Ischemic stroke activates the VE-cadherin promoter and increases VE-cadherin expression in adult mice

Akiko Nakano-Doi, Rika Sakuma, Tomohiro Matsuyama and Takayuki Nakagomi
Institute for Advanced Medical Sciences, Hyogo College of Medicine, Hyogo, Japan

Summary. Endothelial cells (ECs) are a key component of the blood-brain barrier (BBB). Healthy ECs in the BBB form inter-endothelial junctions, including adherens junctions (AJs). Under pathological conditions, such as after ischemic stroke, the BBB may be functionally compromised. However, gene and protein expression patterns involving endothelial AJs have not been well studied. Because expression levels of endothelial AJs are considered to be related to BBB functionality, we investigated the expression pattern of a representative endothelial AJ marker, VE-cadherin, in healthy and diseased mice. We first examined the expression of VE-cadherin in developing mouse brains. In addition, using a mouse model of cerebral infarction, we investigated the expression pattern of VE-cadherin in pathologic brains. Furthermore, using the Cre-LoxP system, we established a strain of mice expressing yellow fluorescent protein (YFP) under the control of the VE-cadherin promoter and investigated the expression pattern of YFP-expressing ECs in developing and pathologic murine brains. VE-cadherin protein and YFP expression driven by the VE-cadherin promoter both showed that VE-cadherin expression was weak during embryonic stages, followed by a steady increase postnatally, which then decreased during adulthood. However, following ischemic stroke, immunohistochemistry of VE-cadherin demonstrated an upregulation in ECs within ischemic regions, concomitant with YFP upregulation. These findings reveal that ischemic stroke activates the VE-cadherin promoter and increases VE-cadherin protein expression, which suggests that endothelial VE-cadherin is involved in the reconstruction of the BBB following ischemic stroke.

Key words: Ischemia, Stroke, VE-cadherin, Endothelial cells, Adherens junction

Introduction

In the central nervous system (CNS), endothelial cells (ECs) are a key element of the blood-brain barrier (BBB). Under normal conditions, ECs in the BBB form inter-endothelial junctions, including tight junctions (TJs) and adherens junctions (AJs). Endothelial cell-to-cell junctions play crucial roles in the maintenance of intercellular adherens and the regulation of cell-cell signaling that modulates cell growth, vessel formation, and cell polarity (Giannotta et al., 2013). Thus, pathological conditions that damage intercellular junctions in the BBB may cause vascular permeability by diminishing endothelial cell-to-cell junctions, as well as changing the features of ECs within and surrounding the BBB.

VE-cadherin is a major component of AJs between ECs. At the early stages of development in mice (embryonic day 7.5 [E7.5]), VE-cadherin transcripts can be detected in mesodermal cells of the yolk-sac mesenchyme. At later embryonic stages, VE-cadherin
mRNA can be detected in vascular cells of various organs, including the brain (Giannotta et al., 2013). VE-cadherin plays multiple important roles, including survival of ECs, stabilization of blood vessel assemblies, and vascular permeability (Vittet et al., 1997; Carmeliet et al., 1999; Gory-Faure et al., 1999; Crosby et al., 2005; Vestweber, 2008; Giannotta et al., 2013; Tietz and Engelhardt, 2015). However, under pathological conditions, such as after ischemic stroke, inactivation of β1-integrin induces BBB leakiness by reducing the binding of P120-catenin to VE-cadherin (Tietz and Engelhardt, 2015; Yamamoto et al., 2015). Hence, VE-cadherin likely plays an essential function in protecting the BBB under ischemic conditions. However, little is known about the expression pattern of VE-cadherin in pathological brains.

Using normal developing and adult mice, we examined VE-cadherin promoter activity and protein expression in the BBB. In addition, using a mouse model of cerebral infarction, we investigated whether the promoter and protein expression of VE-cadherin are altered following induced ischemic stroke.

