Immunolocalization of Tight Junction Proteins, Occludin and ZO-1, and Glucose Transporter GLUT1 in the Cells of the Blood-Nerve Barrier*

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Summary. Facilitated-diffusion glucose transporter GLUT1 is abundant in the blood-nerve barrier. To observe the relationship between glucose transfer across the barrier and the molecular architecture of the barrier, we examined the localization of GLUT1 and tight junction proteins, occludin and zonula occludens-1 (ZO-1), by immunofluorescence microscopy and immunogold electron microscopy in the rat sciatic nerve. GLUT1 was enriched at the whole aspects of the plasma membranes of the cells of the barrier: perineurial cells, and endothelial cells of the blood vessels in the endoneurium. These GLUT1-positive cells were also positive for occludin and ZO-1, both of which were localized at tight junctions. ZO-1 additionally was present in the GLUT1-negative cells not serving as the blood-nerve barrier. These observations suggest that occludin in the tight junctions and GLUT1 at the plasma membranes in the cells of the barrier may constitute a mechanism for the selective transfer of glucose across the barrier while preventing the non-specific flow of blood constituents.

Epithelia and endothelia play a pivotal role in maintaining the specific milieu of a variety of compartments in the mammalian body. At certain sites such as the blood-tissue barriers, the tissue compartments surrounded by epithelia or endothelia are tightly guarded against free access from the outside. In these barriers, epithelial and endothelial cells connected by tight junctions and/or syncytial cell layers prevent the free exchange of substances between the blood and cells encircled by barrier. Specific transporters and channels localized at plasma membranes in these cells of the barrier are involved in the selective

and effective transfer of substances across the barrier (TAKATA, 1996; TAKATA et al., 1997).

Glucose is one of the most ubiquitous and important substances used for ATP production as well as the synthesis of a variety of cellular molecules. Glucose transport across the plasma membrane is mediated by membrane proteins called glucose transporters (WHEELER and HINKLE 1985; BALDWIN, 1993; BELL et al., 1993; TAKATA et al., 1993). These consist of two families: a Na⁺-dependent active glucose transporter family (SGLT family), and a facilitated-diffusion glucose transporter family (GLUT family). We previously showed that GLUT1, an isoform of the GLUT family, is abundant in the cells of blood-tissue barriers (TAKATA et al., 1990, 1997). By high-resolution immunofluorescence and immunogold-electron microscopy, we showed that GLUT1 is localized at both the apical and the basolateral plasma membranes in the barrier epithelial cells. In the barrier endothelial cells, GLUT1 is also localized at both the luminal and contraluminal membranes (TAKATA et al., 1997). Since GLUT1 is a high-affinity glucose transporter and abundant at both domains of these epithelial or endothelial cells, GLUT1 appears to constitute a key machinery in the transcellular passage of glucose through the barrier cell layer, thereby supplying glucose to the cells located inside the barrier (TAKATA et al., 1997).

Tight junctions play a key role in the regulation of the barrier function of epithelia and endothelia (CITI, 1993; ANDERSON and VAN ITALLIE, 1995; DENKER and NIGAM, 1998). Several tight junction proteins have

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been found. ZO-1 (zonula occludens-1), the first to be identified among them (STEVENSON et al., 1986; ANDERSON et al., 1988), is a cytoplasmic protein of 210–225 kDa, and together with the other tight junction proteins ZO-2 (GUMBINER et al., 1991) and ZO-3 (HASKINS et al., 1998), constitutes a membrane-associated guanylate kinase (MAGUK) family. Other cytoplasmic proteins of tight junctions include cingulin (CITI et al., 1988), 7H6 (ZHONG et al., 1993; SATOH et al., 1996), Rab3b (WEBER et al., 1994), Rab13 (ZAHRAOUI et al., 1994), symplekin (KEON et al., 1996), AF-6 (ALL-1 fusion partner from chromosome 6) (YAMAMOTO et al., 1997), and ASIP (atypical PKC-isotype-specific interacting protein) (IZUMI et al., 1998). Occludin, a 4-pass transmembrane protein, had been the only integral membrane protein of tight junctions (FURUSE et al., 1993, 1994; ANDO-AKATSUKA et al., 1996) until recent work led to the identification of claudins which are also 4-pass transmembrane proteins, and their importance in the tight junction formation has been suggested (FURUSE et al., 1998; MORITA et al., 1999). Very recently, paracellin-1, a member of the claudin family, was identified and shown to be involved in the paracellular passage of ions (SIMON et al., 1999). In addition, JAM (junctional adhesion molecule), a single-pass membrane protein of the immunoglobulin gene superfamily, was reported in the tight junctions of endothelial and epithelial cells, where it is suggested to regulate the transmigration of leukocytes (MARTIN-PADURA et al., 1998).

