Astrocyte-Derived Pentraxin 3 Supports Blood–Brain Barrier Integrity Under Acute Phase of Stroke

Akihiro Shindo, MD, PhD; Takakuni Maki, MD, PhD; Emiri T. Mandeville, MD, PhD; Anna C. Liang, MPS; Naohiro Egawa, MD, PhD; Kanako Itoh, MD, PhD; Naoki Itoh, MD; Mia Borlongan; Julie C. Holder, PhD; Tsu Tshen Chuang, PhD; John D. McNeish, PhD†; Hidekazu Tomimoto, MD, PhD; Josephine Lok, MD; Eng H. Lo, PhD; Ken Arai, PhD

Background and Purpose—Pentraxin 3 (PTX3) is released on inflammatory responses in many organs. However, roles of PTX3 in brain are still mostly unknown. Here we asked whether and how PTX3 contributes to blood–brain barrier dysfunction during the acute phase of ischemic stroke.

Methods—In vivo, spontaneously hypertensive rats were subjected to focal cerebral ischemia by transient middle cerebral artery occlusion. At day 3, brains were analyzed to evaluate the cellular origin of PTX3 expression. Correlations with blood–brain barrier breakdown were assessed by IgG staining. In vitro, rat primary astrocytes and rat brain endothelial RBE.4 cells were cultured to study the role of astrocyte-derived PTX3 on vascular endothelial growth factor–mediated endothelial permeability.

Results—During the acute phase of stroke, reactive astrocytes in the peri-infarct area expressed PTX3. There was negative correlation between gradients of IgG leakage and PTX3-positive astrocytes. Cell culture experiments showed that astrocyte-conditioned media increased levels of tight junction proteins and reduced endothelial permeability under normal conditions. Removing PTX3 from astrocyte-conditioned media by immunoprecipitation increased endothelial permeability. PTX3 strongly bound vascular endothelial growth factor in vitro and was able to decrease vascular endothelial growth factor–induced endothelial permeability.

Conclusions—Astrocytes in peri-infarct areas upregulate PTX3, which may support blood–brain barrier integrity by regulating vascular endothelial growth factor–related mechanisms. This response in astrocytes may comprise a compensatory mechanism for maintaining blood–brain barrier function after ischemic stroke. (Stroke. 2016;47:1094-1100. DOI: 10.1161/STROKEAHA.115.012133.)

Key Words: astrocyte ■ blood–brain barrier ■ cell–cell interaction ■ endothelium ■ stroke

Blood–brain barrier (BBB) leakage contributes significantly to the progression of secondary brain injury after ischemic stroke.1 Although the primary substrate of BBB injury may involve dysfunction in tight junction proteins within the affected endothelium, emerging data now suggest that gliovascular interactions may also be important. Under baseline conditions, astrocytes provide trophic support to cerebral endothelial cells to maintain BBB homeostasis.2 However, under ischemic conditions, trophic support from astrocytes may be disrupted and furthermore, reactive/diseased astrocytes may even produce deleterious factors that worsen BBB breakdown.3,4 Although reactive astrocytes often exert deleterious effects on neighboring cells under pathological conditions, they may also protect brain function or promote brain remodeling after brain injury.5,6 In this study, we test our hypothesis that reactive astrocytes may secrete mediators that support BBB function after ischemic stroke. Because one such potential mediator secreted by reactive astrocytes that benefit cerebral endothelial cells, we investigated the roles of pentraxin 3 (PTX3) on BBB integrity. PTX3 is a prototypical member of the long pentraxin family, and it is upregulated in several cardiovascular and cerebrovascular diseases.7–9 Therefore, in this study, we first determined whether reactive astrocytes expressed PTX3 after acute ischemic stroke in vivo. Then, primary cell culture experiments were conducted to assess how astrocyte-derived PTX3 may regulate BBB integrity in vitro.
Materials and Methods

All studies were performed following institutionally approved protocols by Massachusetts General Hospital Subcommittee on Research Animal Care, and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Experiments were randomized and blinded and powered to detect effect sizes in the range of 50% to 60%.

