Role of Central Nervous System Periostin in Cerebral Ischemia

Munehisa Shimamura, MD, PhD; Yoshiaki Taniyama, MD, PhD; Naruto Katsuragi, PhD; Nobutaka Koibuchi, PhD; Mariko Kyutoku, MS; Naoyuki Sato, MD, PhD; Mohammad Allahtavakoli, PhD; Kouji Wakayama, MD, PhD; Hironori Nakagami, MD, PhD; Ryuichi Morishita, MD, PhD

Background and Purpose—Although periostin, an extracellular matrix glycoprotein, plays pivotal roles in survival, migration, and regeneration in various cells, its expression and function in the brain are still unknown. Here, we investigated the expression and role of periostin in the ischemic brain.

Methods—Expression of full-length periostin (periostin 1 [Pn1]) and its splicing variant lacking exon 17 (periostin 2 [Pn2]) was examined by real-time reverse transcription polymerase chain reaction (RT-PCR), Western blotting, and immunohistochemical staining in male C57BL/6J mice. The actions of periostin were examined in adult primary neuronal culture and in a transient middle cerebral artery occlusion (tMCAo) model.

Results—Expression of Pn2, but not of Pn1, mRNA was markedly changed after tMCAo. Pn2 mRNA was decreased in the ischemic core at 3 hours after ischemia. At 24 hours, Pn2 mRNA was significantly increased in both the peri-ischemic and ischemic regions. Periostin was mainly observed in neurons in normal brain. However, neuronal expression of periostin was decreased temporally in the ischemic region, but increased in astrocytes and around endothelial cells at 24 hours after tMCAo. Of importance, intracerebroventricular injection of Pn2 resulted in a significant reduction in infarct volume at 24 hours after tMCAo associated with phosphorylation of Akt. Also, the Pn2-treated mice survived longer until 1 week after tMCAo. Pn2 significantly inhibited neuronal death under hypoxia and stimulated neurite outgrowth.

Conclusions—Here, we demonstrated that periostin was expressed in the brain, and exogenous Pn2 exhibited neuroprotective effects and accelerated neurite outgrowth. Additional studies on periostin may provide new insights into the treatment of ischemic stroke. (Stroke. 2012;43:1108-1114.)

Key Words: periostin ■ ischemia ■ neuroprotection ■ extracellular matrix ■ neurite outgrowth

Periostin is a 93-kDa secreted N-glycoprotein that modulates cell-matrix interactions and cell functions in the extracellular matrix (ECM).1 Although periostin was originally found in osteoblasts,2 recent studies showed that periostin plays pivotal roles in cell survival under hypoxic conditions,3 migration of cancer cells,4 and proliferation of cardiomyocytes after acute myocardial infarction.5 Interestingly, periostin has homology with fasciclin I, which is expressed in grasshoppers and Drosophila. Fasciclin I is a cell adhesion molecule expressed in the central nervous system (CNS) during embryonic CNS development in Drosophila.6 Also, laser inactivation of fasciclin I disrupts axon adhesion of grasshopper pioneer neurons.7 Thus, periostin might have pivotal roles in the CNS in mammals, although its expression and function have not been clarified in the adult CNS.

One of the unique characteristics of periostin is its variable regions in the C-terminal regions, which contain exons 15 to 23 (Figure 1A). The presence of splicing variants of periostin is becoming the center of the interest, given that various splicing variants of periostin, lacking some of exons 15 to 23, have been speculated to have different functions.8 Among the splicing variants, periostin lacking exon 17 was shown to have different roles in cancer metastasis. For example, Kyutoku et al reported that full-length periostin prevented the progression and metastasis of breast cancer,9 whereas Kim et al reported that...
overexpression of a periostin variant that lacked exon 17 suppressed lung metastasis in B16-F10 cells.

From this viewpoint, we examined the expression of full-length periostin (Pn1, Figure 1A) and a splicing variant of periostin that lacked exon 17 (Pn2, Figure 1A) in the adult mouse brain in this study. Also, we studied the actions of periostin using hypoxic cultured neurons and a transient middle cerebral artery (MCA) occlusion model in mice.

