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Evidence for differential changes of junctional complex proteins in murine neurocysticercosis dependent upon CNS vasculature

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Abstract

The delicate balance required to maintain homeostasis of the central nervous system (CNS) is controlled by the blood brain barrier (BBB). Upon injury, the BBB is disrupted compromising the CNS. BBB disruption has been represented as a uniform event. However, our group has shown in a murine model of neurocysticercosis (NCC) that BBB disruption varies depending upon the anatomical site/vascular bed analyzed. In this study further understanding of the mechanisms of BBB disruption were explored in blood vessels located in leptomeninges (pial vessels) and brain parenchyma (parenchymal vessels) by examining the expression of junctional complex proteins in murine brain infected with *Mesocystoides corti*. Both pial and parenchymal vessels from mock infected animals showed significant colocalization of junctional proteins and displayed an organized architecture. Upon infection, the patterned organization was disrupted and in some cases, particular tight junction and adherens junction proteins were undetectable or appeared to be undergoing proteolysis. The extent and timing of these changes differed between both types of vessels (pial vessel disruption within days versus weeks for parenchymal vessels). To approach potential mechanisms, the expression and activity of matrix metalloproteinase-9 (MMP-9) was evaluated by in situ zymography. The results indicated an increase in MMP-9 activity at sites of BBB disruption exhibiting leukocyte infiltration. Moreover, the timing of MMP activity in pial and parenchymal vessels correlated with the timing of permeability disruption. Thus, breakdown of the BBB is a mutable process despite the similar structure of the junctional complex between pial and parenchymal vessels and involvement of MMP activity.

Keywords

neurocysticercosis; blood brain barrier; tight junctions; adherens junctions; pial vessels; matrix metalloproteinases

1. INTRODUCTION

The BBB protects the CNS from compositional fluctuations within the blood stream by limiting the paracellular flux of hydrophilic molecules and the transmigration of leukocytes into the CNS [75]. The BBB is composed of a network of highly specialized endothelial cells

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surrounded by a basal lamina in both pial and parenchymal vessels. In the latter, however, astrocytic end feet processes ensheath the abluminal surface of these cells playing an important role in the maintenance of vessel impermeability [1,28,62]. The paracellular cleft between adjacent lateral endothelial membranes is almost completely sealed because of the presence of a junctional complex that includes tight junctions (TJs) [44,72,75] and adherens junctions (AJs) [67].

Tight junctions (TJs) are the most distinctive molecules in the paracellular cleft because their presence results in a high transendothelial electrical resistance that restricts pinocytic activity and diffusion of polar solutes through the endothelium [31,36]. TJs are located in the most apical section of the plasma membrane, and they are composed of a combination of transmembrane (occludin and claudins) and cytoplasmic molecules including zonula occludens proteins ZO-1, ZO-2, ZO-3 and others [59]. The transmembrane members form heterodimeric bridges that block paracellular diffusion, and these TJ proteins mediate the gate function of the BBB [70]. The TJs are linked and stabilized via the endothelial cell cytoskeleton by complex interactions between transmembrane and cytoplasmic proteins. This linkage is mediated by ZO proteins that serve as a bridge between the cytoplasmic tails of occludin and claudins with the actin cytoskeleton [33]. In the CNS, claudins-1, -3 and -5, together with occludin, have been found in endothelial TJs forming the BBB [49,55]. Another TJ-associated protein is the junctional adhesion molecule (JAM) [51]. Three of these proteins (JAM-A, JAM-B and JAM-C) have been identified, and they belong to the immunoglobulin superfamily [59]. JAM-A is expressed in blood vessels and mediates contact between neighboring cells via homophilic interactions [5,9]. JAM-A is a ligand for LFA-1 and is involved in monocyte and neutrophil transmigration [57].

The adherens junctions (AJs) are ubiquitous in the vasculature and mediate the adhesion of endothelial cells to each other and partially regulate paracellular permeability [8,12]. They consist of the transmembrane proteins cadherins and the cytoplasmic proteins catenins. Catenins are linked with the actin cytoskeleton and support the formation of adhesive contacts between cells. AJs assemble via homophilic interactions between the extracellular domains of calcium-dependent cadherin on the surface of adjacent cells [7]. TJ and AJ components are known to interact, particularly ZO-1 and catenins, and such interplay is known to influence TJ assembly [52].

BBB disruption is a critical process associated with development and progression of several CNS pathologies involving inflammation [59]. Neurocysticercosis (NCC) is one such disease caused by the parasite *T. solium* where inflammatory infiltrates cause a range of symptoms including epileptic seizures, headaches, hydrocephalus, and even death [74]. It is the leading cause of epilepsy worldwide. Recently, we have shown that the kinetics of increased BBB permeability in a murine model of NCC caused by the closely related parasite *Mesocostoides corti* depend upon several parameters including the anatomical area, type of vascular bed, cytokine microenvironment and the subsets of infiltrating leukocytes [2].