Chemicals

Recombinant mouse PTX3 was purchased from R&D systems, recombinant human vascular endothelial growth factor (rVEGF) was purchased from Sigma, and normal rabbit IgG was purchased from Santa Cruz. Mouse PTX3 shows high homology to rat PTX3, and the datasheet describes that mouse PTX3 binds with and activate rat PTX3 receptors.

Animal Model

Ten 3-month-old spontaneous hypertensive male rats (Charles River Laboratories, Wilmington, MA) were used. Rats were anesthetized with isoflurane in 70% N2O and 30% O2 mixture. Rectal temperature was maintained between 36.5°C and 37.5°C with a heating pad. Five rats underwent 75-minute transient middle cerebral artery occlusion (MCAO) by intraluminal filament with ligation of external carotid artery. Five rats underwent 75-minute anesthesia with only ligation of external carotid artery (sham-operated group). Three days after MCAO or sham-operation, rats were deeply anesthetized and perfused with ice-cold saline. Brains were removed and snap frozen. Coronal sections of 20-μm thickness were cut on cryostat (Microm HM505E) at −20°C and collected on the slides and stored at −80°C.

Immunohistochemistry and IgG Staining

Brain sections were fixed by 4% paraformaldehyde and rinsed with PBS. After blocking with 3% BSA, sections were incubated at 4°C overnight in PBS/0.1% Tween solution containing primary antibodies: anti-PTX3 (1:100, Enzo), anti-glial fibrillary acidic protein (GFAP) (astrocyte marker, 1:100, BD Pharm), anti-NeuN (neuron marker, 1:100, Chemicon), anti-platelet-derived growth factor receptor α (oligodendrocyte progenitor cell marker, 1:100, Santa Cruz), anti-platelet-derived growth factor receptor β (pericyte marker, 1:100, R&D systems), anti-nestin (NSC marker, 1:100, abcam), anti–zonula occludens-1 (ZO-1, 1:100, Invitrogen), and anti–vascular endothelial growth factor (VEGF) (1:100, Santa Cruz). For IgG staining, sections were incubated overnight at 4°C with antibody against donkey anti-Rat IgG (1:300, Jackson Immunoresearch Laboratories). Then sections were washed and incubated with secondary antibodies with fluorescence conjugations coupled device camera and an image analysis system. After staining with anti-PTX3 and anti-GFAP antibody and secondary antibodies, MCAO-operated brain section was analyzed on 5 Pascal LSM scanning confocal microscope (Care Zeiss, Germany). Each staining area of PTX3 of peri-infarct area after MCAO was analyzed by quantifying confocal microscope (Care Zeiss, Germany). Each staining area of PTX3 of peri-infarct area after MCAO was analyzed by quantifying mean intensity of divided peri-infarct area with a space of 0.2 mm from the core edge for 3 brain sections of each rat, using Imaged analysis software.

Cell Culture

Rat brain microendothelial cell line RBE.4 cells were maintained in endothelial basal medium-2 (EBM-2) supplemented with EGM-2MV SingleQuots kit (Lonza). Primary astrocyte cultures were prepared from cerebral cortices of 1- to 2-day-old neonatal Sprague–Dawley rats and maintained in DMEM containing 10% fetal bovine serum according to our previous work.10,11

Preparation of Astrocyte-Conditioned Media

Cultured astrocytes were washed with PBS and maintained in DMEM for 24 hours. Culture medium was then collected and centrifuged at 10000g for 5 minutes to remove cells and debris. Control media was prepared from culture wells without astrocytes. Astrocyte-conditioned media were stored at −80°C until use. For media transfer experiments, astrocyte-conditioned media and control media were diluted with endothelial basal medium-2 at the ratio of 1:1.

Immunoprecipitation

According to the manufacturer’s instruction, anti-rabbit PTX3 antibody (Enzo) and Dynabeads Protein G Immunoprecipitation kit (Life technologies) were used to precipitate PTX3 in culture media.

