Tissue-Type Plasminogen Activator Is a Regulator of Monocyte Diapedesis through the Brain Endothelial Barrier

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*J Immunol* 2008; 181:3567-3574; doi: 10.4049/jimmunol.181.5.3567
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Inflammation in the brain contributes to the development of neuronal deficits in a number of brain disorders (1) and is a hallmark of multiple sclerosis (MS), a chronic inflammatory disease of the CNS (2). In MS, infiltrated monocyte-derived macrophages form the major cell type in perivascular infiltrates and are key mediators of demyelination and axonal damage, two characteristic features of MS (2).

Under healthy conditions, brain homeostasis is maintained by the blood-brain barrier (BBB) which limits the entrance of potentially harmful blood components, including cells of the immune system. The restrictive nature of the BBB is due to the specialized brain endothelium and the tight junction complexes between adjacent endothelial cells. Tight junctions are composed of a combination of transmembrane proteins, mainly comprising members of the claudin family and occludin, and cytoplasmic proteins linked to the cytoskeleton (3). This allows the formation of an impermeable cell barrier still capable of rapid regulation. In addition, postcapillary venules in the brain are surrounded by a second impermeable cell barrier still capable of rapid regulation. In addition, the pericyte basal lamina, which is generated by astrocytes (4). To travel into the brain and to exert their detrimental effects, monocytes have to cross the BBB. This requires the active participation of brain endothelial cells to rearrange their cytoskeleton and tight junctions, processes that involve matrix metalloproteinases and intracellular signaling events (5–8).

Increasing evidence indicates that damage to the BBB and inflammation in the brain are linked with extracellular proteolysis (9–13). The extracellular serine protease tissue-type plasminogen activator (tPA) is well known for its function in the elimination of fibrin from the vasculature through activation of the circulating zymogen plasminogen to the primary fibrinolytic enzyme, plasmin (14). Physiologically, tPA is stored in endothelial cell vesicles, which are thought to be the major source of tPA and responsible for rapid release upon distinct types of stimulation (15, 16). In the brain, tPA is mainly present in the cerebrovascular endothelium of smaller vessels (17), in neurons (18, 19), and in microglial cells (20).

Several studies suggest that tPA is implicated in inflammation in the brain. For example, tPA mediates activation of microglia, the resident immune cells of the CNS, upon excitotoxic injury in the brain (20). In addition, in brains of MS patients, tPA is particularly associated with inflammatory cells in the perivascular compartment and high tPA activities in the circulation correlate with the disease progression (21–23). tPA activity is also increased in areas of inflammatory damage in the animal model of MS, experimental autoimmune encephalomyelitis (EAE) (24). Finally, tPA deficiency delays the onset of EAE in mice, which was associated with attenuated microglial activation (25).

The aim of the current study was to elucidate the role of tPA in inflammatory processes in the brain. To investigate the behavior of
tPA in the brain, we used the myeloid basic protein-induced EAE model in rats. In vitro studies on brain endothelial barrier damage and diapedesis of monocytes, which are important hallmarks of MS lesion formation, were performed to elucidate extracellular proteolytic pathways and mechanisms underlying these pathological processes. Together, our results imply that tPA plays a regulatory and specific role in early neuroinflammatory events.

Materials and Methods

Materials and cells

Ham’s F12 medium, RPMI 1640 medium, penicillin, streptomycin, t-glutamine, FCS, and trypsin/EDTA were obtained from Life Technologies. Pefabloc tPA was purchased from Pentapharm. ERK activation inhibitor PD-98059, collagen type I (calf skin) and α-lipoic acid were from Sigma-Aldrich. Human recombinant tPA (Actilys) was purchased from Boehringer Ingelheim. The α4 integrin inhibitor phenylacetyl-t-leucyl-t-aspartyl-t-phenylalanyl-morpholineamide was generated by Cytel. Phalloidin-rhodamine was from Molecular Probes. Abs against occludin (Zymed), ERK1/2 and phosphoThr202/Tyr204)-ERK1/2 (Cell Signaling Technology), and actin (Santa Cruz Biotechnology) were used for immunoblot analyses. Polyonal blocking Abs for rat-tPA (26) were a gift from Dr. J.J. Emeis (TNO Prevention and Health, Leiden, The Netherlands). Recombinant neuroserpin was produced and purified as described (27). The Lewis rat brain endothelial cell line (GP8/3.9) (28) was routinely cultured in collagen type I-coated flasks in Ham’s F12 medium supplemented with 10% FCS (heat inactivated), 2 mM t-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. The immortalized human brain endothelial cell line hCMEC/D3 was cultured as described (29).

