High-Mannose-Type Glycan of Basigin in Endothelial Cells Is Essential for the Opening of the Blood—Brain Barrier Induced by Hypoxia, Cyclophilin A, or Tumor Necrosis Factor-α

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Pathologic opening of the blood—brain barrier accelerates the progression of various neural diseases. Basigin, as an essential molecule for the opening of the blood—brain barrier, is a highly mannosylated transmembrane molecule specified in barrier-forming endothelial cells. This study analyzed the involvement of basigin in the regulation of the blood—brain barrier focusing on its glycosylation forms. First, basigin was found to be expressed as cell surface molecules with complex-type glycan as well as those with high-mannose-type glycan in barrier-forming endothelial cells. Monolayers of endothelial cells with suppressed expression of basigin with high-mannose-type glycan were then prepared and exposed to pathologic stimuli. These monolayers retained their barrier-forming properties even in the presence of pathologic stimuli, although their expression of basigin with complex-type glycan was maintained. Also in vivo, the blood—brain barrier in mice that were pretreated intravenously with endoglycosidase H was protected from the opening under pathologic stimuli. Furthermore, the pathologically opened blood—brain barrier in streptozotocin-injected mice was successfully closed by intravenous injection of endoglycosidase H. These results show that high-mannose-type glycan of the basigin molecule is essential for the opening of the blood—brain barrier and therefore a specific target for protection as well as restoration of pathologic opening of the blood—brain barrier. (Am J Pathol 2023; 189: 1–14; https://doi.org/10.1016/j.ajpath.2023.11.007)

Multicellular organisms have acquired the blood—brain barrier during the evolutionary process to maintain the homeostasis of an optimal tissue microenvironment for neural cells. The blood—brain barrier is induced during embryonic development. Although this barrier in adult brains is basically maintained in the closed state, it is still under the dynamic control in response to the tissue microenvironment to which the vasculature of neural tissues is exposed.1–3 In various intractable neural diseases, pathologic stimuli, including tissue hypoxia and inflammation that reside in the affected tissues, work as triggers to accelerate disease progression through the opening of the blood—brain barrier.4–7 Conversely, the blood—brain barrier in the closed state also serves as a barrier against the delivery of systemically administered drugs to nervous tissues, making the treatments for neural diseases difficult. Therefore, the molecules essential for the dynamic control of the blood—brain barrier function would be the targets of new therapeutic strategies to overcome the intractability of neural diseases by realizing the artificial closing as well as opening of the blood—brain barrier.8,9

We have shown that changes in the microenvironment (including hypoxia) around blood vessels result in opening of the blood—brain barrier through disappearance of claudin-5, an integral molecule for tight junction assembly, from cell membranes of endothelial cells.10–13 We then

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specified three transmembrane molecules, a disintegrin and metalloproteinase-12, a disintegrin and metalloproteinase-17, and basigin, which are expressed in the barrier-forming vascular endothelial cells, as the molecules involved in the pathologic opening of the blood–brain barrier. Of the three, basigin was found to be most available as a target for artificial regulation of the blood–brain barrier function. Inhibition of basigin was shown to lead to the restoration of barrier function in the neural vasculature of intractable neural diseases in which the blood–brain barrier is impaired. We were further successful in showing that an intravenous injection of cyclophilin A (CypA), a ligand of basigin, realizes the transient as well as reversible opening of the blood–brain barrier, which enabled incorporation of systemically administered drug into the parenchyma of neural tissues. Thus, basigin expressed in the barrier-forming endothelial cells is a promising target for establishing artificial regulation of blood–brain barrier function. However, it must be noted that basigin is also expressed in other cell types, including neurons, where it plays important roles in physiological processes. Also, for example, mice deficient in basigin exhibit phenotypes such as developmental abnormality of retinal neurons, delayed wound healing, and so forth. Considering clinical applications, it would be desirable if we could narrow the target within a basigin molecule that is involved more specifically in the control of the blood–brain barrier function.

Basigin has several synonyms due to its diverse histories of discovery with different functions as well as various species: basigin/Gp42 in mouse, HT7/neurothelin/SA11 in chick, extracellular matrix metalloproteinase inducer (EMMPRIN)/CD147 in human, and others. Basigin is a transmembrane molecule belonging to the immunoglobulin superfamily, and it is N-glycosylated at three asparagine residues within two immunoglobulin-like domains of the extracellular region. Basigin is known to be detected by Western blot analysis as two bands of different sizes that are attributed to the variations in glycosylation. Molecules corresponding to a band of higher size and those of lower size were named the high glycosylation form of basigin (basigin-HG) and low glycosylation form of basigin (basigin-LG), respectively, and their functions have been analyzed mainly in the context of immunology and tumor biology. In general, the functions of basigin are believed to be attributed to basigin-HG, which is the fully glycosylated basigin; basigin-LG is regarded as an intermediate product during the process of glycosylation. Ancillary molecules such as caveolin-1 and monocarboxylate transporters reported inhibit or promote the synthesis of basigin-HG by binding to basigin in both endoplasmic reticulum and Golgi apparatus. As for the localization to cell membranes, basigin-HG is detected in cell membranes, whereas the localization of basigin-LG to cell membranes is controversial, with contradictory results reported. Thus, most of the studies on basigin have been focused on basigin-HG, and no essential functions of basigin-LG itself in physiological and/or pathologic phenomena have been defined so far. Here, we present data showing that basigin-LG is essential for opening of the blood–brain barrier and that it can be a specific target for artificial regulation of the blood–brain barrier.

