Microglia Potentiate Damage to Blood–Brain Barrier Constituents
Improvement by Minocycline In Vivo and In Vitro

Midori A. Yenari, MD; Lijun Xu, MD; Xian Nan Tang, MD; Yanli Qiao, MD; Rona G. Giffard, MD, PhD

Background—Blood–brain barrier (BBB) disruption after stroke can worsen ischemic injury by increasing edema and causing hemorrhage. We determined the effect of microglia on the BBB and its primary constituents, endothelial cells (ECs) and astrocytes, after ischemia using in vivo and in vitro models.

Methods and Results—Primary astrocytes, ECs, or cocultures were prepared with or without added microglia. Primary ECs were more resistant to oxygen-glucose deprivation/reperfusion than astrocytes. ECs plus astrocytes showed intermediate vulnerability. Microglia added to cocultures nearly doubled cell death. This increase was prevented by minocycline and apocynin. In vivo, minocycline reduced infarct volume and neurological deficits and markedly reduced BBB disruption and hemorrhage in mice after experimental stroke.

Conclusions—Inhibition of microglial activation may protect the brain after ischemic stroke by improving BBB viability and integrity. Microglial inhibitors may prove to be an important treatment adjunct to fibrinolysis. (Stroke. 2006;37:1087-1093.)

Key Words: blood–brain barrier ■ inflammation ■ ischemia ■ microglia

A fter stroke, a disrupted blood–brain barrier (BBB) can lead to edema, hemorrhage, and transmigration of leukocytes and large toxic molecules into the brain. The BBB consists of endothelial cells (ECs) surrounded by astrocytes. Microglia, considered the resident immune cells of the brain, are also associated with the BBB and are among the first cells to respond to threats to the brain.1,2 When activated, microglia undergo proliferation, chemotaxis, morphological alterations, and generate immunomodulatory molecules. Microglia release proteases, lipases, and glutamate3,4 and are sensitive to hypoxia- and ischemia-like insults in vitro with a vulnerability greater than oligodendrocytes5 and similar to or less than astrocytes,6 depending on the model and species used. Their role in maintaining or interfering with BBB integrity in the context of ischemia is not well studied. Blocking microglial activation may limit BBB disruption and reduce edema and hemorrhagic transformation.

Superoxide is involved in lipid peroxidation, protein oxidation, and DNA damage. Leukocytes and microglia generate superoxide via the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase system.7 ECs also generate reactive oxygen species. Xu et al8 found that inducible NO synthase, NO, and peroxynitrite were increased in bovine ECs after oxygen-glucose deprivation (OGD). Here we show that addition of microglia to ECs and astrocyte cocultures worsens ischemia-like injury, and inhibiting microglial activation with minocycline or inhibiting superoxide production by NADPH oxidase with apocynin preserves BBB constituents in vitro and reduces BBB disruption and hemorrhage in vivo.

Methods

Cell Culture

Primary cerebral microvascular EC cultures were prepared from young mouse brains. Gray matter from 3- to 4-week-old mice was homogenized in calcium and magnesium free Hank’s balanced salt solution (BSS) containing 0.35% collagenase and 0.2% BSA for 2.5 hours at 37°C. Tissue was resuspended in 25% BSA, then centrifuged at 4°C for 20 minutes at 528 g. This pellet containing brain capillaries was incubated in 0.1% collagenase/disprase in bicarbonate containing M199 media (Gibco BRL; 11150-059) for 2 hours at 37°C to remove pericytes. The cells were pelleted, resuspended, then centrifuged on a Percoll gradient (11 mL Percoll in 21 mL Earl’s BSS) at 733 g for 10 minutes at 4°C. The capillary microvessel EC layer was collected, washed in M199 media, and plated on collagen-coated plates at 106 cells/mL in E-STIM EC culture media (Becton Dickinson) supplemented with 10 ng/mL epidermal growth factor plus penicillin/streptomycin (100 U/mL). Cultures were fed every 3 days, subcultured after 2 weeks at ~80% confluence, and used after 3 to 4 passages.

