Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells

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Endothelial cells of the blood-brain barrier form complex tight junctions, which are more frequently associated with the protoplasmic (P-face) than with the exocytoplasmic (E-face) membrane leaflet. The association of tight junctional particles with either membrane leaflet is a result of the expression of various claudins, which are transmembrane constituents of tight junction strands. Mammalian brain endothelial tight junctions exhibit an almost balanced distribution of particles and lose this morphology and barrier function in vitro. Since it was shown that the brain endothelial tight junctions of submammalian species form P-face-associated tight junctions of the epithelial type, the question of which molecular composition underlies the morphological differences and how do these brain endothelial cells behave in vitro arose. Therefore, rat and chicken brain endothelial cells were investigated for the expression of junctional proteins in vivo and in vitro and for the morphology of the tight junctions. In order to visualize morphological differences, the complexity and the P-face association of tight junctions were quantified. Rat and chicken brain endothelial cells form tight junctions which are positive for claudin-1, claudin-5, occludin and ZO-1. In agreement with the higher P-face association of tight junctions in vivo, chicken brain endothelia exhibited a slightly stronger labeling for claudin-1 at membrane contacts. Brain endothelial cells of both species showed a significant alteration of tight junctions in vitro, indicating a loss of barrier function. Rat endothelial cells showed a characteristic switch of tight junction particles from the P-face to the E-face, accompanied by the loss of claudin-1 in immunofluorescence labeling. In contrast, chicken brain endothelial cells did not show such a switch of particles, although they also lost claudin-1 in culture. These results demonstrate that the maintenance of rat and chicken endothelial barrier function depends on the brain microenvironment. Interestingly, the alteration of tight junctions is different in rat and chicken. This implies that the rat and chicken brain endothelial tight junctions are regulated differently.

Abbreviations. CBE Chicken brain endothelial cell. – E-face Exocytoplasmic fracture face. – EFA E-face association. – P-face Protoplasmic fracture face. – PFA P-face association. – RBE Rat brain endothelial cell. – TJ Tight junction. – ZO-1 Zonula occludens protein 1.

Introduction

The blood-brain barrier (BBB) of higher vertebrates is located in the endothelial cells of the brain microvessels. On the one hand, its function is to protect the fragile physiological equilibrium of the brain from blood-borne substances, and on the other to ensure the supply of nutrients via specific transport systems. It is known that the BBB changed phylogenetically from a glial (invertebrates, elasmobranchs) to an endothelial (teleost and higher vertebrates) barrier (Abbott, 1991), which gradually develops during ontogeny under the influence of the neuroectodermal milieu (Engelhardt and Risau, 1995). An intact microenvironment is essential for the maintenance of the endothelial barrier, as was demonstrated by transplantation experiments using chick-quail-chimeras (Stewart and Wiley, 1981).

The basis for the mature BBB are elaborate tight junctions (TJs), which continuously connect the endothelial cells, thereby separating the extracellular compartments of the neural paremchyma and the microvasculature. In freeze-fracture

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replicas, BBB-TJs are characterized by highest structural complexity of the TJ network and predominant association of TJ strands or particles with the protoplasmic (P-face) membrane leaflet (Tsukita and Furuse 1999; Wolburg et al., 1994). In contrast, non-barrier endothelial TJs show low complexity and TJ particles, which are almost exclusively found on the extracellular fracture face of the membrane bilayer (E-face) (Mühleisen et al., 1989).

Wolburg et al. (1994) demonstrated in a bovine in vitro model of the BBB that endothelial cells lose their physiological barrier properties in correlation with a decrease in the P-face association (PFA) of TJ particles and of junctional complexity. Therefore, it is conceivable that the association of particles with either membrane leaflet is a morphological parameter, which reflects the physiological quality of the tight junction. It is known that epithelial cells form TJs, which are almost completely P-face-associated exhibiting a complex junctional network with a smooth appearance of strands (Kniesel and Wolburg, 2000). They also retain their high PFA in vitro and, furthermore, barrier properties indicated by a high transepithelial resistance (TER) are comparable to the in vivo situation.

The hypothesis that a high PFA correlates with the physiological tightness of the TJ is further supported by the characteristics of the two strains of an epithelial cell line. Madine Darby canine kidney (MDCK) cells of strain I (MDCKI) possess a high PFA combined with a high transepithelial resistance (TER), whereas strain II cells (MDCKII) possess a lower PFA and a considerable lower TER (Löhken, 1995). In this context, it is of particular interest that endothelia of lizards and birds form TJs which morphologically would be classified as "epithelial", as they share a high PFA of ≈85% and a smooth, continuous strand appearance (Liebner et al., 1997; Shivers, 1979). Therefore, the question arose as to how chicken brain endothelial cells are regulated in vitro and if they retain BBB properties.

The molecular composition of TJs has been considerably elucidated in recent years. Occludin was the first transmembrane TJ protein discovered. Its cytoplasmic C-terminus was shown to interact with the cytoskeleton via linker proteins of the MAGUK family, zonula occludens protein 1/2/3 (ZO-1/-2/-3) (Furuse et al., 1993, 1994; Haskins et al., 1998; Itoh et al., 1999; Stevenson et al., 1988). However, evidence that occludin is not the major structural TJ protein was corroborated by the finding that occludin-deficient stem cells bear morphologically normal TJs (Saitou et al., 1998). Thereafter, new TJ proteins, claudin-1/-2, were identified, which were shown to belong to a new gene family of at least 16 members (Furuse et al., 1998a,b, 1999; Morita et al., 1999a; Simon et al., 1999). Like occludin, claudin 1–8 have the ability to directly interact with ZO-1/-2/-3 but in contrast, other binding domains are involved (Itoh et al., 1999a,b).

Interestingly, distinct classes of TJs are defined by the expression of different claudins (Furuse et al., 1998a,b, 1999). In mammalian endothelial cells, claudin-1 and claudin-5 were the only claudin members to be identified, of which claudin-5 was shown to be endothelial-specific (Morita et al., 1999b). In a transfection model, claudin-1 induced P-face-associated TJs, whereas claudin-5 supported the formation of E-face-associated TJs (Morita et al., 1999b).

In contrast to the recent advances in knowledge of the molecular composition of TJs, endothelial TJs of submammalian vertebrates are widely unknown, although for example the chicken, and more recently the zebrafish, are accepted models in the field of angiogenesis research.

The aim of this study was to elucidate the differences between rat and chicken endothelial tight junctions on morphological and molecular levels, and to gain further insight into the mechanisms of tight junction regulation by comparing these different models.

Materials and methods

All chemicals were purchased from Merck, Darmstadt, Germany, unless otherwise stated.

Species and developmental stages

Fertilized eggs of White Leghorn chicken (*Gallus domesticus*) were incubated at 37.8 °C and 65% humidity in a forced-draft incubator (Ehret, Emmendingen, Germany). For investigations, freshly hatched chicken (P0) were sacrificed by decapitation. Brains were immediately removed and prepared for immunohistochemistry, immunocytochemistry, biochemistry or cell culture.