Materials and Methods

Cell Culture

A mouse brain microvascular endothelial cell line, bEnd.3, was purchased from ATCC (Manassas, VA). The cell line was cultured in Dulbecco’s modified Eagle’s medium with 4500 mg/L glucose (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum at 37°C in a humidified incubator either with 5% carbon dioxide and 95% atmospheric air for normoxia or with 5% carbon dioxide and 1% oxygen balanced with nitrogen for hypoxia. An oxygen-regulated Personal Multi Gas Incubator (Astec Co., Ltd., Tokyo, Japan) was used to generate the hypoxic culture condition. In all experiments, cells were cultured under normoxia in the confluent state for 5 to 7 days before starting the tests. As pathological stimuli other than hypoxia, cells were incubated for 3 hours with CypA (BioVendor Research and Diagnostic Products, Brno, Czech Republic) or tumor necrosis factor-α (TNF-α) (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan) to the final concentration of 300 ng/mL and 50 ng/mL, respectively. For preincubation with endo-β-N-acetylgalactosaminidase H (Endo H) (New England Biolabs, Ipswich, MA), cells were treated with Endo H (0.25 units/mL) for 1 hour before exposure to hypoxia, CypA, or TNF-α. For the inhibition of conversion from molecules with high-mannose–type glycan to those with complex-type glycan, cells were incubated with kifunensine (20 μg/mL) (Sigma-Aldrich) for 60 hours.

Immunofluorescence Staining of Cultured Cells

Cell monolayers were fixed with 100% methanol at room temperature for 5 minutes and incubated with 10% nonimmune goat serum (Invitrogen, Carlsbad, CA) at room temperature for 15 minutes to block the nonspecific binding of antibodies. The cell monolayers were then reacted with rabbit polyclonal antibody against claudin-5 (1/25 dilution) (Thermo Fisher Scientific Inc., Waltham, MA) at 4°C overnight. After washing in phosphate-buffered saline, they were incubated with Alexa Fluor 488 goat anti-rabbit IgG (1/200 dilution) (Molecular Probes, Eugene, OR) for the staining of claudin-5 at room temperature for 1 hour under light protection. Stained cells were mounted in Fluorescence Mounting Medium (Dako Denmark A/S, Glostrup, Denmark) and observed under the laser confocal microscope.
For quantitative analysis, the fluorescence intensities of claudin-5 on plasma membranes were measured by using an operation menu installed in LSM5 Pascal. Three fields of a cell monolayer were randomly photographed, and five straight lines were drawn on each photograph. Then, fluorescence intensities at the points of cell membranes intersecting with the drawn straight lines were quantified. The mean value of fluorescence intensities was calculated as the level of claudin-5 on cell membrane for each monolayer. All experiments were performed independently in triplicate.

Transendothelial Electrical Resistance

Electrical resistance across a cell monolayer was measured as described previously. In brief, cells were grown on fibronectin-coated inserts of 0.4 nm pore size to the confluent state, and the electrical resistance across the monolayer was measured with the Millicell ERS Voltohmometer (Millipore, Billerica, MA). The transendothelial electrical resistance (TEER) of the monolayers was calculated by subtracting the resistance of blank inserts without cells and multiplying the subtracted values by the surface areas of the inserts. Each experiment was performed in triplicate.

Western Blot Analysis

Cells were lysed in 100 μL of phosphate-buffered saline containing 0.5% Triton X-100, 1% SDS, and Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific). After incubation on ice for 15 minutes, the lysates were centrifuged at 15,000 rpm at 4°C for 15 minutes. Protein concentrations of the supernatant were determined with a Protein Assay Kit II (Bio-Rad, Hercules, CA) and FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). The samples were boiled after addition of Laemmli sample buffer (Bio-Rad) supplemented 5% 2-mercaptoethanol (Sigma-Aldrich). They were separated by SDS-PAGE using 10% gels and transferred to polyvinylidene difluoride membranes (Immobilon P membranes; Millipore, Billerica, MA). Membranes were incubated at room temperature for 1 hour in Tris-buffered saline with 0.1% Tween 20 containing 5% skim milk for blocking. They were then reacted with rabbit polyclonal antibody against basigin (1 μg/mL; generated by Scrum Inc., Tokyo, Japan) at 4°C overnight. After the wash with Tris-buffered saline with 0.1% Tween 20, they were incubated with horseradish peroxidase–conjugated goat anti-rabbit IgG (1/1000 dilution; Dako) at room temperature for 1 hour. They were then reacted with Amersham ECL Prime (GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions, and chemiluminescence was detected by using Amersham Imager 800 (GE Healthcare). For normalization control, the membranes were stripped by using WB Stripping Solution (Nacalai Tesque Inc., Kyoto, Japan) and were subjected to re-blot with β-actin (1/15,000 dilution; Sigma–Aldrich) to ensure equal loading. Experiments were performed independently at least three times.