We previously showed that occludin and GLUT1 were specifically expressed in the cells of the blood-retinal and blood-aqueous barriers in the mouse eye (TSERENTSOODOL et al., 1998). Compared with occludin, ZO-1 was more widely distributed; it was localized in the tight junctions in the cells of barrier as well as in other junctions. We suggested that the presence of occludin in the tight junction and the abundance of GLUT1 in the critical plasma membranes of the cells of the barrier may be essential in the specific glucose transfer across the barrier while preventing the non-specific flow. In order to determine whether occludin and related substances might be present in the cells of other blood-tissue barriers, we immunolocalized occludin, ZO-1, and GLUT1 in the sciatic nerve, a site of the blood-nerve barrier.

MATERIALS AND METHODS

Antibodies

Anti-GLUT1 antibodies were raised in a guinea pig and a rabbit and characterized as previously de-

scribed (TAKATA et al., 1990; SHIN et al., 1996). Rabbit anti-occludin, mouse anti-occludin, and rabbit anti-ZO-1 were purchased from Zymed (San Francisco, CA, USA).

Tissue specimens

Male Wistar rats at 4 weeks of age were obtained from the Animal Breeding Facility, Gunma University (Maebashi, Japan). The rats were anesthetized, killed by an intraperitoneal injection of sodium pentobarbital, and sciatic nerve specimens were removed. For immunoblotting, samples were immediately frozen and stored at -80°C until use. For immunostaining in cryostat sections, fresh specimens were embedded in OCT compound (Sakura Fine Technical, Tokyo, Japan), rapidly frozen in liquid nitrogen, and stored at -80°C until use. For semithin and ultrathin frozen section studies, tissue specimens were fixed in 1% or 3% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 3–24 h at 4°C , and infused with 2.3 M sucrose in 0.1 M sodium phosphate buffer, pH 7.4 (TAKATA and SINGER, 1988).

Immunoblotting

Tissue samples were homogenized in phosphate-buffered saline (PBS) containing protease inhibitors as described (TAKATA et al., 1992). Protein content was measured by the BCA protein assay reagent (Pierce, Rockford, IL, USA). Thirty to 50 μg of protein was electrophoresed through sodium dodecyl sulfate (SDS)-polyacrylamide gels, and transferred to membrane filters (Immobilon-PSQ, Millipore, Bedford, MA, USA). The blotted membranes were first blocked with 3% bovine serum albumin, and sequentially incubated with either of the primary antibodies, and [^{125}I]-protein G (New England Nuclear, Wilmington, DE, USA) or [^{125}I]-protein A (New England Nuclear). Autoradiography was performed with imaging plates, and the plates were processed with a BAS2000 bioimage analyzer (Fuji Film, Tokyo).

Immunofluorescence staining

Immunostaining procedures were performed basically as described previously (TSERENTSOODOL et al., 1998). In short, 4- μm cryostat sections were cut and mounted on poly-L-lysine-coated glass slides. They were immediately fixed in acetone at -20°C for 30 min, treated in 95% ethanol for 1 min at room temperature, and washed with PBS (KIMURA et al., 1996). Semithin frozen sections (1 μm) were cut with an Ultracut S UCT ultramicrotome equipped with a FCS cryokit (Leica, Vienna, Austria) and glass knives, and mounted on glass slides. Rabbit anti-occludin, mouse anti-occludin, rabbit anti-ZO-1, and

guinea pig anti-GLUT1 were used at dilutions of 1:200, 1:500, 1:200, and 1:500, respectively. Fluorescence-labeled secondary antibodies used were Cy3 (indocarbocyanine)- or FITC (fluorescein isothiocyanate)-labeled donkey anti-rabbit IgG (Jackson Immuno-research, West Grove, PA, USA), Cy3-labeled donkey anti-mouse IgG (Jackson Immuno-research), and FITC-labeled donkey anti-guinea pig IgG (Jackson Immuno-research). For nuclear counter-staining, 2 μ g/ml 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Boehringer-Mannheim, Mannheim, Germany) was included in the secondary antibody solution. Double-immunofluorescence staining was carried out by the sequential incubation of a mixture of the primary antibodies raised in different animal species and a mixture of the corresponding species-specific secondary antibodies as described (SHIN et al., 1996). After staining, sections were mounted in 22% polyvinyl alcohol in 56 mM Tris-HCl buffer, pH 9.0, 11% glycerol containing 5% 1,4-diazabicyclo [2, 2, 2] octane (DABCO) as an anti-bleaching reagent (SHIN et al., 1996). Specimens were examined with a BX-50 or an AX-70 microscope equipped with Nomarski differential interference-contrast and epifluorescence optics (Olympus, Tokyo). Some of the images were recorded with a PXL 1400 cooled CCD camera (Photometrics, Tucson, AZ, USA), and processed with an IP Lab Spectrum image analysis software (Signal Analytics, Vienna, VA, USA).