Accordingly, three-week-old rats (Wistar) were sacrificed by inhalation of CO_2. Subsequently, the brains were removed and prepared for immunohistochemistry, immunocytochemistry, biochemistry or cell culture.

Antibodies

To obtain a polyclonal antibody against mouse claudin-5/TMVCF, a polypeptide (SAPRRPTANGDYDKKNYV), which corresponds to the COOH-terminal cytoplasmic domain of mouse claudin-5/TMVCF was synthesized as a MAP (multiple antigenic peptide) construct on an 8-branched resin (Tam, 1988). The immunogen was used to raise antibodies by Charles River Laboratory Service System (Kisslegg, Germany). As the last four amino acids of claudin-5/TMVCF and claudin-6 are identical, the antiserum was affinity purified against a polypeptide (SAPRRPTANGDYDK) lacking the four amino acids. For this purpose, the polypeptide was coupled to a modified Fractogel™ matrix (Merck, Darmstadt, Germany) (Kalbacher et al., 1999). To improve accessibility for the antibody, three spacer molecules (ε-amino caproic acid, β-alanin, β-alanin) were inserted between the peptide and the matrix. The optimized matrix was used for antibody purification.

Additionally, the following specific primary antibodies were used for immunohisto-/-cytochemistry: rabbit polyclonal human-occludin and rabbit polyclonal human-claudin-1 antisera (Zymed; South San Francisco, USA); rat monoclonal chicken-occludin (Oc-2) antibody kindly provided by S. Tsukita.

Secondary antibodies for immunofluorescence labeled with dichlortriazinylamino fluorescein (DTAF) and cyanin-derivative dyes Cy2™ and Cy3™ were purchased from Dianova, Hamburg, Germany.

For controls, the primary antibodies were omitted or substituted by unspecific IgG. For double labeling, controls included crossover incubation in order to exclude cross-reaction.

Immunohistochemistry

Immunostaining on paraformaldehyde-fixed, and on unfixed specimens was performed as described in Gerhardt et al. (1996). Specimens were immersion fixed for 2 hours on ice in 4% freshly depolymerized paraformaldehyde in HMSS (0.1 M HEPES, Sigma, Deisenhofen, Germany; 0.15 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). Subsequently, they were transferred to a graded series of 12%, 15%, and 18% sucrose in HMSS, embedded in Tissue Tek O.C.T., frozen in liquid nitrogen, and stored at −80 °C.

Cryostat sections (8–12 µm thick) were cut using a Reichert-Jung Frigocut 2800 E cryotome (Reichert, Vienna, Austria), before being mounted on glass slides coated with poly-L-lysine (Sigma), and dried. Sections were postfixed in 4% paraformaldehyde/HMSS on ice and washed in Tris-buffered saline (pH 7.6) containing 1 mM $CaCl_2$ (TBS).

Unfixed specimens were sectioned and mounted on glass slides as described above. Sections were fixed for 5 min in ethanol at 4 °C followed by 1 min in acetone at room temperature, and washed in TBS. Labeling procedures were identical for both ethanol/acetone fixed sections and paraformaldehyde fixed sections.

Unspecific binding was minimized by incubation for 20 min in 5% (w/v) skimmed milk, 0.3% (v/v) Triton X-100 (Serva, Heidelberg, Germany), and 0.04% (w/v) NaN_3 in TBS. Antibodies were diluted in the same solution and incubated overnight at 4 °C. Following several washes in TBS, sections were incubated with secondary antibodies for 1 h at room temperature. To visualize cell nuclei, sections were washed in TBS and counterstained with nuclear dye Hoechst 33258 (Sigma). Sections were mounted in 90% glycerol/10% TBS containing 1 mg/ml p-phenylene diamine (Sigma) as anti-bleaching agent. Fluorescence was visualized with a confocal laser-scanning microscope (Zeiss, Oberkochen, Germany). Images were computer processed using Adobe Photoshop (Adobe, Mountain View, CA).

Conventional electron microscopy

For morphological studies, specimens were fixed and embedded as described previously (Gerhardt et al., 1996; Liebner et al., 1997). Briefly, specimens were immersion fixed in 2.5% glutaraldehyde in HMSS, postfixed in 1% OsO_4 in 0.1 M cacodylate buffer and dehydrated in a series of ethanol. The 70% ethanol step was saturated with uranyl acetate for contrast enhancement. Dehydration was completed in propyleneoxide and specimens were embedded in Araldite (Serva, Heidelberg, Germany). Ultrathin sections were mounted on pioloform-coated copper grids and contrasted with lead citrate. Specimens were observed and documented with a CEM 902A electron microscope (LEO, Oberkochen, Germany).

Freeze-fracture analysis

The tissue specimens were immersion-fixed with 2.5% buffered glutaraldehyde, cryoprotected for freeze-fracture in 30% glycerol, and quick-frozen in nitrogen-slush (−210 °C). Subsequently, the specimens were fractured in a Balzer's freeze-fracture unit (BAF 400D) at 5×10^{-6} mbar and −150 °C and the fracture faces were shadowed with platinum/carbon (10:1; 2 nm; 45°) for contrast enhancement, and with carbon (20 nm; 90°) for stabilization. After removal of residual tissue material in 12% sodium hypochlorite, the replicas were cleaned several times in double distilled water and mounted on formvar-coated copper grids. The preparations were investigated using a Zeiss EM10 electron microscope.

Morphometrical analysis of the tight junction network

Quantitative analysis was done at a final magnification of 120000:1. Tight junction complexity was characterized by fractal analysis and complexity index (Kniesel et al., 1994). In brief, for evaluation of the fractal dimension (FD) the electron microscopic image was digitized, five grids of different scaling-levels (grid-sizes) were superimposed and for each scaling-factor (s) the number of boxes containing parts of the tight junction structure were counted (N) in repeated measurements. The grid sizes were 0.2, 0.1, 0.05, 0.025, and 0.0125 μm^2. Since the definition of the FD (box counting) is log N/log (1/s), the values obtained for each scaling-level were inserted into a log N vs. log (1/s) graph for visualization, and the regression curve was calculated. The slope of the curve gives the estimated value for the FD. For Figs. 1 and 3, the FD values which are in the range between 1 and 2, were given in percent (1 corresponds to 0% and 2 to 100%).

The degree of membrane association of tight junction particles was determined as the ratio "total length of E- or P-face-associated tight junction particles or strands" to "total length of the tight junction membrane structure" in percent. For quantification, the digitized images of the tight junctions were analyzed using the morphometric software package "AnalySIS" (SIS, Münster, Germany). The total particle density was given as E- and P-face parts of the TJ strands in percent of the tight junction length.