The aim of this study was to investigate changes in the expression and structural organizations of TJ and AJ proteins in pial and parenchymal vessels during CNS infection using a murine model of NCC. Brain sections were obtained from mice infected with the cestode *M. corti*. Pial vessels and parenchymal vessels were studied using in situ immunofluorescent staining to detect expression of junctional complex proteins. Disruption of junctional complex proteins positively correlated with specific areas of leukocyte extravasation in parasite infected animals. As matrix metalloproteinases (MMPs), particularly MMP-9, have been associated with barrier disruption [27,38,68], in situ zymography was used to determine MMP-9 activity in compromised vessels. The findings suggest that there is a direct correlation between the distinct

temporal changes in the structural alterations of junctional complex proteins in pial and parenchymal vessels and the timing of detectable MMP-9 activity in these vessels.

2. RESULTS

Alterations in the structural organization of tight junction proteins upon infection

Upon infection, analysis of whole brain sections ($n=5$) revealed that $36\% \pm 5$ of pial vessels and $1\% \pm 0.1$ of parenchymal vessels have perivascular immune infiltrates by 1 and 5 wks post-infection ($n=5$), respectively. To test if the expression and spatial pattern of TJ molecules change during the infectious process, several antibodies directed against TJ proteins including ZO-1, occludin, claudin-3, claudin-5 and JAM-A were used for in situ immunofluorescence microscopy. Pial and parenchymal vessels of mock infected animals expressed all of the TJ proteins tested and together they exhibited an ordered structural pattern with extensive colocalization at the junctions between endothelial cells (Fig 1A and 1D). Analysis of TJ proteins in infected animals showed that the junctional complex organization is lost in pial vessels as early as 24 hrs after infection with the TJs appearing thicker and shorter (Fig 1B). In some cases there was an absence of TJ proteins between endothelial cells and areas of leukocyte migration could be observed (Fig 1B). These changes exacerbated after 1 wk and 3 wks of infection (Fig 1C). Disruption in the architecture of the junctional complex correlated with breakdown of the BBB and was accompanied by leukocyte (Fig 1G–I) and serum protein extravasation [2]. In contrast, the TJs in parenchymal vessels at early time points after infection did not exhibit structural alterations consistent with a lack of serum protein and leukocyte extravasation even after 3 weeks of infection (Fig 1E, [2]). Disruption of the tight junctional architecture was observed in some parenchymal vessels at the fifth week of infection (Fig 1F) and such changes correlated with leukocyte diapedesis (Fig 1I, [2]). Analyses of various combinations of the transmembrane (occludin, claudin-3 and -5) and cytoplasmic (ZO-1) TJ proteins by multiple color immunofluorescence exhibited essentially identical results with the same differential kinetics of disruption of pial and parenchymal vessels.

In addition to the infection induced changes observed in the configuration of TJ proteins relative to each other in pial and parenchymal vessels, vessels exhibiting substantial leukocyte extravasation showed signs of TJ protein downregulation/degradation and in some cases; TJ proteins were undetectable at a portion of the junctional complexes. In mock infected animals claudin-3 (Fig 2A), claudin-5 (Fig 2D), occludin and ZO-1 (Fig 2G) colocalize with JAM-A in the junctional complexes of pial and parenchymal vessels. Pial vessels from 1 and 3 day infected animals showed alterations in the organization of TJ proteins, but the proteins were still detected in the junctional complexes (Fig 1B). After 1 wk, expression of claudin-3 (Fig 2B,b''), claudin-5 (Fig 2E,e'), occludin (Fig 1B, 1H) and ZO-1 (Fig 1C, 2H,h') in pial vessels was either undetected or appeared degraded at distinct junctional complexes. In some cases the entire section of the vessel was almost completely devoid of these junctional proteins (Fig 2B, 2E, and 2H). Interestingly, one exception to this was JAM-A which was detected in pial vessels throughout the entire infectious process analyzed (Fig 2B, 2E and 2H). Disruption of the junctional architecture in parenchymal vessels was found after 5 wks of infection with diminished or no expression of claudin-3 (Fig 2C,c'), claudin-5 (Fig 2F,f') and ZO1 (Fig 2I,i') in the junctional complexes. As seen in pial vessels, JAM-A is present on parenchymal vessels lacking expression of other TJ proteins (Fig 2C, 2F and 2I).

Alterations in the structural organization of adherence junctional proteins upon infection

The AJ proteins VE-cadherin, α -catenin and β -catenin exhibit a methodical structural pattern that localizes in the junctional complexes of pial and parenchymal vessels in mock infected animals (Fig 3A, 3D and 3G). Upon infection such organization is lost following the same time dependant disruption and/or downregulation/degradation described for TJ proteins. The

organization of AJ proteins is altered in pial vessels of 1 and 3 day infected animals (data not shown). After 1 and 3 weeks of infection the expression of the 3 AJ proteins analyzed appears more discontinuous, disrupted and in some cases nearly absent in pial vessels (Fig 3B,b', 3E,e' and 3H,h'). In parenchymal vessels, structural alterations or minimal detection was observed at the fifth week of infection (Fig 3C,c', 3F,f' and 3I,i'), but not at the earlier times of infection analyzed. Evidence for the continuous presence of JAM-A in the junctional complexes of both types of vessels relative to AJ proteins is also presented (Fig 3).

