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Tumor necrosis factor-α disruption of brain endothelial cell barrier is mediated through matrix metalloproteinase-9

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Abstract Traumatic brain injuries cause vascular hyperpermeability. Tumor necrosis factor-α (TNF-α), matrix metalloproteinase-9 (MMP-9), and caspase-3 may be important in these processes but the relationship between them has not been investigated. We hypothesized that TNF-α regulates caspase-3-mediated hyperpermeability and blood brain barrier damage and hyperpermeability directly or indirectly via activation of MMP-9. To test this, rat brain microvascular endothelial cells were treated with TNF-α with or without inhibition of MMP-9. Monolayer permeability was measured, zonula occludens-1 and F-actin configuration were examined, and MMP-9 and caspase-3 activities were quantified. TNF-α increased monolayer permeability, damaged zonula occludens-1, induced filamentous-actin stress fiber formation, and increased both MMP-9 and caspase-3 activities. Inhibition of MMP-9 attenuated these changes. These data highlight a novel link between TNF-α and MMP-9 and show that TNF-α regulated caspase-3-mediated hyperpermeability and vascular damage may be linked to MMP-9 in vitro. These findings augment the understanding of traumatic brain injury and pave the way for improved treatment.

In 2010, the CDC reported 2.5 million traumatic brain injuries (TBIs): the costs of such injuries have been estimated to range between $60 and $221 billion.[1,2] Okie[3] also reported that 25% of the soldiers evacuated from the conflicts in Iraq and Afghanistan have suffered TBIs, implying that the actual costs of TBIs may be even higher when factoring effects on society and years of productivity loss. As such, much attention has been paid to understanding the etiology, pathology, mechanisms, and therapeutic options in TBIs.

Unfortunately, this investigation is complicated by the heterogeneity of TBIs. Encompassing a wide range of

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etiologies, the term "TBI" covers a spectrum of injury ranging from extremely mild to completely debilitating or even fatal.[4] Despite the diversity of pathology, the majority of all brain injuries include a primary insult and a secondary injury. The primary injury refers to the initial mechanical harm to the brain, while the secondary injury describes a process initiated by the primary insult that involves perpetuation of damage either by host cellular response or by ongoing physiological insults.[5,6] Although only injury prevention can assuage primary injury, secondary injury can sometimes be amenable to intervention and as such has been the main focus of TBI research of late.[7]

It is known that following the mechanical injury of a TBI, a complex cascade of ischemia, electrolyte disturbances, oxidative stress, lipid peroxidation, and excitotoxicity lead to membrane disruption and mitochondrial dysregulation within the brain.[8,9] Intracellular ATP availability decreases and Ca^{2+} triggers a variety of proteases that, among other things, damage structural proteins important in blood brain barrier (BBB) integrity.[7,10,11] Breakdown of the BBB compromises the protection usually afforded to the brain, allowing extravasation of fluids, proteins, and immune cells from the intravascular compartment into the parenchyma.[12] In addition to increasing intracranial pressure and decreased cranial blood flow, this can lead to activation of both infiltrating and resident immune cells, with release of chemokines, cytokines,[7] and activation of complement.[13]

Pivotal in this multifaceted process is the BBB. Under physiologic conditions, the BBB comprised the capillary basement membrane, brain microvascular endothelial cells, astrocyte endfeet, and pericytes; among these, the endothelial cells are thought to be the most important.[14] Brain endothelial cells possess several features that contribute to the unique protective qualities of the BBB, one of which is their tight junctions (TJs).[14] The TJs are multiprotein complexes, with extracellular proteins such as occludin and claudins tethered to the intracellular cytoskeleton through zonula occluden-1 protein (ZO-1).[14] As such, damage to TJ is thought to be integral in pathophysiology related to injury of the brain[15] and in TBI, derangements of TJ proteins occur through a variety of channels. Matrix metalloproteinases (MMPs), especially MMP-9, have been implicated as important among such mediators.[16–18]