Materials and methods

Animal studies

The Animal Care Committee of the Hyogo College of Medicine approved all experimental procedures (License number: 15-005, 16-084). C57BL/6 mice (Clea Japan, Inc., Tokyo, Japan) at different developmental stages were prepared as previously described (Nakano-Doi et al., 2016). In another set of experiments, VE-cadherin-Cre mice (Kogata et al., 2006) (Laboratory Animal Resource Bank at NIBIO, Osaka, Japan) were crossed with YFP reporter mice [B6;129-Gt(Rosa)26Sortm1(EYFP)/J mice; Jackson Laboratory, Bar Harbor, ME, USA]. The offspring were “VE-cadherin promoter-driven YFP-expressing mice” (VE-cadherin/YFP mice). Animals had access to food ad libitum, and efforts were made to minimize the number of animals used and their suffering. Quantitative analyses were performed by investigators blinded to the experimental protocol and sample identities.

Preparation of tissue from developing brains

C57BL/6 [Embryonic Day 17 (E17), Postnatal Day 1 (P1), Postnatal Day 15 (P15), adolescent animals at 5 weeks, and mature adults at 24 weeks] and VE-cadherin/YFP mice [Embryonic Day 16 (E16), adolescent mice (5 weeks), and mature adults (24 weeks)] at different developmental stages were sacrificed following anesthesia by intraperitoneal injection with sodium pentobarbital as previously described (Nakagomi et al., 2009a,b, 2011; Nakano-Doi et al., 2010). In brief, under isoflurane inhalation, the left MCA was isolated, electro-cauterized, and disconnected just distal to its crossing of the olfactory tract (the distal M1 portion). Following induced ischemic stroke, mice were anesthetized with sodium pentobarbital and transcardially perfused with PLP. As described above, brains were removed, cryoprotected, and sectioned using a cryostat for immunohistochemistry.

Immunohistochemistry

Coronal brain sections (16-μm thick) were subjected to immunohistochemistry as previously described (Nakagomi et al., 2009a,b, 2011; Nakano-Doi et al., 2010). In brief, brain sections were stained using antibodies against VE-cadherin (1:200, rabbit, SC-28644, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), CD31 (1:100, rat, BD 550274, BD Pharmingen, San Diego, CA, USA), CD45 (1:100, rat, MAB1144, R&D systems, Minneapolis, MN, USA), MAP2 (1:500, mouse, M4403, Sigma-Aldrich, St. Louis, MO, USA), GFAP (1:500, mouse, MAB360, Millipore, Temecula, CA, USA), PDGFRβ (1:500, goat, AF1042, R&D systems), CD34 (1:200, rabbit, SC-9095, Santa Cruz Biotechnology), vWF (1:100, rabbit, SC-14014, Santa Cruz Biotechnology), collagen IV (1:500, rabbit, ab6586, Abcam, Cambridge, UK), and albumin (1:200, goat, A90-134A, Bethyl Laboratories, Montgomery, TX, USA). To detect YFP+ cells, an anti-GFP antibody (1:1000, rabbit, ab6556, Abcam) that recognizes YFP was used. Primary antibodies were visualized using Alexa Fluor 488- [1:500, A11006 (goat anti-rat IgG), A11029 (goat anti-mouse IgG), A11034 (goat anti-rabbit IgG), A11055 (donkey anti-goat IgG), Molecular Probes, Eugene, OR, USA]) or 555-conjugated secondary antibodies [1:500, A21424 (goat anti-mouse IgG), A21434 (goat anti-rat IgG), A21429 (goat anti-rabbit IgG), A21432 (donkey anti-goat IgG), Molecular Probes]. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (1:500, 71-03-01, DAPI, Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA). Images of brain sections were captured using a confocal laser microscope (LSM780; Carl Zeiss AG, Oberkochen, Germany). For negative controls of immunohistochemistry experiments, the primary antibodies were omitted and we confirmed that no staining occurred (data not shown). The areas with CD31+ or VE-cadherin+ cells during development (a total of 36 data points, 4 points/section, 3 sections/brain [n=3]) within