MMP expression and activity correlates with BBB breakdown in pial and parenchymal vessels

MMPs have been shown to degrade components in the basal lamina of CNS vessels, leading to disruption of the blood-brain barrier [25,30,64]. MMP-9 expression and activity were analyzed in pial and parenchymal vessels using immunofluorescence and in situ zymography respectively. As pial vessels are located in leptomeninges, they lack the encasement of astrocytic endfeet. This feature allows the study of MMP activity in areas without major glial influence. In mock infected animals minimal expression of inactive MMP-9 was observed in pial (Fig 4A–C) and parenchymal vessels (data not shown). In contrast pial vessels, but not parenchymal vessels exhibited both expression and apparent activity of MMP-9 in cells transmigrating as early as 1 day after infection (Fig 4D–4F). Also, discernable at this very early time point was gelatinolytic activity at the junctional complex that did not appear to colocalize with MMP-9 (Fig 4D–4F).

Some of the cells moving through the vessel displayed gelatinolysis that was oriented away from the lumen and towards the basal lamina (Fig 4D) and colocalized with MMP-9 expression (Fig 4E–F). The temporal expression and activity of MMP-9 in pial vessels correlates with changes in the structural pattern of TJ and AJ proteins (Fig 1–3). MMP-9 expression associated with activity was not detected in endothelial cells and seems to be present exclusively in infiltrating cells (Fig 4F). As the infection progresses there was more expression and apparent activity of MMP-9 in cells infiltrating the CNS through pial vessels (Fig 4G–4I) whereas parenchymal vessels did not show evidence of MMP-9 activity even after 3 weeks (data not shown). MMP activity was seen in parenchymal vessels just after the fifth week of infection (Fig 4J). At this later time point pial vessels exhibited more extensive gelatinolysis in perivascular infiltrates (Fig 4J). In both type of vessels much of the MMP activity was detected in Mac1+ (CD11b) infiltrating cells (Fig 4K–4L). Thus, the MMP-9 activity detected in pial and parenchymal vessels correlates with the kinetics of BBB disruption [2] and suggests that TJ and AJ proteins may be enzymatically degraded during the inflammatory process.

BBB breakdown is reduced in MMP-9 deficient animals

MMP-9 expression and activity correlated with infiltration of leukocytes in pial and parenchymal vessels. To confirm the role of this MMP in disrupting the BBB, we studied the extent of fibrinogen extravasation in pial and parenchymal vessels of wt and MMP-9^{-/-} mice (Fig 5). Pial vessels were analyzed at 1 wk pi and parenchymal vessels at 6 wks pi as these time points coincided with consistent leukocyte extravasation in the two types of vessels respectively. The degree of fibrinogen extravasation through pial and parenchymal vessels of mock infected wt animals was negligible but slightly elevated in MMP-9^{-/-} animals. Fibrinogen leakage in pial vessels increased significantly after 1 wk of infection in both wt and MMP-9^{-/-} mice. The absence of MMP-9 appeared to provide some protection against leakage but the results were not statistically significant. After 6 wks of infection, parenchymal vessels in infected wt animals exhibited a significant increase in the extravasation of fibrinogen but only a minor increase was detected in MMP-9^{-/-} mice, indicating an important role for MMP-9 in BBB permeability.

Leukocyte infiltration through pial and parenchymal vessels is reduced in MMP-9 deficient mice

To confirm the role of MMP-9 in leukocyte extravasation, we studied the extent of cellular infiltration in pial and parenchymal vessels of wt and MMP-9^{-/-} mice at various times post-infection (Figs 6, 7). Pial vessels located in external and internal leptomeninges were analyzed. Significant differences in the number of leukocytes infiltrating through pial vessels of both leptomeninges were found as early as 3 days pi (Fig 6), and perivascular infiltrates in these areas were more prominent in wt than in MMP-9^{-/-} mice ($p < 0.05$). As the infection progressed, leukocytes accumulated in the external and internal leptomeninges of wild type animals (Fig 6, Fig 7A and B) whereas in the MMP-9^{-/-} the extent of infiltration was significantly lower (Fig 6, Fig 7D and E).

Cellular infiltration through parenchymal vessels was not detected in wild type or MMP-9^{-/-} mice until 5 wks pi. At this pi time, substantial leukocyte infiltration was observed in parenchymal vessels of wt mice (Fig 6 and 7C). Infiltration of leukocytes was not completely abrogated in MMP-9^{-/-} mice, but there was a considerable reduction in the number of cells extravasating parenchymal vessels (Fig 6 and 7F) correlating with the lower degree of fibrinogen extravasation seen in these areas (Fig 5).

3. DISCUSSION

Under basal physiological conditions the BBB limits the entrance of leukocytes supporting CNS immunosurveillance. But upon infections such as NCC, bacterial meningitis or HIV encephalitis, disruption of the BBB integrity is associated with infiltration of antigen presenting cells, neutrophils, B cells, $\alpha\beta$ T cells and $\gamma\delta$ T cells [2,11,14,45]. Our data show that the marked differences in the kinetics of leukocyte extravasation between pial and parenchymal vessels are associated with differential changes detected in the structural orderliness of junctional complex proteins in both types of vessels. Moreover, the level of leukocyte infiltration seems to be directly associated with the extent of junctional complex damage. Thus, expression of junctional complex proteins was dislocated in pial and parenchymal vessels showing moderate leukocyte extravasation, whereas claudin-3, -5, occludin, ZO-1, cadherin, α -catenin and β -catenin were absent or barely detectable in vessels displaying greater perivascular infiltration.

