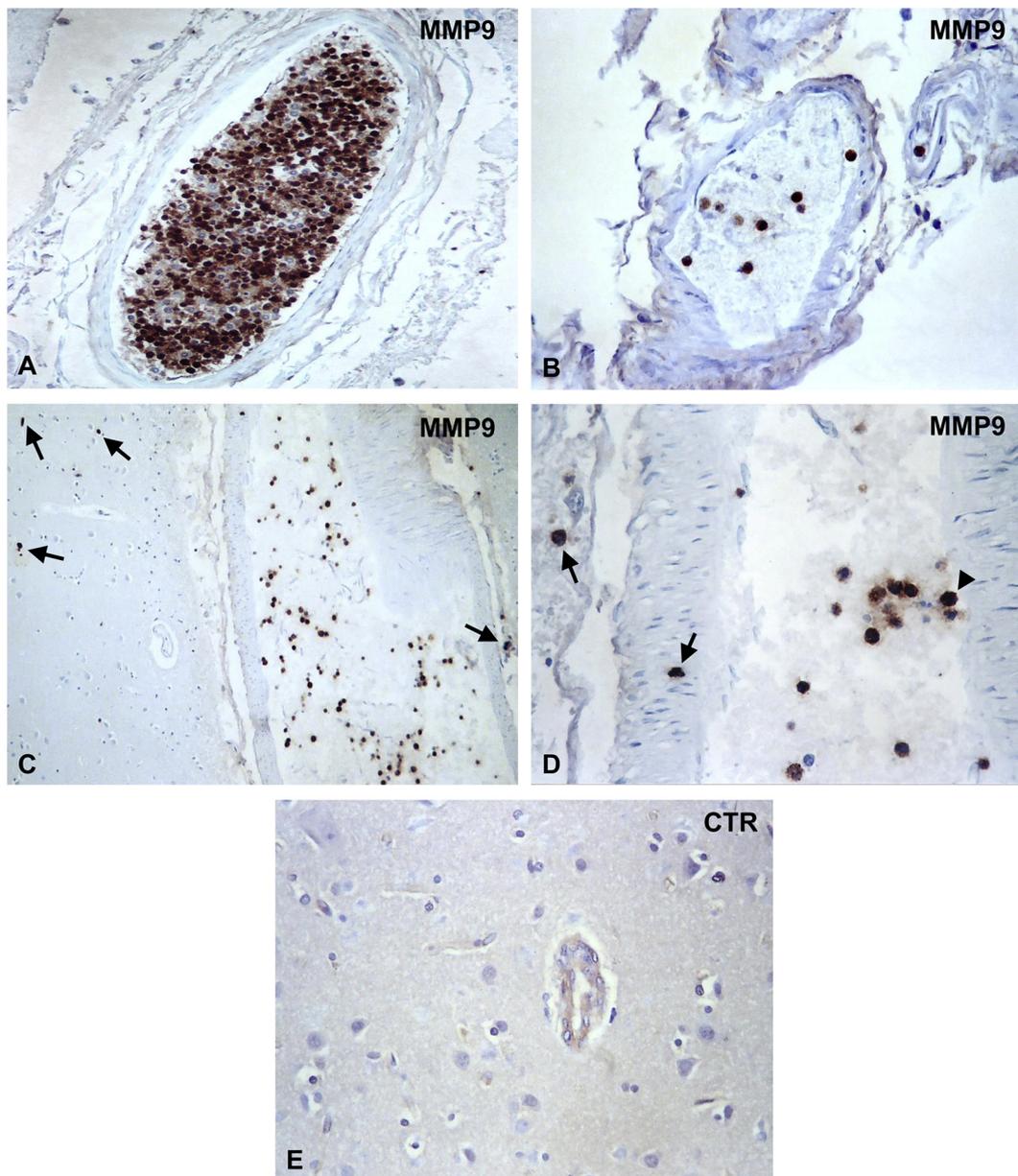

Fig. 3. Immunohistochemical localization of MMP9 in blood vessels in DKA brains.