The avenues through which this occurs in TBI have not been fully elucidated. There is evidence outside trauma demonstrating induction of MMP-9 by various cytokines, such as tumor necrosis factor-α (TNF-α).[19–22] TNF-α is known to be important in the secondary injury seen after TBI[23] and to affect BBB integrity[24]: the relationship between TNF-α and MMP-9 holds promise in better understanding of the vascular permeability of TBI, but is yet unexplored. Additionally, an association between MMP-9 and caspase-3 has also been noted.[25] Furthermore, caspase-3 is a known downstream target of TNF-α and recent evidence from our laboratory has demonstrated that TNF-α increases microvascular endothelial cell permeability via a caspase-3 pathway. These findings and literature call into question the interplay among MMP-9, TNF-α, and caspase-3 in vascular permeability and TJ degradation in TBIs. As this has not been investigated, we hypothesized that in vitro TNF-α regulates caspase-3-mediated BBB disruption and hyperpermeability directly or indirectly via activation of MMP-9 and that MMP-9-mediated breakdown of TJ proteins may be an avenue through which TNF-α affects BBB permeability.

Methods

Chemicals and reagents

Rat brain microvascular endothelial cells (RBMECs) from Cell Applications, Inc (San Diego, CA), fibronectin from bovine serum (.1% solution; Sigma-Aldrich, St. Louis, MO), RBMEC growth media (Cell Applications, Inc), and Trypsin-EDTA solution (.25%; Invitrogen, Grand Island, NY) were used for all in vitro experiments. TNF-α was obtained from Sigma and MMP-9 inhibitor 1 from Calbiochem (San Diego, CA). DMEM-Fluorobrite (Life Technologies, Grand Island, NY) media and Fluorescein isothiocyanate (FITC)-dextran (molecular weight 10,000; Sigma-Aldrich) were used for permeability studies. For chamber slides, polyclonal rabbit antibody against ZO-1 and rhodamine phalloidin were obtained from Life Technologies. VECTASHIELD Mounting Medium was obtained from Vector Laboratories (Burlingame, CA). An MMP-9 Assay Kit from Anaspec (SensoLyte 520 MMP-9 Assay Kit; Anaspec, Fremont, CA) and a Caspase-3 Assay Kit from Calbiochem were utilized for enzyme activity measurements.

Endothelial cell monolayer permeability

RBMECs were cultured until confluent monolayers on Transwell inserts (Corning Life Sciences, Lowell, MA). Wells were divided as follows ($n = 6$): control, TNF-α (10 ng/mL), MMP-9 inhibitor 1 (10 nmol), and TNF-α plus MMP-9 inhibitor 1. A concentration of 10 ng/mL was based on dose response experiments conducted in our laboratory (data not included) and the MMP-9 inhibitor 1 concentration was per the manufactures recommended dose for specific MMP-9 inhibition. (MMP-9 inhibitor 1 is known to inhibit MMP-1 at concentrations 1.05 μM and MMP-13 at 113 nM: 10nmol was selected to avoid inadvertent blockage of these MMPs.) In the MMP-9 inhibitor 1 groups, wells were pretreated at 37°C for 1 hour. Wells were exposed to either TNF-α in media or media alone for 1 hour at 37°C. FITC-dextran was added to the upper chambers and allowed to equilibrate through the monolayer for 30 minutes. Samples obtained from the lower chamber were analyzed using a fluorometric plate reader at excitation/emission (494/520 nm) to quantitate monolayer permeability.

Staining of structural proteins

RBMECs were grown on chamber slides for 24 hours (Thermo Scientific, Rochester, NY) and divided as previously

described. MMP-9 inhibitor 1 pretreatment was performed at 37°C for 1 hour. TNF-α dissolved in media or media alone were applied and incubated for 1 hour at 37°C. Cells were fixed with 4% paraformaldehyde, permeabilized with .5% Triton X-100, and blocked with 2.5% BSA-PBS. For immunofluroescent staining of TJ proteins, slides were exposed to polyclonal rabbit antibody against ZO-1 at 4°C overnight and then secondary anti-rabbit FITC-tagged antibodies for 1 hour. For staining of filamentous-actin (F-actin), rhodamine phalloidin was applied and allowed to incubate for 20 minutes. Cells were mounted utilizing Vectashield DAPI-antifade reagent and imaged with a confocal laser-scanning microscope at 60× (Olympus Fluoview, Center Valley, PA).

