Size-selective loosening of the blood-brain barrier in claudin-5–deficient mice

Takehiro Nitta,1,2 Masaki Hata,3 Shimpei Gotoh,1 Yoshiteru Seo,4 Hiroyuki Sasaki,3,5 Nobuo Hashimoto,2 Mikio Furuse,1 and Shoichiro Tsukita1

1Department of Cell Biology, Faculty of Medicine, Kyoto University, Sakyoku, Kyoto 606-8501, Japan
2Department of Neurosurgery, Faculty of Medicine, Kyoto University, Sakyoku, Kyoto 606-8507, Japan
3KAN Research Institute, Kyoto Research Park, Shimogyoku, Kyoto 606-8317, Japan
4Department of Physiology, Kyoto Prefectural University of Medicine, Kamigyo-ku, Kyoto 602-0841, Japan
5Department of Molecular Cell Biology, Institute of DNA Medicine, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan

Tight junctions are well-developed between adjacent endothelial cells of blood vessels in the central nervous system, and play a central role in establishing the blood-brain barrier (BBB). Claudin-5 is a major cell adhesion molecule of tight junctions in brain endothelial cells. To examine its possible involvement in the BBB, claudin-5–deficient mice were generated. In the brains of these mice, the development and morphology of blood vessels were not altered, showing no bleeding or edema. However, tracer experiments and magnetic resonance imaging revealed that in these mice, the BBB against small molecules (<800 D), but not larger molecules, was selectively affected. This unexpected finding (i.e., the size-selective loosening of the BBB) not only provides new insight into the basic molecular physiology of BBB but also opens a new way to deliver potential drugs across the BBB into the central nervous system.

Introduction

The existence of the blood-brain barrier (BBB)* was first described by Ehrlich (1885) more than 100 yr ago. He found that dyes injected into veins stained all the organs but the brain. Since then, the BBB has been thought to protect the brain from various harmful materials circulating in the blood (for reviews see Pardridge, 1998; Rubin and Staddon, 1999). On the other hand, the BBB prevents many potential drugs from entering the central nervous system (CNS). Therefore, many researchers have tried to loosen the BBB for therapeutic purposes in various CNS disorders (for reviews see Miller, 2002; Pardridge, 2002), but limited information on the molecular basis for BBB has hampered these trials.

To establish BBB, well-developed tight junctions (TJs) between adjacent endothelial cells are indispensable, in addition to various transporters in their plasma membranes (Reese and Karnovsky, 1967; for reviews see Pardridge, 1998; Rubin and Staddon, 1999; Edwards, 2001; Wolburg and Lippoldt, 2002). TJs are one mode of cell–cell adhesion, and play a central role in sealing the intercellular space in epithelial and endothelial cellular sheets (e.g., barrier function; for reviews see Schneeberger and Lynch, 1992; Anderson and van Itallie, 1995). On ultrathin section electron microscopy, TJs appear as a zone where plasma membranes of neighboring cells focally make complete contact (Farquhar and Palade, 1965). On freeze-fracture electron microscopy, TJs are visualized as a continuous, anastomosing network of intramembranous particle strands (TJ strands or fibrils) and complementary grooves (Staehelin, 1974). These observations led to our current understanding of the three-dimensional structure of TJs: at TJs, within the lipid bilayer of each membrane, some specific integral membrane proteins aggregate linearly to constitute “TJ strands.” Individual TJ strands then laterally and tightly associate with those in the apposing membrane of adjacent cells to form paired strands, where the intercellular distance becomes almost zero (so-called “kissing points” of TJs; for review see Tsukita et al., 2001).

Address correspondence to Shoichiro Tsukita, Dept. of Cell Biology, Faculty of Medicine, Kyoto University, Yoshida-Konoe, Sakyoku, Kyoto 606-8501, Japan. Tel.: 81-75-753-4372. Fax: 81-75-753-4660. E-mail: htsukita@mfour.med.kyoto-u.ac.jp

*Abbreviations used in this paper: BBB, blood-brain barrier; Cld, claudin; CNS, central nervous system; ES, embryonic stem; Gd-DTPA, gadolinium–diethylene triamine-\(\text{N}_2\text{N}_2\text{N}_2\text{N}_2\text{N}_2\)pentaacetic acid; MRI, magnetic resonance imaging; pAb, polyclonal antibody; TJ, tight junction.