Isolation and processing of chicken and rat brain microvascular fragments

Chicken brain and rat brain microvascular fragments were processed as described previously (Bowman et al., 1981, 1983; Risau et al., 1990) with some minor modifications. Briefly, telencephalons of at least ten embryonic (E15) or posthatching chicken (P6) were prepared free of the meninges. Tissue was washed several times in buffer A (150 mM NaCl, 0.05 mM KCl, 2.3 mM $CaCl_2$, 2.3 mM $MgCl_2$, 15 mM Hepes; 10 mg/ml BSA), minced and centrifuged at 250*g* for 5 min at 4 °C. The pellet was resuspended in collagenase (0.75%) and incubated at 37 °C for 30–40 min with occasional shaking. Digestion was arrested by adding buffer A. After centrifugation (250*g*, 10 min, 4 °C) the pellet was carefully resuspended in PBS containing 20% bovine serum albumin (BSA) and centrifuged at 1200*g*, 20 min at 4 °C in order to separate capillary fragments from white and gray matter.

The pellet was carefully resuspended in buffer A containing 0.075% collagenase and subsequently digested for 7 min (chicken) or 20 min (rat). Digestion was stopped as mentioned above, the suspension was centrifuged, and the pellet was resuspended in 1 ml of warm washing medium (DMEM, 10% FCS, antibiotics), transferred to a 50% percoll gradient and centrifuged at 1000*g*, 10 min at 20 °C. The band containing small fragments of brain microvessels was harvested from the percoll column and washed in buffer A.

For cell culture experiments, capillary fragments from P6 animals were resuspended in culture medium (DMEM, 50 µg/ml heparin, 100 ng/ml bFGF, 30% egg yolk) and plated at a density of 1.5×10^4 cells/ml. Endothelial cells were cultured for 3–4 days in medium containing 1% yolk, thereafter yolk was omitted from culture medium.

Results

Morphological aspects of rat and chicken endothelial TJs in vivo

In freeze-fracture preparations, tight junctions of rat brain microcapillaries exhibited a high PFA (≈55%) and a high complexity of strands (FD = 1.63) (Fig. 1a). Furthermore, the strands showed a rough, fragmented morphology on the P-face and demonstrated particles on E-face grooves, corresponding to gaps in the P-face strands (Fig. 2a).

The PFA of TJs in chicken brain microcapillaries was considerably higher (≈85%) than in the rat. However, complexity of strands was similar (FD = 1.68) (Fig. 1b). The appearance of endothelial TJ strands in the chicken strongly resembled the situation in epithelial cells, as TJ strands showed smooth ridges on the P-face (Figs. 1b, 2f1) and particle-free grooves on the E-face (Figs. 1b, 2f2).

Expression of tight junction proteins in vivo

In order to find out whether claudin-1/-5, occludin and ZO-1 show a differential expression in endothelial cells of rat and chicken brain, we labeled cryosections with antibodies against these antigens.

Brain endothelial cells of both species were positive for occludin, claudin-1, claudin-5 and ZO-1. Interestingly, in rat cells, claudin-1 and claudin-5 showed an almost identical labeling intensity and junctional localization (Fig. 2b,c). In chicken cells, the labeling of claudin-1 was slightly more intense than that of claudin-5. Moreover, claudin-5 labeling was more fragmented (Fig. 2g,h). Occludin and ZO-1 showed a specific strong continuous junctional labeling of brain endothelial cells which did not differ significantly in either species (Fig. 2d,e,i,k).

Rat peripheral blood vessels showed in agreement with previous reports (Hirase et al., 1997) only traces of anti-

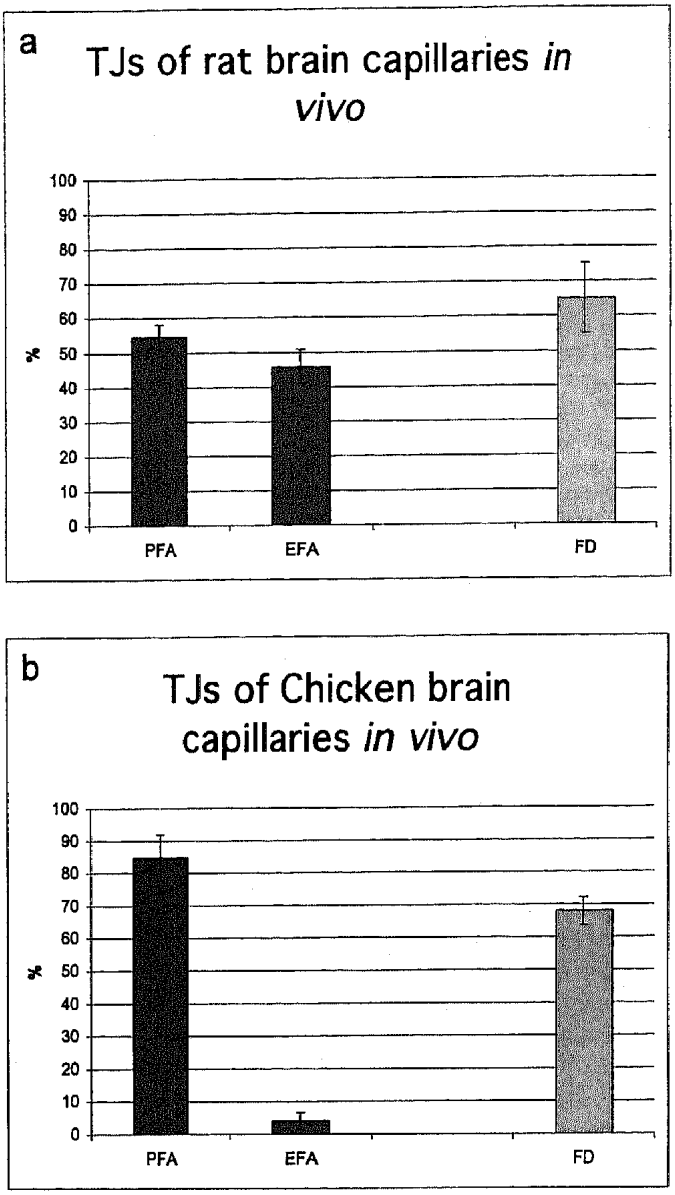


Fig. 1. Quantification of TJ parameters of brain capillary fragments of rat and chicken in vivo. **a** Rat tight junctions exhibited a significant, but only slightly higher P-face (PFA; ≈55%) than E-face association (EFA; ≈45%) of particles. The complexity of the junctional network which was quantified by the fractal dimension (FD) is very high (FD = 1.65; presented as 65%). **b** Chicken tight junctions exhibited a significant and dramatic higher PFA (≈85%) than EFA (≈4%). The complexity of the junctional network was in the same range as those of rat TJs (FD = 1.68; presented as 68%).

occludin immunoreactivity (data not shown). The same result was obtained for the labeling of claudin-1, which was found only faintly in endothelial cells of peripheral blood vessels (data not shown). In contrast, claudin-5 and the constitutive TJ-associated protein ZO-1 showed a strong and specific labeling at the interendothelial junctions (data not shown). Chicken peripheral endothelia showed a significant labeling for occludin, claudin-1/-5 and ZO-1 (data not shown), although the distribution of the antigens appeared to be less continuous at interendothelial junctions when compared to brain microvessels. In particular, in brain endothelia claudin-1 was more profoundly labeled at interendothelial junctions, whereas claudin-5 showed only a faint spot-like labeling.