Preparation of tissue from adult brains following ischemic stroke

Adult C57BL/6, VE-cadherin/YFP, and CB-17/ICR-+/+ Jcl mice (CB-17 mice; Clea Japan Inc., Tokyo, Japan) were subjected to permanent focal cerebral ischemia by ligation and interruption of the distal portion of the left middle cerebral artery (MCA) as previously described (Nakagomi et al., 2009a,b, 2011, 2015a; Nakano-Doi et al., 2010, 2016). In brief, under isoflurane inhalation, the left MCA was isolated, electro-cauterized, and disconnected just distal to its crossing of the olfactory tract (the distal M1 portion). Following induced ischemic stroke, mice were anesthetized with sodium pentobarbital and transcardially perfused with PLP. As described above, brains were removed, cryoprotected, and sectioned using a cryostat for immunohistochemistry.
the region of interest were analyzed using Image J software and subjected to semi-quantitative analysis as previously described (Nakano-Doi et al., 2010; Saino et al., 2010; Sakuma et al., 2016). In this study, we defined the “ischemic area” as the area within the “border of the post-stroke area.” The areas with YFP+ YFP+/CD31+ or VE-cadherin+ cells following ischemia (a total of 27 data points, 3 points/section, 3 sections/brain [n=3]) within the region of interest were analyzed using Image J software. For semi-quantitative analysis, the areas with YFP+ and YFP+/CD31+ cells or VE-cadherin+ cells were analyzed and threshold values were set at ranges of 100-255 or 70-255, respectively.

**Western blot analysis**

Western blot analysis was performed as previously described (Nakano-Doi et al., 2010; Nakagomi et al., 2011). In brief, brain samples isolated from ischemic or non-ischemic areas (10 µg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred onto nitrocellulose membranes. Membranes were incubated with antibodies against VE-cadherin (1:500, SC-28644, Santa Cruz Biotechnology) and albumin (1:2000, A90-134A, Bethyl Laboratories) and then with peroxidase-labeled secondary antibodies (1:1000, PI-1000, Vector Laboratories, Burlingame, CA, USA; 1:1000, 61-1620, Invitrogen, Carsbad, CA, USA). Antibody labeling of protein bands was detected with enhanced chemiluminescence reagents (Chemi-Lumi One, Nacalai Tesque, Kyoto, Japan) according to the manufacturer’s instructions.

**Statistical analysis**

Data are presented as means ± standard deviation. Statistical significance was calculated using a nonparametric test (Mann-Whitney); P values <0.05 were considered statistically significant.

**Results**

**Expression patterns of CD31 and VE-cadherin during development**

To investigate the expression pattern of endothelial AJ markers during development, sections of brains at representative developmental stages (E17, P1, P15, 5 weeks and 24 weeks) (Bifari et al., 2015; Nakano-Doi et al., 2016) were stained with antibodies against CD31 and VE-cadherin. Cell nuclei stained with DAPI showed that cell density was significantly higher during early development (E17 and P1; Fig. 1A-H) compared with later stages (P15, 5 and 24 weeks; Fig. 1I-T). CD31 expression was observed in ECs throughout embryonic (E17; Fig. 1A-C), postnatal (P1; Fig. 1E-G, P15; 1I-K), adolescent (5 weeks; Fig. 1M-O), and adult stages (24 weeks; Fig. 1Q-S). In contrast, VE-cadherin expression in ECs was weak during embryonic (E17; Fig. 1A,B,D) and early postnatal stages (P1; Fig. 1E,F,H), and gradually increased postnatally (P15; Fig. 1I,J,L). However, expression diminished during the adolescent stage (5 weeks; Fig. 1M,N,P) and was rarely observed during adulthood (24 weeks; Fig. 1Q,R,T).

CD31+ (Fig. 1U) and VE-cadherin+ (Fig. 1V) cells were analyzed in the cortical plate during early development and in the cortex during adolescent, young adult, and adult stages. The results demonstrate that expression patterns of CD31 and VE-cadherin differ during different stages of murine development and that VE-cadherin expression is decreased in adults.

**The VE-cadherin promoter is active during embryonic stages but diminishes during adulthood**

Our data show low levels of VE-cadherin protein expression during early embryonic stages (Fig. 1). We investigated expression patterns of the VE-cadherin promoter during development by using the Cre-LoxP system to establish murine strains expressing YFP under the regulation of the VE-cadherin promoter (VE-cadherin/YFP mice) (Fig. 2A). We then examined whether YFP was expressed in ECs during embryonic stages. Immunohistochemistry for YFP using the diaminobenzidine reaction revealed YFP+ cells in embryonic brains (E16; Fig. 2B,C). In addition, some of these cells expressed CD31 (Fig. 2D-F), indicating that the VE-cadherin promoter is activated in brain ECs of embryonic mice, as previously described (Giannotta et al., 2013). Because VE-cadherin protein expression was very weak in adult mice, we investigated whether YFP was expressed during adulthood. Although numerous YFP+ cells were observed among ECs of young mice (5 weeks; Fig. 2G-I), YFP was rarely detected in ECs of adult mice (24 weeks; Fig. 2J-L). These results indicate that the VE-cadherin promoter becomes less active during adulthood, thereby decreasing the abundance of VE-cadherin protein.