Key words: tight junction; central nervous system; endothelial cells; blood vessel; drug delivery
Several peripheral membrane proteins such as ZO-1 were reported to concentrate at the cytoplasmic surface of TJs (for review see Schneeberger and Lynch, 1992; Anderson and van Itallie, 1995; Balda and Matter, 1998; Tsukita et al., 1999, 2001). Until recently, information on TJ adhesion molecules has been lacking, but now three distinct types of integral membrane proteins are known to be localized at TJs: occludin (Furuse et al., 1993), junctional adhesion molecule (JAM) (Martin-Padura et al., 1998), and claudin (Cld; Furuse et al., 1998a). Occludin, an 65-kD integral membrane protein with four transmembrane domains, was identified as the first component of TJ strands (Furuse et al., 1993). However, several studies including gene knockout analyses revealed that TJ strands can be formed without occludin (Balda et al., 1996; Saitou et al., 1998). JAM with a single transmembrane domain was recently shown to associate laterally with TJ strands, but not to constitute the strands per se (Itoh et al., 2001). In contrast, Cld is now believed to be a major constituent of TJ strands (for review see Tsukita et al., 2001). Clds with molecular masses of ~23 kD comprise a multigene family consisting of >20 members (Morita et al., 1999a; Tsukita et al., 2001). Clds also bear four transmembrane domains, but do not show any sequence similarity to occludin. Interestingly, when each Cld species was overexpressed in mouse L fibroblasts lacking endogenous Clds, exogenously expressed Cld molecules were polymerized within the plasma membrane to reconstitute “paired” TJ strands at cell–cell contact regions (Furuse et al., 1998b). Furthermore, through further detailed transfection experiments as well as immunolabeling studies, it is now widely accepted that heterogeneous Cld species constitute the backbone of TJ strands in situ; i.e., TJ strands are copolymers of heterogeneous Cld species (and also occludin; Furuse et al., 1999; Tsukita et al., 2001).

Recently, Cld-5 was found specifically in endothelial cells, in large amounts especially in the brain endothelial cells (Morita et al., 1999b), leading to the idea that Cld-5 may be directly involved in the establishment of the BBB. This pa-

Figure 1. **Generation of Cld-5-deficient mice.** (a) Restriction maps of the wild-type allele, the targeting vector, and the targeted allele of the mouse Cld-5 gene. Only one exon covers the whole open reading frame of Cld-5. The targeting vector contained the pgk neo cassette in its middle portion to delete the exon in the targeted allele. The positions of the 5′ and 3′ probes for Southern blotting are indicated as bars. E, EcoRI; Sa, SacI; K, KpnI; N, NcoI; and S, Sse8387I. (b) Genotype analyses by Southern blotting of Eco RI-digested genomic DNA from wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mice for the mutant Cld-5 gene allele. Southern blotting with 5′ and 3′ probes yielded an 8.4-kb band from the wild-type allele, and a 4.7- and 3.7-kb band from the targeted allele, respectively. (c) Loss of Cld-5 mRNA in the brain of Cld-5−/− mice examined by RT-PCR. As a control, the hypoxanthine phosphoribosyl transferase gene was equally amplified in all samples. (d) Newborn Cld-5+/− and Cld-5−/− mice. Homozygous Cld-5-deficient mice were born in the expected Mendelian ratios, and looked normal macroscopically. However, their movements gradually ceased, and they all died within 10 h of birth.

Figure 2. **Vasculogenesis and the brain histology of Cld5−/− mice.** (a and a′) Whole-mount immunostaining. Mouse 9.5-d wild-type and Cld5−/− embryos were labeled with anti–PECAM-1 mAb. The characteristic treelike and ladderlike staining patterns of blood vessels in the head and vertebrate portions were observed both in wild-type and Cld5−/− embryos. (b, b′, c, and c′) Hematoxylin-eosin-stained paraffin sections (b and b′) and anti–PECAM-1–stained frozen sections (c and c′) of the wild-type and Cld5−/− brains of 18.5-d embryos. No difference was detected. Bars: (a and a′) 300 μm; (b and b′) 100 μm; (c and c′) 40 μm.
per, to evaluate this idea, we generated Cld-5–deficient mice by homologous recombination, and found that in these mice the BBB was loosened in a size-selective manner. We believe that this work marks the first step in developing a new TJ-based method of drug delivery to the CNS.

Results

Generation of Cld-5–deficient mice

We produced mice unable to express Cld-5. Nucleotide sequencing as well as restriction mapping identified only one

Figure 3. Ultrastructure of brain blood vessels in Cld5−/− mice. In the Cld5−/− brains of 18.5-d embryo, ultrathin-section electron microscopy revealed no overt morphological abnormalities in the blood vessels. TJs were observed between adjacent endothelial cells, and at higher magnification, so-called kissing points of TJs were clearly visualized (arrowheads). Bars: (left) 4 μm; (middle) 0.2 μm; (right) 50 nm.