MMP9 was localized by indirect immunoperoxidase in DKA brains. MMP9 was found to be localized in cells present in brain blood vessels (A-D). MMP9 positive cells were seen passing the BBB or to be present in the brain parenchyma (C, D, arrows). Some MMP9+ cells are attached to the blood vessel wall (D, arrowhead). The presence of MMP9 in intravascular cells and that of MMP9+ cells seen passing the BBB indicates a possible role in BBB disruption. Controls of immunoperoxidase reaction were negative (E). Original magnification: x400 (A, B, D) and x200 (C, E).
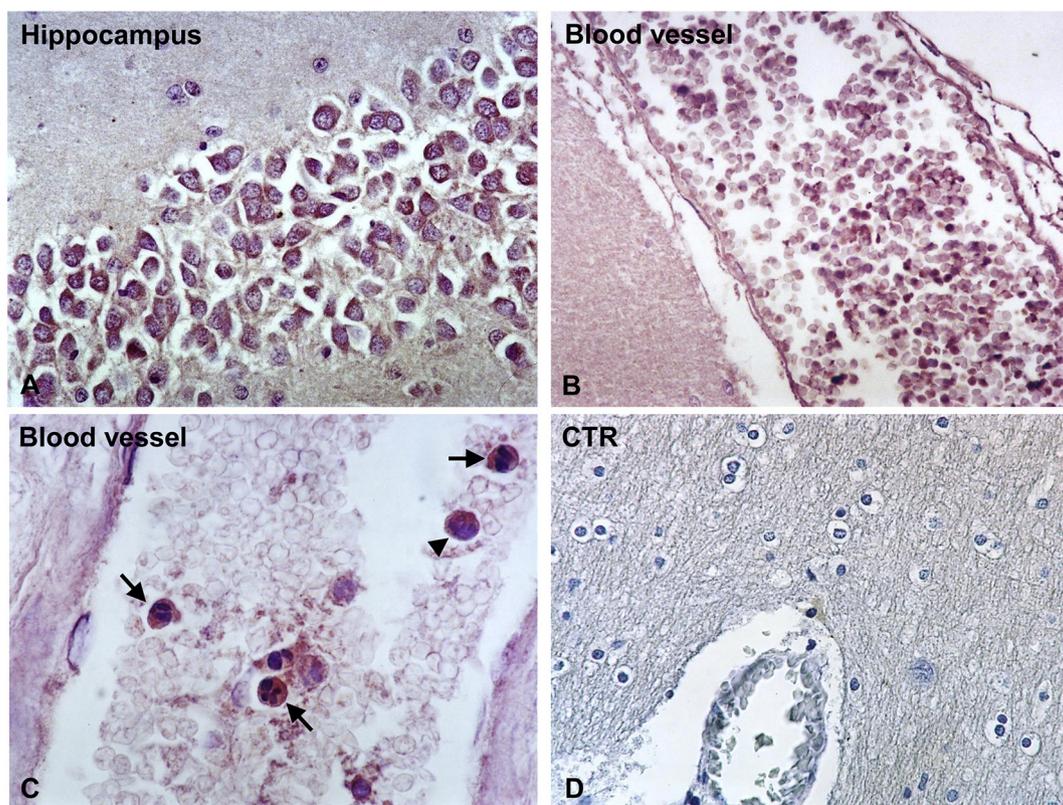
Fig. 4. Immunohistochemical localization of TIMP1 in DKA brains.
TIMP1 was localized by indirect immunoperoxidase in DKA brains as described in Materials and Methods section. Hippocampal neurons exhibit low TIMP1 staining (A). In addition, reduced staining of TIMP1 was found in cells present in the blood vessels (B, C). Some intravascular TIMP1^{+} cells had morphology typical for neutrophils (C, arrows), while others for lymphocytes (C, arrowhead). Controls of Immunoperoxidase were negative (D). Magnification: x400 (in A, C and D) and x200 (in B).

compared to DKA brains ($p = .03$) (Fig. 2B).

3.3. Immunohistochemical localization of C5b-9 and RGC-32 in DKA brains

We investigated the presence of C5b-9 and RGC-32 in hippocampal areas of the brains as these areas also had MMP9 expression. As shown in Fig. 5A and B, C5b-9 (MAC) deposits were found to be present on hippocampal neurons as previously described (Hoffman et al., 2006). In addition, RGC-32 was found to be expressed in the cytoplasm of hippocampal neurons (Fig. 5C, D). Since we previously showed that C5b-9 induces RGC-32 expression (Badea et al., 2002), it is possible that RGC-32 was induced in hippocampal neurons by the C5b-9 assembly.