Biotinylation of Cell Surface Molecules

Cultured cells were biotinylated by incubating them at 4°C for 1 hour with 500 μL of phosphate-buffered saline containing 0.5 mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher Scientific). They were then scraped in lysis buffer containing cocktail inhibitors as described above. After incubation at 4°C for 15 minutes, lysates were centrifuged at 15,000 rpm for 15 minutes at 4°C, and the supernatants were incubated in 60 μL of MagnaBind Streptavidin Beads (Thermo Fisher Scientific) at 4°C for 1 hour. Beads were boiled with 60 μL of Laemmli sample buffer, and 10 μL of the supernatant was loaded on polyacrylamide gels to be processed for Western blot analysis. Experiments were performed independently in triplicate.

Transfection of siRNA

Silencer Select Negative Control siRNA as well as siRNAs specific for basigin [identifiers: s63099 and s63100 (defined as basigin siRNA #1 and #2, respectively)]12 and caveolin-1 [identifiers: s63423 and s63424 (defined as caveolin-1 siRNAs #1 and #2)] were purchased from Thermo Fisher Scientific. Transfection of siRNAs was performed by using Lipofectamine RNAiMAX (Thermo Fisher Scientific) and Opti-MEM I (Thermo Fisher Scientific) according to the manufacturer’s instructions. Final concentration of siRNAs was 10 nmol/L. Cells were incubated with siRNAs for either 36 or 72 hours before the start of experiments.12

Reaction of Cell Lysate with Endo H and Peptide-N-Glycosidase F

Endo H is an endo-type N-acetylgalactosaminidase that digests between the GlcNAc–GlcNAc sequence of innermost high-mannose–type oligosaccharides, and peptide-N-Glycosidase F (PNGase F) is an amidasase that cleaves between the innermost GlcNAc and asparagine residues of high-mannose–type and complex-type oligosaccharides from N-linked glycoproteins. A total of 50 μL of lysate from cells was incubated with 5 μL of glycoprotein denaturing buffer (New England Biolabs) at 100°C for 10 minutes to denature glycoproteins. After the addition of supplied Glycobuffer (New England Biolabs), the lysate was reacted with either Endo H (10 U/μL) or PNGase F (10 U/μL; New England Biolabs) at 37°C for 1 hour, and processed to Western blot analysis.

Animals

Male C57BL/6 N mice (7 weeks old) were obtained from Chiyoda Technol Corporation (Tokyo, Japan). The temperature of room was maintained at 24°C to 26°C, and...
humidity was maintained at 60% to 70%. A standard light–dark cycle of 12 hours’ light exposure was used. Mice were housed in cages with standard bedding and given unlimited access to food and water.

This research was approved by the Institutional Animal Care and Use Committee of Yamaguchi University and conducted under the control of the Rule for the Care and Use of Laboratory Animals of Yamaguchi University and the Law (No.105), Notification (No. 88), and Guideline (No. 71) of the Government.

Exposure of Mice to Hypoxia

For hypoxia stimulation, mice were kept in an airtight chamber (Deuce Co. Ltd., Tokyo, Japan) in which the oxygen concentration was maintained at 4% to 7% for 36 hours. The oxygen concentration inside the chamber could be regulated by controlling the inflow rates of nitrogen and was continuously monitored with the Monitor JKO-25 Version II series (Jik Co. Ltd., Tokyo, Japan). For normoxic condition, mice were housed in cages with atmospheric air. Pre-injection of Endo H (187,500 U/kg) or its vehicle (supplied Glycobuffer) for negative control was performed intravenously immediately before the start of experiments.

Administration of CypA into Mice

CypA was administered intravenously as a dose of 200 μg/kg body weight, while the appropriate control mice received a corresponding volume of saline. Pre-injection of Endo H or its vehicle for negative control was performed intravenously 1 hour before the administration of CypA.

Mice with a Pathologically Opened Blood–Brain Barrier

Mice with a pathologically opened blood–brain barrier were generated by multiple intraperitoneal injections of low-dose streptozotocin (STZ) (50 mg/kg body weight; Sigma-Aldrich) freshly diluted in 0.05 mol/L citrate buffer (pH 4.5).12,32,33 Mice were treated with STZ every second day for three times. The equal volume of citrate buffer (pH 4.5) was injected intraperitoneally into the control group mice. Body weights and blood sugar levels of mice were measured by using Accu-Chek Aviva Nano (Roche, Basel, Switzerland) after a fasting for 4 hours. On the seventh day after the first injection of STZ, blood was collected for determining sugar levels, and mice with hyperglycemia >300 mg/dL were processed for the permeability assay of retinal vasculature. Injection of Endo H or its vehicle for negative control was performed intravenously 4 hours before assessment of retinal vascular permeability.