Figure 4. Cld-12 in brain blood vessels.

(a) Specificity of newly generated anti–Cld-12 pAb. Immunoblotting of total lysates of Escherichia coli expressing GST fusion proteins with cytoplasmic domains of Cld-1–16 confirmed its specificity. CBB, Coomassie brilliant blue staining; anti–Cld-12 pAb, immunoblotting with anti–Cld-12 pAb. (b) Frozen sections of the wild-type and Cld5−/− brains of 18.5-d embryo were double stained with anti–ZO-1 mAb and anti–Cld-5 pAb or anti–Cld-12 pAb. Cld-5 was completely undetectable from the Cld5−/− brain. Cld-12 was concentrated at ZO-1–positive TJs not only in the wild-type but also in the Cld5−/− brain endothelial cells. These Cld-12 signals were abolished when the anti–Cld-12 pAb was preincubated with the GST-Cld-12 fusion protein. There appeared to be no significant difference in the intensity of Cld-12 signal between the wild-type and Cld5−/− brain endothelial cells. The concentration of occludin at TJs of endothelial cells was not affected in the Cld5−/− brain (unpublished data). Bar, 20 μm.
exon that covers the whole open reading frame of Cld-5. We constructed a targeting vector, which was designed to disrupt the Cld-5 gene by replacing its exon with the neo-mycin resistance gene (Fig. 1 a). Two distinct lines of mice were generated from distinct embryonic stem (ES) cell clones in which the Cld-5 gene was disrupted by homologous recombination. Southern blotting confirmed the disruption of the Cld-5 gene in heterozygous as well as homozygous mutant mice (Fig. 1 b), and RT-PCR detected no Cld-5 mRNA from the brains of homozygous mutant mice (Cld5/H11002/H11002 mice; Fig. 1 c). Cld5/H11002/H11002 mice were born in the expected Mendelian ratios, and looked normal macroscopically (Fig. 1 d). However, their movements gradually ceased, and they all died within 10 h of birth. Because both lines of mice showed the same phenotypes, we will mainly present data obtained from one line.

Morphology of brain blood vessels in Cld-5-deficient mice

We examined the brain blood vessels morphologically in Cld5−/− mice. As shown in Fig. 2 (a and a’), whole-mount immunostaining of 9.5-d embryos for PECAM-1, an endothelial cell marker, detected no significant abnormality in the vasculogenesis in Cld5−/− mice. Furthermore, in both hematoxylin-eosin and anti–PECAM-1 mAb stainings of the brain sections of 18.5-d embryos, no difference was discerned between wild-type and Cld5−/− mice (Fig. 2, b, b’, c, and c’). No signs of bleeding or edema were detected in the Cld5−/− brain. Consistently, there was no difference in the brain tissue water content between newborn wild-type (85.6 ± 0.38%, n = 11) and Cld5−/− mice (85.4 ± 0.30%, n = 12).

At the electron microscopic level, the blood vessels of the brain of newborn Cld5−/− mice did not exhibit overt morphological abnormalities (Fig. 3). Close inspection revealed that, even in Cld5−/− mice, TJs with a normal appearance occurred at the endothelial cell–cell contact.
regions, showing the so-called kissing points of TJs. Considering that Cld-5 disappeared from the endothelial cells (Fig. 4), this finding suggested that in endothelial cells of the wild-type brain, TJ strands were not only composed of Cld-5 but also other Cld species. We researched for such endothelial cell-specific Cld among Cld-1–16 using available and newly generated antibodies, and found that, in addition to Cld-5, Cld-12 was expressed in large amounts and colocalized with ZO-1 in the wild-type brain endothelial cells and also in the Cld5−/− brain endothelial cells (Fig. 4). Thus, we concluded that in the Cld5−/− brain, Cld-5 was simply removed from the TJs of endothelial cells, leaving TJs of at least Cld-12.