The temporal differences in junctional complex alterations between pial and parenchymal vessels cannot be explained by the location of the parasites in the CNS. Although a low percentage of parasites is found in parenchyma at the early post infection times [14], parasite antigens are detected in the parenchyma within days after infection and they are continuously found in these areas throughout the infectious process (unpublished data). Thus, pial and parenchymal vessels appear to differ in the mechanisms required for leukocyte ingress into the CNS upon infection. One possibility involves the differential expression of adhesion molecules required for diapedesis in both type of vessels. P- and E-selectin are expressed in pial vessels of non-inflamed CNS but not in parenchymal vessels [43]. In addition, the expression of P-selectin is highly restricted and delayed in parenchymal but not in pial vessels of animals exhibiting high clinical scores of experimental autoimmune encephalomyelitis (EAE) [60]. Thus, in the initial stages of the immune response the endothelium of parenchymal vessels, in contrast to pial vessels, seems less supportive for leukocyte migration perhaps due to the lack of proper activation signals and adhesion molecules.

Another major difference between parenchymal and pial vessels is that the latter lack direct contact with astrocytic endfeet which are known to strengthen barrier function [32,34,53]. In addition, pial vessels lack direct contact with other cell types including neurons and neuronal precursor cells. These cells have been shown to support barrier function and endothelium metabolism in parenchymal vessels [20,33,66]. Our results indicate that both types of vessels

show similar immunoreactivity to specific antibodies directed against distinct junctional complex proteins suggesting similar interendothelial bonds. This is consistent with previous studies that have shown that pial and parenchymal vessels share ultrastructural, biochemical and restricted permeability properties [1]. However, due to the less protective microenvironment surrounding pial vessels, the junctions between pial endothelial cells appear to be more susceptible to disruption than that of parenchymal vessels.

Previous studies have shown changes in the morphology of junctional complexes during diapedesis but largely in vitro. Such analyses have shown collectively that an increase in leukocyte migration alters the molecular organization of the junctional complex including breakdown of occludin, claudin-1, -5, ZO-1, disappearance of AJ proteins and reorganization of the actin cytoskeleton [3,15,37,58]. However, in vitro studies have multiple disadvantages related to the source/phenotype of endothelium, culture conditions, the transendothelial electrical resistance established, and inability to replicate the complex CNS microenvironment [6,13,16,21,46]. To date, in vivo studies of patients have relied on post-mortem samples negating kinetic analyses, and both human and animal studies in vivo have been restricted to the analysis of parenchymal vessels. The results of these studies have been variable. Thus, samples obtained from humans with HIV-1 encephalitis displayed decreased expression of occludin and claudin-5 which correlated with the level of monocyte brain infiltration [58]. Selective loss of claudin-3, but not claudin-5, ZO-1 and occludin have been described in human glioblastoma multiforme and in EAE [76]. In contrast with the findings in the EAE model, abnormalities in the expression of ZO-1 and occludin have been described in multiple sclerosis brains [42,61] suggesting that changes in the expression of junctional complex proteins may be unique to the CNS disorder studied. The studies herein, which allow for a kinetic analysis of multiple tight and adherens junction proteins of both pial and parenchymal vessels as well as permeability changes [2], indicate that the extent of changes/disappearance of junctional proteins correlate with the extent of leukocyte infiltration. This may also help explain differences observed in the published studies.

The only exception to this was JAM-A which was found throughout the infectious process in both pial and parenchymal vessels. JAM-A has been implicated in leukocyte adhesion and migration through the junctional complex and appears to interact with multiple intracellular partners [50,56]. Anti-JAM-A antibody inhibited leukocyte diapedesis in vitro [19]. In contrast, reduced expression and redistribution of JAM-A after TNF- α and IFN- γ pretreatment of endothelial cells does not decrease adhesion or transmigration of leukocytes under flow [69]. Contradictory results were also found in vivo; anti-JAM-A antibody was able to block leukocyte entry into the CNS in a murine meningitis model [19], whereas in another study the same antibody failed to prevent leukocyte influx into the CNS after infection of mice with *Listeria monocytogenes* [47]. Therefore, the role of JAM-A in the BBB remains to be elucidated, but its continuous expression in the junctional complexes shown here may reflect its importance in supporting endothelium structure and morphology even in the absence of other junctional complex proteins. In this regard it is interesting that JAM-A concentrates at intercellular contacts at an early step in the formation of the more complex junctional organization [10]. Moreover, JAM-A null mice exhibit increased hepatic ischemia reperfusion damage despite a decrease in neutrophil infiltration [40].