Neocortical astrocytes were isolated from postnatal day 1 or 2 Swiss Webster mice (Simonsen [Gilroy, Calif] or Charles River [Wilmington, Mass])9 and used for experiments 3 weeks later. Microglia were isolated by shaking off from mixed glial cultures as described previously, and plated at 1 to 3 × 105 cells/mL, and used for experiments 24 hours after plating. ECs or astrocytes were seeded at

Received August 9, 2005; final revision received September 29, 2005; accepted November 8, 2005.
From the Departments of Anesthesia (L.X., X.N.T., Y.Q., R.G.G.) and Neurosurgery (R.G.G.), Stanford University School of Medicine, California; and Department of Neurology (M.A.Y., X.N.T.), University of California, San Francisco, and the San Francisco Veterans Affairs Medical Center, California.
Correspondence to Midori A. Yenari, MD, Dept of Neurology, UCSF, Neurology (127) VAMC, 4150 Clement St, San Francisco, CA 94121. E-mail yenari@alum.mit.edu
© 2006 American Heart Association, Inc.
10^6 cells/mL when used alone or 0.5 × 10^6 cells/mL in EC/astrocyte (EA) cocultures. Cocultures were grown in 50% EC and 50% astrocyte medium and were used for experiments 2 to 4 days later. For tricultures (EC/astrocyte/microglia [EAM]), microglia were seeded onto cocultures at a density of 10^5 cells/mL, and were allowed to stabilize on top of cocultures for 1 day before use.

**OGD and Analysis**

Cultures were subjected to OGD in BSS in an anoxic chamber (Coy Laboratories) with O2 tension <0.02%.11 About 4- to 6-hour OGD for astrocytes,11,12 6 to 12 hours for EC, and 10 to 24 hours for microglia6 induced 50% to 80% death for each cell type. Cocultures were subjected to OGD for 8 hours, then 5.5 mmol/L glucose was added and the cultures returned to the normoxic incubator (ie, reperfusion) for 24 hours. Cell death was quantified by staining with trypan blue and cell counting, expressing stained cells as a percent of total number of cells or assay of released lactate dehydrogenase (LDH),12,13 expressed as a percentage of the total LDH released after freeze-thaw (100%).

**Superoxide and Hydrogen Peroxide Assays**

Superoxide was estimated using iodonitrotetrazolium violet.14 Absorbance at 490 nm was read on a UVmax platereader (Molecular Devices). Optical densities were normalized to the amount of protein in sister cultures treated identically until cell collection.

Hydrogen peroxide (H2O2) levels were determined using Amplex Red (A22180; Molecular Probes) according to manufacturer instructions. The assay uses a 1-step reagent with horseradish peroxidase to produce a red fluorescent product. H2O2 concentrations were calculated using a standard curve (0 to 20 μmol/L) and normalized to the protein concentration in each culture.

**Cytochemistry/Histochemistry**

Cultures were fixed in 100% acetone for 10 minutes for EC markers or 4% paraformaldehyde at room temperature for 20 minutes for all other markers, blocked in normal serum, and immunostained.15 Primary antibodies used were: ECs, purified rat anti-mouse anti-CD31 antibody (557355; BD Pharmingen); astrocytes, anti–glial fibrillary acid protein (GFAP) antibody (G3893; Sigma-Aldrich); fibroblasts, antifibronectin (MAB88904; Chemicon); and pericytes, anti–α2-smooth muscle actin (MAB1501; Chemicon). Microglia were identified using lectin staining by incubating in 10 μg/mL peroxidase-labeled Griffonia simplicifolia B4-isolectin (IB4).16 Brain sections stained with IB4 were counterstained with hematoxylin and eosin.

**ELISA Assays**

ELISA assays of culture supernatants used the OptEIA Kit 558874 (BD Pharmingen) to detect bound and free tumor necrosis factor-α (TNF-α). All samples were run in triplicate. Measurements were normalized to the amount of protein produced per 10^5 cells in the culture supernatants.

**Drug Treatments**

Neither minocycline nor apocynin was toxic to cultures at 200 nmol/L to 2 μmol/L for minocycline and 0.25 to 1.0 mmol/L for apocynin (data not shown). Intermediate concentrations of minocycline (400 nmol/L; Sigma) or apocynin (500 μmol/L; Fluka) were added to culture media at the onset of injury.