**Size-selective loosening of the BBB in Cld5−/− mice**

The important question is whether the barrier function of TJs (i.e., the BBB) is affected in the Cld5−/− brain endothelial cells. To evaluate the BBB in Cld5−/− mice, we performed tracer experiments: Cld5−/− intercross littermates were obtained by Caesarian section at embryonic day 18.5, resuscitated, and perfused with tracers from the left cardiac ventricle. These littermates were later genotyped. First, we used a primary amine-reactive biotinylation reagent (443 D), which covalently cross-links to accessible proteins, as a tracer. This tracer has been successfully used so far to evaluate the permeability of TJs of epithelial cells (Chen et al., 1997; Furuse et al., 2002). 5 min after perfusion, the localization of the biotinylation reagent was examined on sagittal whole-body sections using HRP-conjugated streptavidin (Fig. 5, a and a′). In wild-type embryos, the reagent was specifically excluded from the CNS. This was indeed confirmation of Ehrlich’s work, which first described the BBB (Ehrlich, 1885). In contrast, in the Cld5−/− mice, the brain and the spinal cord showed strong and diffused HRP activity. At a higher magnification in the wild-type brain, the biotinylation reagent was retained in the blood vessels, whereas in the Cld5−/− brain, this reagent was distributed diffusely throughout the brain parenchyma with some concentration in blood vessels (Fig. 5, b, b′, c, and c′). Fig. 6 represents the time course of the leakage of the biotinylation reagent from blood vessels in the Cld5−/− brain. This reagent appeared to have already passed through the BBB 1 min after perfusion. These findings clearly demonstrated that the BBB was severely affected in Cld5−/− mice.

If serum proteins extravasate freely in the brain, vasogenic edema should be induced. However, as mentioned in Fig. 2 b, there was no sign of the edema in the Cld5−/− brain. We checked for the possible leakage of serum albumin (~68 kD) from blood vessels in the Cld5−/− brain (Liu, 1988; Vinores et al., 1989; Fig. 7 a). Interestingly, immunostaining with antialbumin polyclonal antibody (pAb) showed no leakage of serum albumin, which was completely retained in the blood vessels. Furthermore, we performed tracer experiments using microperoxidase (~1.9 kD) as a tracer (Knothe Tate et al., 1998). As shown in Fig. 7 b, no leakage from the brain blood vessels was detected in the Cld5−/− brain. These findings suggested that, in the Cld5−/− brain, the BBB (i.e., the TJ barrier of endothelial cells) was loosened in a size-selective manner. If so, the absence of vasogenic edema in the Cld5−/− brain can be explained. In Fig. 7 c, to confirm this speculation, we perfused a mixture of tetramethylrhodamine-conjugated dextran (~10 kD) and a dye for nuclear staining (Hoechst stain H33258; 562 D) into the heart of resuscitated 18.5-d embryos, and after a 5-min incubation, the brain was fixed. The localization of microperoxidase was visualized by peroxidase activity in frozen sections. No leakage of microperoxidase was observed in the Cld5−/− brain. Bar, 40 μm. (c) A mixture of tetramethylrhodamine-conjugated dextran (~10 kD) and a dye for nuclear staining (Hoechst stain H33258; 562 D) was perfused, and frozen sections were observed under a fluorescence microscope. In the wild-type brain, neither dextran (red) nor Hoechst dye (blue) extravasated, whereas in the Cld5−/− brain, Hoechst dye, but not dextran, extravasated to stain the nuclei of surrounding neurons/glia cells. Bar, 30 μm.

**Magnetic resonance imaging (MRI) analyses of the BBB impairment in Cld5−/− mice**

Finally, we quantitatively examined impairment of the BBB in live Cld5−/− mice using MRI with the longitudinal (T1) relaxation reagent gadolinium–diethylene triamine–N,N,N’,N”-pentaacetic acid (Gd-DTPA; 742 D; Abbott et al., 1999; Caravan et al., 1999; Seo et al., 2002). We injected various dosages of Gd-DTPA into the heart of resuscitated 18.5-d embryos. The sagittal T1-weighted images of the wild-type brain clearly exhibited the existence of the BBB; the signal from most organs except the CNS was significantly enhanced by Gd-DTPA perfusion. In contrast, in the Cld5−/− mice, the signals from all areas of the CNS were markedly enhanced by Gd-DTPA perfusion, as shown in Fig. 8 a. Furthermore, Gd-DTPA perfusion significantly enhanced the signal intensity of the brain, whereas the signal intensity of the blood vessels and organs remained constant. This finding clearly demonstrated that the BBB was severely impaired in Cld5−/− mice.
enhanced, and the degree of enhancement depends on the dosage of injected Gd-DTPA (Fig. 8 a). In Fig. 8 b, we examined the relationship between the averaged $T_1$ relaxation rate of water in the brain and the dosage of injected Gd-DTPA. As $1/T_1$ of the brain, $1/T_1$ values of the five ROIs (156 x 156 x 750 mm) including the cortex, thalamus, hypothalamus, cerebellum, and pons were averaged. The bold and dotted lines are the results of fitting and 95% confidence limits, respectively. As explained in the text, on the assumption of a simple two-compartment exchange model (Fabry and Eisenstadt, 1978; Schwarzbaueer et al., 1997), this relationship allowed us to estimate quantitatively the Gd-DTPA-accessible space per unit volume of the brain.