Matrix metalloproteinase activity assay

SensoLyte 520 MMP-9 Assay Kit (Anaspec) was used for MMP-9 activity assay. RBMECs were grown to confluence on fibronectin-coated plates and divided as previously described. MMP-9 inhibitor 1 was applied for 1 hour and plates were incubated at 37°C. TNF-α dissolved in media or media alone were applied and allowed to incubate for 1 hour at 37°C. Cells were lysed and protein from each sample was estimated with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Equal amounts of protein from each sample were incubated with 1 mM 4-aminophenylmercuric at 37°C for 1 hours to activate MMP-9. 5-FAM/QXL 520 FRET peptide, a substrate that fluoresces when cleaved by MMP-9, was applied to the samples and allowed to incubate for 60 minutes at 37°C. Samples were analyzed using a fluorometric plate reader at excitation (494 and 520 nm) to quantitate MMP-9 activity.

Caspase-3 activity assay

Calbiochem's Caspase-3 Assay kit was used for caspase-3 activity quantification (Calbiochem). RBMECs were grown to confluence and divided as described previously. For 1 hour, plates in the treatment groups were incubated with MMP-9 inhibitor 1 at 37°C. TNF-α dissolved in media or media alone were applied and allowed to incubate for 1 hour at 37°C. Cells were harvested in extraction buffer and incubated on ice for 20 minutes. The suspension was centrifuged at 10,000 rpm for 5 minutes and the supernatant was removed. Sample buffer was added to the cell pellet per kit instructions. Caspase-3 substrate (DEVD-AFC) was added. (Intact DEVD-AFC molecules are not fluorescent, but after cleavage by caspase-3, fluorescence can be measured). Samples were covered, incubated at 37°C for 2 hours, and quantification of caspase-3 activity was performed using a fluorometric plate reader at excitation/emission (494/520 nm).

Statistical analysis

All data are expressed as mean ± standard error. Comparisons between groups were made using analysis of variance or unpaired *t*-test where appropriate. Experimental values were compared with initial baseline value and expressed as percentage change. A P value of less than .05 was considered to indicate a statistically significant difference.

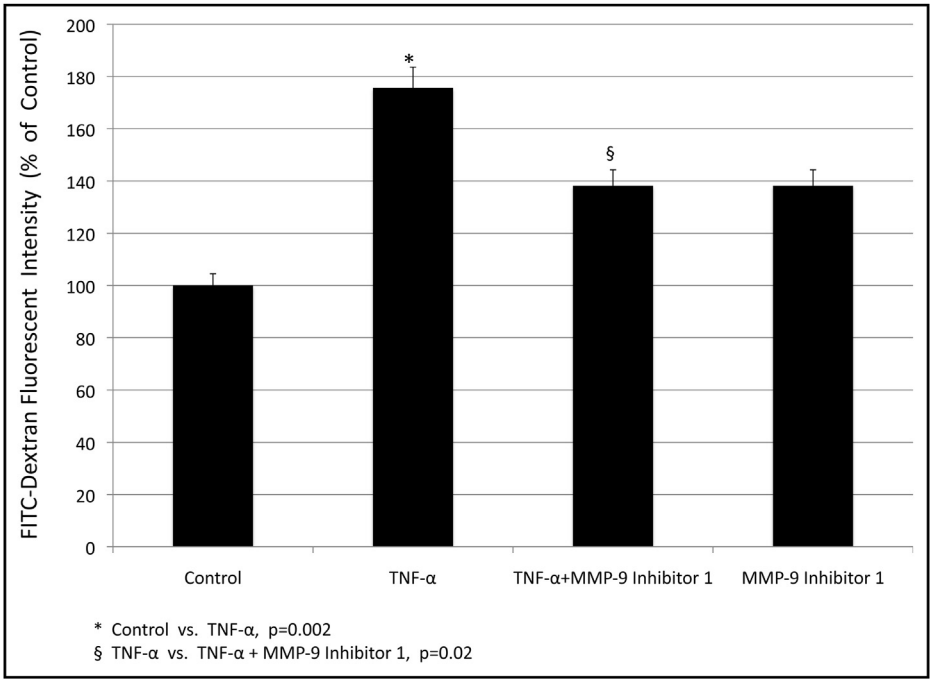

Figure 1 MMP-9 attenuates TNF-α-induced RBMEC monolayer hyperpermeability. Permeability is significantly increased in cells treated with TNF-α cells versus control as indicated by * ($P = 0.002$), and this increase is significantly inhibited by MMP-9 inhibitor 1 as indicated by § ($P = 0.02$).