Various studies suggest that MMPs are involved in the opening of the BBB and MMP-2 and MMP-9 appear to play an important role [63]. To further understand the nature of BBB disruption in pial and parenchymal vessels, the potential expression and activation of MMP-9 was analyzed during the course of infection. Polarized gelatinolytic activity in cells moving from the luminal side of pial vessels to the basal lamina suggests the direct role of this enzymatic activity in its movement through the junctional complex. The temporal expression of MMP-9 and gelatinolytic activity coincided with dislocation and disappearance of junctional complex

proteins implicating the role of MMP-9. In previous studies ZO-1, but not occludin, has been described as a MMP-9 substrate in ischemic brains, and its degradation was significantly reduced in the MMP-9 null background [4]. In vitro studies using human umbilical vein endothelial cells (HUVECs) showed that inhibition of the protein tyrosine phosphatase induced proteolysis of occludin, but not ZO-1, cadherin and β -catenin. MMP involvement in this process was demonstrated when occludin proteolysis was inhibited by the metalloproteinase inhibitor 1,10-phenanthroline [73]. Our study is the first reporting the possible association of MMP-9 activity with the differential disappearance of claudin-3, -5, occludin, ZO-1 and AJ proteins from the junctional complex of pial and parenchymal vessels. However, the role of other MMPs cannot be discounted.

To understand the genesis of BBB disruption, it was important to analyze the potential source of MMP-9 activity. Infiltrating leukocytes have been described as the main source of this MMP [27,38,68]. However, microglia and astrocytes have been also reported to express MMPs and affect the BBB upon insult [24,65]. In this study, we found that enzymatically active MMP-9 is produced by leukocytes, and its polarized activity during the process of exiting pial and parenchymal vessels strongly suggests its involvement in the ability of the leukocyte to extravasate the vessel itself. However, after extravasation cells exhibiting MMP-9 activity continue to accumulate in infiltrates without noticeable polarization suggesting an additional role for MMP-9 in supporting the inflammatory process induced by infection, e.g. cytokine and chemokine activation [26,71].

Further understanding of the role played by MMP-9 in the breakdown of the BBB was obtained by studying fibrinogen extravasation and immune cell infiltration in MMP-9 deficient animals. Decreases were found in the relative extent of fibrinogen extravasation and the number of leukocytes infiltrating the brain of MMP-9 deficient compared to wild type animals during the infection process. These findings correlate with results from a mouse model of ischemia, where the magnitude of BBB breakdown was reduced to a similar extent in MMP-9 deficient mice and in chimeras lacking leukocytic MMP-9, but not in chimeras with MMP-9 containing leukocytes [27]. Our data suggest that in murine NCC the source of MMP-9 activity is from infiltrating leukocytes. As the kinetics of MMP-9 directly correlate with BBB breakdown, leukocyte infiltration and degradation of junctional complex proteins, it is likely that immune cell derived MMP-9 is an active participant in BBB disruption during infection. However, slight fibrinogen leakage in mock infected MMP-9 deficient mice was found in the absence of leukocyte infiltration. Thus, MMP-9 activity in the normal brain apparently plays a role in maintaining BBB permeability correlating with the known multifunctional nature of individual MMPs.

Like MMP-9, MMP-2 has been the focus of studies dealing with the breakdown of the BBB during various CNS disorders [23,35]. This MMP could be playing a role in the disruption of the BBB in the murine model of NCC as BBB breakdown and leukocyte infiltration are not completely abrogated in the brain of MMP-9 deficient animals. However, it has been observed that MMP-9 activation but not MMP-2 may worsen the pathology of cerebral ischemia when pharmacotherapy with tissue plasminogen activator is supplied [39]. Also potentially relevant are MMP-8 and MMP-3 as they have been shown to affect the permeability of the barrier under distinct CNS conditions [29,41,48]. Thus, a better understanding of the mechanisms directing BBB disruption during the course of murine NCC will be determining the differential roles of relevant MMPs and the extent of redundancy.

The distinct kinetics of leukocyte extravasation through pial and parenchymal vessels can be correlated with the symptomatology of NCC. Metacestodes lodged in leptomeningeal spaces are commonly associated with development of meningitis, hydrocephalus and intracranial hypertension [18]. Whereas, more severe forms of NCC such as epilepsy and encephalitis are

more frequent in patients with parenchymal metacestodes [17,54]. Thus, leukocyte extravasation and inflammation in leptomeninges appear to be less detrimental to the host and the delay of such processes in the parenchyma suggests an inherent mechanism of protection against exacerbated immune responses in the nervous tissue.

4. EXPERIMENTAL PROCEDURE

Animals

Female BALB/c mice 3–5 wk old were purchased from the National Cancer Institute Animal Program (Bethesda, MD). Wild-type FVB mice and MMP-9 null (MMP-9 deficient) mice 3–5 wk old were purchased from Jackson Laboratory (Bar Harbor, ME). Animal experiments were conducted under the guidelines of the University of Texas System, the U.S. Department of Agriculture, and the National Institutes of Health.