For the whole-mount observations sciatic nerve specimens fixed in 1% paraformaldehyde were washed with PBS and digested for 1 h at 37°C with 0.5 mg/ml collagenase (type II, Sigma, St. Louis, MO, USA) in 119 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 5 mM glucose, and 0.25% bovine serum albumin, pH 7.4. After digestion, specimens were permeabilized with three changes of 0.1% Triton X-100-PBS (T-PBS) for 5 min each and blocked with 5% normal goat serum in T-PBS for 1 h.

Subsequent procedures for immunolabeling were basically the same as those in the frozen sections except that specimens were incubated with the primary antibodies for 2 h, and with the secondary antibodies for 1.5 h. Two μ M of TO-PRO-3 (Molecular Probes, Eugene, OR, USA) was used instead of DAPI for nuclear counterstaining (SUZUKI et al., 1998). Specimens were observed with an Olympus BX-50 fluorescence microscope equipped with a Bio-Rad MRC-1024 laser confocal system (Bio-Rad Laboratories, Hercules, CA, USA). Images were processed with Laser Sharp (Bio-Rad) and Photoshop (Adobe Systems, San Jose, CA, USA) softwares.

Immunoelectron microscopy

Ultrathin frozen sections were cut with a diamond knife, mounted on nickel grids coated with Formvar and carbon, and immunolabeled essentially as described previously (TAKATA et al., 1992; SHIN et al., 1996). In short, sections were treated with 5% normal goat serum containing 1% gelatin in PBS for 10 min. They were then sequentially incubated with either of the primary antibodies for 1.5 h, and the affinity-purified goat anti-rabbit IgG or the goat anti-guinea pig IgG (Jackson Immuno-research)-colloidal gold (diameter 10 nm) conjugate (prepared according to SLOT and GEUZE, 1985, and DEMEY, 1984) for 1 h. After washing with PBS, the specimens were refixed in 2% glutaraldehyde, stained with uranyl acetate, and ultrathin-embedded in 1.8% methylcellulose containing 0.5% uranyl acetate. Specimens were observed with a JEM-1010 transmission electron microscope (JEOL, Tokyo).

Immunohistochemical controls

As immunohistochemical controls, either one or both of the primary antibodies were replaced with normal serum. These controls were carried out in parallel with the experimental studies. None of the controls gave positive staining, confirming the specificity of the staining.

Fig. 1. Immunoblotting of the rat sciatic nerve with antibodies against GLUT1, occludin, and ZO-1. Sciatic nerve homogenate (50 μ g each) was applied to SDS-polyacrylamide gel electrophoresis, and subjected to immunoblotting with guinea pig anti-GLUT1 (a), rabbit anti-occludin (b) and rabbit anti-ZO-1 (c).

Fig. 2. Immunofluorescence localization of GLUT1, occludin, and ZO-1 in the sciatic nerve. Cryostat (a-c) semithin frozen sections (d, e), and a whole-mount specimen (f) were stained for occludin and GLUT1 (a, b, d, f), or ZO-1 and GLUT1 (c, e). Nuclear DNA was labeled with DAPI (a, b, d, e), or TO-PRO3 (f) and shown in blue. Bars: 50 μ m (a, b, c, f); 5 μ m (d, e). **a.** Occludin (red) and GLUT1 (green) are colocalized in the perineurium (P) and the blood vessel (BV). Arrows and arrowheads indicate occludin in the perineurium and the blood vessel, respectively. **b.** Occludin (red) and GLUT1 (green) are positive in the blood vessels (double arrowhead) in the connective tissue surrounded by and adjacent to the perineurium (P). **c-f.** Legends on the next page.