**Ischemic stroke activates the VE-cadherin promoter in adult mice**

Next, we investigated whether ischemic stroke could reactivate the VE-cadherin promoter in brain ECs. To study this, ischemic stroke was induced in VE-cadherin/YFP adult mice at 24 weeks. On post-stroke day 3, YFP expression under the regulation of the VE-cadherin promoter was elevated within ischemic regions (Fig. 3A-C). In contrast, YFP+ cells were infrequently observed in non-ischemic areas (Fig. 3A,D). Similar to these findings, immunohistochemistry at post-stroke day 5 revealed many YFP+ cells in ischemic areas (Fig. 3E-L), and a small number of YFP+ cells in non-ischemic regions (Fig. 3M-P). Semi-quantitative analysis indicated that the areas with YFP+ (Fig. 3Q) and YFP+/CD31+ cells (Fig. 3R) were significantly increased within ischemic regions at post-stroke days 3 and 5.

**Ischemic stroke and VE-cadherin**

Ischemic stroke activates the VE-cadherin promoter in adult mice.
Fig. 1. Expression patterns of CD31 and VE-cadherin in C57BL/6 mice at developmental stages (A-T). CD31 expression was observed in ECs throughout development (E17, P1, P15, 5 weeks, and 24 weeks). In contrast, expression patterns of VE-cadherin were variable and VE-cadherin was rarely observed during adulthood. [CD31 (B, C, F, J, K, N, O, R, and S: red), VE-cadherin (B, D, H, J, L, N, P, R, and T: green), and DAPI (B-D, F-H, J-L, N-P, R-T: blue)]. Areas with CD31+ (U) and VE-cadherin+ cells (V) at each developmental stage. Scale bars: 25 µm.
Fig. 2. Using the Cre-LoxP system, murine strains expressing YFP under the control of the VE-cadherin promoter were engineered (A). Immunohistochemistry for YFP using the dianobenzidine reaction shows that YFP is expressed in embryonic brains (E16; B, C). Some YFP+ cells at this stage also express CD31 [YFP (D, E: green), CD31 (D, F: red), and DAPI (D-F: blue)]. Multiple YFP+ cells were observed in ECs of adolescent mice (5 weeks) [YFP (G, H: green), CD31 (G, I: red), and DAPI (G-I: blue)]. However, YFP was infrequently observed in ECs of adult mice (24 weeks) [YFP (J, K: green), CD31 (J, L: red), and DAPI (J-L: blue)]. Scale bars: B, 200 µm; C, 50 µm; D, G, J, 25 µm.
Fig. 3. Expression pattern of YFP<sup>+</sup> cells in VE-cadherin/YFP adult mice at post-stroke days 3 (A-D) and 5 (E-P). On day 3 after stroke induction, numerous YFP<sup>+</sup> cells were observed within ischemic areas (B, C), and they were rare in non-ischemic regions (D) [YFP (B-D: green), CD31 (B-D: red), and DAPI (B-D: blue)]. Similarly, on day 5 post-stroke, a significant number of YFP<sup>+</sup> cells were detected within ischemic areas (E-L) [YFP (F, G, J, and K: green), CD31 (F, H, J, and L: red), and DAPI (F-H and J-L: blue)]. In contrast, these were rarely observed in non-ischemic regions (M-P) [YFP (N and O: green), CD31 (N and P: red), and DAPI (N-P: blue)]. The areas with YFP<sup>+</sup> (Q) and YFP<sup>+</sup>/CD31<sup>+</sup> cells (R) within ischemic areas were significantly higher at post-stroke days 3 and 5 compared with contralateral non-ischemic areas. *P<0.05 versus non-ischemic areas (n=3) (Q, R). Scale bars: 25 µm.
Ischemic stroke and VE-cadherin

... compared with contralateral, non-ischemic areas. These findings demonstrate that ischemia reactivates VE-cadherin promoter activity that is normally decreased during adulthood.