Results

Matrix metalloproteinase-9 inhibitor 1 decreases tumor necrosis factor-α-induced monolayer hyperpermeability

Fluorescent intensity was measured and values are expressed as a percentage of control. RBMEC monolayers exposed to TNF-α displayed significantly increased permeability evidenced by increased fluorescent intensity when compared with those measured in the control group ($P = .002$). MMP-9 inhibitor 1 significantly attenuated this increase ($P = .02$; Fig. 1).

Tumor necrosis factor-α-induced disruption to tight junction protein zonula occluden-1 is ameliorated with matrix metalloproteinase-9 inhibitor 1

Confocal images of RBMECs with immunofluorescence staining of the endothelial TJ protein ZO-1 are illustrated in Fig. 2A, demonstrating loss and rearrangement of ZO-1 in cells treated with TNF-α when compared with those in the control group (Fig. 2A, white arrow). Pretreatment with MMP-9 inhibitor 1 mitigated TNF-α-induced damage and reorganization of ZO-1 proteins and preserved baseline TJ integrity (Fig. 2A, yellow arrow).

Matrix metalloproteinase-9 inhibitor 1 decreases filamentous-actin stress fiber formation after tumor necrosis factor-α exposure

Fig. 2B displays rhodamine phalloidin staining for the cytoskeletal protein F-actin. Cells exposed to TNF-α demonstrated increased polymerization of actin and formation of F-actin stress fibers when compared with those in the control group, indicating cytoskeletal rearrangement associated with TJ damage (Fig. 2B, white arrow). In cells pretreated with MMP-9 inhibitor 1, baseline actin cytoskeletal configuration was maintained even after exposure to TNF-α (Fig. 2B, yellow arrow).

Tumor necrosis factor-α increases matrix metalloproteinase-9 activity and matrix metalloproteinase-9 inhibitor 1 attenuates the increase

RBMEC MMP-9 activity was quantified and results are expressed as a percentage of negative control (Fig. 3).