Induction of acute experimental autoimmune encephalomyelitis in rats

EAE was induced in male Lewis rats (200–250 g; Harlan Sprague Dawley) and clinical symptoms were scored as described previously (30). Experimental procedures were approved by the Ethical Committee for Animal Experiments of the VU University Medical Centre, Amsterdam, The Netherlands. Animals were kept under standard laboratory conditions and received food and water ad libitum. Monocyte infiltration was detected with the monocyte/macrophage marker ED1 (produced at the Department of Molecular Cell Biology and Immunology, VU University Medical Center). VCAM-1 was stained with 5F10 (a gift from Dr. R. Lobh, Biogen, Cambridge, MA). Abs against perlecan (10B2) and agrin (GR-14) were a gift from Dr. J.J. Emeis (TNO Prevention and Health, Leiden, The Netherlands). Animals were subjected to SDS-PAGE. Live imaging of GFP-occludin was performed with the Leica DMIL microscope (Leica Microsystems). Images were analyzed with the ImageJ software (Wayne Rasband, National Institutes of Health). Statistical analysis was performed with the Student’s t test (Prism 4.0; GraphPad Software), and results were considered significant if \( p < 0.05 \).

Coculture experiments

GP8/3.9 cells were cultured to confluence in collagen-coated tissue culture 48-well plates. The endothelial monolayer was washed with complete RPMI 1640 medium and different amounts of rat primary monocytes (suspended in complete RPMI 1640) were added and cocultured for 3 h at 37°C under 5% CO\(_2\). As a control, monocytes were incubated with formalin-fixed endothelial cells or collagen-coated empty wells. Supernatants were collected, stored at −20°C and then assayed for tPA Ag by ELISA as described (36). Cell homogenates were prepared by replacing the culture medium with SDS sample buffer containing 5% 2-ME, and subsequent heating at 95°C for 5 min.

Detection of reactive oxygen species (ROS)

ROS production in a coculture of primary rat monocytes and GP8/3.9 was quantified using Amplex Red (Molecular Probes) as described previously (37). In short, primary rat monocytes and GP8/3.9 cells were preincubated with or without 100 µg/ml heparin in 132 mM NaCl, 20 mM HEPES, 6 mM KCl, 1 mM MgSO\(_4\), 1.2 mM K\(_2\)PO\(_4\), 1 mM CaCl\(_2\), and 0.5% (weight-to-volume ratio) BSA. After 1 h, primary rat monocytes (3 × 10\(^4\)/cm\(^2\)) and Amplex Red reaction mixture were added to GP8/3.9 cells cultured in a 96-well plate. ROS formation was assayed in real time using a FLUOstar Galaxy microplate reader (BMG Labtechnologies) at 37°C, excitation 550 nm and emission 590 nm. Increase in fluorescence intensity was used as a measure for ROS.

ROS treatment

Monolayers of GFP-occludin expressing GP8/3.9 endothelial cells (5) were cultured in collagen-coated 24-well plates and washed with UltraCulture serum-free medium (Cambrex Corporation). ROS were continuously generated by adding 0.08 U/ml xanthine oxidase and 100 µM hypoxanthine (Sigma-Aldrich) in UltraCulture serum-free medium to the endothelial cells as previously described (35). After 2 h, cell supernatants were harvested. The cells were extracted in SDS sample buffer containing 5% 2-ME, subsequently heated at 95°C for 5 min, and subjected to SDS-PAGE. Live imaging of GFP-occludin was performed as described previously (5, 6). In short, GFP-occludin-expressing GP8/3.9 endothelial cells were cultured in µSlide V1 (Ibidi) coated with collagen. At confluency, the effect of ROS treatment (15 µM/ml xanthine oxidase and 100 µM hypoxanthine) was imaged using the Olympus CellIR real-time live-imaging station (type IX81, UPLFLN 40 × O/1.3 lens).