**Methods**

**Surgical Procedure and Measurement of Infarct Volume**

All procedures were approved by the Institutional Animal Care and Use Committee of Osaka University. Experiments were performed in 6- to 8-week-old male C57BL/6 mice (CLEA Japan Inc.). Some mice were exposed to transient MCA occlusion model (online-only Supplemental Methods, http://stroke.ahajournals.org). The variation in infarct volume is presented in online-only Supplemental Figures S4B and S4C. Overall mortality was 14% at 24 hours after ischemia and 67% at 7 days after MCA occlusion. Ischemic damage was evaluated using sections stained with cresyl violet (online-only Supplemental Methods).

**Administration of Recombinant Periostin**

Recombinant human Pn1 as full-length periostin and mouse Pn2 periostin that lacks exon 17 (R&D Systems) were injected intracerebroventricularly (online-only Supplemental Methods).

**Behavioral Test**

Neurological deficit was assessed using a modification of the Bederson neurological scale, as previously described (online-only Supplemental Methods).

![Figure 1. Expression of Periostin mRNA and Protein](image)

**Real-Time Reverse Transcription Polymerase Chain Reaction**

The cerebral cortex was collected using a punch (FST No.18035–80). RNA of the punched brain was isolated using a QIAGEN RNeasy Lipid TissueMini Kit (Qiagen), according to the manufacturer’s recommendations. Each quantitative PCR analysis was performed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Applera Co.) with SYBR green staining of DNA double strands. Primer pairs were shown in the online-only Supplemental Methods. Each mRNA value (relative quantification) was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RNase Cocktail (Applied Biosystems) was used to degrade RNA.

**Cell Culture**

To examine the neuroprotective effects of periostin in hypoxic conditions, cultured neurons, which were pretreated with Pn2 (10 μg/mL) or bovine serum albumin (10 μg/mL), were exposed to hypoxia. To examine the effects of periostin on neurite outgrowth, neurons were cultured in medium containing Pn1, Pn2, or bovine serum albumin (online-only Supplemental Methods).

**Immunohistochemical Staining**

Immunohistochemical staining was performed in frozen sections (online-only Supplemental Methods). The images were examined with a Nikon A1 confocal scanning laser microscope, and images were analyzed using NIS Elements software (Nikon). All parameters were set in a similar manner when the signal intensity was compared.

**Western Blotting**

Tissue samples were lysed in radio immunoprecipitation assay buffer containing 150 mmol/L NaF, 2 mmol/L sodium orthovanadate, and protease inhibitors (protease inhibitor mixture; Roche Applied Science). Protein of the total lysate (20 μg) was loaded and blotted.
Primary antibodies against periostin (1:25; goat polyclonal; Santa Cruz, SC-49480), phospho-Akt (Ser473; 1:1000, Cell Signaling Technology, #9271) and total Akt (1:1000, Cell Signaling, #9272) were used.

Statistical Analysis
All values are expressed as mean±SEM. Multiple comparisons were performed by ANOVA followed by Dunnett’s Test. Two groups were compared by unpaired t test. Survival rates were evaluated by log-rank test. Differences were considered significant at P<0.05.

Results
Expression of Periostin in Adult Brain
To examine the expression of periostin, we first studied the expression of periostin mRNA in the adult mouse brain. Among the various variants of periostin,12 we analyzed full-length periostin (Pn1) and a spliced variant form of periostin (Pn2) that lacked exon 17. Using specific primers (online-only Supplemental Figure S1), mRNA expression of Pn1 and Pn2 was successfully detected in extracts from normal cerebral cortex (Figure 1B, 1C). The relative ratio of Pn1/GAPDH and Pn2/GAPDH mRNA calculated by the relative quantification was 0.46±0.11 and 0.47±0.11, respectively. Consistent with real-time reverse transcription polymerase chain reaction (RT-PCR), Western blotting using S-15 antibody against an epitope (amino acids 110–160) of periostin to recognize both Pn1 and Pn2 showed a 90-kDa band, which was identical to the size of periostin, in the cerebral cortex and hippocampus (Figure 1D). Although a 65-kDa band was also detected, this 65-kDa band might have been the spliced form of periostin via proteolytic processing, given that previous reports also exhibited the same findings.13,14 Immunohistochemical study showed that periostin was widely expressed in the brain (online-only Supplemental Figure S2A), and its expression was observed in MAP2-positive cells (mature neurons), whereas GFAP-positive (astrocytes), Iba-1-positive (microglia), and CD-31-positive (endothelial cells) cells did not express periostin (Figure 2). In the hippocampus, periostin was expressed in MAP2-positive cells and partially in GFAP-positive cells (online-only Supplemental Figure S2C), but not in Iba-1-positive or CD-31-positive cells (data not shown). In the striatum, some cells expressed periostin, and their signals were weak as compared with those in the cerebral cortex (online-only Supplemental Figure S2B). Overall, periostin was expressed mainly in neurons in the adult mouse brain.