Parasites and inoculations

M. corti metacestodes were maintained by serial intraperitoneal inoculation of 8 to 12 wk old female BALB/c mice. For intracranial inoculations, parasites were aseptically collected from the peritoneal cavity and extensively washed in HBSS. Then, metacestodes (50 microorganisms) were suspended in 50 μ l of HBSS and injected intracranially into 3–5-wk-old female mice from the different genotypes using a 1-ml syringe and a 25-gauge needle [14,22]. The needle was inserted to a 2-mm depth at the junction of the superior sagittal and the transverse sutures. This allows insertion of the needle into a protective cuff avoiding penetration of the brain tissue. Control mice were injected with 50 μ l sterile of HBSS using the same protocol. Before intracranial inoculation, mice were anesthetized intramuscularly with 20 μ l mouse mixture containing 100 mg/ml ketamine and 20 mg/ml rompum (Laboratory Animal Resource, University of Texas Health Science Center, and San Antonio, TX). Animals were sacrificed at 1 d, 3 d, 1 wk, 3 wk and 5 wk after inoculation and analyzed for parasite burden and immune cell infiltration. Before sacrifice, animals were anesthetized with 25 μ l of mouse mixture and perfused through the left ventricle with 15 ml of cold PBS. Five or more animals were analyzed for each post-infection time with multiple sections analyzed per animal.

Tissue processing

Perfused brains were immediately removed, embedded in O.C.T. resin (Sakura, Torrance, CA) and snap frozen. Serial horizontal cryosections of 10 μ m in thickness were placed on silane prep slides (Sigma-Aldrich, St. Louis, MO). One in every five slides was fixed in formalin for 10 min at room temperature and stained with Hematoxylin and Eosin (H&E). The remainder of the slides was air dried overnight and fixed in fresh acetone for 20 seconds at room temperature (rt). Acetone-fixed sections were wrapped in aluminum foil and stored at -80°C or processed immediately for immunofluorescence.

Antibodies

Antibodies purchased from Zymed (San Francisco, CA) and generated in rabbits included: anti- β -catenin, anti- α -catenin, anti-occludin, anti-claudin-3 and anti-claudin-5. Goat-anti-JAM-A was obtained from R&D systems (Minneapolis, MN). Antibodies purchased from BD PharMingen (San Diego, CA) include 11D4.1 (rat anti-VE-cadherin), M1/70 (rat anti-Mac1 phycoerythrin (PE)-conjugated), GL3 (hamster anti-panTCR $\gamma\delta$ PE-conjugated), H57–597 (hamster anti-TCR β chain PE-conjugated). The purified rabbit anti-fibronectin and rat anti-Zonula Occludens 1 (ZO1) antibodies were purchased from Chemicon International (Temecula, CA). Rabbit anti-MMP-9 was purchased from Triple Point Biologics (Forest Grove, OR). Rabbit primary antibodies were detected with a Cy2-conjugated AffiniPure anti rabbit IgG and JAM-A with a Rhodamine red X (RRX)-conjugated AffiniPure anti goat IgG.

ZO-1 was detected with a RRX and Cy2 conjugated AffiniPure anti rat IgG purchased from Jackson ImmunoResearch (West Grove, PA). All antibodies were titrated with brain sections from 2 wk infected animals to determine optimal concentrations prior to use. Spleen sections were used as positive controls in titration experiments.

Immunofluorescence microscopy

Double immunofluorescence staining was performed as previously described [2]. Briefly, tissues were fixed in -20°C acetone for 10 min and then hydrated in PBS. Non-specific immunoglobulin binding was blocked by 30 min incubation at rt with serum from the same species that the fluorochrome conjugated antibodies were derived. Sections were incubated for 40 min with primary antibodies diluted in species specific serum. Sections were washed 7X for 3 min each after incubation with specified antibodies. Secondary antibodies were incubated for 30 min at rt when necessary. The second set of primary antibodies was then incubated with their respective secondary antibody. Negative controls using secondary antibodies alone were included in each experiment and found to be negative for staining. After washing, sections were mounted using fluorsave reagent (Calbiochem, La Jolla, CA) containing $0.3\text{ }\mu\text{M}$ 4', 6'-diamidino-2-phenylindole dilactate-DAPI (Molecular Probes, Eugene, OR). Fluorescence was visualized using a Leica DMR epifluorescent microscope (Leica Microsystems, Wetzlar Germany). Images were acquired using a cooled CCD SPOT RT camera (Diagnostic Instruments Inc, Sterling Heights, MI), and they were processed and analyzed using Adobe Photoshop 7.0 (Adobe, Mountain View, CA).

In situ zymography

In situ zymography was performed to detect and localize gelatinolytic activity in brain sections. Sections that had been stored at -80°C were fixed and hydrated as described for immunofluorescent microscopy. A solution containing DQ-gelatin (Molecular probes, Eugene, OR) a MMP substrate heavily labeled with fluorescein, was used to determine activity of MMP-9 in situ. The increase in fluorescence upon digestion is proportional to MMP activity. Sections were incubated 40 minutes at rt with $1\text{ }\mu\text{g/ml}$ of DQ-gelatin in reaction buffer (0.05 M Tris-HCl, 0.15 M NaCl, 5 mM CaCl_2 , pH 7.6), washed and subsequently followed by immunofluorescent staining to correlate the levels of active and inactive MMP-9 in the samples. Images were acquired and processed as previously described.