Permeability Assay of Retinal Vasculature

To evaluate the retinal vascular permeability, 500 μL of phosphate-buffered saline containing 100 μg/mL Hoechst stain H33258 (molecular mass, 534 Da; Sigma-Aldrich) and 1 mg/mL of tetramethylrhodamine-conjugated lysine-fixable dextran (molecular mass, 10,000 Da; Thermo Fisher Scientific) were injected into the left ventricle according to the procedures described previously.10–12,14 The eyes were enucleated and immediately fixed in 4% paraformaldehyde at room temperature for 15 minutes under light protection. Retinal flat mounts were then prepared and mounted in fluorescent mounting medium for observation under a Zeiss LSM510 META laser confocal microscope (Carl Zeiss). For quantitative analysis, three areas in a retina were randomly selected, and the fluorescence intensity of Hoechst dye of each area was quantified with an operation menu installed in an LSM510 META laser confocal microscope to calculate the average of three areas for the value of permeability of a retina. Experiments were performed independently at least three times.

Statistical Analysis

All data are presented as means ± SD. Data were compared by using Student’s t-test, as the variance was shown to be equal with an F test between the study groups that were to be compared. Differences were considered statistically significant at P < 0.05.

Results

Basigin-LG Is Essential for the Hypoxia-Induced Disappearance of Claudin-5 from Cell Membranes of Brain Microvascular Endothelial Cells

Changes in microenvironment, including hypoxia, around blood vessels cause opening of the blood–brain barrier through disappearance of claudin-5 from the surface of endothelial cells. We thus examined the expression of claudin-5 as the index of barrier-forming properties of endothelial cells.10–13 Basigin-specific siRNAs were introduced into mouse brain microvascular endothelial cells, bEnd.3 cells, which express both basigin-HG and basigin-LG, and subsequently the expression level of basigin was determined 36 hours and 72 hours after the introduction of siRNAs (Figure 1A). As reported previously, there was a difference in the time course of the decreased expression level between basigin-HG and basigin-LG.12 After 36 hours of siRNA treatment, the expression of basigin-LG was already negligible, whereas the level of basigin-HG expression was almost unchanged (Figure 1A). By contrast, the expression of both basigin-HG and basigin-LG clearly decreased 72 hours after the introduction of siRNAs, although there was a difference in the effect of suppression between siRNA #1 and siRNA #2 (Figure 1A).
Taking advantage of the difference in the time course of expression levels between basigin-HG and basigin-LG after the introduction of basigin-specific siRNAs, we could obtain the bEnd.3 cells that lost only the expression of basigin-LG with sustained expression of basigin-HG, as well as the bEnd.3 cells that lost both basigin-HG and basigin-LG expressions after the treatment of siRNAs for 36 hours and 72 hours, respectively. The monolayers of those cells were exposed to hypoxia to determine if the loss of their barrier-forming properties by hypoxia is influenced by the difference in glycosylation forms of basigin. In bEnd.3 cells in which only the basigin-LG expression was suppressed 36 hours after the introduction of siRNAs, the hypoxia-induced disappearance of claudin-5 from cell membranes, a critical step for the loss of barrier-forming properties, was found to be inhibited to the same degree as in bEnd.3 cells in which both basigin-HG and basigin-LG expressions were suppressed 72 hours after the

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**Figure 1** Essential role of the low glycosylation form of basigin (basigin-LG) in the hypoxia-induced disappearance of claudin-5 from cell membranes of brain microvascular endothelial cell. A: On Western blot analysis, in cells that are incubated with basigin-specific siRNA for 36 hours, the expression of basigin-LG is suppressed while the expression of the high glycosylation form of basigin (basigin-HG) is retained. Both basigin-LG as well as basigin-HG expressions are suppressed in cells 72 hours after the transfection of siRNAs. B and C: Immunofluorescence images (B) and their corresponding quantitative analysis (C) of claudin-5 levels on cell membranes of bEnd.3 monolayers show that hypoxia-induced disappearance of claudin-5 from cell membranes is inhibited in both cells that are incubated with basigin-specific siRNA for 36 hours as well as 72 hours. Error bars indicate means ± SD. *P < 0.01. Scale bars: 10 μm (B). NC siRNA, nonsilencing siRNA for negative control; ns, not significant.
introduction of siRNAs (Figure 1, B and C). These results indicate that loss of basigin-LG expression is essential to maintaining barrier-forming properties of endothelial cells under hypoxic conditions and therefore could be a specific target for artificial regulation of the opening of the blood–brain barrier.