Change in Expression of Periostin in Ischemic Brain
To clarify the functional roles of periostin in pathological conditions, we analyzed the temporal profile of mRNA expression in the cerebral cortex after transient MCA occlusion. Although the expression of Pn1 and Pn2 was changed by ischemia, the change in Pn2 mRNA was greater (Figure 3A). At 3 hours after MCA occlusion, the expression of Pn1 and Pn2 mRNA was significantly decreased in the ischemic core. At 24 hours, the expression of Pn2 mRNA was significantly increased in both the peri-ischemic and ischemic regions, whereas the expression of Pn1 mRNA was not changed (Figure 3A). Immunohistochemical staining showed that the expression of periostin was very weak in the ischemic region (region I in Figure 3B and online-only Supplemental Figure S3A) at 3 hours after MCA occlusion. Astrocytes, microglia, and endothelial cells did not express periostin at this time (data not shown). At 24 hours after ischemia, expression of periostin emerged in the infarct region (online-only Supplemental Figure S3B), and the expression was observed around CD-31-positive cells (Figure 3C). In the peri-ischemic region, periostin was expressed in astrocytes (Figure 3D, online-only Supplemental Figure S3B) and neurons, whereas microglia did not express periostin (data not shown). The present study demonstrated that the expression of periostin was decreased in the ischemic region at 3 hours, but was increased in the peri-ischemic and ischemic regions at 24 hours after MCA occlusion, suggesting that periostin might play roles in neuroprotection and/or the healing process of the ischemic brain.

Exogenous Pn2, but Not Pn1, Attenuated Ischemic Injury In Vivo
Given that the expression of periostin was decreased in the ischemic region at 3 hours and increased in the peri-ischemic region at 24 hours after reperfusion, we hypothesized that exogenous supplementation of periostin in the acute stage of ischemia would prevent neuronal damage after ischemia-reperfusion. Pn1 or Pn2 was injected intracerebroventricularly at 30 minutes before ischemia and 5 minutes after reperfusion (1.5 hours after ischemia). First, we examined the effect of 10 μg/mL of periostin because this concentration of periostin was reported to have effects in cardiomyocytes, fibroblasts,15 and 293T cells.16 Although intracerebroventricular injection of Pn1 did not affect the infarct volume, treatment with Pn2 significantly reduced infarct volume at 24...
hours after reperfusion, with decreased neurological deficit (Figure 4A, 4B). Injection of Pn1 or Pn2 before ischemia had no influence on cerebral blood flow during ischemia and reperfusion (online-only Supplemental Figure S4A). Next, we examined the 1 μg/mL of Pn2 because another article showed that 1 μg/mL of periostin exhibited effects in cancer cells. However, 1 μg/mL of Pn2 did not show any effect on infarct volume (Figure 4A). Of importance, Pn2 (10 μg/mL)

Figure 3. Expression of Periostin After Transient MCA Occlusion in Brain. A, Real-time RT-PCR from sham-operated mice (SH), ischemic region (1), peri-ischemic region (2), and contralateral intact regions corresponding to ischemic hemisphere (3,4). n=4. *P<0.05, **P<0.01 vs sham-operated mice. B-D, Double immunohistochemical staining for MAP2 at 3 hours, CD-31 at 24 hours and GFAP at 24 hours. B, Peri-ischemic region; C, and ischemic core, are shown. I, ischemic region; PI, peri-ischemic region. These images coincide with the region shown in online-only Supplemental Figure S3. B, yellow box 1; C, yellow box 2; D, yellow box 3 in online-only Supplemental Figure S3. Bar=50 μm B; 100 μm C; 25 μm D.