In Vitro Endothelial Permeability Assay

RBE.4 monolayer was prepared on collagen-I–coated transwells (6.5-mm diameter, 3.0-μm pore size polycarbonate filter, Corning). When RBE.4 became confluent on the transwell, cells were treated with astrocyte-conditioned media or control media for 24 hours. Then, fluorescein isothiocyanate–labeled dextran (molecular weight, 40,000; Sigma) was added to the upper chamber. After 60-minute incubation, 100 μL of culture media from the lower compartment was corrected and measured for fluorescence (excitation, 488 nm and emission, 516 nm) with a spectrophotometer.

Cell Proliferation Assay

Cells were incubated with 10% water soluble tetrazolium solution for 1 hour at 37°C, and then the absorbance of the culture medium was measured using a spectrophotometer.

Figure 1. Pentraxin 3 (PTX3) expression in ischemic brain. Male spontaneously hypertensive rats were subjected to 75-minute middle cerebral artery occlusion (MCAO) followed by 3-day reperfusion. A and B, Western blots showed that PTX3 expression level increased in the MCAO group. Data are represented means±SD from n=5 per each group. *P<0.05. C, Immunostaining (n=5 each group) confirmed that in the peri-infarct area (ie, cerebral cortex), PTX3 expression was upregulated after MCAO. Scale bar, 50 μm. D, Confocal microscopic observation confirmed that in the peri-infarct area after MCAO, the major cell type for PTX3 production was glial fibrillary acidic protein (GFAP)-positive reactive astrocytes. Scale bar, 50 μm.
measured by microplate reader with test wavelength of 450 nm and reference wavelength of 630 nm.

**Cell Death Assay**

Cytotoxicity was quantified by a standard measurement of lactate dehydrogenase using lactate dehydrogenase assay kit (Roche).

**Western Blot**

Brain samples of ipsilateral hemisphere from MCAO and sham-operated rats (n=5 each) were homogenized in Pro-PREP Protein Extraction solution (Boca scientific). Cell cultures rinsed twice with ice-cold PBS and then collected into the Pro-PREP. Astrocyte-conditioned media were concentrated 10x by using Vivaspin 500 centrifugal concentrator (Vivaproducts). Immunoprecipitation beads were processed according to the manufacturer’s instruction for Western blot analysis. Western blot samples were mixed with equal volumes of SDS (Novex) sample buffer containing 10% 2-mercaptoethanol (Sigma). After electrophoresis and transferring to nitrocellulose membranes (Novex), the membranes were blocked in 1 block (Tropix) for 60 minutes and then incubated overnight at 4°C with primary antibodies with ZO-1 (1:1000), claudin 5 (1:500, Santa Cruz), PTX3 (1:1000), VEGF (1:500, Santa Cruz), or β-actin (1:5000, Sigma Aldrich) followed by incubation with peroxidase-conjugated secondary antibodies and visualization with chemiluminescence (GE Healthcare). Optical density was assessed using ImageJ analysis software.

**Statistical Analysis**

Results were expressed as mean±SD Statistical significance was evaluated using Mann–Whitney U test to compare between 2 groups and Kruskal–Wallis test for multiple comparisons. A P value of <0.05 was considered to be statistically significant.

**Results**

First, we examined the expression of PTX3 during the acute phase of stroke in vivo. Brain samples from spontaneously hypertensive rats with 75-minute MCAO followed by 3-day reperfusion were subjected to Western blot analysis. PTX3 expression level significantly increased in the MCAO group compared with the sham-operated group (Figure 1A and 1B). Then, we conducted immunohistochemistry to identify which cell type(s) expressed PTX3 using cell-specific antibodies such as NeuN for neuron, GFAP for astrocyte, platelet-derived growth factor receptor α for oligodendrocyte progenitor cell, platelet-derived growth factor receptor β for pericyte, and
nestin for NSPC. We found that the majority of PTX3-positive cells (87.8±3.7%) was coimmunostained with GFAP in the peri-infarct area (Figure 1C), consistent with an astrocytic cellular origin. Furthermore, the confocal microscopic observation confirmed that the GFAP-positive cells exhibited stellate appearance, which is generally thought as a typical morphology of reactive astrocytes (Figure 1D).