Analysis of fibrinogen extravasation

To establish the status of the BBB in pial and parenchymal vessels of FVB (wild type) and MMP-9 deficient genotypes, brain cryosections from infected and non-infected animals were stained with antibodies against JAM-A and fibrinogen. JAM-A was used to provide a frame of reference for the location of blood vessels. Images of a number of blood vessels from mock and infected animals were taken using identical camera settings so that the number and intensity of pixels were solely due to differences in fibrinogen extravasation. Disruption of the BBB in both types of vessels was determined by measuring the area (number of pixels) and fluorescent intensity (average intensity of pixels) of fibrinogen surrounding vessels exhibiting leakage. This was done using the imaging software IP lab 4.0 (BD Biosciences bioimaging, Rockville MD). The relative extent of fibrinogen extravasation was calculated by multiplying the number of pixels (area) by the average intensity of pixels. Statistical analysis was performed with a student's t-test using SIGMA PLOT 8.0 (Systat Software, San Jose CA).

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Figure 1. Kinetics of TJ protein expression changes in pial and parenchymal vessels during murine NCC

Nuclei are stained blue with DAPI. ZO1 (Fig 1A–F) and leukocyte subtypes (Fig 1G–I) are shown in green (Alexa 488). Occludin is shown in red (Rhodamine red X). (A) Parallel organization of TJs in pial vessels (asterisks) of mock infected animal. X1000 (B) Disruption of TJ architecture in pial vessel of 1 day infected animal. Arrowheads indicate cells migrating through the vessel. X1000 (C) Massive leukocyte migration, perivascular infiltrates (i) and disruption of TJ architecture in pial vessel of 3 wk infected animal. X400 (D) Alignment of TJ proteins in parenchymal vessel (asterisk) of mock infected animal. X1000 (E) Expression and morphology of TJ proteins remain intact in parenchymal vessel (asterisk) of 3 wk infected animal. X1000 (F) Disruption of TJs in parenchymal vessel (asterisk) after 5 wk of infection. X1000 (G) Occludin expression showing parallel organization in junctional complexes of pial vessels (asterisks). Perivascular macrophages (green) are found in vicinity of the vessels. Mock infected animal. X400 (H) Disruption of TJ architecture shown by occludin staining in pial vessel of 1 wk infected animal. Arrowheads represent $\gamma\delta$ T cells (green) migrating through the vessel. X1000 (I) Migration of $\alpha\beta$ T cells (green) through parenchymal vessel that displayed disrupted expression of occludin. 5 wks after infection. X1000

Figure 2. Downregulation/degradation of TJ proteins in murine NCC

Nuclei are stained blue with DAPI. JAM-A is shown in **green** (Alexa 488) and claudin-3 (A to C), claudin-5 (D to F) and ZO1 (G to I) in **red** (Rhodamine red X). For each image a portion of it; demarcated by dotted lines and labeled a', b', b'', c', etc is shown below in which only claudin-3, claudin-5 and ZO1 are included so that changes in expression can be more easily observed (in red). **(A)** Claudin-3 and JAM-A expression in the junctional complexes of pial vessels in a mock infected animal. X400 **(B)** Claudin-3 and JAM-A colocalize in parenchymal vessel (arrowhead), but claudin-3 expression is almost undetectable in pial vessel (asterisk) after 1 wk of infection (b''). X1000 **(C)** Parenchymal vessel of 5 wk infected animal expressing JAM-A; low to undetectable levels of claudin-3 are detected (c'). X1000 **(D)** Claudin-5 and JAM-A expression in parenchymal vessel of mock infected animal. X400 **(E)** JAM-A, but no claudin-5 (e') is detected in an inflamed pial vessel after 3 wks of infection. X630 **(F)** Parenchymal vessel of 5 wk infected animal expressing JAM-A; disrupted and low expression of claudin-5 (f') is detected. X630 **(G)** ZO1 and JAM-A expression in parenchymal vessel of mock infected animal. X1000 **(H)** ZO-1 expression is low and undetectable in pial vessels (small arrowheads). A parenchymal vessel (large arrowhead) in the vicinity shows low ZO1 expression in area (red arrowhead) close to the subarachnoid space. JAM-A is detected in all the vessels. 1 wk post-infection. X630 **(I)** ZO1 expression is lost in parenchymal vessel (asterisk) after 5 wks of infection (i'). X630

Figure 3. Changes in the expression of AJ proteins during the course of murine NCC

Blue represents the nuclei stained with DAPI. JAM-A is represented in **green** (Alexa 488) and VE-cadherin (A to C), α -catenin (D to F) and β -catenin (G to I) in **red** (Rhodamine red X). For each picture a section (between dashed lines and labeled a', b', etc) only showing the AJ proteins was included to better appreciate changes in expression during infection. All pictures were taken with a 63X objective (A) VE-cadherin and JAM-A expression in pial (arrowheads) and parenchymal vessels of mock infected animal (B) Disruption and loss of VE-cadherin in junctional complexes of pial vessel (asterisk) after 3 wks of infection (b') (C) Parenchymal vessels (arrowheads) of 5 wk infected animal stained with JAM-A; low to undetectable levels of VE-cadherin are found (c') (D) α -catenin and JAM-A expression in pial and parenchymal

vessels (asterisks) of mock infected animal α (**E**) Disruption and loss of α -catenin (e') in pial vessel (asterisk) of 3 wk infected animal. JAM-A is present in the vessel (**F**) Disruption of the TJ architecture accompanied of low to undetectable levels of α -catenin (f') in parenchymal vessel (asterisk) of 5 wk infected animal (**G**) β -catenin and JAM-A expression in pial (arrowheads) and parenchymal vessels (asterisks) of mock infected animal (**H**) Pial vessel (asterisk) of 3 wk infected animal showing low to undetectable expression of β -catenin (h') (**I**) Parenchymal vessel (asterisk) of 5 wk infected animal expressing low to undetectable levels of β -catenin (i').