**Experimental Stroke Model**

Male C57/BL6 mice (Jackson Laboratories; Bar Harbor, Maine) weighing 25 to 30 g were anesthetized and subjected to transient middle cerebral artery occlusion (MCAO)12 using an uncoated monofilament suture for 2 hours. Mice were euthanized with an isoflurane overdose at 1 day. Mice (n=12) received 2 injections of 45 mg/kg minocycline HCl (Sigma) intra-peritoneally at 30 minutes and 12 hours after MCAO17,18 or vehicle (n=9). Neurological deficits were assessed 24 hours after ischemia onset using a modified scale where 0 indicates no detectable deficit; 1, unable to extend the contralateral
forelimb; 2, flexion of the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side.  

Infarct Size and Hemorrhage Assessment

After euthanasia, brains were removed and cut into 2-mm coronal slices, and sections were inspected for gross hemorrhage and then immersed in 2,3,5-triphenyltetrazolium chloride (Sigma) at 37°C for 20 minutes. Infarct volume was determined using methods published previously. Afterward, brains were fixed and prepared for histochemistry.

Detection of BBB Disruption

BBB permeability was evaluated by detection of extravasated Evans blue dye. A total of 100 μL of 4% Evans blue in PBS was infused via the tail vein 3 hours before euthanasia (n=6 per group). At the time of euthanasia, mice were anesthetized and transcardially perfused with ice-cold PBS. The volume of dye extravasation was determined by tracing the region of blue-stained tissue from coronal sections and normalized to the infarct volume to correct for smaller lesion sizes among treated animals. This method is highly correlated with a previously used spectrophotometric method (data not shown).

Statistics

ANOVA followed by Neuman–Keuls was used for studies involving 2 groups of normally distributed data, Student t test for studies involving 2 groups and nonparametric tests (Mann–Whitney) were applied when data were not normally distributed. Fisher exact test was applied to hemorrhage scores and mortality data. In vitro experiments were conducted in triplicate on cells from ≥3 separate dissections. Data were expressed as means±SE. Values of P<0.05 were considered significant.

Results

Characterization of Primary Astrocytes, ECs, and Microglia Cultures

EC cultures are shown in Figure 1A and 1B. Immunostaining for von Willebrand factor indicates that the majority of cells are ECs (Figure 1B). Fibroblasts, pericytes, and microglia accounted for <5% of cells (data not shown). Representative EA cocultures were stained for GFAP (brown) to identify astrocytes (Figure 1C). A representative triculture (Figure 1D) shows phase-bright microglia on a bed of astrocytes and ECs by phase contrast. Microglia are identified by staining with isoelectin IB4 (Figure 1E).

Microglia Increase Susceptibility to OGD

The addition of astrocytes to EC cultures somewhat increased susceptibility to OGD compared with ECs alone, although this was not statistically significant (Figure 2A and 2B). No cell death was observed in microglial cultures after 8-hour OGD (data not shown). When EA cocultures were subjected to 8-hour OGD and 24-hour reperfusion, ~30% cell death occurred (Figure 2A through 2D). When microglia were added to the cocultures (EAM), cell death nearly doubled.

Figure 2. Microglia potentiate OGD-induced cell death of EA cocultures, and minocycline and apocynin block this potentiation. Cell death was assessed using LDH released into the media (A and C) or by cell counting after trypan blue staining (B). The addition of astrocytes (EA) to EC cultures (E) somewhat increased susceptibility to OGD (NS). The addition of microglia (EAM) approximately doubled the extent of cell death compared with EA. B, Trypan blue staining revealed similar results (*P<0.05 vs EA; #P<0.001 vs E; n=10 to 12 cultures per condition). C, EA and EAM were subjected to 8-hour OGD followed by 24-hour reperfusion in the presence or absence of inhibitors. Minocycline (+Mi) reduced cell death to that of EA cocultures. Apocynin (+Apo) also prevented the microglial-induced exacerbation of EA death. (*P<0.01 vs EAM). D, Cultures were stained with Trypan blue after OGD and reperfusion. Photomicrographs of EA cocultures show limited cell death after 8-hour OGD. In the presence of microglia (EAM), the amount of injury is increased. Treatment of EAM with minocycline (+400 nmo/L Mi) reduced the extent of cell death to levels similar to that of EA.
Minocycline, used here to inhibit microglial activation, reduced the microglia-induced injury after OGD in tricultures (Figure 2C and 2D) to levels similar to EA cocultures but did not protect EC or EA cocultures. Apocynin reduced cell death in EAM tricultures to a level similar to that in untreated EA cultures subjected to OGD (Figure 2C).

