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Molecular anatomy of interendothelial junctions in human blood-brain barrier microvessels

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Abstract: Immunogold cytochemical procedure was used to study the localization at the ultrastructural level of interendothelial junction-associated protein molecules in the human brain blood microvessels, representing the anatomic site of the blood-brain barrier (BBB). Ultrathin sections of Lowicryl K4M-embedded biopsy specimens of human cerebral cortex obtained during surgical procedures were exposed to specific antibodies, followed by colloidal gold-labeled secondary antibodies. All tight junction-specific integral membrane (transmembrane) proteins - occludin, junctional adhesion molecule (JAM-1), and claudin-5 - as well as peripheral zonula occludens protein (ZO-1) were highly expressed. Immunoreactivity of the adherens junction-specific transmembrane protein VE-cadherin was of almost similar intensity. Immunolabeling of the adherens junction-associated peripheral proteins - α-catenin, β-catenin, and p120 catenin - although positive, was evidently less intense. The expression of γ-catenin (plakoglobin) was considered questionable because solitary immunosignals (gold particles) appeared in only a few microvascular profiles. Double labeling of some sections made possible to observe strict colocalization of the junctional molecules, such as occludin and ZO-1 or JAM-1 and VE-cadherin, in the interendothelial junctions. We found that in human brain microvessels, the interendothelial junctional complexes contain molecular components specific for both tight and adherens junctions. It is assumed that the data obtained can help us find the immunodetectable junctional molecules that can serve as sensitive markers of normal or abnormal function of the BBB.

Key words: Interendothelial junctions - Blood-brain barrier - Immunogold cytochemistry - Tight junctions - Adherens junctions

Introduction

Interendothelial junctional complexes in brain blood microvessels are believed to be one of the main factors responsible for the tightness of the blood-brain barrier (BBB). Morphologically, this tightness is manifested by the presence of characteristic intimate contacts between plasma membranes of adjacent endothelial cells (ECs) visible at the level of ultrastructure and known as zonula occludens. As a result of the existence of continuous rows of these contacts, the intercellular cleft between adjacent ECs is obstructed. In effect, tight junctions (TJs) are formed that impose a passive, nonselective obstruction to solute exchange between blood and brain interstitial fluid [7]. It was shown that BBB maturation during development is paralleled by the formation of complex TJs between microvascular ECs [34].

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The presence of TJs in the brain microvascular endothelial layer is presumably responsible not only for its barrier function but also for the maintenance of cell polarity. The functional polarity (or asymmetry) of the brain vascular ECs is manifested morphologically by the different distribution of several enzymes and carriers between luminal and abluminal plasma membranes [10, 39]. Molecular components of TJs are considered important factors engaged in their fence function controlling the intramembrane diffusion and consequently maintaining the structural and functional polarity of the ECs [3, 4 , 7, 8].

There are several molecular components present in the TJs of various endothelial and epithelial cells. The most important among them are intrinsic, transmembrane proteins such as occludin, the family of junctional adhesion molecules (JAMs) found in different types of cells [2, 24, 31], and claudins [26, 27]. Occludin is the first and best-known integral membrane protein of TJs, which is believed to be directly involved in their barrier and fence functions [12, 13]. The TJ-associated peripheral zonula occludens protein (ZO-1) establishes a link

between the transmembrane proteins (e.g., occludin) and the actin cytoskeleton [11, 37].

In addition to the TJ-associated protein molecules, proteins are also associated with zonula adherens that form the adherens junctions (AJs). Several AJ-associated proteins were found in the endothelium of the rat brain blood microvessels representing the BBB [35]. These data indicate that in the endothelium of the BBB microvessels, not only TJs but also AJs are present. The structural and functional relations of both types of interendothelial junctions and their involvement in the formation and maintenance of the barrier function of brain blood microvessels are not yet well known [20, 25].

Similar to TJs, in the AJs, both transmembrane and peripheral protein molecules are present. The large family of cadherins represents the transmembrane proteins of AJs that interact homotypically in the presence of calcium ions [36]. Vascular endothelium cadherin (VE-cadherin or cadherin-5) is an important determinant of microvascular integrity both *in vitro* and *in vivo* and, together with catenins, forms the complex that functions as an early-recognition mechanism between ECs [29]. Although VE-cadherin is expressed in the blood vessel endothelium, its specific association with the BBB remains unclear, as emphasized by Rubin and Staddon [32].