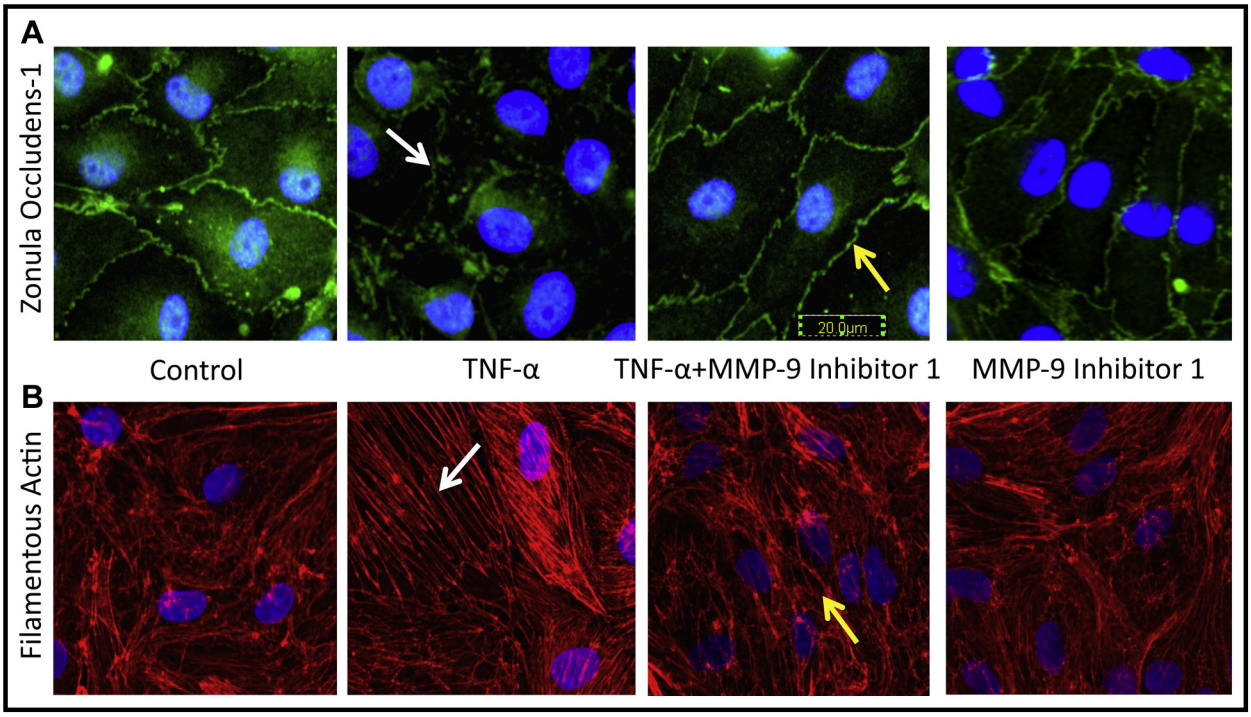

Figure 2 (A) Immunofluorescence localization of ZO-1 (green) with nuclear stain (blue) in RBMECs after exposure to TNF-α. TNF-α-exposed cells show discontinuity of ZO-1 immunofluorescence (white arrow), demonstrating tight junction disruption. In contrast, MMP-9 pretreatment followed by TNF-α shows preservation of the tight junctions (yellow arrow). (B) Rhodamine phalloidin staining for F-actin stress fiber formation (red) and DAPI nuclear staining (blue) in RBMECs after exposure to TNF-α. TNF-α treated cells show an increase in F-actin stress fiber formation (white arrow), demonstrating stress-induced cytoskeletal rearrangement. In contrast, MMP-9 inhibitor 1 pretreatment followed by TNF-α treatment shows preservation of baseline cytoskeletal actin arrangement (yellow arrow). (For interpretation of the references to color in this Figure, the reader is referred to the web version of this article.)

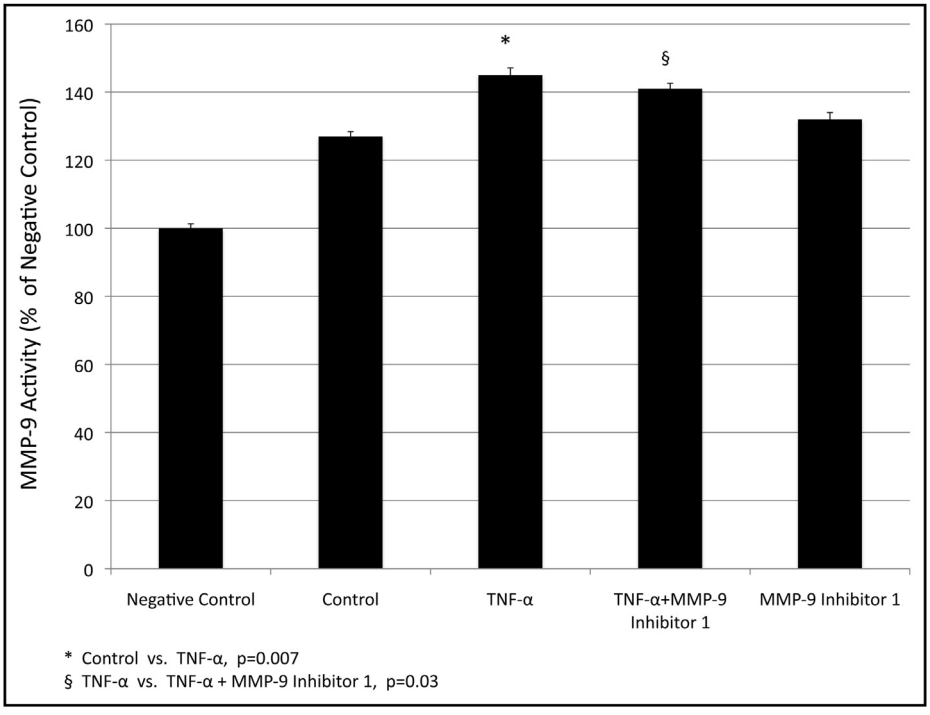

Figure 3 RBMEC MMP-9 activity assay. RBMECs exposed to TNF-α revealed significantly elevated MMP-9 activity levels when compared with cells in the control group ($P = .007$). MMP-9 activity was significantly decreased after TNF-α exposure with MMP-9 inhibitor 1 pretreatment ($P = .03$).