Statistical analysis

Statistical analysis was performed with the Student’s t test (Prism 4.0; GraphPad Software), and results were considered significant if \( p < 0.05 \).

Results

tPA levels in plasma of rats correlate with EAE

Blood samples were collected from control rats and rats suffering from EAE at different time points upon induction, i.e., immediately before onset, at the peak, and after full recovery of the disease (Fig. 1A, inset). Analysis of circulating levels of tPA at the different time points revealed that tPA coincided with clinical symptoms and was increased more than 2-fold at the peak of the disease (Fig. 1A, control: 0.99 ng/ml tPA). Moreover, elevated tPA levels were associated with increased circulating amounts of monocytes (relative to total PBMC, control: 9.8 ± 1.2%; EAE: 39.7 ± 1.6%, \( n = 3, p < 0.0001 \); Fig. 1B). To define the presence and localization of tPA in the brain of EAE rats, day 14 brains were stained for tPA and ED1 (monocytes), VCAM-1 (activated brain endothelium), perlecan (endothelial basement membrane) or agrin (endothelial and parenchymal basement membranes). Control stainings with secondary Abs were negative (data not shown). The results show that tPA was present in inflamed areas in the brain, and particularly localized to the brain endothelium and the brain endothelial and parenchymal basement membranes (Fig. 1C).
monocytes were incubated with formalin-fixed GP8/3.9 cells indicated that monocyte-endothelial cell interaction induced the release of tPA by these endothelial cells, which was blocked by the protein export pathway inhibitor brefeldin A (5 μg/ml) and the α4 integrin inhibitor phenylacetyl-l-leucyl-l-aspartyl-l-phenylalanyl-morpholineamide (20 μg/ml) (C). Data are expressed as the mean ± SEM; n = 3; **, p < 0.005; *, p < 0.05 vs control.

**FIGURE 2.** tPA is released by GP8/3.9 cells upon monocyte interaction. Confluent monolayers of GP8/3.9 rat brain endothelial cells were cocultured with different concentrations of monocytes. A. Immunofluorescent staining of tPA (in green). Actin was stained with with phalloidin-rhodamine (in red). N, nucleus; C, cytoplasm; arrow, vesicular tPA. B. ELISA analyses of supernatants after incubation of live or fixed GP8/3.9 cells indicated that monocyte-endothelial cell interaction induced the release of tPA. C. Data are expressed as the mean ± SEM; n = 3; **, p < 0.005; *, p < 0.05 vs control.

**tPA is released by brain endothelial cells upon monocyte interaction**

Endothelial cells are the major source of tPA in the blood circulation. Immunofluorescence analysis showed a vesicular staining of tPA in rat brain endothelial cells (Fig. 2A). We first investigated the effect of monocytes on the release of tPA from rat brain endothelial cells. In culture, the brain endothelial cells constitutively secreted tPA. The addition of monocytes caused a dose-dependent increase of tPA in the medium (control: 0.56 ± 0.07 ng/ml; 3.0 × 10^5 monocytes/cm²: 0.98 ± 0.06 ng/ml; 6.0 × 10^5 monocytes/cm²: 1.40 ± 0.17 ng/ml, n = 3, p < 0.05, Fig. 2B). tPA levels were not increased when monocytes were incubated with formalin-fixed brain endothelial cells. This indicated that tPA was secreted by brain endothelial cells. Disruption of the classical protein export pathway in endothelial cells by brefeldin A (5 μg/ml) completely blocked the monocyte-induced release of tPA in the extracellular medium (n = 3, p < 0.05; Fig. 2C), indicating that exocytosis-related cell structures are involved in this process. Monocyte to endothelial cell adhesion is mediated by α4-integrins, including VLA-4 (38, 39). The α4 integrin inhibitor phenylacetyl-l-leucyl-l-aspartyl-l-phenylalanyl-morpholineamide, which prevented monocyte to endothelial cell adhesion (data not shown), blocked the release of tPA (n = 3, p = 0.98; Fig. 2C). These results demonstrated that this process was dependent on the close interaction of monocytes with brain endothelial cells.