A major role of catenins is to anchor the cadherin complex to the actin cytoskeleton, and in this respect, their role as peripheral proteins in AJs bears resemblance to that of ZO-1 in TJs. It is believed that β-catenin and γ-catenin (plakoglobin) link cadherin to α-catenin, which in turn couples the complex to the actin microfilament network of the cell cytoskeleton [30]. A new p120 catenin (pp120 or p120 cas) was recently identified whose role remains controversial in contrast to the above-mentioned classical catenins. High-affinity binding of the p120 catenin to VE-cadherin suggests that it can be engaged in regulation of vascular permeability and can in some way affect BBB function [1]. This problem is of great interest and needs further experimental work.

One should bear in mind that the data related to the presence and localization of the protein molecules (transmembrane and peripheral) associated with TJs and AJs were obtained on different types of epithelial and endothelial cells cultivated *in vitro* or obtained as biopsy specimens. Thus, it is not clear whether all new data on the molecular composition of intercellular junctions also relate to highly specialized cells that form the endothelial lining in the BBB type of brain blood microvessels.

Consideration of the data presented above prompted us to study the distribution at the ultrastructural level of the interendothelial junction-associated molecules in human brain blood microvessels representing the BBB. We applied the postembedding immunogold procedure to study the localization of TJ- and AJ-associated protein molecules with commercially available specific antibodies.

Materials and methods

Material. Biopsy specimens of human cerebral cortex, obtained during surgical procedures on four patients with brain tumors (meningioma), used previously for our cytochemical studies [38, 40], were also utilized for the present immunocytochemical trials. Small samples (1-2 mm^3) of normal-looking brain tissue were immersion-fixed at room temperature (RT; ca 22°C) in freshly prepared fixative containing 4% formaldehyde (prepared from paraformaldehyde), 0.1% glutaraldehyde, 0.15 M sucrose in 0.1 M cacodylate buffer, pH 7.4. After fixation (up to 2 h), tissue blocks were washed overnight in ice-cold 7.5% sucrose, dehydrated in ethanol with concomitant lowering of temperature to -35°C, and finally embedded in hydrophilic resin Lowicryl K4M. After polymerization under UV lamp in the low-temperature embedding apparatus TTP 010 (Balzers Union; Liechtenstein), the samples were cut with a diamond knife using an ultramicrotome (Sorvall MT-5000; DuPont).

Immunocytochemical procedure. Ultrathin sections attached to formvar-carbon-coated nickel grids were placed in a moist chamber at RT on a drop of 0.1 M glycine for 15 min to quench free aldehyde groups. The sections were incubated in a refrigerator (4°C) overnight (approximately 16 h) on a drop of primary antibody (see below) solution in TBS (pH 8.0) containing 1% bovine serum albumin. After washing with TBS, the sections were exposed for 1 h at RT to the solution of secondary antibody labeled with colloidal gold (see below). The diameters of gold particles used were as follows: 10 nm (shortly G10); 12 nm (G12); 15 nm (G15); and 18 nm (G18).

Occasionally, sections were immunostained with two different primary antibodies that were labeled with secondary antibodies of different size (diameter) of the colloidal gold particles. After several washings with TBS and glass distilled water, and staining with 4% uranyl acetate (3 min) and Reynold's lead citrate (1 min), the sections were examined in a Hitachi 7000 electron microscope.

Controls for the specificity of the immunostaining consisted of sections incubated with normal rabbit preimmune serum or with secondary antibodies only.

The following primary antibodies were used: rabbit polyclonal anti-occludin and anti-ZO-1, mouse monoclonal anti-γ-catenin (plakoglobin) and anti-claudin-5, all from Zymed Laboratories, Inc., San Francisco, CA, USA; rabbit polyclonal anti-α-catenin and anti-β-catenin, from Chemicon International, Inc. Temecula, CA, USA; goat polyclonal anti-human JAM-1 from R&D Systems, Inc., Minneapolis, MN, USA; rabbit polyclonal anti-VE-cadherin from Cayman Chem., Ann Arbor, MI, USA; and mouse monoclonal anti-p120 catenin (p120 cas) from BD Biosciences, San Diego, CA, USA. The following colloidal gold-labeled secondary antibodies were used: goat anti-rabbit (GAR-G15) and goat anti-mouse (GAM-G15), from Amersham Biosciences UK Ltd.; donkey anti-goat (DAG-G12 and DAG-G18) and goat anti-rabbit (GAR-G18), from Jackson Immunoresearch Lab. Inc., West Grove, PA , USA; and GAR-G10, from Sigma-Aldrich, St. Louis, MO, USA.