We next focused on the distribution of PTX3 in the peri-infarct area (≈0.8 mm from the core edge). Within that region, GFAP-positive cells were uniformly distributed (Figure I in the online-only Data Supplement). However, IgG staining indicative of BBB leakage was mainly observed in the peri-infarct areas close to the edge of the infarct core, whereas PTX3 was strongly immunostained in the peri-infarct areas farthest away from the edge of the infarct core (Figure 2A–2C). The level of PTX3 expression exhibited significant negative correlation with the IgG leakage in the peri-infarct area (Figure 2D), raising the possibility that astrocytic PTX3 may be involved in the regulation of BBB permeability during acute stroke in vivo.

To further examine how astrocyte-derived PTX3 may regulate BBB function, we conducted in vitro cell culture experiments using primary rat astrocytes and RBE.4 rat brain endothelial cells. Western blots of astrocyte-conditioned media (ACM) confirmed that cultured astrocytes secreted PTX3 (Figure 3A; left lane). To investigate the role of astrocyte-derived PTX3 in the context of BBB permeability, we measured endothelial permeability in a standard transwell system, either in the presence of normal ACM or PTX3-depleted ACM (ACM$_{PTX3}$) for 24 hours. Then cells were subjected to Western blot analyses with anti–zonula occludens-1 (ZO-1) and anti-claudin 5 antibodies. A, Representative images for Western blotting. β-actin was used as an internal control. B and C, Quantitative data for ZO-1 and claudin 5 expressions in RBE.4 cells. Data are represented means±SD from n=4. *P<0.05.

PTX3 is known to bind to VEGF, which is a key regulator of endothelial leakage during stroke. Therefore, we asked whether BBB-protecting effects of PTX3 may also involve VEGF in our model systems. Indeed, in our in vivo rat stroke model, VEGF expression increased after ischemia and reactive astrocytes indeed produced both VEGF and PTX3 (Figure V in the online-only Data Supplement). Immunoprecipitation assays...
confirmed that recombinant PTX3 directly bound to VEGF in cell-free conditions (Figure 6A). Similarly, we confirmed that astrocyte-derived PTX3 bound to VEGF in the conditioned media from astrocyte cultures (Figure 6B). Finally, exogenous PTX3 suppressed the VEGF-mediated increase in cerebral endothelial cell permeability in vitro (Figure 6C), suggesting that PTX3 may negatively regulate VEGF actions on BBB permeability by directly binding and capturing VEGF.

Discussion

In this study, we described a novel role of astrocytes in supporting BBB integrity during the acute phase of ischemic stroke. Within the neurovascular unit, astrocytes are the major cell type to maintain cerebrovascular homeostasis. In the brain, astrocytes enfold blood vessels with multiple endfeet and release several trophic factors that support BBB function under normal conditions. For example, astrocyte-derived glial-derived neurotrophic factor enhances the barrier function of BBB. On the other hand, after brain injury, reactive astrocytes secrete deleterious factors, such as VEGF, that cause BBB dysfunction as well. Our findings may provide an important insight for a compensatory response of reactive astrocytes after brain injury.