Morphological aspects of rat and chicken endothelial TJs in vitro

Rat and chicken brain endothelial cells showed considerable differences in their kinetics of growth and differentiation in vitro. Rat brain endothelial cells (RBEs) needed several days to reach confluency on collagen G-coated Petri dishes. They were easily transferred over several passages, without significantly changing their growth pattern (data not shown). Four days after the onset of cultivation, RBEs showed a low PFA but a high EFA of endothelial TJs (Figs. 3a, 4a,c). Compared to the in vivo situation, the complexity of endothelial TJs was dramatically reduced (Figs. 1a, 3a). After 7 days in vitro, only backbones of TJs were found on the P-face (Fig. 4b), whereas the E-face grooves were regularly occupied by particles (Figs. 4d). Interestingly, particle-free ridges on the P-face and grooves on the E-face were occasionally found (data not shown) underlining that the degradation of tight junctions during cultivation takes place as a reversal of their formation (Kniesel et al., 1996).

Chicken brain endothelial cells (CBEs) attached and sprouted very rapidly after seeding and reached confluency already two days later. In contrast to their mammalian counterparts, CBEs were not suitable for passaging. After only two days of cultivation, CBEs formed complex tight junctions in vitro, which were highly P-face associated (Figs. 3b, 4e). Nevertheless, TJ complexity was reduced in comparison to the in vivo situation (Figs. 1b, 3b). Unexpectedly, and in clear contrast to RBEs, we did not observe a switch of TJ particles from P- to E-face: the PFA was found to be considerably higher than the EFA (Figs. 1b, 3b, 4g,h). Nevertheless, there was a loss of particles, resulting in fragmented and incomplete strands of low interconnectivity (Table I). After longer periods of cultivation (5 days), the PFA was still found to be higher than the EFA and the TJ complexity was further decreased (Fig. 4f,h; data of quantification not shown). Additionally, TJ strands did not show a smooth appearance and single particles could be identified on the P-face (Fig. 4f).

Expression of tight junction proteins in vitro

In order to obtain information about the expression of junctional proteins, RBEs were labeled for claudin-1, claudin-5, occludin and ZO-1 at different stages of cultivation. After a few days of growth in vitro, patches of RBEs were positive for claudin-1 and claudin-5, occludin and ZO-1 (Fig. 5a,c,e,g). Most antigens were located at interendothelial junctions, only claudin-5 was additionally detected intracellularly. Interestingly, during cultivation, primary attached cell aggregates were shown to be positive for claudin-1 (Fig. 5a,b), whereas the cells beside these aggregates were not labeled for claudin-1. The only claudin labeled in these descendants was claudin-5 (Fig. 5c,d). Occludin and ZO-1 remained positive throughout cultivation time, but the continuous belt-like labeling around the cells showed an increasingly interrupted appearance (Fig. 5e–h). After 7 days in vitro RBEs reached confluency. At that time, claudin-1 was reduced at intercellular contacts (Fig. 5b). In contrast, the labeling of claudin-5, occludin and ZO-1 did not change significantly from early to later stages of cultivation (Fig. 5d,f,h).

With respect to the different growth characteristics, chicken brain endothelial cells (CBEs) were also tested for the expression of the TJ antigens claudin-1, claudin-5, occludin

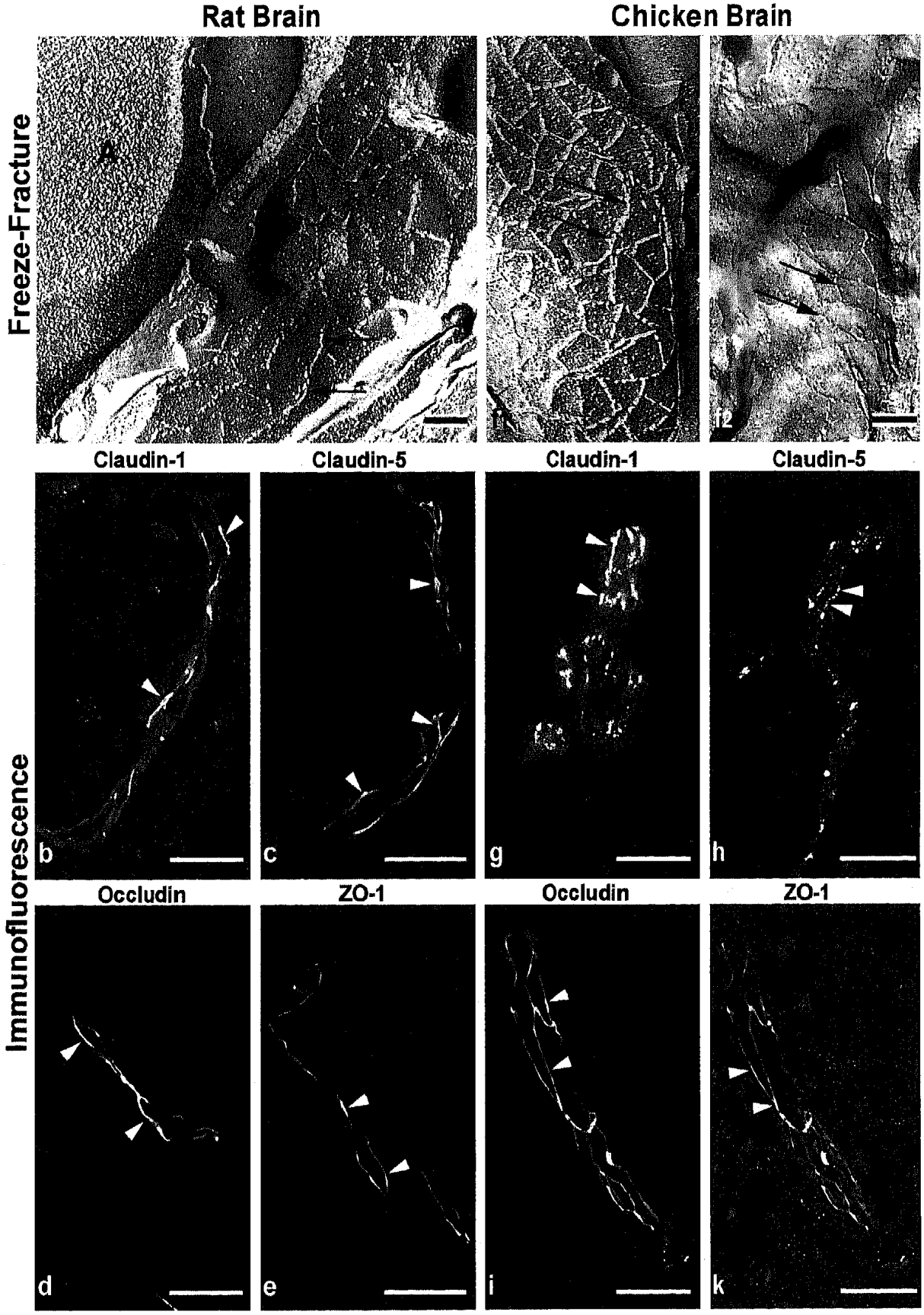
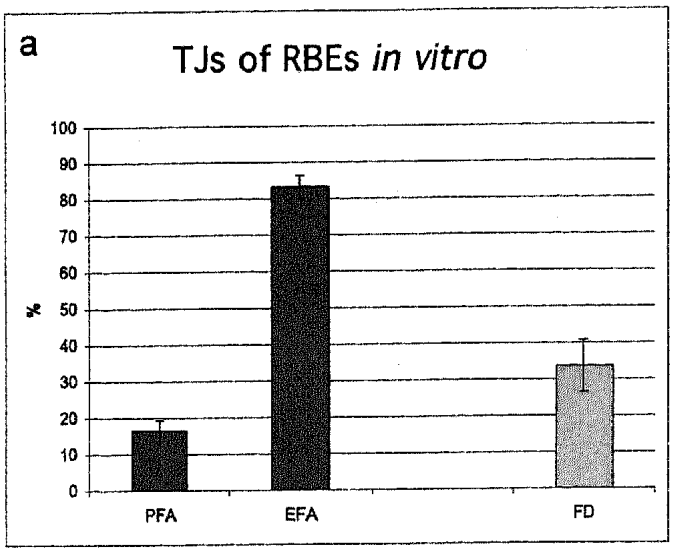