RESULTS

Immunoblotting

By immunoblotting of the homogenate of the sciatic nerve with anti-GLUT1 antibody, a 46-kDa protein was detected (Fig. 1a). With anti-occludin and anti-ZO-1 antibodies, a 58-kDa protein and a 208-kDa protein were detected respectively (Fig. 1b, c). The apparent molecular weights of these bands were within the range of GLUT1 (TAKATA et al., 1990), occludin (SAITOU et al., 1997), and ZO-1 (STEVENSON et al., 1986). These results showed the presence of GLUT1, occludin, and ZO-1 proteins in the sciatic nerve, and their specific recognition by the antibodies used in the present work.

Immunofluorescence localization of GLUT1, occludin, and ZO-1 in the sciatic nerve

In cryostat sections, GLUT1 was abundant in the perineurium covering the surface of nerve fiber bundles as well as in blood vessels in the endoneurium.

Double-immunofluorescence observation showed that occludin and ZO-1 were colocalized in these GLUT1-positive regions (Fig. 2a-e). The epineurium, the connective tissue covering the surface of the sciatic nerve, was negative for GLUT1 and occludin. Blood vessels in the epineurium were positive for ZO-1, but were negative for GLUT1 and occludin (Fig. 3). Blood vessels in the connective tissue surrounded by and adjacent to the outer surface of the perineuria, the inward extension of the epineurium, were all positive for GLUT1, occludin, and ZO-1, a similar staining pattern for these also appearing in the endoneurium (Fig. 2b). Examinations of semithin frozen sections at higher magnification revealed that GLUT1-positive signals in the perineurium comprised 4-7 lines each forming thin layers (Fig. 2d, e). Positive signals for occludin and ZO-1 were observed as dots and bars between adjacent perineurial cells. Examination of the surface of the perineurium in laser confocal microscopy revealed that occludin was localized in the cell borders of GLUT1-positive perineurial cells, forming stacks of meshworks (Fig. 2f).

Fig. 3. Immunofluorescence localization of occludin, ZO-1, and GLUT1 in the epineurium. Fluorescence micrographs (a, b, d, e) and corresponding Nomarski differential interference-contrast images (c, f) are shown. a-c. Double-labeling for occludin (a) and ZO-1 (b). The perineurium (P) is positive for occludin and ZO-1. A blood vessel in the surrounding epineurium is positive for ZO-1 but negative for occludin (arrowheads). d-f. Double-labeling for GLUT1 (d) and ZO-1 (e). The perineurium (P) is positive for GLUT1 and ZO-1. A blood vessel in the surrounding epineurium is positive for ZO-1 but negative for GLUT1 (arrowheads). Bars: 50 μ m

Fig. 2 c. ZO-1 (red) and GLUT1 (green) are colocalized in the perineurium (P) and the blood vessel (BV). Arrows and arrowheads indicate ZO-1 in the perineurium and blood vessels, respectively. d and e. Higher magnifications of the perineuria stained for occludin (red) and GLUT1 (green) (d), and for ZO-1 (red) and GLUT1 (green) (e). GLUT1-positive lines with dots for occludin (d) or ZO-1 (e) are seen (arrows). f. A laser confocal image for GLUT1 and occludin in the whole-mount sciatic nerve specimen. Consecutive optical sections were projected to generate an image from the nerve surface. Occludin (red) is seen as a meshwork surrounding GLUT1-positive (green) cells.

Fig. 4. Ultrastructural localization of GLUT1 in the sciatic nerve. Bars: 0.5 μm . **a.** Gold particles representing GLUT1 are seen along the plasma membranes of perineurial cells (*arrowheads*). **b.** GLUT1 is seen along the plasma membranes of endothelial cells of the blood vessel (*arrowheads*). *L* lumen

Immunoelectron microscopic localization of GLUT1, occludin, and ZO-1 in the sciatic nerve

Ultrathin frozen sections were immunolabeled by the colloidal gold method. In the perineurium, GLUT1 was localized along the plasma membranes of stacked layers of perineurial cells interlaced with collagen fibers (Fig. 4a). All the cell layers of perineurial cells individually connected by tight junctions were positive for GLUT1. In the blood vessels in the endoneurium, plasma membranes of endothelial cells were positive for GLUT1 (Fig. 4b). Occludin was located in

the tight junctions connecting the perineurial cells side by side (Fig. 5a). It was also present in the tight junctions between endothelial cells in the blood vessels in the endoneurium (Fig. 5b). ZO-1 was present in the tight junctions of perineurial cells and endothelial cells (Fig. 6).