Basigin-LG and Basigin-HG Have Different N-Glycans, but Both Are Localized on the Cell Surface

Total cell lysate of bEnd.3 cells was prepared and incubated with Endo H or PNGase F and then evaluated by Western blot analysis using anti-basigin antibody. Endo H is an

Figure 2 Expressions of basigin with complex-type glycan and basigin with high-mannose-type glycan as well as their localization to cell membranes in brain microvascular endothelial cells. A–C: Total cell lysate from bEnd.3 cells was reacted with endo-β-N-acetylglucosaminidase H (Endo H) or peptide-N-Glycosidase F (PNGase F) and processed for Western blot analysis (A), and intensities of bands for high glycosylation form of basigin (basigin-HG) (B) as well as the low glycosylation form of basigin (basigin-LG) (C) were determined. D and E: Total cell lysate from bEnd.3 cells that were cultured in the absence or presence of kifunensine (Kif) was reacted with Endo H and processed for Western blot analysis (D), and intensities of bands for basigin-LG were determined (E). A–E: The results show that basigin-HG and basigin-LG correspond to basigin molecules with complex-type glycan and high-mannose—type glycan, respectively. F–H: Total cell lysate from bEnd.3 cells cultured in the absence or presence of Endo H (lanes 1 and 2 in F) as well as the biotinylated cell surface molecules from bEnd.3 cells cultured in the absence or presence of Endo H (lanes 3 and 4 in F) were processed for Western blot analysis (F), and intensities of bands for basigin-LG were determined (G and H). F–H: The results show the localization of basigin with high-mannose—type glycan to the cell surface and the availability of Endo H for the removal of basigin with high-mannose-type glycan from cell surface. Buffer is a supplied Glycobuffer for negative control of Endo H (nsb) in (A, D and F), nonspecific bands. Data in B, C, E, G, and H are expressed as means ± SD from three independent experiments. *P < 0.01. ns, not significant.
enzyme that specifically digests the high-mannose—type glycan, whereas PNGase F digests both high-mannose—type as well as complex-type glycans. On Western blot analysis with quantitative analysis, the bands of both basigin-LG and basigin-HG disappeared with PNGase F treatment and shifted to lower molecular weight bands corresponding to de-N-glycosylated basigin (Figure 2, A–C). Conversely, treatment with Endo H showed that the bands of basigin-LG (but not those of basigin-HG) shifted to lower molecular weight bands (Figure 2, A–C). Thus, both basigin-LG and basigin-HG were sensitive to PNGase F, whereas basigin-LG (and not basigin-HG) was sensitive to Endo H, indicating that basigin-LG and basigin-HG in bEnd.3 monolayers have high-mannose—type glycan and complex-type glycan, respectively (Figure 2, A–C). These findings were further confirmed by Western blot analysis with total cell lysates from bEnd.3 cells treated with kifunensine, which inhibits the conversion from high-mannose—type glycan to complex-type glycan. In bEnd.3 cells cultured in the presence of kifunensine, the bands of basigin-HG shifted to those of basigin-LG, which further shifted with Endo H treatment to lower molecular weight bands corresponding to de-N-glycosylated basigin (Figure 2, D and E).

We then determined if the basigin with high-mannose—type glycan is expressed on cell membranes of bEnd.3 cells, as molecules with high-mannose—type glycan are generally the intermediate products to be processed into the molecules with complex-type glycan within endoplasmic reticulum as well as Golgi apparatus and are not transferred to cell surfaces, with a very few exceptions.34 Cell surface molecules of the bEnd.3 cells were labeled with biotin, and total cell lysate was precipitated with streptavidin. Precipitated cell surface molecules were evaluated by using Western blot analysis. Basigin-LG with high-mannose—type glycan was confirmed to be localized on cell membranes, although its amount was much smaller than that of the basigin-HG with complex-type glycan (Figure 2, F–H). It is also noteworthy that basigin-LG remained in the total cell lysate, which was prepared from non-biotinylated bEnd.3 cells cultured in the medium with Endo H (Figure 2B); this finding indicates that basigin-LG molecules in bEnd.3 cells are not only processed for cell surface molecules but also used as intermediate molecules for the synthesis of basigin-HG.

**Basigin with High-Mannose—Type Glycan at the Cell Surface of Endothelial Cells Is Commonly Involved in the Loss of Barrier-forming Properties of Brain Microvascular Endothelial Cells**

The aforementioned findings suggest a certain role of basigin with high-mannose—type glycan in endothelial cells, and Endo H would be available as a useful tool to eliminate the high-mannose—type glycan from basigin at the cell surface to determine its role in the blood—brain barrier. Endo H was added into the culture medium of bEnd.3 cells, and their surface molecules were then labeled with biotin. Precipitated molecules with streptavidin were shown to contain no detectable basigin molecules with high-mannose—type glycan, whereas they contained almost same amount of basigin molecules with complex-type glycan as the sample from bEnd.3 cells without Endo H treatment (Figure 2, F and H); this finding indicates that the high-mannose—type glycan of basigin at the surface of cells can be removed by Endo H treatment.