**Discussion**

Blood vessels are lined with a single layer of endothelial cellular sheets. These cellular sheets function as barriers to maintain the internal environment of blood vessels, but various materials must be selectively transported across these sheets. There are two pathways through which materials cross endothelial cellular sheets: the transcellular pathway including transcytosis through the cell and the paracellular pathway through TJs (for reviews see Spring, 1998; Tsukita et al., 2001). In the blood vessels in the CNS, the endothelial cellular sheets function as very tight barriers, constituting the so-called BBB (for reviews see Pardridge, 1998; Rubin and Staddon, 1999). For the establishment of the BBB, first, the transport of materials through transcellular pathways should be suppressed so completely that specialized transport systems are required in the plasma membranes of endothelial cells for the uptake of even small molecules such as glucose and amino acids by the CNS (for reviews see Pardridge, 1998; Rubin, 2001; Miller, 2002). Furthermore, when some materials are leaked into the parenchyma of the CNS, the multi-drug resistance transporter mdr1a (P-glycoprotein 1), which is concentrated in the endothelial cell plasma membranes, redistributes them out of the brain parenchyma into endothelial cells and, hence, back to the blood (Cordon-Cardo et al., 1989; Schinkel et al., 1994; Edwards, 2001). Second, the paracellular route also should be tightly sealed. TJs are markedly developed compared with those in endothelial cells in nonneuronal tissues (Reese and Karnovsky, 1967; Wollburg and Lipold, 2002). Taking these cellular bases for the BBB into consideration, TJs have been considered attractive targets for transient breakdown of the BBB in therapies for various CNS disorders.

We found that TJs in the brain blood vessels are primarily composed of at least two distinct species of Clds: Cld-5 and -12. Anti–Cld-1 pAb also stained the blood vessels in the wild-type brain as previously reported (Liebner et al., 2000), but this staining was still positive in the Cld-1–deficient mice (unpublished data). We examined the roles of Cld-5 in the BBB by generating Cld-5–deficient mice. In the Cld5−/− brain, Cld-5 was simply removed from the TJs of endothelial cells, leaving morphologically normal blood vessels as well as the Cld-12–based TJs. However, in terms of the barrier function, these endothelial TJs showed a peculiar abnormality. Tracer experiments and MRI revealed that in these mice, the BBB is severely affected against small molecules (less than ~800 D), but not larger molecules. In other words, the Cld-12–based TJs in Cld5−/− brain blood vessels would function as a molecular sieve. They allow only small molecules (less than ~800 D) to pass across TJs. Of course,
this molecular mass cut-off (~800 D) is tentative because the tracers used in this work have all a different chemical structure and differ in charge and hydrophilicity.

The size-selective loosening of TJ in Cld5<sup>−/−</sup> brain blood vessels is consistent with the previous data on epithelial TJ. The TJs of MDCK I epithelial cells were primarily composed of Cld-1 and -4, and when Cld-4 was removed from these TJs using a Cld-4–binding peptide, the continuous Cld-1–based TJs still persisted, which leaked 4 kD/10 kD dextran, but not 40 kD dextran (Sonoda et al., 1999). Furthermore, we recently found that the TJs in the epidermis were also composed of Cld-1 and -4. In Cld-1–deficient mice, there still remained the continuous Cld-4–based TJs in the granular layer of the epidermis, and the layered organization of keratinocytes in the epidermis was not affected (Furuse et al., 2002). However, interestingly, the epidermal barrier against water was severely affected, resulting in the dehydration of newborn mice. In general, it is, thus, safe to say that, when TJs are composed of more than two distinct species of Cls, the removal of one Cld species changes the barrier function of TJs markedly while keeping their continuous structural integrity.