**Monocyte passage of a brain endothelial barrier involves tPA**

Next, we tested whether tPA has a functional role during traversal of primary monocytes through a monolayer of brain endothelial cells. Transendothelial migration of monocytes was studied in the absence or presence of specific inhibitors of tPA (Fig. 3A). Diapedesis through rat brain endothelial cells was reduced in the presence of Pefabloc tPA, a synthetic and nontoxic inhibitor with high affinity for tPA (K_i = 0.035 μM; control: 26.1% ± 0.9; Pefabloc tPA: 16.9% ± 1.0; n = 4, P = 0.001) and a blocking Ab against α4-integrins, including α4β1 integrin (15.6% ± 0.3; n = 4; p = 0.001 vs control). Notably, the effect of Pefabloc tPA on coagulation factor Xa and thrombin is unlikely given the absence of these proteases in our experimental system. Control polyclonal Abs against von Willebrand factor had no effect (data not shown). Brefeldin A, which blocked tPA release by brain endothelial cells (Fig. 1B) also reduced transendothelial migration of monocytes (control: 26.1% ± 1.3; brefeldin A: 18.5% ± 0.4, n = 4; P = 0.003 vs control; Fig. 3B), without affecting...
monocyte to endothelial cell adhesion (data not shown). Human neuroserpin (control: 14.3% ± 0.7; neuroserpin: 8.5% ± 0.7; n = 3; p = 0.0025 vs control), a physiological inhibitor of tPA, and Pefabloc tPA (8.0% ± 0.7; n = 4; p = 0.0015 vs control) similarly reduced the migration of human monocytes over human brain endothelial cell monolayers, indicating that the observed effects with rat brain endothelial cells was not a peculiarity of that cell line (Fig. 3C). Recently, both in vitro (40) and in vivo (41) experiments have shown that leukocytes not only extravasate through endothelial cell junctions (the paracellular route) but have revealed that they also can traverse by a route through endothelial cells (the transcellular route), which we showed is dependent on proteolytic activity (5). To further delineate the role of tPA in diapedesis, we therefore performed paracellular permeability analyses of human brain endothelial monolayers during monocyte transmigration in the absence or presence of Pefabloc tPA. Results indicated that monocytes increase monolayer permeability toward large molecules (150 kD), which is indicative of the loss of cell-cell contacts (Fig. 3D; control: 100.0% ± 8.2; monocytes: 131.9% ± 4.5; n = 3, p < 0.05 vs control). This increase was abolished in the presence of Pefabloc tPA (control: 83.4% ± 11.4, p = 0.30; monocytes: 102.3% ± 8, p = 0.85), which indicated that tPA regulates the paracellular route of monocyte transmigration.