Characterization of YFP+ cells following ischemia

Although YFP+ cells largely express CD31 following ischemia (Fig. 3Q,R), some YFP+ cells lacking CD31 expression were present within ischemic areas (Fig. 3B,C,F,J). Thus, the precise phenotypes of YFP+ cells following ischemia remain unclear. Because previous studies showed that a subpopulation of VE-cadherin+ cells expressed the hematopoietic marker CD45 (Kogata et al., 2006; Fraser et al., 2002), we examined whether YFP expressed CD45. YFP+/CD45+ cells were rarely observed within ischemic regions at post-stroke day 3 (Fig. 4A-D). However, after post-stroke day 5, some YFP+ cells within ischemic areas expressed CD45 (Fig. 4E-H). Because recent studies showed that EC traits are variable under pathological conditions (Zeisberg et al., 2007; Susienka and Medici, 2013; Yu et al., 2014), we investigated whether the VE-cadherin promoter was activated in cells other than ECs. We examined YFP expression in the neurons, astrocytes, and pericytes that, together with ECs, form the neurovascular unit (NVU). Immunohistochemistry at post-stroke days 3 (data not shown) and 5 showed that YFP+ cells did not express the neuronal marker, MAP2 (Fig. 4I-L), the astrocytic marker, GFAP (Fig. 4M-P), or the pericytic marker, PDGFRβ (Fig. 4Q-T). These data suggest that ECs do not transform into these cell types.

Expression patterns of VE-cadherin at various time points following ischemia

Although the precise traits of YFP+ cells remain unclear, our results show that ischemia activates the VE-cadherin promoter. Thus, we next examined whether ischemic stroke increased VE-cadherin expression at the protein level. The expression pattern of VE-cadherin at various time points following ischemic stroke was investigated in CB17 adult mice (8 weeks) because the infarct area of CB17 mice is highly reproducible compared with C57BL/6 mice (Taguchi et al., 2010). On post-stroke days 1, 3, and 5, mice were sacrificed and the brains were subjected to immunohistochemical analysis. The neuronal marker MAP2 was observed in brain sections of sham-operated mice, whereas VE-cadherin was rarely observed (Fig. 5A-D). However, most MAP2+ cells disappeared within 1 day of ischemia (Fig. 5E-G), and similar findings were observed at post-stroke days 3 (Fig. 5I-K) and 5 (Fig. 5M-O). These findings were consistent with previous reports that mature neurons rapidly undergo cell death following severe ischemic insult (Nakata et al., 2017; Tachibana et al., 2017; Tatebayashi et al., 2017). In contrast, VE-cadherin expression slightly increased as early as day 1 following ischemia, mainly in ischemic regions where mature neuronal cells could not survive (Fig. 5E,F,H), and the increase was more apparent at post-stroke days 3 (Fig. 5I,J,L) and 5 (Fig. 5M,N,P).

The relationship between VE-cadherin expression and BBB integrity following ischemia

We then investigated BBB function following ischemia by examining the vascular leakage of albumin in brain sections at post-stroke days 1, 3, and 5, as previously described (Garbuzova-Davis et al., 2011). Immunohistochemistry studies of the brains of CB17 adult mice (8 weeks) showed that albumin was mainly present within CD31+ blood vessels in sham-operated mice (Fig. 6A-D). However, following ischemia, albumin extravasation was observed within ischemic areas at post-stroke day 1 (Fig. 6E-H), and increased at post-stroke days 3 (Fig. 6I-L) and 5 (Fig. 6M-P). These results suggest that ischemia increased albumin leakage, presumably through functional impairment of the BBB.

To determine whether expression patterns of VE-cadherin are related to the function of the BBB, we performed Western blot studies using brain samples from ischemic regions at post-stroke days 1, 3, and 5. As a control, brain tissues were obtained from the same regions (MCA areas) of sham-operated mice. Western blot analysis showed that VE-cadherin expression gradually increased following ischemia (Fig. 6Q), consistent with immunohistochemistry findings (Fig. 5A-P). In addition, Western blots showed that VE-cadherin expression increased in parallel with albumin following ischemia (Fig. 6Q).