The labeling density of immunosignals represented by colloidal gold particles (GPs) was expressed as the number of GPs per µm of length of interendothelial junction. The details of the label quantification were described previously [41]. The results of the labeling density quantification are presented in Table 1. Due to small number of vascular profiles that were perfectly suitable for morphometry, the significance of differences between labeling densities of the particular junctional molecules was not statistically evaluated.

Results

Tight junction-associated protein components

Occludin. Expression of occludin, evidenced by the density of immunosignals represented by colloidal GPs,

was the highest among all junctional proteins under study. Morphometric analysis of 10 randomly chosen interendothelial junctional complexes gave values from 3.16 to 6.9 GPs per μm of junctional length (see Table 1). In longitudinally (Fig. 1) or more obliquely sectioned interendothelial junctions (Fig. 2), numerous GPs were associated with the plasma membranes of adjacent ECs or appeared inside the junctional clefts. The GPs located inside the junctional cleft were especially conspicuous in short segments of transversely sectioned junctions (Fig. 3). In the majority of examined junctional complexes, the shorter or longer segments of the EC plasma membranes delimiting the interendothelial clefts, together with adjacent cytoplasm, revealed increased density and were recognized as perijunctional plaques (*e.g.*, in Fig. 3).

Junctional adhesion molecules (JAM-1). Immunosignals for JAM-1 represented by colloidal GPs were distributed unevenly in the interendothelial junctions. In some junctional segments, numerous GPs were located inside the interendothelial clefts. Some GPs were scattered singly, whereas others formed small clusters composed of three to six particles (Fig. 4). In many vascular profiles, however, only a few GPs were associated with perijunctional plaques and with adjacent plasma membranes, or were located inside the interendothelial cleft (Fig. 5). Morphometric analysis gave a mean value for labeling density of 3.31 GPs/μm, *i.e.*, somewhat lower than that for occludin (Table 1).

Claudin-5. The distribution of immunosignals for claudin-5 was similar to that for occludin and JAM-1, although the density of immunolabeling was slightly lower (2.94 GP/μm). In some vascular profiles, solitary GPs were almost uniformly at relatively even space intervals, scattered along the interendothelial junctions (Fig. 6). A few GPs were occasionally clustered inside or in close proximity to the junctional cleft (Fig. 7). In several longitudinally sectioned junctions, long segments were unlabeled, whereas a few GPs were present in the vicinity of luminal or abluminal estuary of the interendothelial cleft.

Zonula occludens protein (ZO-1). Immunosignals for ZO-1 were frequently grouped in short segments of the interendothelial junctions, leaving long segments of these junctions unlabeled (Fig. 8). In many vascular profiles, however, the entire length of the sectioned junction was labeled with several GPs, located usually in the perijunctional plasma membranes and in cytoplasmic plaques (Fig. 9). The density of immunolabeling was relatively high, giving mean value of 6.48 GPs/μm.

Colocalization of ZO-1 and occludin. The structural relations of the transmembrane proteins represented by occludin immunolabeled with G10 and peripheral protein represented by ZO-1 labeled with G18 was judged by double immunogold labeling. As shown in Figure 10, immunosignals for occludin were mainly located inside or in close proximity to the interendothelial cleft. In contrast, immunosignals for ZO-1 represented by larger GPs (18 nm) were located mostly more peripherally, although a few of them were occasionally present inside the interendothelial cleft in close association with occludin.

Adherens junction-associated protein components

VE-Cadherin. Immunosignals for VE-cadherin were numerous, but they were irregularly scattered along the interendothelial junctions, leaving their shorter or longer segments unlabeled (Figs. 11 and 12). The majority of GPs were located inside or in close proximity to the interendothelial cleft. In some capillary profiles, several GPs were associated only with a relatively short segment of the junctional complex (Fig. 12). In spite of this irregularity, the mean value for the labeling density of 5.07 GPs/μm was relatively high.