Another important finding of our current study is that PTX3 may support BBB integrity in both direct and indirect ways. Under stroke conditions, several deleterious factors, such as VEGF, are released to cause BBB dysfunction. As one of the early events after central nervous system injury and inflammation, VEGF breaks down BBB integrity by downregulating protein and mRNA levels of claudin 5 and occludin, which are important components of the BBB. Our findings may arouse the novel concept that PTX3 counteracts the negative effects of VEGF on the BBB. Importantly, Rusnati et al previously demonstrated that PTX3 has high affinity to fibroblast growth factor (FGF) 2 and blocks FGF2-induced angiogenesis in vitro. The same study showed that PTX3 has low or no affinities to a panel of cytokines and growth factors, including FGF-1, nerve growth factor, or IL-1. But interestingly, PTX3 exhibited an affinity to VEGF to some extent, which supports our idea that PTX3 interacts with VEGF and supports the BBB integrity during the acute phase of stroke. However, our current study only demonstrated the interaction of PTX3 with VEGF. To understand the roles of PTX3 in ischemic brains more precisely, we may need to carefully examine whether PTX3 interacts with other BBB-damaging factors in future studies.
Although our findings describe a novel role of astrocytic PTX3 in cerebral ischemia, we need to note a few caveats and limitations in this proof-of-concept study. First, PTX3 expression pattern in peri-infarct area was negatively correlated to the IgG leakage (ie, BBB damage) in our stroke rats. But strictly speaking, we have not provided a direct proof that astrocytic PTX3 protects BBB against ischemic stress in vivo. Future studies may require gain and loss-of-function studies of PTX3 in astrocytes in vivo to confirm our in vitro findings that astrocyte-derived PTX3 increases tight junction protein expressions and decreases the endothelial permeability. Second, not all the reactive astrocytes expressed PTX3 in the peri-infarct area; consistent with the well-known heterogeneous nature of astrocytes. Which factors or signaling pathways regulate PTX3 expression in reactive astrocytes would be an important research topic in future. Third, reactive astrocytes are one of the major cell types for PTX3 production in brain parenchyma, but as mentioned above, circulating cells such as monocytes and macrophages also secrete PTX3. How circulating cell-derived PTX3 contribute to cerebrovascular function under ischemic stroke is an important area of future investigations. Fourth, our cell culture experiments described an important role of PTX3 on paracellular pathway of BBB breakdown, but after stroke, transcellular pathways may also be important. Whether and how PTX3 contributes to the transcellular pathway of BBB should also be examined in future to understand the roles of PTX3 on cerebrovascular function more carefully. Finally, our current study focused only on the acute phase of stroke. Although VEGF (or other BBB-damaging factors) is deleterious during the acute phase of stroke, it is beneficial during the chronic phase because it promotes brain remodeling. Because PTX3 inhibited VEGF effects in endothelial cultures, it is possible that astrocytic PTX3 would, in turn, inhibit brain repair, especially compensatory angiogenesis, after stroke. In fact, past studies demonstrate that PTX3 inhibits in vitro angiogenesis by FGF2 or FGF8 treatment. Therefore, the effects and dynamic roles of PTX3 on angiogenesis should be carefully assessed to further delineate the therapeutic potential of PTX3 for stroke or other cerebrovascular diseases.

In summary, our present study demonstrates that PTX3 is an important mediator from reactive astrocytes that acts on cerebral endothelial cells and supports BBB integrity after ischemic stroke. This finding reveals a novel role of reactive astrocytes under pathological conditions and suggests that astrocytic PTX3 may be an effective therapeutic target for acute ischemic stroke.

Acknowledgments
We thank Dr Igor Bagayev for technical help in confocal microscopic analysis.

Sources of Funding
This study was supported, in part, by grants from National Institutes of Health (P01-NS55104 and R01-NS065089) and the Glaxo-Smith-Kline-Harvard-Stem-Cell-Institute consortium.

Disclosures
Salaries for authors with primary appointments with Glaxo-Smith-Kline (Drs Holder, Chuang, and McNeish) were paid by Glaxo-Smith-Kline. These authors (Drs Holder, Chuang, and McNeish) participated in study design and data analysis. The other authors report no conflicts.