Ischemic stroke increases the expression of VE-cadherin protein in adult mice

Because the VE-cadherin/YFP mice were in a C57BL/6 background, we used C57BL/6 adult mice (young adults at 8 weeks and mature adults at 24 weeks) to further investigate whether ischemic stroke was followed by an increase in VE-cadherin expression at the protein level. On post-stroke day 4, VE-cadherin expression was elevated within ischemic areas in 8 week-old mice (Fig. 7A-H), although VE-cadherin was rarely observed in non-ischemic regions (Fig. 7I-P). In mature adult mice at 24 weeks, immunohistochemical findings showed similar expression levels of VE-cadherin (data not shown). Semi-quantitative analysis showed that VE-cadherin+ ECs were significantly increased in ischemic areas compared with contralateral non-ischemic locations in mice at 8 weeks (Fig. 7Q) and 24 weeks (Fig. 7R). These results indicate that ischemia increases the expression of VE-cadherin in adult mice at both the promoter and protein levels.

Characterization of CD31+ cells following ischemia

Although VE-cadherin+ cells largely express CD31 following ischemia, the precise traits of CD31+ cells
Fig. 4. Traits of YFP⁺ cells in VE-cadherin/YFP adult mice at post-stroke days 3 (A-D) and 5 (E-T). Although YFP⁺ cells rarely expressed CD45 at post-stroke day 3 (B-D), some YFP⁺ cells expressed CD45 at post-stroke day 5 (F-H) (YFP (B, C, F, and G: green), CD45 (B, D, F, and H: red), and DAPI (B-D and F-H: blue)). YFP⁺ expression was not observed in MAP2⁺ neurons (YFP (J and K: green), MAP2 (J and L: red), and DAPI (J-L: blue)), GFAP⁺ astrocytes (YFP (N and O: green), GFAP (N and P: red), and DAPI (N-P: blue)), and PDGFRβ⁺ pericytes (YFP (R and S: green), PDGFRβ (R and T: red), and DAPI (R-T: blue)). Scale bars: 25 µm.
Ischemic stroke and VE-cadherin

remain unclear. A previous report showed that CD31 is expressed in ECs and non-ECs, such as hematopoietic stem cells (HSC) (Kim et al., 2010). Thus, using adult mice in the same background (C57BL/6 mice at 8 weeks), we examined the traits of CD31+ cells within ischemic regions. Although CD31+ cells rarely expressed the HSC marker, CD34 (Fig. 8A-D), some CD31+ cells expressed an alternative EC marker, vWF.
Fig. 6. BBB integrity in adult CB17 mice (8 weeks): sham-operated (A-D) and post-stroke days 1 (E-H), 3 (I-L), and 5 (M-P). Immunohistochemistry shows albumin in CD31⁺ blood vessels in sham-operated mice (B-D). Following ischemia, albumin leakage was observed in ischemic areas at post-stroke day 1 (F-H). Leakage was increased at post-stroke day 3 (J-L) and 5 (N-P) [albumin (B, C, F, G, J, K, N, and O: green), CD31 (B, D, F, H, J, L, N, and P: red), and DAPI (B-D, F-H, J-L, N-P: blue)]. Western blot shows increased expression of VE-cadherin and albumin following ischemia (Q). Scale bars: 25 µm (B, F, J, and N). Results shown are representative of three repetitions of the experimental protocol (Q). Scale bars: 25 µm.
Fig. 7. Expression patterns of VE-cadherin in C57BL/6 adult mice (young adults at 8 weeks and mature adults at 24 weeks) at post-stroke day 4. In young adult mice, VE-cadherin expression was increased in ischemic areas (A-H), and it was rarely observed in non-ischemic regions (I-P) [VE-cadherin (B, C, F, G, J, K, N, and O: green), CD31 (B, D, F, H, J, L, N, and P: red), and DAPI (B-D, F-H, J-L, and N-P: blue)]. The abundance of VE-cadherin+ ECs within ischemic areas was significantly higher than in contralateral non-ischemic areas [(8 weeks (Q), 24 weeks (R)]. *P<0.05 versus non-ischemic areas (n=3) (Q, R). Scale bars: 25 µm.
(Fig. 8E-H). In addition, almost all CD31+ cells in ischemic regions were localized near PDGFRβ+ pericytes (Fig. 8I-L) and collagen IV (Fig. 8M-P), which are expressed in the extracellular matrix surrounding brain ECs (Baeten and Akassoglou, 2011). These results show that CD31 within ischemic regions is likely expressed in cells from a vascular lineage and that most of those cells are ECs.