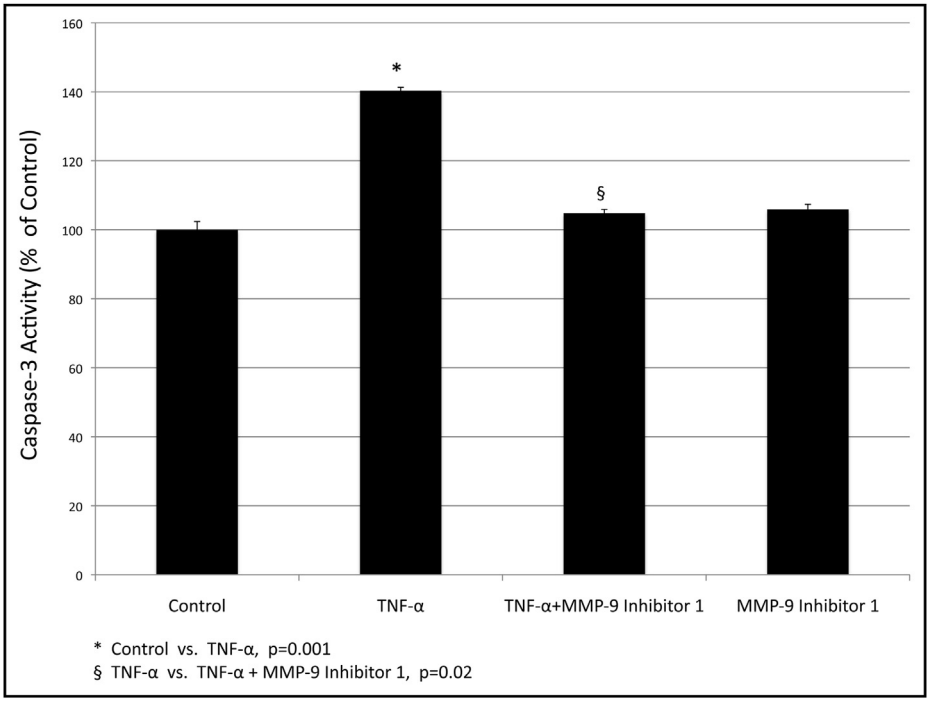

Figure 4 Caspase-3 activity assay. RBMECs exposed to TNF-α revealed significantly elevated caspase-3 activity levels when compared with cells in the control group ($P = .001$). Caspase-3 activity was significantly decreased after TNF-α exposure with MMP-9 inhibitor 1 pretreatment ($P = .02$).

Significant elevation of MMP-9 activity was illustrated in cells treated with TNF-α when compared with cells in the control group ($P = .0007$). With MMP-9 pretreatment, this increase was significantly attenuated ($P = .03$).

Tumor necrosis factor-α stimulates caspase-3 activity and matrix metalloproteinase-9 inhibitor 1 attenuates the increase

RBMEC caspase-3 was quantified and results are expressed as a percentage of control (Fig. 4). Analysis revealed significantly elevated caspase-3 activity in cells treated with TNF-α compared with cells in the control group ($P = .001$). The increased activity was significantly mitigated with pretreatment of MMP-9 inhibitor 1 as illustrated in Fig. 3 ($P = .02$).

Comments

The complexity of the human brain is paralleled only by the pathology that afflicts it. Traumatic injuries are no exception, and the role of TNF-α therein is equally as complex. As a testament to this, a PubMed query for 'TNF and brain injury' returns over 1,000 results. In this literature, TNF-α has been shown, among other things, to stimulate apoptosis of brain microvascular endothelium, increase vasogenic brain edema, and correlate with breakdown of the BBB.[7,23,24,26] On the contrary, there is also evidence suggesting that the role of TNF-α may not be fully detrimental. Genetically modified mice that lack TNF show evidence of increased lesion size and worsened breakdown of BBB after TBIs.[27] Additional studies have illuminated TNF-α-mediated induction of cerebral microvascular repair processes, activation of anti-apoptotic pathways, and neuroprotective properties.[26,28]