4. Discussion

MMP9 expression in the pathogenesis of acute cerebrovascular accidents unrelated to DKA has been well studied. Recently, it has been hypothesized that MMP9 is a candidate for the disruption of BBB tight junction proteins (TJP) in DKA (Woo et al., 2016a, 2016b; Garro et al., 2017). Important contributors to this perturbation include: 1) systemic leukocytosis with neutrophil predominance, as seen in DKA, which can result in increased MMP9 levels (Turner and Sharp, 2016); 2) MMP9 production by astrocytes, important cellular components of the neurovascular unit of the BBB (Hsieh et al., 2013, 2014); 3) activation by increased oxidative stress (Pun et al., 2009), a major product of DKA's dysfunctional metabolic state that initiates the superoxide-MMP9 cascade; 4) activation by the endothelin (ET) system, along with vascular endothelial growth factor (VEGF) (a perturbator of the BBB) (Koyama and Michinaga, 2012); and 5) modulation of both systemic and local inflammation (Manicone and McGuire, 2008; Nissinen and Kahari, 2014). Unfortunately, the metabolic (Lee et al., 2002; Carl et al., 2002; Hoffman et al., 2003a) and immunologic crisis of DKA (Hoffman et al., 2003b; Jerath et al., 2005; Karavanaki et al., 2011; Close et al., 2013; Hoffman et al., 2013) can occur frequently in young patients, resulting in insults to the brain and other organs and leading to early cognitive deficits (Jessup et al., 2015; Nunley et al., 2015) and progressive chronic subclinical renal complications. Both comorbidities have much a greater frequency (Hursh et al., 2017) than does the tragic acute complication of clinical BE (Tasker and Acerini, 2014; Patel et al., 2016; Peeters et al., 2018).