OGD Increases Superoxide, H$_2$O$_2$, and TNF-α in Activated Microglia

Superoxide and H$_2$O$_2$ were assayed after only 4-hour OGD followed by 3-hour reperfusion in EAM tricultures to avoid the confound of having a large number of cells already lysed. Superoxide and H$_2$O$_2$ were increased over basal levels ($P < 0.05$). Minocycline and apocynin reduced superoxide after OGD/reperfusion compared with untreated controls ($P < 0.05$; Figure 3A), and H$_2$O$_2$ was also reduced by apocynin (Figure 3B). TNF-α levels in uninjured microglial cultures were below the limits of detection, indicating that these cells were not activated but increased markedly (to 1500 pg/mL/10$^5$ cells) after 8-hour OGD and 16-hour reperfusion.

Neither minocycline nor apocynin significantly reduced TNF-α production from this level (data not shown).

Minocycline Improves Neurological Outcome and Reduces BBB Disruption, Hemorrhage, and Perivascular Microglial Accumulation In Vivo

After MCAO and reperfusion, mice treated with minocycline had significantly smaller infarcts and better neurological scores than vehicle-treated mice (Figure 4). Furthermore, mortality was no different between groups (minocycline 2 of 12 died; vehicle 2 of 9 died; NS). Minocycline reduced Evan’s Blue extravasation (Figure 5A and 5B). Because minocycline may lessen the extent of BBB disruption by reducing infarct size, we corrected for this potential confound by expressing the extent of BBB disruption relative to the infarct size and found that minocycline still reduced the proportionate amount of Evan’s Blue dye extravasation (Figure 5C). Minocycline reduced the occurrence of hemorrhagic transformation, assessed by scoring each brain for macroscopic hemorrhage at 24 hours, from 70% of animals in the control group to only 17% in the treated group ($P < 0.01$; Figure 6A and 6B). Lectin histochemistry to identify microglia/monocytes showed that cerebral vessels in cortex within the ischemic core were surrounded by microglia (Figure 6C). In contrast, vessels in comparable ischemic brain regions in a minocycline-treated animal showed fewer microglia/monocytes associated with vessels (Figure 6D).

**Discussion**

The BBB consists of a continuous capillary endothelium with tight junctions between cells, surrounded by astrocytes and perivascular microglia. This barrier restricts access of molecules and cells into the brain. Its disruption can lead to vasogenic edema and central nervous system pathology in conditions such as stroke and brain tumors. Hemorrhagic transformation and edema after ischemic stroke are thought to occur in the setting of BBB disruption and often lead to worsening of neurologic damage. Free radicals decrease EC electrical resistance and increase permeability.$^{23}$ Astrocytes strengthen the BBB, increasing electrical resistance almost 2-fold.$^{24-26}$ The role of other cellular constituents, such as microglia, has not been studied extensively.
It is now appreciated that excessive or improperly regulated inflammatory responses may contribute to damage. Microglia are stimulated within hours after ischemia onset, followed by upregulation of a variety of inflammatory factors. Microglia can generate similar substances under ischemia-like and ischemic conditions. Lynch et al correlated microglial activation to BBB disruption in a model of 3-chloropropanediol toxicity. Although they showed increased complement and inflammatory cytokine expression in microglia where BBB disruption occurred, their study did not prove a direct detrimental role. Using a model system in which microglia can be systematically added, we demonstrate for the first time that microglia clearly worsen ischemia-like injury to components of the BBB (i.e., astrocytes and ECs).