Colocalization of VE-cadherin and JAM-1. The structural relation of transmembrane proteins representing different types of junctions was judged by examining the localization of immunosignals for VE-cadherin (G18) representing the AJs, and JAM-1 (G12) representing the TJs. As shown in Figure 13, both proteins were closely associated with the interendothelial junctions, although larger GPs (VE-cadherin) revealed some tendency to more peripheral distribution than smaller immunosignals for JAM-1 which were located mainly in the interendothelial cleft.

α-Catenin. The density of immunogold labeling for α-catenin was significantly lower than that for VE-cadherin. In the majority of microvascular profiles, only 2-4 GPs per sectioned interendothelial junction were present. These immunosignals were located mainly peripherally in perijunctional plaques or even in the vicinity of the EC plasma membrane (Fig. 14). The mean value of the labeling density for α-catenin was 1.79 GPs per μm of junctional length.

β-Catenin. The distribution of immunosignals for β-catenin was similar to that for α-catenin, although the density for immunogold labeling was somewhat lower, yielding the value of 1.41 GPs/μm. The immunosignals represented by a few GPs were usually scattered along the short segments of the junctional cleft (Fig. 15) and frequently were associated with the EC plasma membranes or with perijunctional plaques.

γ-Catenin (Plakoglobin). Immunoreactivity of γ-catenin appeared in some microvascular profiles only and was represented by one or two GPs located inside or in close proximity to the interendothelial cleft (Fig. 16). In these microvessels, the density of immunolabeling was low, approximately 0.77 GP/μm. Since in the majority of microvascular profiles no immunosignals were present, these results were considered questionable.

p120 Catenin. Immunosignals for p120 were found in almost all microvascular profiles examined, although the density of immunolabeling was low, yielding values between 0.86 and 0.92 GP/μm. The localization of GPs was similar to that observed for α-catenin and β-catenin, *i.e.*, they were scattered irregularly along the interendothelial junctions (Fig. 17). Occasionally, solitary GPs were located inside the junctional cleft or were associated with EC plasma membranes or perijunctional plaques.

Discussion

The main findings of our studies are as follows. (a) The interendothelial junctional complexes of the human brain blood microvessels representing the BBB contain all three TJ-specific integral membrane (transmembrane) proteins. The protein molecules occludin, JAM-1, and claudin-5 colocalize with ZO-1, which is considered the primary junctional peripheral membrane protein. (b) In these junctional complexes, several AJ-specific molecular components, such as transmembrane protein VE-cadherin, and the peripheral proteins α-catenin, β-catenin, and p120 catenin are also present. (c) The presence of the above-mentioned, highly expressed proteins in the microvascular interendothelial junctions allows us to assume that some of them can serve as sensitive biomarkers of normal or abnormal function of the BBB. These points deserve additional comments and discussion.

(a) The presence of all of the above-mentioned transmembrane components of TJs in the ECs of human BBB microvessels is of great interest because these proteins were found originally in other species and in other types of cells. Occludin, the first known TJ-specific integral membrane protein, was identified, characterized biochemically, and localized at the ultrastructural level in chicken intestinal epithelial and liver cells [12]. Its ultrastructural localization in the interendothelial junctions of BBB microvessels of animals (porcine) was observed later by Hirase *et al.* [17]. JAM was originally identified in vascular ECs from mouse heart and lungs and in epithelial cells growing *in vitro.* This protein was also obtained from mouse duodenum, where its ultrastructural localization was observed for the first time [24]. Claudin family members were originally identified in cultured MDCK transfectants and in sections of mouse liver and kidney [26], whereas claudin-5 was found in mouse lungs, kidney, intestine, and brain [27].