The hippocampus was the only location in which neuronal expression of MMP9 was found. This hippocampal location of MMP9 expression is in keeping with the expression of the other inflammatory components, particularly C5b-9 and RGC-32, along with the increased neuronal deficits and neuronal loss occurring in this region (Fig. 6) (Hoffman et al., 2006, 2008, 2009; Cameron et al., 2014). C5b-9 has been shown to induce MMP9 expression (Wu et al., 2010), which is also dependent on RGC-32 (Xu et al., 2014). It is possible that MMP9 expression in hippocampal neurons during DKA is induced by C5b-9 assembly (Hoffman et al., 2006), with a possible contribution from RGC-32. RGC-32 mRNA and protein levels have been found to be upregulated in the brains of patients with mild cognitive impairment and Alzheimer's disease. In addition, high brain levels of RGC-32 protein are associated with poorer antemortem global cognitive performance (Counts and Mufson, 2017). The increased expression of RGC-32 found in hippocampal neurons may reflect neuronal degeneration during DKA. Published reports indicate that neurons are capable of synthesizing MMP9 (Kaplan et al., 2014), and this synthesis may result in an increased stress on vulnerable neurons, which could trigger the
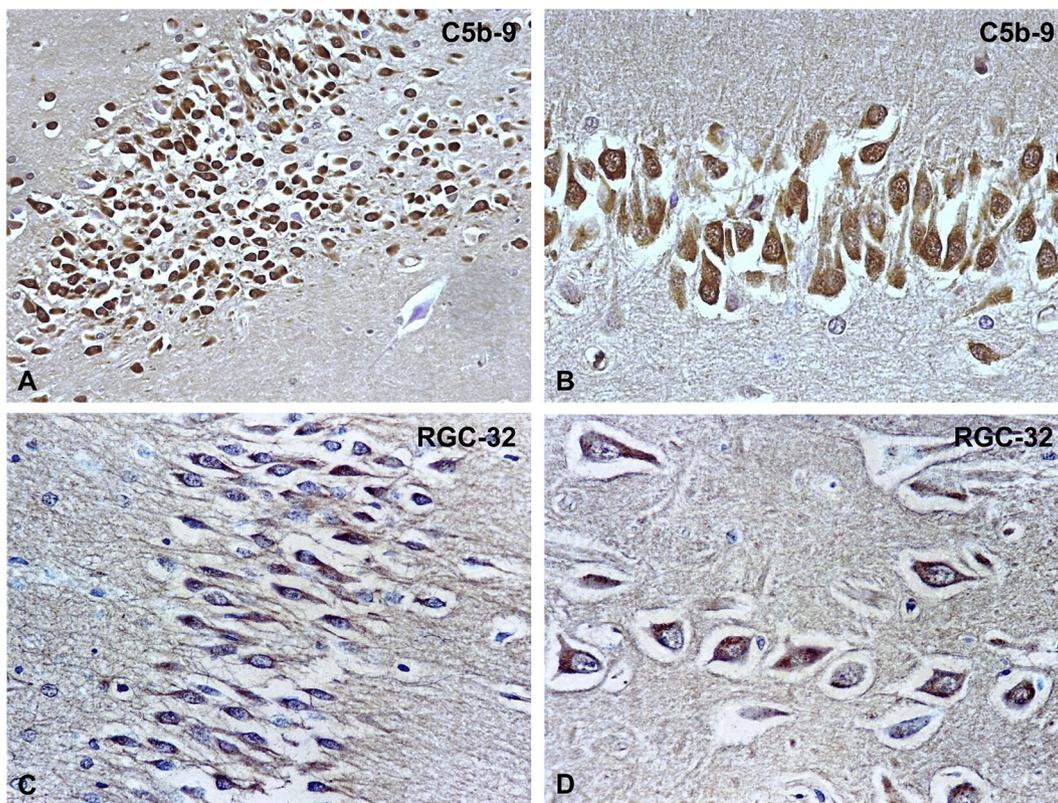
Fig. 5. Immunohistochemical localization of C5b-9 neoantigens and RGC-32 in DKA brains.
C5b-9 and RGC-32 were localized by indirect immunoperoxidase in DKA brains. We found that hippocampal neurons exhibit extensive intracytoplasmatic C5b-9 deposits (A, B); RGC-32 was found to be highly expressed in cytoplasm of hippocampal neurons (C, D). Magnification: x200 (A, C); x400 (B, D).
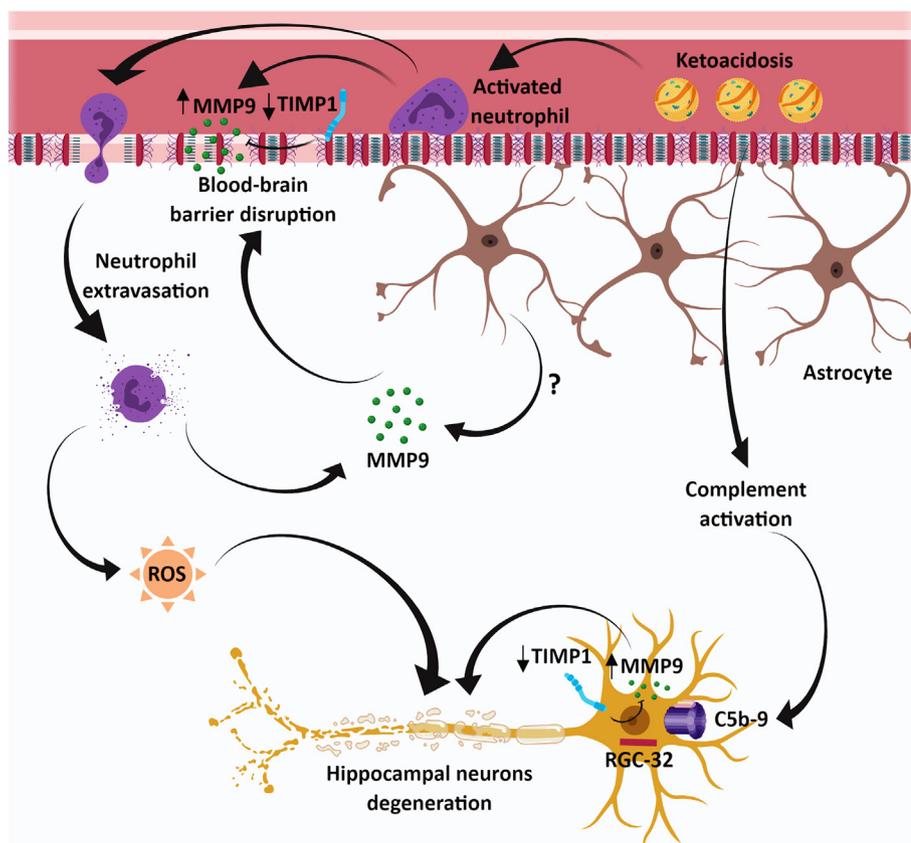