Fig. 5. Ultrastructural localization of occludin in the sciatic nerve. **a.** Gold particles representing occludin are seen in the tight junction between perineurial cells (*arrowheads*). Bar: 0.5 μm . **b.** Occludin is localized in the tight junction of the endothelial cells of the blood vessel (*arrowheads*). L Lumen. Bar: 0.1 μm

DISCUSSION

We have demonstrated in this work the colocalization of tight junction proteins, occludin, and ZO-1, and a glucose transporter GLUT1, in the cells comprising the blood-nerve barrier, the perineurial cells and endothelial cells, in the rat sciatic nerve. Immunogold electron microscopy revealed that occludin and ZO-1 were localized to tight junctions in these cells, where GLUT1 was abundant along the plasma membranes.

Peripheral nerves are isolated from the free access of blood constituents by the blood-nerve barrier. Bundles of nerve fibers are covered by the multi-layered perineurial cells, which are connected by tight junctions (SHANTHAVEERAPPA and BOURNE, 1962; THOMAS, 1963). Tracer experiments using horseradish peroxidase showed that the perineurium serves

as a barrier layer (BÖCK and HANAK, 1971; OLSSON and REESE, 1971; WELCH and DAVSON, 1972). In the endoneurium of thick nerve fibers, endothelial cells of blood vessels connected by tight junctions also serve as barriers, similar to those in the brain (OLSSON, 1966; BÖCK and HANAK, 1971; OLSSON and REESE, 1971). These observations indicated that the perineurial cells and endothelial cells, each connected by tight junctions, represent the principal sites of the blood-nerve barrier. GLUT1 was found to be abundant in these cells (FROEHNER et al., 1988; HANDBERG et al., 1992). We have shown that presence of occludin is mostly restricted to the cells of these barriers: the perineurial cells and the endothelial cells of the blood vessels in the endoneurium. The presence of occludin in the cells of the barrier was reported in the microvessels in the brain (HIRASE et al., 1997). We also showed previously that pigment epithelial cells in the

Fig. 6. Ultrastructural localization of ZO-1 in the sciatic nerve. Bars: 0.5 μm . **a.** Gold particles representing ZO-1 are seen in tight junctions of perineurial cells (*arrowheads*). **b.** ZO-1 is localized in tight junctions of the endothelial cells of the blood vessel (*arrowheads*). *L* lumen

retina and non-pigmented epithelial cells in the ciliary body, both of which constitute parts of the blood-ocular barrier, were positive for occludin and GLUT1 (TSERENTSOODOL et al., 1998). Endothelial cells in the retina, another site of the blood-retinal barrier, were also positive for occludin and GLUT1, whereas blood vessels of non-barrier property were negative for these molecules (TSERENTSOODOL et al., 1998). These observations suggest occludin to be one of the key molecules of the barrier function of tight junctions, and the coexpression of occludin and GLUT1 in the

cells of the blood-tissue barrier may be important in the selective transfer of glucose across the barrier.

ZO-1 was colocalized with occludin and GLUT1 in the cells of the blood-nerve barrier. In addition, ZO-1 was present in the endothelial cells not serving as a barrier. The presence of ZO-1 in such non-barrier cells was reported in the mouse eye (TSERENTSOODOL et al., 1998). These observations suggest that ZO-1 may not be directly involved in the barrier function of the tight junction.

Cells not serving as barriers were negative for

GLUT1 and occludin except for endothelial cells in the connective tissue extension of the epineurium surrounded by perineuria. The permeability of these blood vessels has not been characterized, and the significance of GLUT1, occludin, and ZO-1 in these endothelial cells is unclear. One possible explanation for the resemblance of these cells to those of the barrier may be that the blood vessels surrounding bundles of perineuria could be provided with a milieu for the preferential expression of GLUT1 and occludin similar to the cells of the barrier.

Analyses of occludin-deficient cells have recently indicated that occludin is not the only integral membrane protein of tight junctions (SAITOU et al., 1998; FURUSE et al., 1998). In the occludin-null epithelial cells, tight junctions were formed, where ZO-1 was recruited. Functionally, these tight junctions serve as a diffusion barrier to small molecules (SAITOU et al., 1998). These observations have led to the discovery of claudins, which comprise another family of tight junction integral membrane proteins (FURUSE et al., 1998; MORITA et al., 1999; TSUKITA and FURUSE 1999). Recent observations suggests that a combination of occludin and claudin(s) may determine the characteristics of tight junctions, although the precise tissue distribution and the roles of claudins in the tight junction function remain to be investigated (TSUKITA and FURUSE, 1999). Although occludin may not be the prime molecule of the tight junction, it has been shown that the barrier function of the tight junction is affected by occludin (CHEN et al., 1997; WONG and GUMBINER, 1997). The present observations suggest that occludin may also be important in the function of the blood-nerve barrier.