Minocycline, a member of the tetracycline antibiotic family, will inhibit microglial activation and protect cultured neurons from excitotoxic insults by preventing microglial generation of glutamate, IL-1β and NO. Minocycline also limits brain injury in experimental models of focal and global ischemia and Parkinson’s disease. It has also been associated with decreased BBB disruption after intranigral lipopolysaccharide injection and collagenase-induced intracerebral hemorrhage. We observed that inhibiting microglial activation with minocycline limited ischemia-like damage in an in vitro BBB model. We also found that minocycline reduced the release of superoxide after OGD but did not prevent TNF-α release by microglia. In vivo, we reproduced previous observations that minocycline improved neurological outcome after experimental stroke. We now show that minocycline reduces BBB disruption, findings consistent with our in vitro observations. Furthermore, vascular and perivascular microglia were decreased by minocycline treatment, consistent with a possible detrimental role in BBB integrity. Together, these findings suggest that activated microglia potentiate injury to BBB elements, likely at least in part by superoxide generation. We also demonstrate parallel observations at the in vivo level, where inhibiting microglial activation led to decreased BBB disruption and hemorrhage.

The anti-inflammatory properties of minocycline are thought to be through its ability to inhibit p38 mitogen-activated protein kinase and matrix metalloproteinase. Although minocycline can inhibit other aspects of cell death, we know of no other agent with a more selective profile for microglia. In support of minocycline as a relatively specific inhibitor of microglial activation, we observed complete reversal of microglia-exacerbated injury to ECs and

Figure 5. Minocycline reduces BBB disruption. A, Representative sections of animals perfused with Evan’s Blue dye. Extravasation is observed in a vehicle-treated, control animal (Con), but this is markedly reduced in an animal given minocycline (Mi). B, The total volumes of Evan’s Blue dye extravasation were reduced by minocycline as well as the relative proportion of extravasation normalized to the infarct volume (C).

Figure 6. Minocycline reduces the incidence of hemorrhage after MCAO. Brains were sectioned and inspected for gross hemorrhage 24 hours after ischemia onset. The majority of control animals (70%) had some amount of hemorrhage (A), but most minocycline-treated animals (83%) had no hemorrhage (B). Lectin histochemistry shows that perivascular microglia/monocytes are abundant in the vicinity of cerebral vessels within ischemic cortex in a vehicle-treated animal (C) but are reduced by minocycline treatment (D). Arrows indicate microglia; arrowheads, cerebral vessels. Bar=25 μm.
astrocytes and reduced numbers of perivascular microglia/monocytes.

Leukocytes and microglia secrete superoxide using NADPH oxidase as a defense against microorganisms. Although largely found in inflammatory cells, some reports suggest NADPH oxidase may also generate superoxide in neurons and astrocytes. After experimental stroke and meningitis, NADPH oxidase is found primarily in microglia and peripheral inflammatory cells. We show that blocking superoxide generation using apocynin also prevented microglia-induced exacerbation of cell death and superoxide generation. Other microglial factors may also potentiate injury and superoxide may upregulate additional damaging factors, which would be suppressed by inhibiting NADPH oxidase. Furthermore, neuroprotective factors produced by microglia could be downregulated by OGD.

In conclusion, microglia potentiate damage to components of the BBB under ischemia-like conditions, and this worsened injury is prevented by inhibiting microglial activation and generation of superoxide. Minocycline reduces BBB disruption and hemorrhage in experimental ischemic stroke. Thrombosis, although indicated for the treatment of acute ischemic stroke at the clinical level, is still infrequently used because of the short temporal therapeutic window and risk of hemorrhage. Although further studies are needed to clarify the role of minocycline in reducing thrombolytic-induced cerebral hemorrhage, these data suggest a novel reason to target microglia for treatment of cerebral ischemia.

Acknowledgments

This work was supported by National Institutes of Health grants R01 NS40516 (M.A.Y.), P50 NS14543, and P01 NS37520 (R.G.G., M.A.Y.), and R01 GM49831 (R.G.G.). The authors thank David Schaaf for editorial assistance, Elizabeth Hoyte for preparing the figures, and Maya Koike and Lipping Liu for expert technical assistance.

References


Microglia Potentiate Damage to Blood–Brain Barrier Constituents: Improvement by Minocycline In Vivo and In Vitro
Midori A. Yenari, Lijun Xu, Xian Nan Tang, Yanli Qiao and Rona G. Giffard

Stroke. 2006;37:1087-1093; originally published online February 23, 2006; doi: 10.1161/01.STR.0000206281.77178.ac
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2006 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/37/4/1087

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org/subscriptions/