Fig. 2. **a** Freeze-fracture image of rat brain endothelial TJs in vivo. Note that the strands on the P-face are occasionally interrupted. *Arrows* point to strands on the P-face; A, astrocyte. Bar 300 nm. **b–e** Confocal immunofluorescent images of rat brain endothelia in vivo. Claudin-1 (**b**) and claudin-5 (**c**) showed a similar distribution and labeling intensity at interendothelial junctions. Occludin (**d**) and ZO-1 (**e**) were also specifically and continuously labeled at cell-cell contacts. *Arrowheads* point to junctional labeling. Bar 25 μm. **f** Freeze-fracture image of chicken brain endothelial TJs in vivo. Note that in contrast to the rat, the strands on the P-face (**f1**) were more continuous and smooth (*arrows*) and E-face (**f2**) grooves are devoid of particles (*arrows*). Bar 300 nm. **g–k.** Confocal immunofluorescent images of chicken brain endothelia in vivo. In contrast to the rat, claudin-1 (**g**) was more continuously labeled at interendothelial junctions than claudin-5 (**h**). Double-labeling against occludin and ZO-1 showed no difference to the labeling in rat. *Arrowheads* point to junctional labeling. Bar 25 μm.



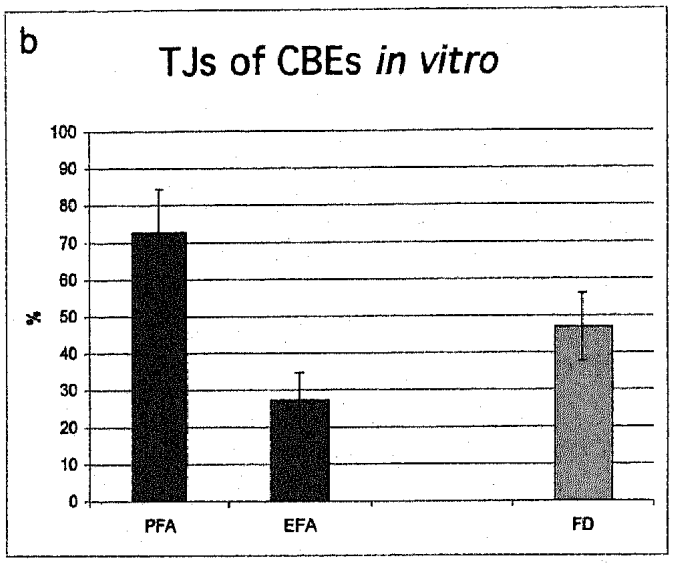


Fig. 3. Quantification of tight junction (TJ) parameters of brain endothelial cells of rat and chicken after 4 and 2 days in vitro, respectively. **a** Rat brain endothelial (RBE) TJs in vitro showed a dramatic decrease in P-face-associated particles (PFA; ≈16%) compared to the in vivo situation, whereas in parallel the E-face-associated particles (EFA; ≈83%) increased in the same way. This P-face switch was accompanied by a decrease in TJ complexity (FD = 1.34; presented as 34%). **b** Chicken brain endothelial (CBE) TJs in vitro didn't show a P-face switch of particles. Nevertheless, PFA (≈73%) was slightly decreased, whereas the EFA (≈27%) significantly increased compared to the in vivo situation. Like in rat, the changes in particle association were accompanied by a significant reduction of TJ complexity (FD = 1.47; presented as 47%).

and ZO-1. Two days after seeding, CBEs were almost confluent and exhibited a positive labeling at interendothelial junctions for all antigens tested (Fig. 5i, l, n, p). Besides a strong specific labeling of the cell-cell contacts, an intracellular spot-like labeling for claudin-1/-5 around the nuclei was observed (Fig. 5i,l). After 5 days in vitro, claudin-1 vanished at interendothelial contacts, whereas a punctuate labeling of the entire endothelial cell was maintained (Fig. 5k). Claudin-5, occludin and ZO-1 exhibited an increasingly patchy labeling at intercellular junctions (Fig. 5m, o, q).

Discussion

The present work provides substantial evidence that TJs of rat and chicken brain endothelial cells differ in morphology, molecular architecture and regulation. In order to elucidate the molecular basis of the difference in TJ morphology between rat and chicken brain endothelial cells, which was reported previously (Liebner et al., 1997), we characterized the expression of tight junction molecules in combination with a morphological analysis of TJs in brain endothelial cells in vivo and in vitro. The goal of the study was to compare the distribution of junctional proteins in rat and chicken brain endothelial cells with the distribution of intramembranous particles in the different leaflets of the membranes rather than to quantify tight junctional proteins in different species under different conditions.

Tight junctions in vivo

First, the morphology of brain microvascular endothelial TJs in vivo was investigated by means of freeze-fracturing and immunocytochemistry. The difference in PFA between rat (≈55%) and chicken (≈85%) brain capillary fragments correlated with the differential expression of claudin-1 and claudin-5 (Table I). Whereas in rat ECs, the anti-claudin-1 and -5 immunoreactivities were found to be similar, in chicken ECs, claudin-1 showed a more intense labelling, indicating an expression level higher than that of claudin-5. This result corresponds to the findings of Furuse et al. (1998b) and Morita et al. (1999b), who demonstrated that claudin-1 forms P-face and claudin-5 E-face associated TJs. Since the complexity of TJs was found to be almost identical in rat and chicken brain endothelial cells, the differences in immunoreactivity for claudin-1 and claudin-5 most likely corresponded to the predominant E- or P-face association of TJ particles in the different species.