Figure 4. Effects of Intracerebroventricular Injection of Exogenous Periostin Into Ischemic Brain. A, Infarct volume at 24 hours after reperfusion when different doses of periostin were injected 30 minutes before MCA occlusion and 5 minutes after reperfusion. B, Bederson neurological scale at 2.5 hours after MCA occlusion (1 hour after reperfusion) and 24 hours after MCA occlusion. C, Infarct volume at 24 h after reperfusion when 10 μg/mL Pn2 was injected 1.5 hours after ischemia (5 min after reperfusion) or 3.5 h after ischemia (2 h after reperfusion). *P<0.05, **P<0.01 vs mice treated with vehicle, #P<0.05 vs mice treated with Pn1. n=9. D, Survival rate of Pn2-treated mice. *P<0.05 vs mice treated with vehicle. n=12.
injected at 1.5 hours after ischemia (5 minutes after reperfusion), but not at 3.5 hours after ischemia (2 hours after reperfusion), resulted in a significant reduction in the infarct volume (Figure 4C). Finally, we checked whether the effects of injection of Pn2 lasted for 7 days. When mice were treated with Pn2 (10 μg/mL) at 1.5 hours after MCA occlusion, they survived significantly longer (Figure 4D). Given that previous studies showed that periostin activated the p-Akt/PI-3 kinase pathway through activation of the integrin receptor, we examined expression of p-Akt in the injured hemisphere using Western blot analysis (Figure 5). Although the expression of p-Akt was slightly increased at 75 minutes after reperfusion, its expression was much higher in mice treated with Pn2 as compared with PBS-treated mice. Level of p-Akt was the same in mice treated with Pn1 and vehicle-treated mice. These data suggest that Pn2 prevented neuronal death through activation of the Akt signaling pathway.

**Pn2 Promoted Neuroprotection and Neurite Outgrowth in Cultured Adult Neurons**

To confirm the neuroprotective action of Pn2, we employed primary adult neuronal culture. Addition of recombinant Pn2 (10 μg/mL) to cultured neurons under hypoxia significantly inhibited cell death, whereas a hypoxic condition strongly promoted cell death (Figure 6A, 6B); this indicates that Pn2 might directly act on neurons and protect them from ischemic injury in vivo.

Finally, we checked whether periostin had effects on neurite outgrowth, given that other extracellular matrix glycoproteins, such as fibronectin and tenascin-C, accelerated neurite outgrowth after ischemic injury or spinal cord injury. When cultured neurons were treated with Pn2 after plating, the average length of the maximum neurite at 48 hours was significantly longer than that of neurons treated with bovine serum albumin (10 μg/mL) or with Pn1 (10 μg/mL; Figure 6C, D). These data indicate that Pn2 has the potential to promote neurite outgrowth.

**Figure 5.** Expression of p-Akt in ischemic hemisphere at 75 minutes after reperfusion. *P*<0.05 vs sham mice, #P*<0.05 vs mice treated with vehicle, %P*<0.05 vs mice treated with Pn1. n=6.

**Figure 6.** Effects of Periostin on Survival and Neurite Growth in Cultured Adult Neurons. A, B, Effects of periostin on hypoxic neurons. Most neurons died under a hypoxic condition, whereas neurons treated with Pn2 were protected from hypoxic damage. Green: MAP2; Blue: DAPI. *P*<0.05 vs neurons without Pn2 in hypoxic condition, #P*<0.05 vs normal neurons. n=6. C, D, Effects of periostin on neurite outgrowth at 48 hours after plating. *P*<0.05 vs neurons treated with Pn1, #P*<0.05 vs nontreated neurons. Green: MAP2. Bar=50 μm. n=6.
Discussion

Recently, the role of ECM in the CNS has been a focus of interest, because glycoproteins in ECM, such as fibronectin and tenasin-C, have various effects in neuronal protection, neurite outgrowth, and the migration of neural stem cells. However, peristin has not yet been studied in the brain. In the present study, we demonstrated that peristin was expressed in neurons in the brain, and Pn2 showed a marked change of expression after cerebral ischemia. Exogenous Pn2 revealed neuroprotective effects in the ischemic brain as well as in cultured neurons, and Pn2 also promoted neurite outgrowth in cultured neurons. Because peristin is also an important glycoprotein in other tissues, such as the heart and cancers, we speculate that it also has a critical role in the brain. Given that the expression of Pn1 mRNA did not dynamically change after MCA occlusion, and exogenous Pn1 showed no effect on p-Akt expression in the ischemic brain, it follows that Pn1 and Pn2 might have different roles in the brain.