Fig. 6. Schematic representation of the hypothetical role of MMP9 in brain dysfunction during DKA.
Our data suggest that during DKA, MMP9 is secreted by inflammatory cells and may be involved in BBB disruption, which leads to neutrophil infiltration into the brain parenchyma. In addition, activation of complement system with the assembly of terminal complement complex C5b-9 occurs both in plasma and in the brain tissue. It is possible that C5b-9 induces the expression of MMP9 particularly in hippocampal neurons, possibly by activating RGC-32. This increase in MMP9 expression may lead to hippocampal neuron dysfunction and degeneration. MMP9 intravascular and neuronal expression may also be facilitated also by the decreased expression of its natural inhibitor, TIMP1.

degeneration of these cells (Kaplan et al., 2014). Thus, is possible that MMP9 inhibition or modulation can be beneficial for neuronal preservation. The goal of protecting against MMP9's harmful effects in DKA is in line with therapeutic strategies used in inflammatory diseases and cancer that would block the damaging effects of MMP9 without inhibiting its beneficial actions.

We have also reported significant neuroinflammation of the choroid plexus (CP) (Hoffman et al., 2007), a site of major importance in view of its role in bioactive organic anion and neuro-immune regulation (Strazielle et al., 2003). Unfortunately, studying the CP was precluded because of limited tissue availability. The previous MMP9/DKA studies (Woo et al., 2016a, 2016b; Garro et al., 2017) did not allow for identifying a peak time for the systemic concentration in relation to the 6- to 12-h duration of the SIR following the initiation of insulin.

Although ischemia of the cerebrovascular tight junctions and the presence of MMP9 have been extensively researched (Turner and Sharp, 2016), we were unable to identify any histopathologic evidence of hemorrhage to indicate the occurrence of vascular compromise in either of the two brains, suggesting that there are other metabolic and immunologic initiating/activating factors of MMP9 in DKA. The intracerebral presence of MMP9 in DKA extends the previously described inflammatory phenotype and adds another mediator for consideration as a participant in BBB perturbation during DKA (Fig. 6). This study is limited by the fact that it relies only on immunohistochemical analysis, and therefore there are no functional data to show a role in alerting BBB or neuronal function. In addition, while we believe that our study extends the previous observations by Woo et al. (Woo et al., 2016a, 2016b) and Garro et al. (Garro et al., 2017), our investigation is limited to brain samples obtained postmortem from two DKA patients, and therefore the results must be interpreted with caution.

The reduction in TIMP1 expression associated with other possible inhibitors may affect MMP9 levels. Other possible MMP9 inhibitors include: 1) heat shock protein (HSP) 70 (Lee et al., 2002), which is increased in DKA, paralleling changes in serum glucose levels (Oglesbee et al., 2005); and 2) IL-10, an anti-inflammatory molecule, which, like HSP70 is elevated prior to DKA treatment; both these anti-inflammatory molecules decrease rapidly with the initiation of insulin treatment (Hoffman et al., 2003a; Oglesbee et al., 2005). In contrast, insulin treatment is reported to increase MMP9 expression (Mostafa Mtairag et al., 2001), akin to the increase in inflammatory cytokines that occurs during SIR/DKA (Hoffman et al., 2003b; Karavanaki et al., 2011; Close et al., 2013; Hoffman et al., 2013) and complement activation (Jerath et al., 2005) during DKA treatment.

In conclusion, we speculate that MMP9 plays a dual role during DKA/BE by perturbing BBB permeability and possibly contributing to neuronal dysfunction and loss. Further studies are necessary to establish that MMP9 plays a definite role in the pathogenesis of subclinical and clinical BE in DKA.

Acknowledgements

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