Therefore, in the Cld5<sup>−/−</sup> brain, the existence of the Cld-12–based TJs would keep the structural integrity (and the polarity) of endothelial cells, showing no bleeding. Furthermore, the size selectivity of the Cld-12–based TJs did not allow most serum proteins to extravasate, resulting in no vasogenic edema. From the viewpoint of the drug delivery, the lack of bleeding and edema is very important and advantageous for the delivery of therapeutic agents to the brain. Therefore, Cld-5 can be regarded as a potential target for developing a new drug delivery method for CNS disorders. Of course, it is possible that impairment of the BBB, even if temporary or in a size-selective manner, would be harmful to CNS activity to some extent. Indeed, Cld5<sup>−/−</sup> mice died within 10 h of birth. Cld-5 is expressed in large amounts in all segments of the blood vessels in the brain, but it is also detected in some segments of the blood vessels of nonneural tissues such as the lung and kidney (Morita et al., 1999b). Various tissues were examined histologically in hematoxylin-eosin–stained sections of Cld5<sup>−/−</sup> mice, but no significant abnormalities were detected (unpublished data). Therefore, at this moment it is not easy to discuss a causal sequence between BBB impairment and death in Cld5<sup>−/−</sup> mice. To answer this question, as well as to better understand the basic physiology of the BBB, we should generate mice in which Cld-5 gene can be conditionally knocked out in the endothelial cells of the brain blood vessels in adult mice.

**Materials and methods**

**Antibodies**

Rat anti–PECAM-1 mAb (BD Biosciences), rat anti–mouse ZO-1 mAb (CHEMICON International, Inc.), rabbit anti–Cld-5 pAb (Zymed Laboratories), and rabbit anti–mouse albumin pAb (Inter-Cell Technology, Inc.) were purchased. Anti-Cld-12 pAb was raised as follows. A polypeptide corresponding to the COOH-terminal cytoplasmic domain of mouse Cld-12 (a cysteine residue was added at its NH terminus) was synthesized and coupled via the cysteine residue to keyhole limpet hemocyanin. This peptide was injected into rabbits as an antigen. Rabbit antisera were affinity-purified on nitrocellulose membranes with GST fusion proteins with Cld-12 before use.

**Generation of Cld5<sup>−/−</sup> mice**

Two overlapping clones encoding mouse Cld-5 were obtained by screening a 129/Sv genomic library. Using them, the targeting vector was constructed as shown in Fig. 1a. The diphthelia toxin A expression cassette (MC1pDT-A) was placed outside the 3′ arm of homology for negative selection. As shown in Fig. 1a, only one exon covered the whole open reading frame of Cld-5. Thus, this targeting vector was designed to delete this exon by replacing with the polylinker cassette in ES cells electroporated with the targeting vector and selected for ~9 d in the presence of G418. The G418-resistant colonies were removed and screened by Southern blotting with the 5′ and 3′ external probes (Fig. 1a). When digested with EcoRI, correctly targeted ES clones were identified by an additional 4.7-kbp band together with the 8.4-kbp band of the wild-type allele with the 5′ probe, and by an additional 3.7-kbp band together with the 8.4-kbp band of the wild-type allele with the 3′ probe. The targeted ES cells obtained were injected into C57BL/6 blastocysts, which were, in turn, transferred into BALB/c foster mothers to obtain chimeric mice. Male chimeras were mated with C57BL/6 females, and agouti offspring were genotyped to confirm the germine transmission of the targeted allele. The littermates were genotyped by Southern blotting. Next, heterozygous mice were interbred to produce homozygous mice.

**Immunostaining**

For whole-mount staining, mouse 9.5-d embryos were killed. Samples were pretreated by microwaving in PBS for 20 s and fixed in 4% PFA/PBS for 30 min. They were dehydrated in methanol and bleached with 30% H2O2. Samples were then rehydrated, blocked with PBS-0.1% Triton X-100 and 1% skimmed milk/PBS, and incubated overnight with rat anti-PECAM-1 mAb followed HRP-conjugated goat anti–rat IgG (CHEMICON International, Inc.). Next, they were washed with PBS-MT and PBS-T (0.2% Triton X-100/PBS) each for 5 h. Bound antibodies were visualized by incubating with 0.025% DAB, 0.08% NiCl2, and 30% H2O2 in PBS-T. Immunofluorescence staining/peroxidase histochemistry for frozen and paraffin sections, and ultra-thin section electron microscopy were performed as previously described (Morita et al., 1999b).