Interestingly, a recent study showed that peristin promoted incorporation of tenasin-C into the ECM and organized the meshwork architecture of the ECM because of adjacent domains banded to tenasin-C and other ECM proteins, acting as a bridge between tenasin-C and other ECM proteins. Tenasin-C is also reported to be linked with lecticans and to form perineuronal nets, which protect neurons from damage, such as oxidative stress. From this viewpoint, it is possible that the linkage of tenasin-C to other ECM proteins was accelerated by exogenous peristin, which augmented the formation of a perineuronal net to protect neurons.

Integrin receptors (αvβ1, αvβ3, or αvβ5) have been proposed as the receptor for peristin in the heart and cancer cells. Peristin activated the Akt signaling pathway through αvβ3 integrin to increase cellular survival, and αvβ1, β3, and β5 integrins induced the proliferation of differentiated cardiomyocytes. Importantly, in neurons, integrin αv promoted neuronal protection and neurite outgrowth, and integrin β1 protected neurons through the Akt/PKB signaling pathway, as well as controlled the migration of neuroblasts. Phosphorylation of Akt by Pn2 might explain the effects of Pn2 on neurons, probably through activation of integrin receptors in neurons.

The mechanisms of the different effects among splicing variants have not yet been reported. Given that no increase in p-Akt level was observed in Pn1-treated mice, one of the mechanisms might be related to the different actions of splicing variants on the p-Akt signaling pathway. One possibility is that the different splicing variants might activate a different type of integrin receptors. Another possibility is that the exon 17 in peristin might have an inhibitory effect on integrin-binding affinity, considering that alternatively spliced segments in fibronectin modulated integrin-binding affinity of fibronectin. Lack of exon 17 in Pn2 might upregulate integrin-binding affinity. Additional studies on the signaling pathways of splicing variants are necessary for clarification of the mechanisms.

Recently, peristin was reported to induce matrix metalloproteinase-2 (MMP-2) in atherosclerotic and rheumatic cardiac valves. In the ischemic brain, MMP-2 is one of the key factors promoting the breakdown of extracellular matrix, resulting in opening of the blood-brain barrier. In the present study, we speculate that MMP-2 might not have been increased because of the better outcome in Pn2-treated mice, although additional studies are necessary to clarify the relationship between Pn2 and MMP-2.

One limitation of this study is that administration of Pn2 at 3.5 hours after MCA occlusion did not show an effect on infarct volume. This result suggests the existence of a therapeutic time window of treatment with Pn2. Additional studies are necessary to examine the possibility of extending the therapeutic time window by changing the route of injection or injection dose. Another limitation is that differential induction of Pn1 or Pn2 in the astrocytes and around endothelial cells in ischemic brain was not clarified in the present study. Specific antibodies that recognize Pn1 or Pn2 should be developed to address this issue.

Overall, the present study demonstrated that peristin was expressed in the brain and its expression changed after cerebral ischemia. Also, exogenous Pn2, but not Pn1, promoted neuroprotection and neurite outgrowth. Although we focused on Pn1 and Pn2 in the present study, additional studies to clarify the function of peristins, including other splicing variants, might provide new insights into the treatment of ischemic stroke.

Acknowledgments

Mohammad Allahavakoli has joined to this study as a fellow of Japan’s Matsumae International Foundation.

Sources of Funding

This work was supported by Takeda Science Foundation and a Japan Heart Foundation/Novartis Grant for Research Award on Molecular and Cellular Cardiology, 2011.

Disclosures

None.

References


Role of Central Nervous System Periostin in Cerebral Ischemia
Munehisa Shimamura, Yoshiaki Taniyama, Naruto Katsuragi, Nobutaka Koibuchi, Mariko Kyutoku, Naoyuki Sato, Mohammad Allahtavakoli, Kouji Wakayama, Hironori Nakagami and Ryuichi Morishita

Stroke. 2012;43:1108-1114; originally published online February 2, 2012;
doi: 10.1161/STROKEAHA.111.636662
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2012 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/43/4/1108

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2012/02/10/STROKEAHA.111.636662.DC1.html