**tPA release by brain endothelial cells is dependent of ROS**

TtPA is stored in endothelial cells and can rapidly be released upon distinct types of stimulation including ROS (15, 16). To investigate whether ROS are responsible for monocyte-induced secretion of tPA, we first determined the generation of ROS in monocyte-endothelial cell cocultures. Analysis of ROS using the fluorescent probe Amplex Red revealed that monocyte-endothelial cell interaction enhanced the levels of extracellular ROS (184.7% ± 12.9, n = 6; p = 0.0002 vs control; Fig. 4A). Heparin, which blocks monocyte adhesion to brain endothelial cells (42), reduced ROS production (140.6% ± 12.2, n = 6, p = 0.03) indicating that this process was dependent on the close interaction of monocytes with brain endothelial cells. Next, we treated rat brain endothelial cells with ROS directly. Interestingly, the results revealed that this induced a tremendous increase of extracellular tPA (Fig. 4B). Together, these results suggested a possible role of ROS in monocyte-induced tPA secretion. Cotreatment with the ROS scavenger α-lipoic acid (previously shown to inhibit transcellular migration of monocytes and not affecting monocyte adhesion to endothelial cells (37) blocked monocyte-induced endothelial secretion of tPA indeed indicating an important role for ROS (Fig. 4C). In conjuction with ROS, reactive nitrogen species can be produced by nitric oxide synthase (NOS). However, the NOS inhibitor nitro-L-arginine methyl ester had no effect on monocyte-induced tPA secretion by brain endothelial cells, showing that this process is independent of NOS. Together, these results show that secretion of tPA by brain endothelial cells, which has a functional role during monocyte diapedesis, is under the control of oxidative stress.

**tPA regulates ROS-induced breakdown of the tight junction protein occludin**

An important characteristic of the BBB is the presence of interendothelial tight junctions, intricate complexes of several proteins including the transmembrane protein occludin. Previously, we showed that ROS can induce ruffling of endothelial cell-cell contacts, disrupt the tight junction structure and regulate monocyte diapedesis (6, 35). Because ROS treatment of rat brain endothelial cells caused a remarkable increase of extracellular tPA (Fig. 4B), we next investigated whether tPA is implicated in ROS-induced occludin redistribution and degradation and thereby may regulate monocyte diapedesis. Therefore, we performed live cell imaging...
and immunoblotting analyses of GFP-occludin in rat brain endothelial monolayers in the presence or absence of Pefabloc tPA to inhibit tPA activity. Live cell imaging of GFP-occludin revealed that ROS induce loss of cell-cell contacts, the formation of gaps between brain endothelial cells and disappearance of junctional occludin within 15 min (Fig. 5A, upper). Immunoblot analyses of GFP-occludin after ROS treatment revealed a second band of apparent size of ∼50 kDa, a proteolytic fragment of occludin (Fig. 5B). Inhibition of tPA almost completely blocked redistribution (Fig. 5A, lower panel) and degradation (Fig. 5, B–D) of GFP-occludin.

tPA regulates ROS-induced activation of ERK in brain endothelial cells

Previous studies from our group revealed that ROS-induced TJ disengagement in rat brain endothelial cells involve signaling processes including Ca²⁺ mobilization, inositol (1, 4, 5)-trisphosphate formation and phospholipase C (35), and protein kinase B activation (6). Activation of the ERK pathway has been related to occludin distribution (43). Therefore, phosphorylation of ERK1/2 at Thr202/Tyr204 was studied in ROS-treated rat brain endothelial cells. The results demonstrated that ROS induce activation of the ERK pathway within minutes (Fig. 6A), which could be blocked by an inhibitor of MEK1/2 (PD-98059, 10 μM) a signal transduction protein upstream of ERK1/2 (Fig. 6B). Next, we studied the role of tPA in this process. Interestingly, blockade of tPA almost completely prevented ROS-induced ERK1/2 phosphorylation (Fig. 6B). Similar results were obtained in human brain endothelial cells (data not shown). Moreover, incubation of these cells with human recombinant tPA caused activation of ERK1/2 in a time frame similar to that of ROS (Fig. 6C).

Finally, we investigated the role of ERK activation in ROS-induced degradation of occludin. The results showed that occludin breakdown was partially abolished by inhibition of ERK1/2 activation with PD-98059 (Fig. 5, B–D).