The ambiguity and dichotomy surrounding TNF-α's action may lie in the fact that little is known of the manner in which its actions on endothelial cells are mediated. Consequently, the aim of this study was to investigate the relationship among TNF-α, MMP-9, and caspase-3 in regard to microvascular permeability. To this end, RBMEC monolayers exposed to TNF-α show increased permeability in vitro and demonstrate that such fluid leak can be reversed with MMP-9 inhibitor 1. Immunofluoroescence staining illuminated disruption of the TJ protein ZO-1 after exposure to TNF-α. MMP-9 inhibitor 1 pretreatment attenuated these alterations, preserving typical configuration of cell-to-cell attachments. In addition, F-actin stress fibers were provoked by application of TNF-α, indicating cytoskeletal reorganization and TJ damage. With MMP-9 inhibitor 1 pretreatment, baseline actin composition was maintained. Cells exposed to TNF-α exhibited significantly increased MMP-9 activity, while pretreatment with MMP-9 inhibitor 1 neutralized this escalation. Additionally, after application of TNF-α, RBMECs exhibited increased caspase-3 enzyme activity and this was mitigated with MMP-9 inhibitor 1. These findings confirm our hypothesis and demonstrate an in vitro relationship between TNF-α and MMP-9 in promoting barrier disruption and hyperpermeability, illustrating that TNF-α-mediated microvascular endothelial disruption occurs via activation of MMP-9 either directly or through caspase-3.

Additional studies are warranted to examine application to human injury, as these data are an in vitro study of endothelial cells. Despite this, our findings augment the understanding of MMPs' role in BBB vascular permeability and suggest a novel interplay among TNF-α, MMP-9, and caspase-3 therein. This study shows that, in vitro, inhibition of MMP-9 attenuates hyperpermeability of vascular endothelial monolayers, preserves TJs, and reduces F-actin stress fiber formation after exposure to TNF-α. Furthermore, we show that enzymatic activity of both caspase-3 and MMP-9 can be induced with TNF-α and illustrate that an MMP-9 antagonist can ameliorate these increases. These data highlight the role of TNF-α, MMP-9, and caspase-3 in the pathophysiology of BBB microvascular hyperpermeability. As there is currently no available direct therapy for secondary injuries in TBI, these finding offer insight into an important facet of the mechanisms of secondary injuries within TBI and illuminate targets for potential therapies.

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Discussion

Discussant: Dr Josh Mammen (Kansas City, KS). As the authors mentioned in their presentation, the complex series of events involved in traumatic brain injury are just now being well understood. Their study focuses on endothelial cells, a critical component of the blood-brain barrier. In this study, the authors were able to demonstrate the important role of TNF-a in increasing endothelial cell permeability via MMP-9 and caspase-3. The study suggested a potential therapeutic target for the treatment of traumatic brain injury.

I have 3 areas that I would like the authors to address in the discussion of this study.

The first is, MMP-9 inhibitor pretreatment was required to avoid TNF-a mediated endothelial permeability. Were studies performed that evaluated the role of MMP-9 inhibitor treatment after TNF-a administration, which might be more clinically relevant? Could the increased endothelial cell permeability be potentially reversed?

The second question is MMP-9 inhibitor by itself, surprisingly the first figure, leads to increased endothelial cell permeability as compared to controls. Do the authors have a theory on how the MMP-9 inhibitor itself leads to a paradoxical increase in permeability?

The final question is, endothelial cells are polarized in vivo. The authors did use Transwells in their experiments. Did the authors evaluate whether the extent of polarization was essential for the observed loss of tight junction integrity? Did the results vary with the application of TNF-a on the apical surface versus the basal surface?

Dr Katie Wiggins-Dohlvik: As far as using MMP-9 as a post-treatment versus a pretreatment, we have not done that yet. As this is a novel area of research, we chose to use a pretreatment group to identify whether or not this relationship was even there in a very simplistic manner. We think that when you get into post-treatment, there may be more than one thing that you can look at and more than one thing that affects that. By blocking this initially, we know that we are focusing on blocking MMP-9. Our further studies will, of course, have post-treatment.

You had a question about MMP-9 versus the control in the permeability group, and the graph is a little misleading. That was not significantly different. We always look at that to ensure that our results are saying what they say. It was just that the bar looked higher. There was no significant difference between the control and the inhibitor alone group.

As far as the polarization of the cells in our mono layers, this particular study didn't include applying any agents to the lower well, but future studies we are already conducting will do that as well to examine that facet of this relationship.