Tab. I. Summary of freeze-fracture and immunocytochemical investigations on rat and chicken brain endothelial cells in vivo and in vitro

		TJ morphology			Expression of TJ antigens			
		Complexity	PFA	EFA	Claudin-1	Claudin-5	Occludin	ZO-1
Rat	in vivo	+++	++(+)	++	++(+)	+++	+++	+++
	in vitro	+(+)	(+)	++(+)	(+)*	++	++	++
Chicken	in vivo	+++	+++	(+)	+++	++	+++	+++
	in vitro	++	++	+	++	++	++	++

The symbols of the morphological parameters reflect the data from Figs. 1, 3. The symbols of the immunocytochemical parameters are estimations from relative fluorescence intensities which, of course, do not allow a real quantification.
* The decrease in claudin-1 in vitro does not concern intensity of immunofluorescence but the number of labeled cells (Fig. 5a, b). PFA, P-face association; EFA, E-face association; TJ, tight junction.

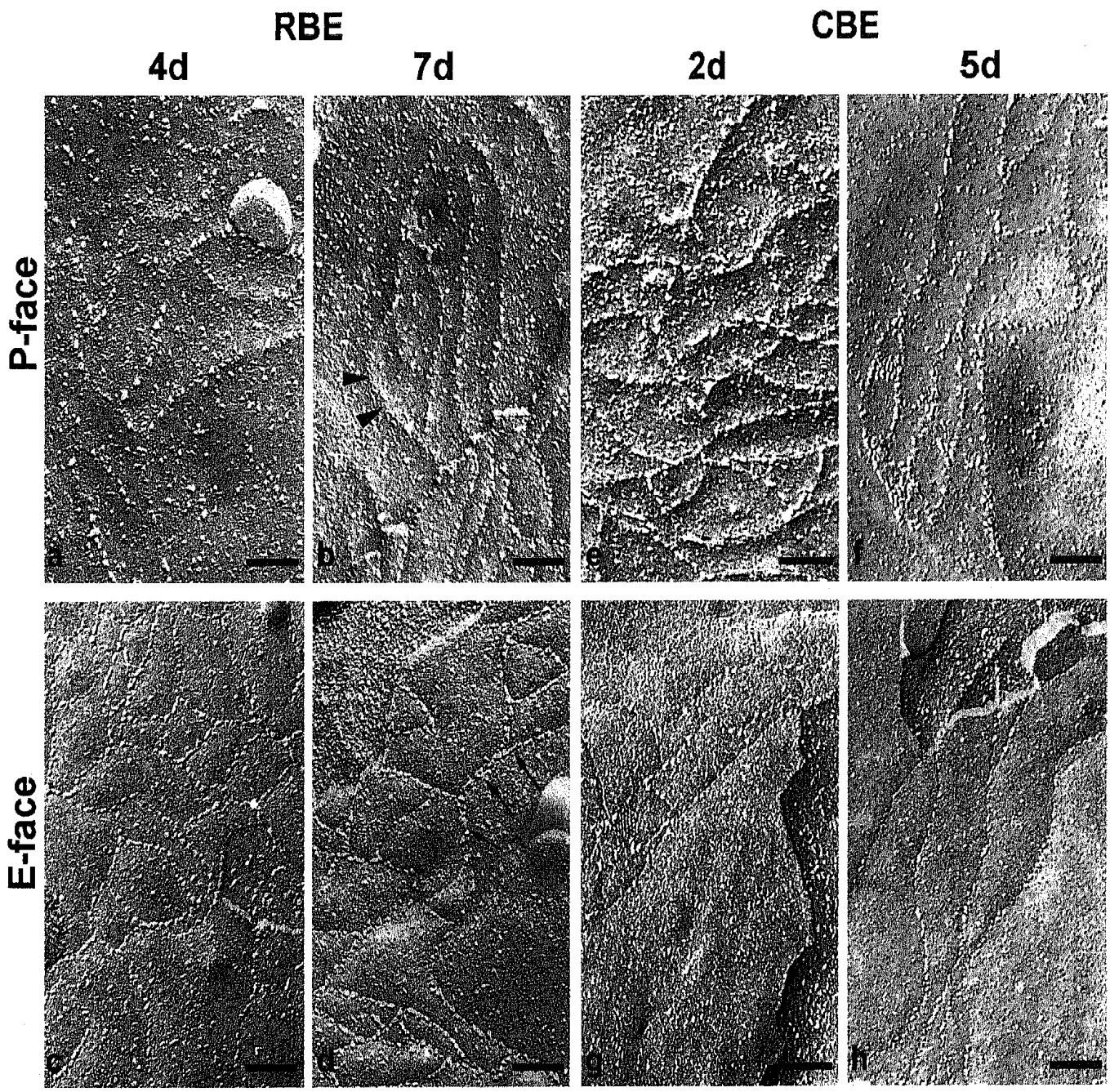
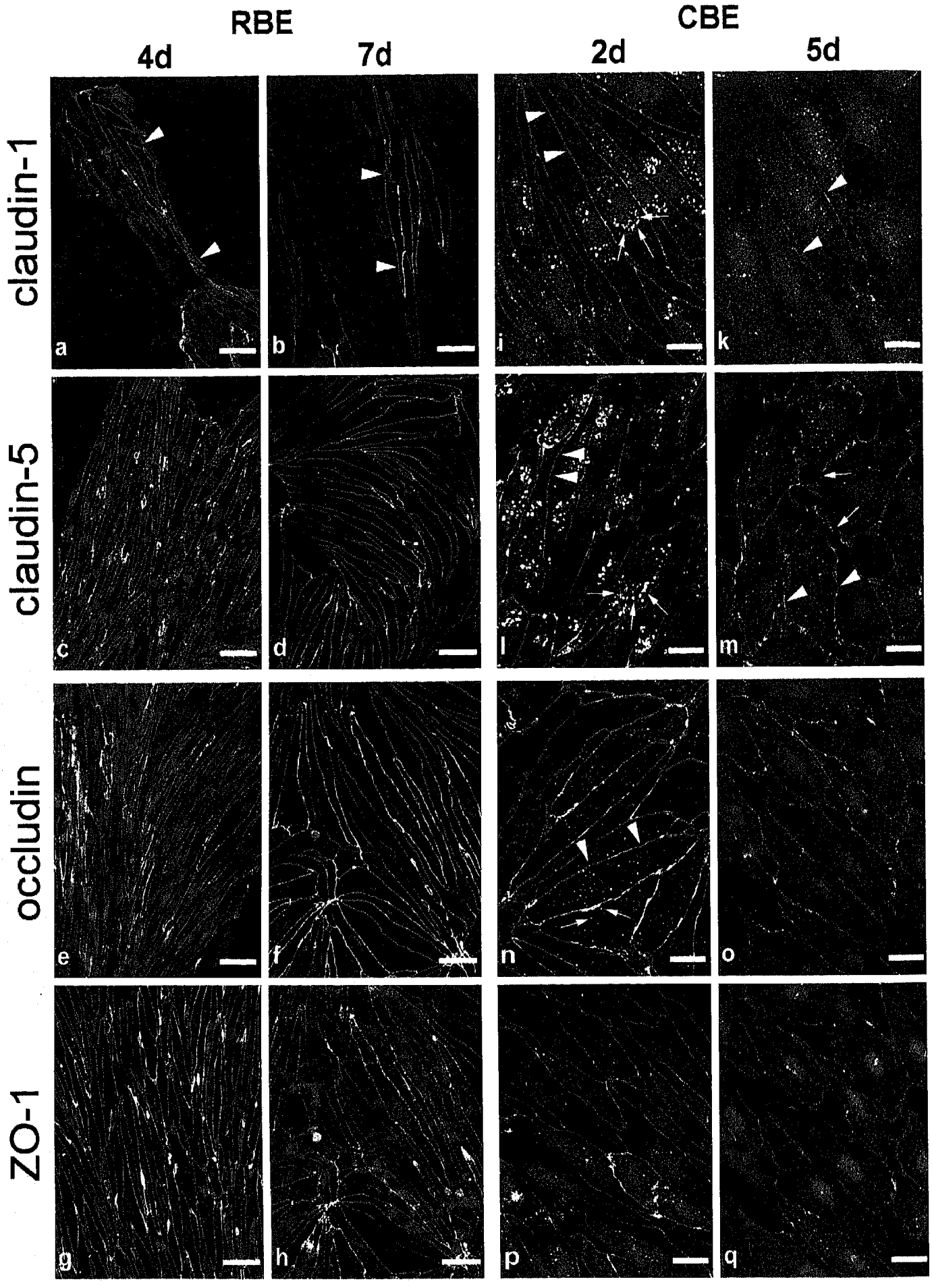