**Tracer experiments**

Cld5<sup>−/−</sup> intercross littermates were obtained by Caesarian section at embryonic day 18.5 and resuscitated. The following solutions were perfused from the left cardiac ventricle under a stereoscopic microscope using a low-pressure perfusion apparatus (Terumo) within 1 min; 5 μl/g body wt of 25 mg/ml microperoxidase (MP-11; 1,862 D; Sigma-Aldrich) in PBS containing 1 mM CaCl2 (Knothe Tate et al., 1998); 1 mg/l body wt of 2 mg/ml EZ-LinkTM Sulfo-NHS-Biotin (443 D; Pierce Chemical Co.) in PBS containing 1 mM CaCl2 (Chen et al., 1997; Furuse et al., 2002); and 1 mg/l body wt of 100 μg/ml Hoechst stain (H33258; 562 D; Calbiochem-Novabiochem) in PBS containing 1 mM CaCl2 with or without 1 mg/ml tetramethylrhodamine-conjugated lysine-fixable dextran (10 kD; Molecular Probes; Chang-Ling et al., 1992; Hu et al., 2000). 1–5 min after perfusion, the whole brain was removed, fixed with 3.7% formaldehyde (Sonoda et al., 1999). Furures et al. 659

**MRI**

Mouse 18.5-d embryos were resuscitated and anesthetized with pentobarbital (50 μg/g body wt), and Gd-DTPA solution (~7 μl in total) was injected transcardially. The mice were placed in the head-up position on a polystyrene sledge, the position of the head was fixed with adhesive tape, and the sledge temperature was maintained at 34 ± 1°C using a warming air flow. 1H magnetic resonance images were obtained using an NMR spectrometer (7.05 T; model AMX-300; Bruker) with an active shielded gradient (microm5.2) and an 1H birdcage resonator (15-mm diam). The T-weighted sagittal gradient-echo imaging (repeat time [TR] = 100 ms, echo-time [TE] = 4.4 ms, flip angle = 45°) was obtained with 78 μm in-plane resolution and 0.75-mm slice thickness. The T relaxations times were measured using an inversion recovery fast-imaging sequence (Haase et al., 1989) with a series of 16 detection pulses (12° flip angle), 10 inversion recovery delays (40–7,030 ms), 156-μm in-plane resolution, 0.75-mm slice thickness, 3,000-ms TR, 2.4-ms TE, and 2 accumulations. On the assumption of a two-compartment model with water exchange between Gd-DTPA solution and extracellular space, the following equation was used and solved numerically (Sonoda et al., 1978; Schwarzbauer et al., 1997), the slow relaxation rate (R<sub>s</sub>) given by R<sub>s</sub> = 0.5(R<sub>r</sub> + R<sub>c</sub> + k<sub>d</sub>/l) − (R<sub>r</sub> − R<sub>c</sub> + k<sub>d</sub>/l − 200/l<sup>2</sup> + 4k<sub>d</sub>l/(1 − l/l<sup>2</sup>)), where R<sub>r</sub> is the intrinsic 1/T1 of water in the brain, k<sub>d</sub> is the rate constant of...
diffusive water influx from the Gd-DTPA-nonaccessible space into the accessible space, f and Rf are the volume fractions of water, and 1/T1 for water in the Gd-DTPA-accessible space, respectively. The Rf was estimated from the relativity of Gd-DTPA (Caravan et al., 1999), the dosage of the Gd-DTPA and the volume fraction of extracellular fluid.

We thank Drs. T. Noda and Y. Sugitani for technical advices in producing and analyzing Chd5+/− mice, and Dr. T. Aoki for helpful discussions and encouragements.

This work was supported in part by a Grant-in-Aid for Cancer Research and a Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Science and Culture of Japan to S. Tsukita, and by the Japan Society for the Promotion of Science Research for the Future Program to M. Furuse.