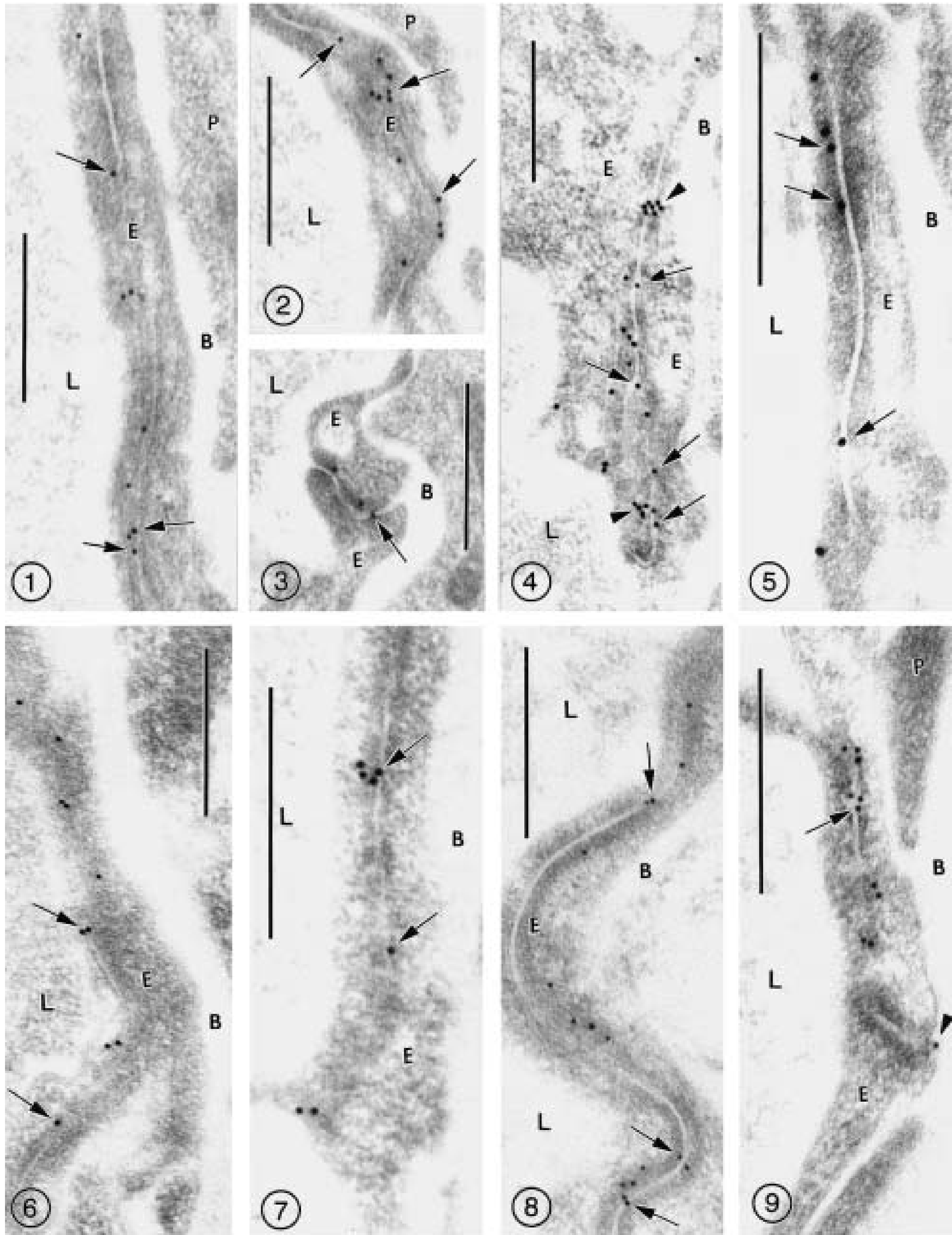
Our observation of double-labeled brain sections revealed a close structural relation of TJ-specific transmembrane protein occludin to membrane peripheral protein ZO-1. Closely related to ZO-1, which is a phosphoprotein with a molecular mass of 210-225 kD, are 160-kD molecules known as ZO-2 [16], and 130-kD molecules, known as ZO-3 [19]. Only the localization of ZO-1 was studied in our present work because the efficient antibodies for ZO-2 and ZO-3 were not com-

Table 1. Cumulative results of immunogold labeling density distribution for molecular components of interendothelial junctions in human brain capillaries

Type of junction	Junctional protein	Labeling density*
Tight Junctions	*Transmembrane:*	
	Occludin	5.24 (3.16 - 6.90)
	JAM-1	3.31 (2.38 - 4.33)
	Claudin-5	2.94 (2.03 - 4.40)
	Peripheral:	
	ZO-1	6.48 (5.61 - 7.75)
Adherens Junctions	*Transmembrane:*	
	VE-Cadherin	5.07 (3.90 - 6.50)
	Peripheral:	
	α-Catenin	1.79 (1.45 - 2.51)
	β-Catenin	1.41 (1.36 - 1.47)
	γ-Catenin	0.77 (0.71 - 0.86)
	p120	0.88 (0.86 - 0.92)

*Means and ranges (in parentheses) of gold particle counts per one μm of the length of the interendothelial junctions are shown. Each value is based on 10 microvascular profiles examined.