Monolayers of bEnd.3 cells were exposed to hypoxia without or with pretreatment of Endo H, and their expression of claudin-5 at cell surfaces (a critical phenomenon for assembly of the barrier10–12) as well as their TEER were determined (Figure 3, A–C). Interestingly, in bEnd.3 cells pretreated with Endo H, neither the disappearance of claudin-5 from cell membranes (Figure 3, A and B) nor the consequent decrease in TEER (Figure 3C) of monolayers was detected under hypoxia. These results would deepen our previous discovery of basigin as a molecule involved in the opening of the blood—brain barrier12,14 indicating that the specific type of basigin molecule with high-mannose—type glycan at the surface of endothelial cells is essential for the hypoxia-triggered opening of the barrier of bEnd.3 monolayers.

We then investigated if the high-mannose—type glycan of basigin is also involved in the loss of barrier-forming properties of bEnd.3 monolayers under stimuli other than hypoxia. Previously, we reported that both CypA as well as TNF-α open the blood—brain barrier in the basigin-dependent manner.12,14 Monolayers of bEnd.3 cells without and with pretreatment of Endo H were stimulated by CypA (Figure 3, D–F) or TNF-α (Figure 3, G–I), and their barrier-forming properties were monitored. As is the case of bEnd.3 cells under hypoxia, CypA- and TNF-α—induced disappearance of claudin-5 from cell membranes (Figure 3, D, E, G, and H) and a decrease in TEER (Figure 3, F and I) were inhibited in the bEnd.3 monolayers by pretreatment of Endo H. Therefore, it can be true that the basigin with high-mannose—type glycan at the cell surface is commonly involved in the loss of barrier properties in response to various stimuli.

We further examined the specific involvement of cell surface basigin with high-mannose—type glycan (not the other cell surface molecules with high-mannose—type glycan, which can also be substrates of Endo H). An in vitro experiment was designed focusing on caveolin-1, which is reported to be a candidate molecule involved in the movement of basigin-LG from the endoplasmic reticulum and Golgi apparatus to the cell surface. First, the localization of basigin with high-mannose—type glycan to the cell surface of bEnd.3 cells was confirmed to be suppressed by treatment of bEnd.3 cells with caveolin-1—specific siRNAs (Figure 4, A and B). Those cells treated with caveolin-1—specific siRNAs were regarded as bEnd.3 cells, which specifically lack cell membrane—localized
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Figure 3  Inhibitory effect of endo-β-N-acetylglucosaminidase H (Endo H) on the hypoxia-, cyclophilin A (CypA), and tumor necrosis factor α (TNF-α)—induced loss of barrier properties of brain microvascular endothelial cells in vitro. Effect of Endo H on the barrier properties of bEnd.3 cells under hypoxia (A–C), under stimulation with CypA (D–F), and under stimulation with TNF-α (G–I) were analyzed. Immunofluorescence images (A, D, and G) with their corresponding quantitative analyses (B, E, and H) of cell membrane-located claudin-5 as well as the transendothelial electrical resistance (TEER) of bEnd.3 cell monolayers (C, F, and I) show that hypoxia-induced (A–C), CypA-induced (D–F), and TNF-α–induced (G–I) disappearance of claudin-5 from cell membranes and consequent loss of barrier properties of monolayers are inhibited by the pretreatment of cells with Endo H. Buffer is a supplied Glycobuffer for negative control of Endo H. Data in (B, C, E, F, H, and I) are expressed as means ± SD from three independent experiments. **P < 0.05. Scale bars: 10 μm (A, D, and G). ns, not significant.

basigin with high-mannose—type glycan without any changes in the expression of the other cell surface molecules with high-mannose—type glycan. Then, those bEnd.3 cells with suppressed expression of cell surface basigin with high-mannose—type glycan were stimulated by hypoxia, CypA, or TNF-α. They showed no significant decrease in the levels of membrane-localized claudin-5 as well as the TEER of monolayers (Figure 4, C–E). Together with the data from the Endo H treatment (Figure 3), it can be concluded that the basigin with high-mannose—type glycan at the cell surface is essential for the loss of barrier properties of bEnd.3 cell monolayers.

High-Mannose—Type Glycan of Basigin Is Essential for the Opening of the Blood—Brain Barrier In Vivo

The essential role of high-mannose—type glycan of basigin in the in vivo opening of the blood—brain barrier was verified through monitoring the permeability of mouse retinal vasculature. Mice without or with an intravenous pre-injection of Endo H were kept under normoxic or hypoxic conditions for 36 hours, and the permeability of their retinal vasculature was then determined by intracardiac injection of fluorescence dyes as described previously.10–12,14 (Figure 5A). Without pre-injection of Endo H, the leakage of Hoechst dye from the vasculature was negligible in retina under normoxic conditions, whereas the significant extravasation of Hoechst dye was observed in retina under hypoxic conditions. By contrast, in retina of mice with pre-injection of Endo H, hypoxia-enhanced leakage of Hoechst dye was undetectable (Figure 5B). Significant suppression of hypoxia-enhanced leakage of Hoechst dye by pre-injection of Endo H was verified through quantitative analysis (Figure 5C).