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/
ONLINE SUPPLEMENT

Role of Central Nervous System Periostin in Cerebral Ischemia

Munehisa Shimamura, M.D., Ph.D.¹, Yoshiaki Taniyama, M.D., Ph.D.²,
Naruto Katsuragi, Ph.D.³, Nobutaka Koibuchi, Ph.D.⁴, Mariko Kyutoku, M.S.¹,
Naoyuki Sato, M.D., Ph.D.², Mohammad Allahtavakoli, Ph.D.⁵,
Kouji Wakayama, M.D., Ph.D.⁴, Hironori Nakagami, M.D., Ph.D.¹,
and Ryuichi Morishita, M.D., Ph.D.²

1 Division of Vascular Medicine and Epigenetics, Department of Child Development, United Graduate School of Child Development, Osaka University, Kanazawa University and Hamamatsu University School of Medicine, Japan, 2 Department of Clinical Gene Therapy, Graduate School of Medicine, Osaka University, Japan, 3 Biomedical Research Laboratories, Asubio Pharma Co., Ltd., Japan, 4 Department of Advanced Clinical Science and Therapeutics, Graduate School of Medicine, The University of Tokyo, Japan, 5 Department of Physiology, Rafsanjan University of Medical Sciences, Iran

Cover title: Role of Periostin in Ischemic Brain

Corresponding author: Ryuichi Morishita, M.D., Ph.D., Professor
Department of Clinical Gene Therapy, Osaka University, Graduate School of Medicine
2-2 Yamada-oka, Suita, Osaka 565-0871, Japan
Tel: 81-6-6879-3406, Fax: 81-6-6879-3409, E-mail: morishit@cgt.med.osaka-u.ac.jp

Key Words: periostin, ischemia, neuroprotection, extracellular matrix, neurite outgrowth

Code: [44] Acute Cerebral Infarction, [72] Neuroprotectors, [151] Ischemic biology - basic studies
Supplemental Methods

Transient middle cerebral artery occlusion

Mice were anesthetized with isoflurane (1.4%). Cerebral blood flow was measured using a laser Doppler flowmeter (Unique Acquisition software; Unique Medical, Japan). A 6.0 monofilament surgical suture was advanced into the internal carotid artery to obstruct the origin of the MCA. The filament was left in place for 90 minutes and then withdrawn. Only animals that exhibited a typical reduction pattern and more than 80% reduction in CBF during MCA occlusion in which CBF recovered by 50% after 5 min of reperfusion were included in the study. In all mice, rectal temperature was kept at 37.0 ± 0.5 °C during surgery and in the recovery period until animals regained consciousness.

Cell culture

Adult cerebral cortex neurons were obtained from 8-week-old C57BL/6J mice as described before1. Briefly, the cerebral cortex was dissected, and individual cells were isolated by treatment with papain, and neurons were separated using an Optiprep gradient (Sigma-Aldrich, Saint Louis, MO, USA). The isolated neurons were plated on poly-D-lysine-coated 12-mm glass coverslips in 24-well plastic culture dishes with Neurobasal A (Invitrogen, San Diego, CA, USA)/B-27 (Invitrogen)/Glutamax (Invitrogen) at 37 °C. The rate of immuno-positive cells for MAP2 on the fourth day was 77.2 ± 5.2%, which was almost the same as that in a previous report1. To examine the neuroprotective effects of periostin in hypoxic conditions, cultured neurons (please see Supplemental Methods) were changed to fresh medium containing recombinant Pn2 (10 µg/ml) or BSA (10 µg/ml) at 4 days after being plated. The cells were cultured for 2 hrs, and then 200 µM cobalt chloride was added to the medium2. At 21 hours after being exposed to hypoxia, the number of surviving MAP2-positive cells whose nucleus was not condensed and shrunken was counted.

To examine the effects of periostin on neurite outgrowth, neurons plated on a glass coverslip were cultured in medium containing Pn1 (10 µg/ml), Pn2 (10 µg/ml), or BSA (10 µg/ml). The maximum neurite length was measured in 50 neurons that did not contact each other.

Immunohistochemical staining

Mice were perfused with 4 % PFA, and the brain was cut at 12 µm thickness. Sections were incubated in anti-periostin antibody (1:25; goat polyclonal; Santa Cruz, CA, USA, #SC-49480). This antibody was previously used in another paper to examine the expression of periostin in skin wounds3. As negative controls, normal control IgG (Santa Cruz, #SC-2028) was applied instead of anti-periostin antibody. For double immunostaining, these sections were fixed again, and blocked. The sections were incubated with anti-MAP2 (1:1000; mouse monoclonal; Sigma-Aldrich), GFAP (1:1000; mouse monoclonal; Sigma-Aldrich), Iba-1 (1:1000; rabbit polyclonal; Wako, Japan), or CD31 (1:100; rat monoclonal; CA, USA).