Fig. 1. A longitudinal section of the wall of a blood microvessel (capillary) from the human cerebral cortex showing many immunosignals for occludin, represented by colloidal gold particles of 15-nm diameter (G15). The majority of immunosignals are located inside or at close proximity to the interendothelial junctional cleft (arrows). The following abbreviations (symbols) are used in this and in all other figures: B, basement membrane; E, endothelial cell; L, vessel lumen; P, pericyte; R, red blood cell. Scale bars = 0.5 μm. × 60,000. **Fig. 2.** Cross-sectioned capillary shows numerous immunosignals for occludin associated with the interendothelial junction (arrows). × 60,000. **Fig. 3.** In this cross-sectioned capillary wall, the immunosignals for occludin are located inside (arrow) or in close proximity to the interendothelial junctional cleft. The cytoplasm of the endothelial cell (E) adjacent to the interendothelial junction forms characteristic plaques showing increased density. × 60,000. **Fig. 4.** Section of the capillary wall with numerous immunosignals for JAM-1. Several gold particles (G18) are located in the junctional cleft (arrows). Gold particles occasionally form small aggregates (arrowheads). × 51,000. **Fig. 5.** A longitudinally sectioned interendothelial junction of the capillary incubated for immunogold localization of JAM-1 is shown under high magnification. Immunosignals represented by colloidal gold particles (G18) are closely associated with the interendothelial cleft (arrows). × 90,000. **Fig. 6.** A portion of the capillary wall with several immunosignals (arrows) for claudin-5 scattered along slightly tangentially sectioned interendothelial junction. × 60,000. **Fig. 7.** Immunosignals (G15) for claudin-5 (arrows) associated with the interendothelial junction in the capillary wall. × 90,000. **Fig. 8.** Longitudinally sectioned wall of the capillary showing numerous immunosignals for ZO-1 (G10) associated with some segments of the interendothelial junction (arrows). × 68,000. **Fig. 9.** In this cross-sectioned capillary wall, several immunosignals for ZO-1 are located in close proximity to the interendothelial junction (arrows). Single gold particle appears also at the abluminal estuary of the interendothelial cleft (arrowhead). × 80,000.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mercially available. Presumably, this limitation does not affect the value of our findings because both satellite molecules ZO-2 and ZO-3 are smaller and are attached to the larger molecule of ZO-1. Thus, one can expect that the localization of all these ZO molecules is identical. It is noteworthy that strict colocalization of ZO-1 with TJ-specific transmembrane proteins such as occludin, claudin-5, and ESAM (JAM-related endothelial cell-selective adhesion molecule) in mouse brain capillaries was recently documented by immunogold electron microscopy [28].

(b) Our observations revealed the presence of AJ-specific proteins in human BBB-type brain blood microvessels. The most interesting is the high expression of transmembrane protein VE-cadherin, which is considered an important determinant of microvascular integrity both *in vivo* and *in vitro*. Together with catenins, VE-cadherin forms the complex that functions as an early-recognition mechanism between endothelial cells [21]. In spite of its high expression in human brain microvessels, VE-cadherin is not specifically associated with BBB function, as emphasized by Rubin and Staddon [32], but instead, appears to be a generally occurring component of interendothelial junctions in various types of vessels, including retothelial cells of lymph nodes [33].