Figure 4. Expression and activity of MMP-9 in pial and parenchymal vessels at different post-infection times

Enzymatic activity of MMPs is visualized by in situ proteolysis of gelatin conjugated with Alexa 488 (green). MMP-9 and inflammatory cells were detected using antibodies conjugated with rhodamine red X (red). Nuclei were stained with DAPI (blue). DAPI staining was excluded in C, F, I and L for clarity. (A) Pial vessel (asterisk) displaying minimal gelatinolysis in mock infected animal. X1000 (B) Same field as A showing low expression of MMP-9 in cells associated with pial vessel (asterisk) (C) Merged image of A and B showing very low expression of active MMP-9 in cells (arrowheads) extravasating pial vessel (asterisk) (D) Pial vessel (asterisk) with extravasating cells (arrowheads) showing polarized gelatinolysis from the lumen towards the basal lamina and the perivascular area. 1 day infected animal. X1000 (E) Same field as A, but showing MMP-9 expression in extravasating cells (F) Merged image of D and E. MMP-9 expression (red) and gelatinolysis (green) is seen in cells (arrowheads) infiltrating into leptomeninges through a pial vessel (asterisk) (G) Increase of gelatinolytic activity in cells extravasating pial vessels (asterisks) after 1 wk of infection. X400 (H) Same field as G showing MMP-9 expression (I) Merged image of G and H. Gelatinolysis (green) and MMP-9 expression (red) increase in cells (arrows) extravasating pial vessels (asterisks) (J) Gelatinolytic activity in cells infiltrating in the periphery of pial (asterisk) and parenchymal (arrow) vessels after 5 wks of infection. X400 (K) Same field as G, showing Mac1⁺ cells (CD11b) (L) Merged image of J and K. Gelatinolysis in Mac1⁺ cells infiltrating through pial vessel (asterisk) and parenchymal vessel (arrow).

Figure 5. Measure of BBB permeability in pial and parenchymal vessels of wild type and MMP-9^{-/-} animals

Quantitative analysis of fibrinogen extravasation in both genotypes was done in pial (1 wk) and parenchymal vessels (6 wks) of mock and *M. corti* infected animals ($n = 5$). The relative levels of fibrinogen extravasation were calculated as described in Experimental Procedures. The extent of fibrinogen extravasation was higher in pial and parenchymal vessels of mock infected MMP-9^{-/-} mice compared to wt. Upon infection, the extent of fibrinogen measured outside of vessels was higher in wild type than MMP-9^{-/-} animals, particularly in parenchymal vessels ($p < 0.01$). Error bar: standard error of the mean (SEM). The data obtained was compared using a student's t-test.

Figure 6. Infiltrating leukocytes in perivascular space of vessels located in leptomeninges and parenchyma of wild type and MMP-9^{-/-} animals infected with *M. corti*

Semiquantitative analysis of cellular infiltrates was done by counting the number of leukocytes perivascular to pial vessels in both the external and internal leptomeninges and parenchymal vessels ($n = 12$ vessels per analyzed area) at different post-infection times. With the exception of the occasional perivascular macrophages normally present in pial and parenchymal vessels of mock infected mice (Fig 1G), the number of perivascular leukocytes in these animals is essentially zero as multiple sections needed to be screened to find 1 or 2 leukocytes by fluorescent staining for specific immune cell subsets. Error bar: standard error of the mean (SEM), the number of cells in the perivascular infiltrates of both type of vessels is statistically different comparing wild type and MMP-9 deficient mice in the distinct post-infection times, $P < 0.05$ (paired t -test). LM: leptomeninges, Parench: parenchymal.

Figure 7. Leukocyte infiltration in wild type and MMP-9^{-/-} mice

H&E staining of mouse brain sections at different post-infection times. A to C are samples from wild type mice and D to F are from the MMP-9^{-/-} phenotype (A) Leukocyte infiltrates (arrowhead) in external leptomeninges. Wild type – 1 wk, X200 (B) Leukocyte infiltrate (i) in periphery of pial vessels (asterisks) located in internal leptomeninges. Wild type – 3 wk, X400 (C) Massive leukocyte infiltration (arrowhead) around parenchymal vessel. Wild type – 5 wk, X200 (D) External leptomeninges (arrowheads) without apparent leukocyte infiltration. MMP-9^{-/-} – 1 wk, X200 (E) Scarce leukocyte infiltration in periphery of pial vessels (asterisks) located in internal leptomeninges. MMP-9^{-/-} – 3wk, X400 (F) Low perivascular infiltration (arrowheads) in parenchymal vessels. MMP-9^{-/-} – 5wk, X200.