We next evaluated the effects of pre-injection of Endo H on the permeability of retinal vasculature in mice with intravenous administration of CypA, which was shown in our previous study14 to open the blood–brain barrier (Figure 5D). Without pre-injection of Endo H, the leakage of Hoechst dye from the vasculature was negligible in the retina of mice in which CypA were not administered, whereas Hoechst dye was significantly extravasated to retinal parenchyma of mice with CypA administration, as described previously.14 As with the case of hypoxic
stimulus, CypA-induced leakage of Hoechst dye was inhibited in the retina with pre-injection of Endo H (Figure 5E). Significant inhibition of CypA-induced leakage of Hoechst dye by pre-injection of Endo H was verified through quantitative analysis (Figure 5F). These results are consistent with data obtained in vitro, indicating that the high-mannose-type glycan of basigin at the cell surface of endothelial cells is involved in the opening of the blood–brain barrier in vivo as the common regulator downstream of various stimuli.

To further discuss the availability of high-mannose-type glycan of basigin as a therapeutic target, we investigated if the pathologically opened blood–brain barrier in disease conditions could be closed to restore the barrier function. In STZ-injected mice (Figure 6 and Table 1), the leakage of Hoechst dye from the vasculature was observed in the retina.
without injection of Endo H as reported previously, whereas no significant leakage of dye was detectable in the retina of mice with injection of Endo H (Figure 6, B and C). A suppressive effect of Endo H on the leakage of dye from retinal vasculature was verified through quantitative analysis (Figure 6D). These results indicate that the basigin with high-mannose-type glycan is expected to be the therapeutic target to restore the blood-brain barrier function to the neural vasculature, which loses the barrier-forming properties in neural diseases.
Discussion

Blood–brain barrier function in adult neural tissues depends on physiological as well as pathological stimuli to which the vascular endothelial cells are exposed. In general, pathologic stimuli existing in the affected regions of neural diseases such as cerebral infarction open the blood–brain barrier, allowing harmful molecules to enter the neural tissue parenchyma and consequently accelerate disease progression. As the target available for the artificial closing of pathologically opened barrier, basigin, which is a highly glycosylated transmembrane molecule expressed in barrier-forming brain vascular endothelial cells, is specified here.

Basigin has been shown to be essential for opening of the blood–brain barrier, and the inhibition of basigin results in closing the opened barrier under various stimuli such as hypoxia and inflammation. However, because basigin is known to be expressed in other cell types for their physiological functions, it would be desirable for future clinical applications to narrow down the target for artificial regulation of the blood–brain barrier function. In the present study, we were successful in selecting the basigin with high-mannose–type glycan (not the basigin with vascular endothelial cells exposed. In general, pathologic stimuli existing in the affected regions of neural diseases such as cerebral infarction open the blood–brain barrier, allowing harmful molecules to enter the neural tissue parenchyma and consequently accelerate disease progression. As the target available for the artificial closing of pathologically opened barrier, basigin, which is a highly glycosylated transmembrane molecule expressed in barrier-forming brain vascular endothelial cells, is specified here.

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complex-type glycan) as a specific target for the artificial closing of a pathologically opened barrier; minimal influence was shown on most of the physiological functions of basigin, which are attributable to the basigin with complex-type glycan.  

There was a difference in the time course of expression levels between the basigin with high-mannose—type glycan and the basigin with complex-type glycan after the introduction of basigin-specific siRNAs. Although the mechanisms underlying this phenomenon have not been specified, it is possible that the difference in ancillary molecules which depends on the glycosylation form of basigin causes the different intracellular movement and/or stability between the basigin with high-mannose—type glycan and the basigin with complex-type glycan (Figure 7). Identification of ancillary molecules of basigin would clarify the intracellular dynamics of basigin molecules, including the glycosylation form—dependent dynamics. Aside from the mechanisms, taking advantage of this phenomenon, we could obtain two types of bEnd.3 cells depending on the period of incubation with basigin-specific siRNAs; one lost only the expression of the basigin molecule with high-mannose—type glycan and the other lost the expression of both basigin molecules with high-mannose—type glycan as well as complex-type glycan. Interestingly, there were no significant differences between those two types of bEnd.3 cells as for the opening of the barrier in response to hypoxia, showing that neither type of cells lost barrier-forming properties under hypoxia. These results prompted us to consider that the specific inhibition of the basigin molecule with high-mannose—type glycan would be enough for the protection as well as the restoration of the blood—brain barrier function under pathologic stimuli. However, in general, molecules with high-mannose—type glycan appear in endoplasmic reticulum and Golgi apparatus as the intermediate products for those with complex-type glycan, and their localization to cell membranes is exceptional.  