Fig. 4. Freeze-fracture micrographs of rat (RBE) and chicken brain endothelial (CBE) cells in vitro. **a–d** P-face strands of rat brain endothelial cells showed a significant decrease in inserted particles after 4 (**a**) and 7 (**b**) days in vitro. Note that the TJ-structure was still visible after 7 days by particle-free ridges on the P-face (**b**; *arrowheads*). In parallel to the P-face switch of particles the complexity of the TJ network became reduced during cultivation, what was more obvious in micrographs of the E-face (**c**, **d**). As a result of the P-face switch the grooves on the E-face were densely occupied by particles (*arrows*). Bars 300 nm. **e–h** Chicken brain endothelial cells showed only a slight decrease in particles in P-face strands, whereas the complexity became reduced from 2 (**e**) to 5 (**f**) days in vitro. Although the frequency of particles in E-face grooves was increased in vitro, neither after 2 (**g**), nor after 5 (**h**) days in vitro a switch of particles from the P- to the E-face was observed. Bar 300 nm.

The finding that the expression pattern of occludin and ZO-1 did not differ between brain endothelia of the two species, may support the notion of Saitou et al. (1998) that these proteins are not directly involved in the determination of P- or E-face associated TJs, although occludin is an integral part of them (Furuse et al., 1993). On the other hand, it was shown that occludin formed short tight junctional strands lacking the typical network arrangement when transfected in insect cells lacking TJs, implying that occludin has the potential to essentially contribute to TJ morphology (Bamforth et al., 1999; Furuse et al., 1996).

Tight junctions in vitro

Interestingly, the very high (≈85%) PFA of chicken brain endothelial TJs in vivo was largely reestablished in vitro (73%), whereas TJ complexity was considerably reduced. In contrast, the high association of particles with the P-face (≈55%) of BBB-TJs in rat brain endothelial cells in vivo dramatically decreased in vitro to ≈14%. This was accompanied by a complementary increase in E-face particles resulting in a so-called P-face switch, comparable to that found in a bovine blood-brain barrier in vitro model by Wolburg et al. (1994). Additionally, TJ complexity was also


RBE
CBE
4d
7d
2d
5d
claudin-1
claudin-5
occludin
ZO-1
a
b
i
k
c
d
l
m
e
f
n
o
g
h
p
q

◀ **Fig. 5.** **a, c, e, g** Immunofluorescence micrographs of rat brain endothelial (RBE) cells after 4 days in vitro. Claudin-1 (**a**) was only labeled in small, rarely found clusters of cells (*arrowheads*). Beside these clusters, claudin-1-negative endothelial cells are present, but invisible in this figure. In contrast, claudin-5 (**c**) showed a specific junctional labeling in all endothelial cells grown so far. Occludin (**e**) and ZO-1 (**g**) showed a distribution similar to claudin-5 at interendothelial junctions. Bar 50 μm. **b, d, f, h** Immunofluorescence micrographs of rat brain endothelial (RBE) cells after 7 days in vitro. Claudin-1 (**b**) did not show any changes in its distribution in comparison to the 4-day stage. Claudin-5 (**d**) showed a slight reduction of diffuse labeling accompanied by a condensation of immunoreactivity at interendothelial junctions. Also after 7 days in vitro, the labeling of occludin (**f**) and ZO-1 (**h**) were almost identical to the labeling of claudin-5. Both antigens also showed a decrease in diffuse labeling. Bar 50 μm. **i, l, n, p** Immunofluorescence micrographs of chicken brain endothelial (CBE) cells after 2 days in vitro. In contrast to the rat cells, claudin-1 (**i**) and claudin-5 (**l**) showed a specific labeling at interendothelial junctions (*arrowheads*) of all cells. Interestingly, both antigens showed a considerable vesicular labeling around the nucleus (*arrows*). Occludin (**n**) showed a labeling almost restricted to interendothelial junctions (*arrowheads*), and additionally, some evenly distributed vesicles were positive for the antigen (*arrows*). ZO-1 (**p**) grossly showed the same distribution like occludin. Bar 12.5 μm. **k, m, o, q** Immunofluorescence micrographs of chicken brain endothelial (CBE) cells after 5 days in vitro. After 5 days in vitro claudin-1 (**k**) was only barely detectable in some chicken brain endothelial cells (*arrowheads*). In contrast, claudin-5 (**m**) was still localized at all endothelial cell-cell junctions (*arrowheads*). However, the continuity of claudin-5 at the interendothelial junction was reduced (*arrows* point to gaps). The same was observed for occludin (**o**) and ZO-1 (**q**) which also still were localized at interendothelial junctions but became discontinuous. Bar 12.5 μm.

reduced. This result is of particular interest, since it is generally accepted that brain endothelial cells lose their barrier properties in vitro and a high PFA is believed to indicate the quality of the tight junctional seal (Kniesel and Wolburg, 2000; Tsukita and Furuse, 1999; Wolburg et al., 1994).