**tPA mediates ERK1/2 activation upon coculture of monocytes and brain endothelial cells**

Our results suggest that a potential pathway by which ROS promotes degradation of occludin involves tPA and ERK1/2 signaling in brain endothelial cells. We next investigated whether this pathway could mediate the promigratory capacity of tPA. First, we assessed ERK1/2 activation and the role of ERK1/2 during transendothelial migration of monocytes. Immunoblot analyses indicated that phosphorylation of ERK1/2 at Thr202/Tyr204 (163.4% ± 19.7 of control) is increased in a coculture of primary rat monocytes and rat brain endothelial cells. ERK1/2 phosphorylation upon monocyte-endothelial cell interaction was reduced to control levels (111.4% ± 2.3 of control) when coculture was performed in the presence of Pefabloc tPA (Fig. 6D). These data do not allow the discrimination between ERK1/2 activation in monocytes and/or brain endothelial cells. To further delineate the cellular specificity and function of tPA-mediated ERK1/2 activation, we therefore differentially blocked ERK1/2 activation in rat monocytes and rat brain endothelial cells and assessed the transmigration capacity. Fig. 6E shows that pretreatment of the endothelial cells with PD-98059 (1 h, 10 μM) potently decreased monocyte traversal (control: 16.3% ± 0.9; pretreated rat brain endothelial cells, EC:

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**FIGURE 5.** tPA regulates ROS-induced breakdown of the tight junction protein occludin in GP8/3.9 rat brain endothelial cells. A, Time-lapse observation of GFP-occludin during ROS treatment in the absence (upper panel) and presence (lower panel) of Pefabloc tPA. Time is indicated in minutes. Bars, 10 μm. B, Immunoblot of GFP-occludin showing full length (∼90 kDa) and a cleaved fragment (∼50 kDa) of occludin after incubation without and with ROS (control), ROS plus 100 μM Pefabloc tPA and ROS plus 10 μM PD-98059. C, Densitometric quantification of full length occludin. D, Densitometric quantification of the 50 kDa cleaved fragment showing that inhibitors of tPA and ERK (PD-98059) significantly reduced occludin degradation (mean ± SEM; n = 3; *** p < 0.0001; * p < 0.05 vs control).
Pefabloc tPA for 3 h. Graphs on the upon treatment with 40 of activated and total ERK1/2 in hCMEC/D3 human brain endothelial cells.

10.2% ± 1.0, n = 4, p = 0.0039 vs control). In contrast, preincubation of monocytes with PD-98059 did not affect their transendothelial passage (14.3% ± 0.3, n = 4, p = 0.09 vs control).

These data demonstrate that a potential molecular mechanism underlying monocyte passage through the brain endothelial barrier is through activation of the ERK1/2 pathway by tPA in brain endothelial cells.

Discussion

tPA has been associated with inflammation in the brain. Our results represent the first evidence that tPA regulates the dynamics of the brain endothelial barrier protein occludin and diapedesis of monocytes through this barrier. Moreover, our data point to a potential working mechanism for tPA in brain endothelial cells. tPA, which is secreted by brain endothelial cells upon interaction with monocytes or under oxidative stress is responsible for the activation of ERK1/2, a signal transduction protein which was involved in the regulation of occludin degradation and paracellular passage of monocytes through a monolayer of brain endothelial cells.

In EAE, an animal model of MS, monocytes accumulate in the perivascular space, which is defined by the inner brain endothelial monolayer and the outer parenchymal basement membrane. Our in vitro studies clearly indicated that tPA regulates monocyte diapedesis through the brain endothelial barrier. Interestingly, in animals, tPA was not only present at the brain endothelium but also marked the second barrier, which is formed by the parenchymal basement membrane. Although transmigration of this barrier was not investigated in this study our data suggest that, like has been shown for MMP-2 and −9 (44), tPA could also contribute to monocyte migration through the parenchymal basement membrane.

We show that circulating tPA levels in the blood correlate with the clinical symptoms of EAE, which confirms previous results showing increased tPA in spinal cord homogenates of EAE animals (25). Our studies further revealed that monocytes, through the activity of ROS, strongly contribute to the release of brain endothelial tPA, which appeared to be involved in subsequent diapedesis (37). In endothelial cells, tPA is stored in specific granules called Weibel-Palade bodies (45). Interestingly, Weibel-Palade bodies also contain a number of other bioactive components known to be implicated in inflammation, including cell adhesion mediators (P-selectin, von Willebrand factor) and chemokines (46). In view of this it is anticipated that Weibel-Palade bodies are of pathophysiological importance, playing a central role in monocyte diapedesis across the blood-brain barrier.