References


Astrocyte-Derived Pentraxin 3 Supports Blood–Brain Barrier Integrity Under Acute Phase of Stroke

Stroke. 2016;47:1094-1100; originally published online March 10, 2016; doi: 10.1161/STROKEAHA.115.012133

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 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/47/4/1094

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2016/03/10/STROKEAHA.115.012133.DC1

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/
SUPPLEMENTAL MATERIAL

Astrocyte-derived pentraxin 3 supports blood-brain barrier integrity under acute phase of stroke

Akihiro Shindo, MD, PhD 1,3; Takakuni Maki, MD, PhD 1; Emiri T. Mandeville, MD, PhD 1; Anna C. Liang, BS 1; Naohiro Egawa, MD, PhD 1; Kanako Itoh, MD, PhD 1; Naoki Itoh, MD 1; Mia Borlongan 1; Julie C. Holder, PhD 2; Tsu Tshen Chuang, PhD 2; John D. McNeish, PhD 2; Hidekazu Tomimoto, MD, PhD 3; Josephine Lok, MD 1,4; Eng H. Lo, PhD 1; Ken Arai, PhD 1

1 Neuroprotection Research Laboratory, Departments of Radiology and Neurology, Massachusetts General Hospital and Harvard Medical School, Charlestown, Massachusetts, USA
2 Regenerative Medicine Discovery Performance Unit, GlaxoSmithKline, Cambridge, USA
3 Department of Neurology, Mie University Graduate School of Medicine, Japan
4 Department of Pediatrics, Massachusetts General Hospital and Harvard Medical School, USA.

Corresponding author: Ken Arai, Neuroprotection Research Laboratory, MGH East 149-2401, Charlestown, MA 02129, USA. Tel: 617.724.9503, Email: karai@partners.org
Supplementary Figure I: GFAP-positive astrocytes in peri-infarct area.
Male SHRs were subjected to 75-min MCAO followed by 3-day reperfusion. (a) Representative image for GFAP immunostaining. Scale bar = 100 μm. (b) Quantitative data for GFAP signal showed that GFAP-positive astrocytes uniformly distributed in the peri-infarct area. Data are represented mean ± SD from n=5.

Shindo et al., Suppl Figure I
Supplementary Figure II: Astrocytic PTX3 protected in vitro BBB integrity against hypoxic stress.

Brain endothelial RBE.4 cells were treated with control media, ACM, or PTX3-depleted ACM. After that, cells were subjected to 6-hr hypoxia, and then the in vitro endothelial permeability assay was conducted. Hypoxic stress increased the in vitro endothelial permeability (i.e. breakdown in vitro BBB tightness), but ACM protected the tightness. However, PTX3-depleted ACM was not supportive for cerebral endothelial cells. Data are represented mean ± SD from n=4. *P<0.05. n.s. stands for “not significant”.

Shindo et al., Suppl Figure II
Supplementary Figure III: Recombinant PTX3 and cell viability in RBE.4 cells.
Brain endothelial RBE.4 cells were treated with recombinant PTX3 for 24 hrs. Then cells were subjected to WST (a) or LDH (b) assays. PTX3 (1, 10, 100 ng/mL) did not change cell proliferation and viability in RBE.4 cells. Data are represented mean ± SD from n=4.

Shindo et al., Suppl Figure III
Supplementary Figure IV: PTX3 and ZO-1 expression in ischemic brain.

Five male SHRs were subjected to 75-min MCAO followed by 3-day reperfusion. (a) Representative images for PTX3 (green) and ZO-1 (red). (b) The scattered plots showed mild correlation between PTX3 and ZO-1 expressions in the peri-infarct area.

Shindo et al., Suppl Figure IV
Supplementary Figure V: VEGF expression increased after stroke.

Male SHRs were subjected to 75-min MCAO followed by 3-day reperfusion. (a-b) Western blots showed that VEGF expression level increased in the MCAO group. Data are represented mean ± SD from n=5 each. *P<0.05. (c) Immunostaining (N=5 each group) confirmed that in the peri-infarct area after MCAO, GFAP-positive reactive astrocytes expressed both PTX3 and VEGF.

Shindo et al., Suppl Figure V