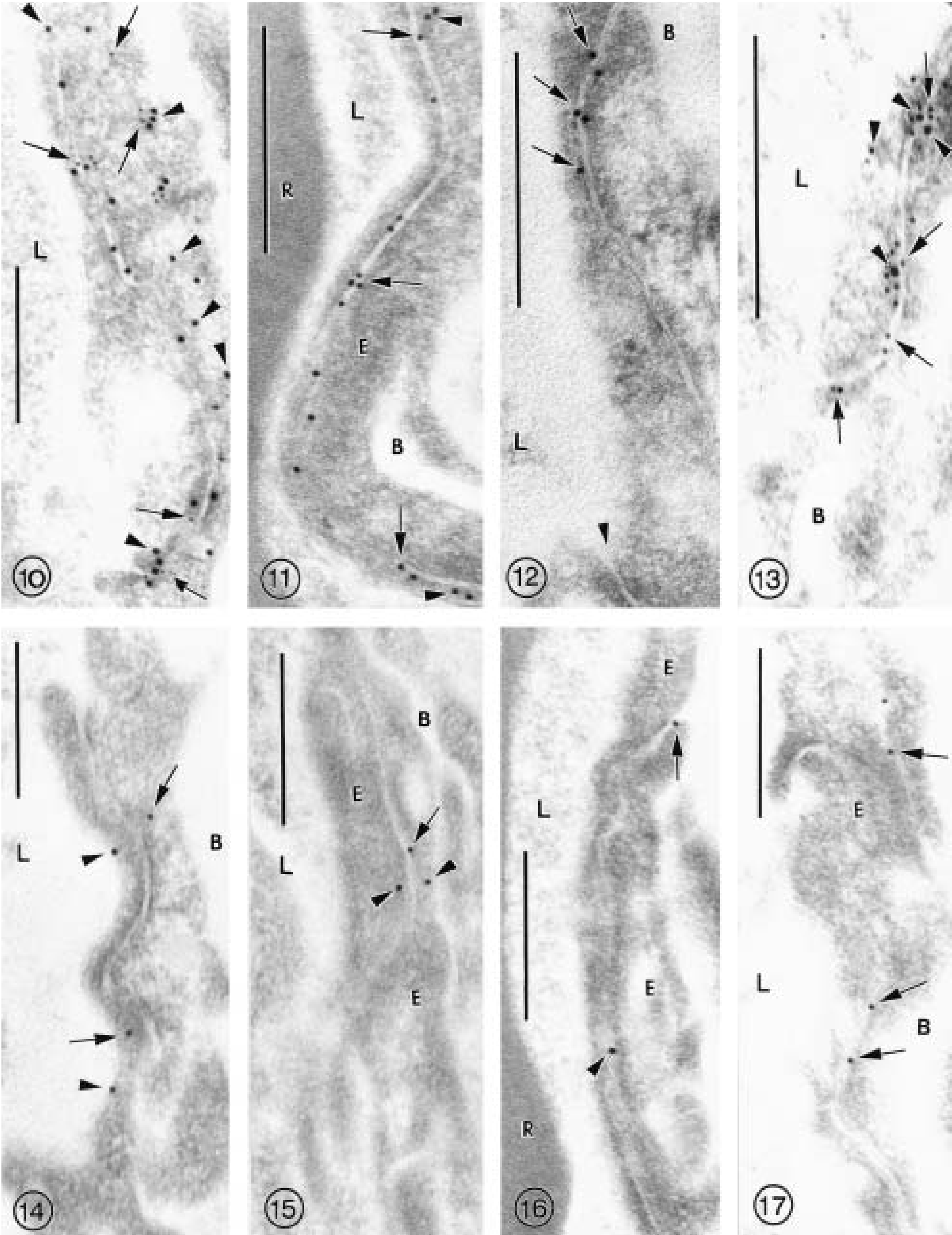
The AJ-specific membrane peripheral proteins of the catenin family (α-catenin, β-catenin, and p120) are expressed in human BBB capillaries less intensely than VE-cadherin. Presumably, the epitopes of these antigens are more sensitive to the fixatives used or to the embedding procedure than is VE-cadherin.

The close spatial association of AJ-specific and TJ-specific proteins indicates that both types of junctions are present in the endothelial lining of BBB-type human brain vasculature. This assumption is compatible with the results of immunogold study of Schulze and Firth [35] on rat BBB microvessels. Although these authors used antibodies to other AJ-associated proteins such as pan-cadherin, N-cadherin, α-actinin, vinculin, and zyxin, they concluded that in BBB endothelium, the complex TJ is embedded in an AJ that occupies the entire length of the junctional cleft. The results of our previous studies performed on mouse, rat, and human brain samples [41, 42] also led us to the conclusion that both junctional types, *i.e.*, TJs and AJs, are intermingled in the interendothelial junctions present in BBB-type blood microvessels.