The mechanisms that mediate ROS signaling at the BBB are relatively obscure. ROS can react with cellular lipids directly or indirectly, generating a spectrum of products, many of which contain functional groups capable of modifying proteins, ultimately leading to the activation of particular cell-signaling pathways (6, 35, 47). Which pathways are implicated in ROS-induced tPA secretion is currently under investigation.

We demonstrate a novel and specific role for tPA in occludin degradation. Occludin is a component of the tight junction complex and fulfills an important function in maintaining BBB integrity. We recently showed that paracellular diapedesis of monocytes requires redistribution of junctional occludin (5) and failure of the BBB caused by ROS has been attributed to the disruption of tight junctions (35, 48). Our current results show that ROS induce loss of endothelial cell-cell contacts, redistribution of junctional occludin (corroborating our earlier observations (6), and occludin degradation.

To further explore the mechanism of tPA-mediated monocyte diapedesis through brain endothelium and occludin degradation, we performed intracellular signal transduction analysis. Our results demonstrate that tPA is responsible for activation of a signaling cascade toward ERK1/2 either induced upon monocyte-endothelial...
cell interaction or by ROS treatment. These data are in accordance with the results of recent studies showing that tPA can activate ERK1/2 in neuronal cells (49). Because coculture studies of ERK1/2 activation did not reveal whether ERK1/2 activation was initiated in monocytes, brain endothelial cells or both, cell specific function of ERK1/2 activation was dissected in transmigration studies in which we differentially blocked ERK1/2 activation in monocytes and brain endothelial cells. These analyses provided evidence that functionally activated ERK1/2 is restricted to the brain endothelial cells and that blockade of ERK1/2 activation in monocytes did not affect their passage through the brain endothelial monolayer.

ERK1/2 mediates a range of activities from metabolism, motility, and inflammation to cell death and survival and activated ERK1/2 is associated with both beneficial and detrimental effects in the brain (50, 51). Conceivably, these dual effects of ERK1/2 actions in the brain are related to its responses to a diverse array of stimuli and cell surface receptors. ERK1 and ERK2 do serve different functions, but there is very little information on the contribution of individual forms of ERK to inflammatory processes. Our results indicate that ERK1/2 activation in brain endothelial cells is detrimental to integrity of the blood-brain barrier associated with oxidative stress and proinflammatory processes. Evidence supporting the protective effects of ERK1 activity is derived from experiments in animals lacking ERK1 (52). Increased susceptibility to EAE in these animals, which expressed ERK2, was due to a bias toward a Th1 type immune response. Future experiments should be conducted to scrutinize the dual function of ERK1/2 activation in disease processes.

It is currently unknown which brain endothelial receptors regulate the function of tPA during monocyte transmigration. Potential candidates are the diverse receptors for tPA possessing cell signaling properties toward ERK1/2 which are present in brain endothelial cells, including annexin II (49, 53), the N-methyl-D-aspartate receptor (54, 55) and low-density lipoprotein receptor-related protein (56). Interestingly, recent literature has indicated that the N-methyl-D-aspartate receptor is involved in glutamate-induced alterations of occludin expression in brain endothelial cells (57). Identification of cellular receptors for tPA regulating its function in inflammation in the brain is under current investigation. Together, our findings support the conclusion that tPA has a specific function during diapedesis of monocytes in the brain. Our results provide novel insight into the regulation of the tight junction component occludin and indicate an important contribution of the ROS-tPA-ERK1/2-axis to BBB damage and neuroinflammation. Particularly, the extracellular protease tPA mediates ROS-induced breakdown of the tight junction protein occludin, possibly via activation of ERK1/2. Knowledge of proteolytic mechanisms at the BBB that contribute to inflammation in the brain may be crucial for treatment of MS.

Acknowledgments
We thank Dr. J.J. Emeis for quantities of purified rat tPA and anti-rat tPA Ab.

Disclosures
The authors have no financial conflict of interest.

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