In summary, glucose transporter GLUT1 and tight junction protein occludin are present in the cells of the perineurium and blood vessels in the endoneurium. They appear to constitute a mechanism for the selective transfer of glucose across the barrier while preventing the nonspecific flow of blood constituents.

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REFERENCES

- ANDERSON, J. M. and C. M. VAN ITALLIE: Tight junctions and the molecular basis for regulation of paracellular permeability. *Amer. J. Physiol.* 269: G467-G475 (1995).
- ANDERSON, J. M., B. R. STEVENSON, L. A. JESAITIS, D. A. GOODENOUGH and M. S. MOOSEKER: Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* 106: 1141-1149 (1988).
- ANDO-AKATSUKA, Y., M. SAITOU, T. HIRASE, M. KISHI, A. SAKAKIBARA, M. ITOH, S. YONEMURA, M. FURUSE and S. TSUKITA: Interspecies diversity of the occludin sequence: cDNA cloning of human, mouse, dog, and rat-kangaroo homologues. *J. Cell Biol.* 133: 43-47 (1996).
- BALDWIN, S. A.: Mammalian passive glucose transporters: members of an ubiquitous family of active and passive transport proteins. *Biochim. Biophys. Acta* 1154: 17-49 (1993).
- BELL, G. I., C. F. BURANT, J. TAKEDA and G. W. GOULD: Structure and function of mammalian facilitative sugar transporters. *J. Biol. Chem.* 268: 19161-19164 (1993).
- BÖCK, P. and H. HANAK: The distribution of exogenous peroxidase within the endoneural space. *Histochemie* 25: 361-371 (1971).
- CHEN, Y., C. MERZDORF, D. L. PAUL, and D. A. GOODENOUGH: COOH terminus of occludin is required for tight junction barrier in early *Xenopus* embryos. *J. Cell Biol.* 138: 891-899 (1997).
- CITI, S.: The molecular organization of tight junctions. *J. Cell Biol.* 121: 485-489 (1993).
- CITI, S., H. SABANAY, R. JAKES, B. GEIGER and J. KENDRICK-JONES: Cingulin, a new peripheral component of tight junctions. *Nature* 333: 272-276 (1988).
- DEMEY, J.: Colloidal gold as marker and tracer in light and electron microscopy. *EMSA Bull.* 14: 54-66 (1984).
- DENKER, B. M. and S. K. NIGAM: Molecular structure and assembly of the tight junction. *Amer. J. Physiol.* 274: F1-F9 (1998).
- FROEHNER, S. C., A. DAVIES, S. A. BALDWIN and G. E. LIENHARD: The blood-nerve barrier is rich in glucose transporter. *J. Neurocytol.* 17: 173-178 (1988).
- FURUSE, M., T. HIRASE, M. ITOH, A. NAGAFUCHI, S. YONEMURA, S. TSUKITA and S. TSUKITA: Occludin: a novel integral membrane protein localizing at tight junctions. *J. Cell Biol.* 123: 1777-1788 (1993).
- FURUSE, M., M. ITOH, T. HIRASE, A. NAGAFUCHI, S. YONEMURA, S. A. TSUKITA and S. H. TSUKITA: Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* 127: 1617-1626 (1994).
- FURUSE, M., K. FUJITA, T. HIRAGI, K. FUJIMOTO and S. TSUKITA: Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J. Cell Biol.* 141: 1539-1550 (1998).
- GUMBINER, B., T. LOWENKOPF and D. APATIRA: Identification of a 160-kDa polypeptide that binds to the tight junction protein ZO-1. *Proc. Nat. Acad. Sci. USA* 88: 3460-3464 (1991).
- HANDBERG, A., L. KAYSER, P. E. HØYER and J. VINTEN: A substantial part of GLUT-1 in crude membranes from muscle originates from perineurial sheaths. *Amer. J. Physiol.* 262: E721-E727 (1992).
- HASKINS, J., L. GU, E. S. WITTCHE, J. HIBBARD and B. R. STEVENSON: ZO-3, a novel member of the MAGUK protein family found at the tight junction, interacts with ZO-1 and occludin. *J. Cell Biol.* 141: 199-208 (1998).
- HIRASE, T., J. M. STADDON, M. SAITOU, Y. ANDO-AKATSUKA, M. ITOH, M. FURUSE, K. FUJIMOTO, S. TSUKITA