**Discussion**

The NVU is composed of ECs from the BBB, neural cells (neurons and astrocytes), and vascular pericytes in the CNS. Under pathological conditions, such as after ischemic stroke, mature neural cells within ischemic areas undergo cell death in a matter of days (Nakata et al., 2017; Tatebayashi et al., 2017). In contrast, cells constituting the NVU, such as ECs (Nakano-Doi et al., 2010; Nakagomi et al., 2015a; Tachibana et al., 2017) and vascular pericytes (Nakagomi et al., 2015a; Nakano-Doi et al., 2016; Tachibana et al., 2017), can survive even under severe ischemic conditions. This indicates that, in contrast to mature neural cells, ECs and vascular pericytes in the NVU are resistant to extreme ischemia/hypoxia and can maintain inherent functionality in damaged tissues. In support of this concept, we demonstrated that ECs in ischemic areas can act as a stem cell niche, playing a pivotal role in CNS regeneration following ischemia (Nakagomi et al., 2009a; Nakano-Doi et al., 2010). In addition, we showed that the expression patterns and phenotypes of vascular pericytes are variable following ischemic stroke, and that brain pericytes can also function as stem cells following ischemia (Nakagomi et al., 2015a-c; Nakano-Doi et al., 2016; Sakuma et al., 2016). However, recent studies showed the plasticity of ECs; their features are variable under pathological conditions induced by a variety of stimuli (Zeisberg et al., 2007; Susienka and Medici, 2013; Yu et al., 2014). Thus, ECs may also alter their traits in response to ischemia/hypoxia. However, changes in the expression pattern and phenotype of ECs after ischemic stroke are not well-documented.

In the present study, we investigated expression patterns of VE-cadherin in normal and pathological mouse brain ECs. VE-cadherin helps to maintain BBB function by acting as AJs, an endothelial-specific key component of the BBB (Tietz and Engelhardt, 2015; Yamamoto et al., 2015). At both promoter and protein levels, VE-cadherin expression was detectable in embryonic brains and young mice. However, VE-cadherin protein expression was very weak in young mice compared with YFP expression induced by the VE-cadherin promoter. Because all YFP+ cells in adolescent mice (5 weeks) express CD31, YFP is likely expressed in ECs under normal conditions. Thus, the different patterns of VE-cadherin expression at the promoter and protein levels suggests that VE-cadherin protein is degraded or not expressed at the same time as the VE-cadherin promoter is active. Alternatively, we employed the Cre-LoxP system to determine the localization of cells in which the VE-cadherin promoter was activated. Once the VE-cadherin promoter was activated, YFP expression could be detected in ECs unless YFP+ ECs were removed by cell turnover. Thus, it is possible that YFP+ cells in adolescent mice (5 weeks) included ECs with YFP expression occurring prior to this developmental stage.

However, VE-cadherin expression was diminished at both the promoter and protein levels during adulthood and was barely detectable by immunohistochemistry. This indicates that the expression of VE-cadherin differs during different developmental stages and decreases during adulthood. The precise roles of VE-cadherin remain unclear, but it is well-established that VE-cadherin is essential for the organization of a proper, stable vascular system during embryogenesis, whereas it mainly regulates vascular permeability and inhibits unrestrained vascular growth during adulthood (Vittet et al., 1997; Carmeliet et al., 1999; Gory-Faure et al., 1999; Crosby et al., 2005; Vestweber, 2008; Giannotta et al., 2013; Tietz and Engelhardt, 2015). Thus, phenotypic changes in VE-cadherin+ cells may be accompanied by functional changes that alter VE-cadherin expression during development.