There is a controversy among published reports regarding the basigin molecule with high-mannose—type glycan as to whether it localizes to the cell surface. In our present analyses with bEnd.3 cells, endothelial cells with barrier-forming properties expressed the basigin molecule with high-mannose—type glycan as a cell surface molecule, which disappears from the cell surface by the addition of Enol H from the outside of cells. A report showed that caveolin-1 inhibits the conversion of basigin-LG to basigin-HG in Golgi apparatus as well as enhances the localization of basigin-LG to cell membranes. Basigin-LG and basigin-HG correspond to basigin molecules with high-mannose—type glycan and those with complex-type glycan, respectively. Previously, we published interesting data showing that the disappearance of claudin-5 from cell membranes of bEnd.3 cells under pathologic stimuli can be inhibited by the suppression of caveolin-1 expression, although no significant change in the basigin-LG/basigin-HG ratio was observed. These findings indicate the essential role of cell surface basigin molecules with high-mannose—type glycan (not those in the endoplasmic reticulum and Golgi apparatus) for barrier-forming endothelial cells to open the barrier in response to stimuli. In the present study, we added Endol H to the culture medium of bEnd.3 cells to remove the high-mannose—type glycan, and bEnd.3 cells without cell surface basigin with high-mannose—type glycan were found to retain their barrier properties even under hypoxic conditions. Together with the data obtained using basigin-specific siRNAs as well as the data using caveolin-1—specific siRNAs, the basigin with high-mannose—type glycan at the surface of endothelial cells is considered to be essential for the opening of the blood—brain barrier by hypoxia. Furthermore, also under stimuli other than hypoxia, including CypA and TNF-α, bEnd.3 cells that lose the cell surface basigin with high-mannose—type glycan retained the barrier-forming properties; this finding shows that the high-mannose—type glycan of basigin molecules at the cell surface is indispensable commonly for the pathologic opening of the blood—brain barrier. These findings are important for future availability of high-mannose—type glycan of basigin as the target for artificial closing of the pathologically opened blood—brain barrier, as the barrier opening in neural diseases is triggered by the admixture of various stimuli.  

To verify if our in vitro findings are also true in vivo, pathologic opening of the blood—brain barrier in vivo was induced in mice by exposure to hypoxia or the intravenous...
administration of CypA. The pathologic opening of the blood–brain barrier by hypoxia as well as CypA was prevented by the pretreatment of mice with Endo H, indicating the essential role, also in vivo, of cell surface high-mannose–type glycan of basigin in the opening of the blood–brain barrier. In addition, the availability of basigin with high-mannose–type glycan as a target to restore the barrier function to endothelial cells which had lost barrier-forming properties under pathologial stimuli was determined. In STZ-injected mice, a pathologically opened blood–brain barrier that was caused by hyperglycemia and/or STZ toxicity was successfully closed by an intravenous injection of Endo H, indicating that the cell surface high-mannose–type glycan of basigin can be a therapeutic target to restoring barrier function. Thus, the cell surface basigin molecule with high-mannose–type glycan is expected to be a specific target for the establishment of new therapy to prevent the pathologic opening of the blood–brain barrier as well as restore barrier function to neural vasculature with a pathologically opened blood–brain barrier in neural diseases.

Our findings in the present study are also expected to be a clue to new aspects of regulatory mechanisms of the blood–brain barrier function in multicellular organisms. Because the localization of molecules with high-mannose–type glycan to the cell surface is exceptional, the movement of basigin molecules with high-mannose–type glycan from the endoplasmic reticulum and Golgi apparatus to cell membranes might be a crucial step for determining the status of the blood–brain barrier function. Data in the present study indicate that basigin molecules with high-mannose–type glycan express not only as cell surface molecules but also as intracellular molecules in barrier-forming endothelial cells. The latter molecules are thought to be localized in the endoplasmic reticulum and Golgi apparatus as the intermediate molecules for synthesis of basigin molecules with complex-type glycan. There must be some ancillary molecules that inhibit or promote the processing of basigin molecules with high-mannose–type glycan to those with complex-type glycan and consequently control their movement to the cell surface. One of the candidates for those ancillary molecules regulating the blood–brain barrier function is caveolin-1 (Figure 7), based on the reports (including our previous study) showing that caveolin-1 is involved in the localization of basigin-LG to cell membranes and is indispensable for the pathologic opening of the blood–brain barrier.12 Also in the present study, caveolin-1 was shown to be indispensable for the localization of basigin molecules with high-mannose–type glycan to cell membranes of brain microvascular endothelial cells (Figure 4A). In addition, we have reported the data showing that caveolin-1 as well as claudin-5 combine with basigin, and furthermore that caveolin-1 and claudin-5 become co-localized on cell membranes under pathologic stimuli, including TNF-α, in a manner dependent on basigin.12

Considering all of these findings, the data of the present study might lead us to the hypothesis that cell surface basigin molecules with high mannose–type glycan provide the scaffolding for caveolin-1 to meet with claudin-5 to remove it from cell membranes. Further studies on the molecules associated with basigin with high-mannose–type glycan would reveal new aspects of mechanisms for regulating the blood–brain barrier function.

Author Contributions

E.I. conceived the project and designed the experiments with D.C.; D.C. performed most of the experiments and statistical analysis; K.Y. co-supervised E.I. and helped conceptualize the project; and D.C. and E.I. wrote the manuscript.

Disclosure Statement

None declared.

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