- and **L. L. RUBIN**: Occludin as a possible determinant of tight junction permeability in endothelial cells. *J. Cell Sci.* 110: 1603-1613 (1997).
- IZUMI, Y., T. HIROSE, Y. TAMAI, S. HIRAI, Y. NAGASHIMA, T. FUJIMOTO, Y. TABUSE, K. J. KEMPHUES and S. OHNO**: An atypical PKC directly associated and colocalizes at the epithelial tight junction with ASIP, a mammalian homologue of *Caenorhabditis elegans* polarity protein PAR-3. *J. Cell Biol.* 143: 95-106 (1998).
- KEON, B. H. S. SCHÄFER, C. KUHN, C. GRUND and W. W. FRANKE**: Symplekin, a novel type of tight junction plaque protein. *J. Cell Biol.* 134: 1003-1018 (1996).
- KIMURA, M., N. SAWADA, H. KIMURA, H. ISOMURA, K. HIRATA and M. MORI**: Comparison between the distribution of 7H6 tight junction-associated antigen and occludin during the development of chick intestine. *Cell Struct. Func.* 21: 91-96 (1996).
- MARTIN-PADURA, I., S. LOSTAGLIO, M. SCHNEEMANN, L. WILLIAMS, M. ROMANO, P., FRUSCELLA, C. PANZERI, A. STOPPACCIARO, L. RUCCO, A. VILLA, D. SIMMONS and E. DEJANA**: Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J. Cell Biol.* 142: 117-127 (1998).
- MORITA, K., M. FURUSE, K. FUJIMOTO and S. TSUKITA**: Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands. *Proc. Nat. Acad. Sci. USA* 96: 511-516 (1999).
- OLSSON, Y.**: Studies on vascular permeability in peripheral nerves. I. Distribution of circulating fluorescent serum albumin in normal, crushed and sectioned rat sciatic nerve. *Acta Neuropathol.* 7: 1-15 (1966).
- OLSSON, Y. and T. S. REESE**: Permeability of vasa nervorum and perineurium in mouse sciatic nerve studied by fluorescence and electron microscopy. *J. Neuropathol. Exp. Neurol.* 30: 105-119 (1971).
- SAITOU, M., Y. ANDO-AKATSUKA, M. ITOH, M. FURUSE, J. INAZAWA, K. FUJIMOTO and S. TSUKITA**: Mammalian occludin in epithelial cells: its expression and subcellular distribution. *Eur. J. Cell Biol.* 73: 222-231 (1997).
- SAITOU, M., K. FUJIMOTO, Y. DOI, M. ITOH, T. FUJIMOTO, M. FURUSE, H. TAKANO, T. NODA and S. TSUKITA**: Occludin-deficient embryonic stem cells can differentiate into polarized epithelial cells bearing tight junctions. *J. Cell Biol.* 141: 397-408 (1998).
- SATO, H., Y. ZHONG, H. ISOMURA, M. SAITOH, K. ENOMOTO, N. SAWADA and M. MORI**: Localization of 7H6 tight junction-associated antigen along the cell border of vascular endothelial cells correlates with paracellular barrier function against ions, large molecules, and cancer cells. *Exp. Cell Res.* 222: 269-274 (1996).
- SHANTHAVEERAPPA, T. R. and G. H. BOURNE**: The 'perineural epithelium', a metabolically active, continuous, protoplasmic cell barrier surrounding peripheral nerve fasciculi. *J. Anat. (Lond.)* 96: 527-537 (1962).
- SHIN, B.-C., T. SUZUKI, S. TANAKA, A. KURAOKA, Y. SHIBATA and K. TAKATA**: Connexin 43 and the glucose transporter, GLUT1, in the ciliary body of the rat. *Histochem. Cell Biol.* 106: 209-214 (1996).
- SIMON, D. B., Y. LU, K. A. CHOATE, H. VELAZQUEZ, E. AL-SABBAN, M. PRAGA, G. CASARI, A. BETTINELLI, G. COLUSSI, J. RODRIGUEZ-SORIANO, D. MCCREDIE, D. MILFORD, S. SANJAD and R. P. LIFTON**: Paracellin-1, a renal tight junction protein required for paracellular Mg^{2+} resorption. *Science* 285: 103-106 (1999).
- SLOT, J. W. and H. J. GEUZE**: A new method of preparing gold probes for multiple-labeling cytochemistry. *Europ. J. Cell Biol.* 38: 87-93 (1985).
- STEVENSON, B. R., J. D. SILICIANO, M. S. MOOSEDER and D. A. GOODENOUGH**: Identification of ZO-1: a high molecular weight polypeptide associated with the tight junction (Zonula occludens) in a variety of epithelia. *J. Cell Biol.* 103: 755-766 (1986).
- SUZUKI, T., T. MATSUZAKI and K. TAKATA**: Fluorescence counter-staining of cell nuclear DNA for multi-color laser confocal microscopy. *Acta Histochem. Cytochem.* 31: 297-301 (1998).
- TAKATA, K.**: Glucose transporters in the transepithelial transport of glucose. *J. Electron Microsc.* 45: 275-284 (1996).
- TAKATA, K. and S. J. SINGER**: Phosphotyrosine-modified proteins are concentrated at the membranes of epithelial and endothelial cells during tissue development in chick embryos. *J. Cell Biol.* 106: 1757-1764 (1988).
- TAKATA, K., T. KASAHARA, M. KASAHARA, O. EZAKI and H. HIRANO**: Erythrocyte/HepG2-type glucose transporter is concentrated in cells of blood-tissue barriers. *Biochem. Biophys. Res. Commun.* 173: 67-73 (1990).
- TAKATA, K., T. KASAHARA, M. KASAHARA, O. EZAKI and H. HIRANO**: Ultracytochemical localization of the erythrocyte/HepG2-type glucose transporter (GLUT1) in cells of the blood-retinal barrier in the rat. *Invest. Ophthalmol. Vis. Sci.* 33: 377-383 (1992).
- TAKATA, K., M. KASAHARA, Y. OKA and H. HIRANO**: Mammalian sugar transporters: their localization and link to cellular functions. *Acta Histochem. Cytochem.* 26: 165-178 (1993).
- TAKATA, K., H. HIRANO and M. KASAHARA**: Transport of glucose across the blood-tissue barriers. *Int. Rev. Cytol.* 172: 1-53 (1997).
- THOMAS, P. K.**: The connective tissue of peripheral nerve: an electron microscope study. *J. Anat. (Lond.)* 97: 35-44 (1963).
- TSERENTSOODOL, N., B.-C. SHIN, T. SUZUKI and K. TAKATA**: Colocalization of tight junction proteins, occludin and ZO-1, and glucose transporter GLUT1 in cells of the blood-ocular barrier in the mouse eye. *Histochem. Cell Biol.* 110: 543-551 (1998).
- TSUKITA, S. and M. FURUSE**: Occludin and claudins in tight-junction strands: leading or supporting players? *Trends Cell Biol.* 9: 268-273 (1999).
- WEBER, E., G. BERTA, A. TOUSSON, St. P. JOHN, M. W. GREEN, U. GOPALAKRISHNAN, T. JILLING, E. J. SORSCHER, T. S. ELTON, D. R. ABRAHAMSON and K. L. KIRK**: Expression and polarized targeting of a rab3 isoform in epithelial cells. *J. Cell Biol.* 125: 583-594 (1994).