Behavioral test

Neurological deficit was assessed using a modification of the Bederson neurological scale, as previously described4: 0 (normal motor function), 1 (flexion of torso and contralateral forelimb when mouse is lifted by the tail), 2 (circling to the contralateral side when mouse is held by the tail on a flat surface, but normal posture at rest), 3 (leaning to the contralateral side at rest), and 4 (no spontaneous motor activity).
Measurement of infarct volume

Coronal sections (12 µm thick) were made at -1.4, -0.7, 0, 0.7, and 1.4 mm from the bregma, mounted on a stereomicroscope, and proceeded to cresyl violet staining. The hemispheric lesion area (HLA) in coronal sections was calculated. Corrected HLA was calculated as HLA (%) = [LT-(RT- RI)]/LT×100, where LT is the area of the left hemisphere, RT is the area of the right hemisphere, and RI is the infarcted area5.

Primer pairs for realtime RT-PCR

The following primer pairs were used: Pn1 (sense: 5-ataaccaagtcctggaacc-3, antisense: 5-tgtctccgtaagcagtcttt-3, 415 bp), Pn2 (sense: 5-ccatgacctcttagacactg-3, antisense: 5-tgtctccgtaagcagtcttt-3, 360 bp), mouse GAPDH (sense: 5-gggtggagccaaaaggtc-3, anti-sense: 5-ggagttgctgttgaagtcga-3, 534 bp) (Supplemental Fig. 1).

Administration of recombinant periostin

Recombinant human Pn1 as full length periostin and mouse Pn2 periostin that lacks exon 17 were purchased from R&D Systems (Minneapolis, MN, USA). An intracerebroventricular injection cannula (Plastic One Inc., Roanoke, VA, USA) was implanted into the right lateral ventricle at 7 days before MCA occlusion. Recombinant Pn1, Pn2 (10 or 1 µg/ml in 1.5 µl artificial cerebrospinal fluid; aCSF), or aCSF was administered 30 minutes before ischemia and/or 5 min - 3.5 hours after reperfusion.
Supplemental Figures and Figure Legends

Supplemental Figure S1: Region where primers were selected in order to differentiate full length and splice variant.

- **Region where primers were selected in order to differentiate full length and splice variant.**
  - **Exon 17 region (arrow),** whereas the sense-primer in **Pn2 mRNA recognized the region before and after the Exon 17 region (dotted arrow).**

---

**Supplemental Figure S1:** Region where primers were selected in order to differentiate full length and splice variant.

- **Regions in italics and bold characters are categories of exon 17.** The sense-primer in **Pn1 mRNA recognized the exon 17 region (arrow),** whereas the sense-primer in **Pn2 mRNA recognized the region before and after the exon 17 region (dotted arrow).**
Supplemental Figure S2: Immunohistochemical staining for periostin in cerebral cortex and striatum.

Periostin was widely expressed in the cerebral cortex, whereas the normal IgG control showed no expression of periostin (A). In the striatum, periostin was also expressed, but the signal was much weaker than that in the cerebral cortex (B). In the hippocampus CA1 region, periostin was expressed in neurons (C-a) and partly in astrocytes (C-b, arrow). Confocal analysis showed that periostin co-localized with GFAP-positive cells (C-c). Bar = 100 µm (A, B), 50 µm (C-a, C-b) and 25 µm (C-c).
Supplemental Figure S3: Expression of periostin after MCA occlusion

Immunohistochemical staining for Pn at 3 hrs (A) and 24 hrs (B) after MCA occlusion. I: ischemic region; PI: peri-ischemic region. These images coincide with the region shown in Figure 3. Yellow box 1: B; yellow box 2: C; yellow box 3: D in Fig. 3. Bar = 100 µm.
Supplemental Figure S4: Cerebral blood flow (CBF) during cerebral ischemia, variation of infarct volume

(A) CBF recorded from right occipital bone when 10 µg/ml of Pn1 or Pn2 was injected before and after MCA occlusion. The reduction and recovery of CBF were not different between the groups. n = 9 in each group. (B, C) Variation of infarct volume. * P<0.05 vs. mice treated with vehicle. n = 9.
Supplemental References