Both VE-cadherin promoter activity and VE-cadherin protein expression were up-regulated in ECs within ischemic areas in adult brains. These results are consistent with a recent study showing increased levels of endothelial VE-cadherin mRNA in injured brain tissues following ischemia/reperfusion (Gertz et al., 2016). Although the precise mechanism(s) by which the VE-cadherin promoter is activated following ischemia remains unclear, previous studies have revealed that hypoxia inducible factors (HIFs), including HIF-1α and HIF-2α activate VE-cadherin expression (Le Bras et al., 2007; Tang et al., 2014). Furthermore, similar up-regulation of the VE-cadherin promoter was observed in ECs in ischemic heart tissue after myocardial infarction (Kogata et al., 2006). Thus, it is likely that ischemia/hypoxia stimulates the VE-cadherin promoter in ECs regardless of organ type.

In the present study, YFP was also observed in CD45+ cells within ischemic regions, as previously described (Kogata et al., 2006). Although YFP+/CD45+ cells may originate from circulating cells rather than the CNS (e.g., bone marrow), ECs at early developmental stages have the potential to produce hematopoietic cells (Adamo and Garcia-Cardena, 2012). In addition, Fraser and colleagues proposed that VE-cadherin+/CD45+ cells are hemogenic ECs (Fraser et al., 2002). Thus, we cannot rule out the possibility that ischemic stimuli induce some ECs to acquire the traits of hemogenic ECs, leading to the expression of YFP and CD45. In this study, some CD31+ cells at early embryonic day (E16) did not express YFP. Although it is possible that some CD31+ ECs had not yet activated the VE-cadherin promoter at that time, we did not find any CD31+ cells expressing vWF. The discrepancy between CD31 and
vWF expression patterns may be due to the restricted expression of vWF during endothelial maturation (e.g., early and late endothelial stages). Alternatively, it is possible that some CD31+ cells contain non-EC lineages.

However, the exact traits of VE-cadherin+ and CD31+ cells should be clarified by further investigations. In addition, we could not selectively analyze the traits of capillary endothelial cells expressing CD31, vWF, and...
VE-cadherin within the BBB. Thus, future studies examining the expression patterns of these markers between brain micro- and macrovessels are required.

In the present study, we found that VE-cadherin expression increased in parallel with albumin extravasation in ischemic areas. This suggests that VE-cadherin expression may be activated when the BBB is functionally impaired by induced ischemic stroke. Moreover, ECs in the BBB form inter-endothelial junctions through TJs as well as AJs. Although AJs and TJs have different functions, increasing evidence shows they function coordinately (Tietz and Engelhardt, 2015; Tornavaca et al., 2015). In addition, a recent study showed that mRNA of T3-related molecules, such as occludin and claudin5, was up-regulated along with VE-cadherin following ischemic stroke (Gertz et al., 2016). Thus, expression patterns of VE-cadherin may be influenced by TJs during development and under pathological conditions.

In the present study, we mainly used C57BL/6 mice because this is the background strain of the transgenic mice. However, C57BL/6 mice have more cerebral arterial branches and collateral vessels than CB17 mice. Therefore, there is a large degree of sample variation in the post-stroke areas of brains of C57BL/6 mice, while the brains of CB17 mice are more consistent across samples as we have reported previously (Taguchi et al., 2010), which made it difficult to collect brain samples of the same size from C57BL/6 mice. Therefore, we used CB17 mice in addition to C57BL/6 mice.

Although the significance of increased expression of VE-cadherin under ischemic conditions remains unclear, VE-cadherin may play an essential role in the reorganization of the vascular system in damaged tissues following ischemia. Further studies using transgenic mice and endothelial-specific genes, including VE-cadherin (Vittet et al., 1997; Gory-Faure et al., 1999; Gertz et al., 2016), would provide valuable insights on the multiple roles of ECs under pathological conditions.

Conclusion

We demonstrated that there are variable patterns of VE-cadherin expression during murine development. In addition, we showed that ischemic stroke activates VE-cadherin, both at the promoter and protein levels, suggesting that VE-cadherin may be involved in the reconstruction and restoration of the BBB following ischemic stroke. The precise roles of VE-cadherin and the phenotypic changes of ECs under ischemic conditions remain to be clarified in detailed future studies.

References

neural stem/progenitor cells after cerebral infarction. Stem Cells 27, 2185-2195.


Accepted December 5, 2017