- WELCH, K.** and **H. DAVSON**: The permeability of capillaries of the sciatic nerve of the rabbit to several materials. *J. Neurosurg.* 36: 21-26 (1972).
- WHEELER, T.J.** and **P.C. HINKLE**: The glucose transporter of mammalian cells. *Annu. Rev. Physiol.* 47: 503-517 (1985).
- WONG, V.** and **B.M. GUMBINER**: A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. *J. Cell Biol.* 136: 399-409 (1997).
- YAMAMOTO, T., N. HARADA, K. KANO, S. TAYA, E. CANAANI, Y. MATSUURA, A. MIZOGUCHI, C. IDE and K. KAIBUCHI**: The ras target AF-6 interacts with ZO-1 and serves as a peripheral component of tight junctions in epithelial cells. *J. Cell Biol.* 139: 785-795 (1997).
- ZAHRAOUI, A., G. JOBERTY, M. ARPIN, J. J. FONTAINE, R. HELLIO, A. TAVITIAN and D. LOUVARD**: A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. *J. Cell Biol.* 124: 101-115 (1994).
- ZHONG, Y., T. SAITOH, T. MINASE, N. SAWADA, K. ENOMOTO and M. MORI**: Monoclonal antibody 7H6 reacts with a novel tight junction-associated protein distinct from ZO-1, cingulin and ZO-2. *J. Cell Biol.* 120: 477-483 (1993).

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