This conclusion relates at least to mammalian brain capillaries, because in chicken BBB microvessels, the location of cadherin and catenins is different. Gerhardt *et al.* [14] observed that in developing and maturing chicken BBB, N-cadherin is localized in the EC abluminal plasma membrane. Because the similar location of β-catenin in chicken BBB was observed by Liebner *et al.* [23], one can assume that during development and maturation of the BBB, these proteins are responsible for anchorage of the endothelium to other perivascular cell types such as pericytes or smooth muscle cells. The γ-catenin, whose expression was found to be very low (even questionable) in human brain microvessels, is highly expressed in the interendothelial junctions of chicken BBB vasculature [23]. These data suggest the existence of some species-related differences in the presence and localization of molecular components of interendothelial junctional complexes in BBB-type blood microvessels. The elucidation of this interesting problem requires further experimental studies.

(c) One of the objectives of this work was to determine the interendothelial junction-associated protein molecules that can serve as sensitive markers of normal and/or disturbed barrier function of brain microvessels. Understanding of the structural-functional correlations between molecular components of the junctional complexes in normal, experimental, and pathological conditions can provide important information on the pathogenesis of various neurodegenerative diseases. One can assume that the pathological insult that disturbs the barrier function also destroys or changes the molecular architecture of the interendothelial junctional complexes in the affected vessel. As emphasized by Gloor *et al.* [15], there is little information about the involvement of endothelial TJ proteins in diseases of the central nervous system. On the other hand, however, several

Fig. 10. A portion of the capillary wall showing colocalization of occludin (G10) and ZO-1 (G18). Immunosignals for occludin (arrows) are located mainly inside or in close proximity to the interendothelial cleft, whereas immunosignals for ZO-1 (arrowheads) are located mostly more peripherally. × 75,000. **Fig. 11.** In this longitudinally sectioned interendothelial junction of brain capillary, the immunosignals for VE-cadherin are localized inside the interendothelial cleft (arrows) or in the adjacent perijunctional plaques (arrowheads). × 80,000. **Fig. 12.** Immunosignals for VE-cadherin are gathered in the segment of the interendothelial junction (arrows), whereas the remaining segment of the junctional cleft remains unlabeled, including its luminal estuary (arrowhead). × 90,000. **Fig. 13.** Colocalization of JAM-1 (G12) and VE-cadherin (G18) in the wall of brain capillary. The immunosignals for JAM-1 (arrows) are located mostly inside the interendothelial cleft, whereas some immunosignals for VE-cadherin (arrowheads) are scattered more peripherally. × 100,000. **Fig. 14.** In this capillary profile, a few immunosignals for α-catenin are associated with the interendothelial junction (arrows) or with the EC plasma membrane (arrowheads). × 75,000. **Fig. 15.** In this capillary profile, only solitary immunosignals for β-catenin appear inside the junctional cleft (arrow) or in the perijunctional EC cytoplasm (arrowheads). × 60,000. **Fig. 16.** Longitudinally sectioned capillary wall showing only two immunosignals for γ-catenin (plakoglobin) in the abluminal estuary of the interendothelial junctional cleft (arrow) and in the perijunctional EC cytoplasm (arrowhead). × 60,000. **Fig. 17.** In this capillary profile, a few immunosignals for p120 catenin are scattered along the interendothelial cleft (arrows). × 60,000.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interesting data indicate that diseases and conditions that affect brain function, such as multiple sclerosis, HIV encephalitis, Alzheimer's disease, and stroke, perturb BBB integrity with subsequent increase in vascular permeability [18].

The above-mentioned markers are indeed in demand in the light of growing evidence indicating that disturbed barrier function of brain microvessels is paralleled by altered expression of junctional proteins, *e.g.*, occludin and ZO-1 [5], claudin-1 and claudin-5 [22], or mainly ZO-1 [6, 9].

The data presented by the authors mentioned above as well as our own observations suggest that TJ-specific transmembrane proteins such as occludin, claudin-5 and JAM-1, together with peripheral protein ZO-1, can be considered valuable and efficient markers of the integrity of the interendothelial junctional complex in human BBB. Occludin and ZO-1 appear to be the most reliable because these proteins survive fixation and embedding at low temperature in Lowicryl K4M and give reproducible and intense immunogold labeling at the ultrastructural level. The validity of these markers should be evaluated in the course of further studies performed on human brain biopsy specimens and on laboratory (eventually transgenic) animals.

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