Submitted: 11 February 2003
Revised: 18 March 2003
Accepted: 26 March 2003

References

Abbott, N.J., D.C. Chugani, G. Zaharchuk, and B.R. Rose. 1999. Delivery of imaging agents into brain. Adv. Drug Deliv. Rev. 37:253–277.
Anderson, J.M., and C.M. van Itallie. 1995. Tight junctions and the molecular basis for regulation of paracellular permeability. Am. J. Physiol. 269:G467–G475.
Balda, M.S., and K. Matter. 1998. Tight junctions. J. Cell Sci. 111:541–547.
Balda, M.S., J.A. Whitney, C. Flores, S. Gonzalez, M. Cerrejio, and K. Matter. 1996. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical–basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. J. Cell Biol. 134:1031–1049.
Caravan, P., J.J. Ilson, T.J. McMurry, and R.B. Lauffer. 1999. Gadolinium(III) chelates as MRI contrast agents: structure, dynamics, and applications. Chem. Rev. 99:2293–2352.
Chang-Ling, T.-L., A.L. Neill, and N.H. Hunt. 1992. Early microvascular changes in murine cerebral malaria detected in retinal wholemounts. Am. J. Pathol. 140: 1121–1130.
Chen, Y.-H., C. Mezirdof, D.L. Paul, and D.A. Goodenough. 1997. COOH terminus of occludin is required for tight junction barrier function in early Xenopus embryos. J. Cell Biol. 138:891–899.
Cordon-Cardo, C., J.P. O’Brien, D. Casals, L. Rittman-Grauer, J.L. Biedler, M.R. Melamed, and J.R. Bertino. 1989. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-barrier sites. Proc. Natl. Acad. Sci. USA. 86:695–698.
Edwards, R.H. 2001. Drug delivery via the blood-brain barrier. Nat. Rev. Neurosci. 2:285–293.
Ehrlich, P. Ed. 1885. Das Sauerstoff-Bedurfnis des Organismus. Eine Farbenanalyse ed.
Furuse, M., H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, and S. Tsukita. 1998. Claudin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J. Cell Biol. 141:397–408.
Furuse, M., K. Fujimoto, Y. Doi, M. Itoh, T. Fujimoto, M. Furuse, H. Takano, T. Noda, and S. Tsukita. 1998. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J. Cell Biol. 141:397–408.
Furuse, M., K. Fujimoto, Y. Doi, M. Itoh, T. Fujimoto, M. Furuse, H. Takano, T. Noda, and S. Tsukita. 1998. Claudin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J. Cell Biol. 141:397–408.
Furuse, M., H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, and S. Tsukita. 1999. Claudin multi gene family encoding four-transmembrane domain protein components of tight junction strands. Proc. Natl. Acad. Sci. USA. 96:511–516.
Furuse, M., H. Sasaki, M. Furuse, and S. Tsukita. 1999b. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. J. Cell Biol. 147:185–194.
Pardridge, W.M. 1998. Introduction to the Blood-Brain Barrier. Cambridge University Press, Cambridge. 486 pp.
Pardridge, W.M. 2002. Drug and gene targeting to the brain with molecular Trojan horses. Nat. Rev. Drug Discov. 1:131–139.
Reese, T.S., and M.J. Karnovsky. 1967. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J. Cell Biol. 34:207–217.
Rubin, L.L., and J.M. Staddon. 1999. The cell biology of the blood-brain barrier. Annu. Rev. Neurosci. 22:11–28.
Saitou, M., K. Fujimoto, Y. Doi, M. Itoh, T. Fujimoto, M. Furuse, H. Takano, T. Noda, and S. Tsukita. 1998. Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. J. Cell Biol. 141:397–408.
Schinkel, A.H., J.J. Smit, O. van Tellingen, J.H. Beijnen, E. Wagenlaar, L. van Deemter, C.A. Mol, M.A. van der Valk, E.C. Reubans-Maandag, H.P. te Riele, et al. 1994. Disruption of the mouse mdr1a P-glycoprotein gene leads to a deficiency in the blood-brain barrier and to increased sensitivity to drugs. Cell. 77:491–502.
Schneedeberger, E.E., and R.D. Lynch. 1992. Structure, function, and regulation of cellular tight junctions. Annu. Rev. Physiol. 26:167–1661.
Schwarzfuehr, C., S.P. Morrissey, R. Deichmann, C. Hillenbrand, J. Syha, H. Adol, U. Noth, and A. Haase. 1997. Quantitative magnetic resonance imaging of capillary water permeability and regional blood volume with an intravascular MR contrast agent. Magn. Reson. Med. 37:769–777.
Sey, Y., A. Takamata, T. Ogino, H. Morita, S. Nakamura, and M. Murakami. 2002. Water permeability of capillaries in the subfornical organ of rats determined by Gd-DTPA enhanced ‘H magnetic resonance imaging. J. Physiol. 545:217–228.
Sonoda, N., M. Furuse, H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, and S. Tsukita. 1999. Claudin perfringens enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier. J. Cell Biol. 147:195–204.
Spring, K. 1998. Routes and mechanism of fluid transport by epithelia. Annu. Rev. Physiol. 60:105–119.
Stachelm, L.A. 1974. Structure and function of intercellular junctions. Int. Rev. Cytol. 39:191–283.
Tsukita, S., M. Itoh, and M. Furuse. 1999. Structural and signaling molecules come together at tight junctions. Curr. Opin. Cell Biol. 11:628–633.
Vinores, S.A., C. Gadegbeku, P.A. Campochiaro, and W.R. Green. 1989. Immunohistochemical localization of blood-retinal barrier breakdown in human diabetics. Am. J. Pathol. 134:231–235.
Wolburg, H., and A. Lippoldt. 2002. Tight junctions of the blood-brain barrier: development, composition and regulation. Vascul. Pharmacol. 38:323–337.