In order to follow up the processes of junctional reorganization in vitro, ECs of both species were labeled for TJ proteins. The result that already at the early cultivation stage of RBEs (4d), claudin-1 is detectable only in small clusters of endothelial cells, favored the interpretation that claudin-1 is localized in primary attached capillary fragments, which may exhibit a residual labeling for this antigen. In contrast, at this cultivation stage, claudin-5, occludin and ZO-1 showed a similar distribution at cellular contacts of all endothelial cells. These results support the interpretation that the switch of particles from P- to E-face, subsequently after cultivation, is a consequence of a low claudin-1 expression level in proliferating endothelial cells in vitro. Importantly, according to the finding that small colonies of endothelial cells were positive for claudin-1 (Fig. 5a,b) occasionally, P-face-associated tight junctions were found in freeze-fracture preparations (data not shown). In agreement with previous reports describing endothelial TJ morphology in vitro (Wolburg et al., 1994), both, high PFA and claudin-1 expression were not reinduced in RBEs during culture (see Fig. 5a,b). Therefore, it is conceivable that cultured RBEs are not able to pass the state of proliferation to further differentiation. Thus, rat brain endothelial cells in culture form TJs resembling those of peripheral endothelial cells (Mühleisen et al., 1989; Wolburg et al., 1994).

Claudin-5 and ZO-1 seem to be constitutively located at interendothelial junctions of rat and chicken endothelial cells, contributing to contacts which are not sufficient for a restrictive diffusion barrier. The fragmentation of claudin-5 and ZO-1 during cultivation may be due to the parallel decrease in TJ complexity. Interestingly, peripheral blood vessels of the chicken form low-complex but P-face-associated TJs which are positive for claudin-1, claudin-5 and occludin (our unpublished observation). In mammalian peripheral blood vessels, occludin (Hirase et al., 1997) as well as claudin-1 (our unpublished observation) expression does not reach significant levels. Therefore, at least in vivo, the expression of occludin suggests a close correlation with the expression of claudin-1 and the formation of P-face-dominated TJs. Nevertheless, the role of occludin for TJ morphology and regulation remains to be elucidated especially as differently phosphorylated as well as alternatively spliced forms of occludin have been identified (Muresan et al., 2000; Sakakibara et al., 1997).

The finding that CBEs, after two days (2d) in vitro, retained a high PFA and proved to be positive for claudin-1 at all intercellular junctions supports the idea that TJs of the "epithelial type" share a different molecular composition and regulation in comparison to the "endothelial type". Furthermore, CBEs seem to have only a low proliferative capacity, as they have to be seeded in high density, and after detachment from the substrate, they fail to grow to confluency again, so that splitting and reculturing is not possible.

The observation that after 5 days in vitro chicken brain endothelial cell TJs lose claudin-1 but maintain their high PFA demonstrates that there is no strict correlation between PFA and claudin-1 expression as suggested for rat endothelial cells. Hence, it is conceivable that other determinants may contribute to the high PFA in chicken endothelial cells. In any case, as claimed previously for mammalian brain endothelial cells, chicken brain endothelial cells in vitro also require the morphological and molecular organization of brain capillaries to maintain blood-brain barrier characteristics. Therefore, CBEs combine morphological and regulatory properties of epithelial and mammalian endothelial TJs.

Furthermore, these results favor the suggestion that the high PFA of barrier TJs is necessary, but not sufficient for the formation of occlusive TJs (Bentzel et al., 1980). Evidence for this theory originates from results on the development of the leptomeningeal blood-cerebrospinal fluid (CSF) barrier. Rascher and Wolburg (1997) showed that rat arachnoideal TJs are highly E-face associated but form an expanded and complex network of strands. In contrast, chicken arachnoideal TJs are dominantly P-face associated but form only narrow networks of similar complexity. However, the arachnoidea of both species is believed to be a comparably restrictive CSF barrier. Together with the results of the present work, therefore it is likely that the tight junctional seal is determined by at least two morphological parameters which are the PFA and the complexity. However, the knowledge of how are these parameters are precisely regulated still is at its beginning.

Implications for TJ regulation

In addition to different PFA levels in vivo, endothelial TJs of rat and chicken brain also showed a contrary morphology in vitro. Particles of rat brain endothelial TJs switched very early and almost completely to the E-face, whereas their chicken counterparts retained more greatly the in vivo PFA and only slowly lost a certain ratio of particles on the P-face. Although the E-face association of particles was also enhanced in the chicken system, a considerably high PFA was maintained

throughout the observed time of cultivation and most importantly, no P-face switch was observed. Taken together, there is clear evidence for distinct regulatory systems of blood-brain barrier TJs in both species.

This is also supported by results obtained from studies on the development of the BBB in both species. Kniesel et al. (1996) showed that in the rat BBB, endothelial TJs develop from particle-free grooves via E-face-associated TJs to the P-face-dominated mature morphology. In contrast, Liebner et al. (1997) reported that during the maturation of blood-retina barrier TJs in the pecten oculi of the chicken, TJ particles were consecutively inserted on the P-face. A stage dominated by an abundant particle distribution on E-face grooves was not observed. Both schemes of TJ genesis can be applied in the reverse manner for the TJ degradation under culture conditions.

Indeed, culturing rat brain endothelial cells led to the downregulation of claudin-1 and to a reduction of PFA with a concomitant increase in EFA. Culturing chicken brain endothelial cells also provoked downregulation of claudin-1 but no dramatic increase in EFA. In addition, the P-face-associated strands became successively fragmented. However, a switch from P- to E-face as found in the rat (Fig. 3a) could not be demonstrated in the chicken (Fig. 3b). Therefore, the observation that CBE-TJs after 5 d in vitro still exhibit a high amount of P-face-associated particles but no claudin-1 immunoreactivity obviously demands an other explanation than a strict correlation between claudin-1 and PFA. Possibly, chicken endothelial cells possess additional claudin member(s). Alternatively, claudins and/or occludin may be differently regulated and posttranslationally modified in order to form substitutional molecule aggregates. In any case, as a consequence of the species-specific regulatory events, the cytoskeletal attachment of TJ particles seems to be affected as indicated by TJ morphology. It was previously speculated that the distribution of particles on either membrane leaflet is a consequence of cytoskeletal tethering (Wolburg et al., 1994). Recently, Itoh et al. (1999a,b) demonstrated that most members of the claudin family are able to bind to the tight junction-associated proteins ZO-1/-2/-3, of which ZO-1 and -2 have the ability to mediate actin binding. The incapacity of ZO-3 for actin binding and/or the regulation of ZO-1/-2 may be of particular significance for altered cytoskeletal tethering and hence for particle distribution in freeze-fracture preparations. The suggestion seems reasonable, since the mammalian epithelial and endothelial cells express different splice variants of ZO-1, which are termed ZO-1 $\alpha+$ and ZO-1 $\alpha-$, respectively (Balda and Anderson, 1992, 1993). As chicken endothelial cells form epithelial-like TJs, an epithelial-like endowment with cytoskeletal linker proteins may contribute to this morphology.

Furthermore, many signaling events affect the phosphorylation of ZO-1 and occludin, and, depending on the cell type and the involved pathway, the attachment or detachment of these proteins from the membrane lead to a reinforcement or breakdown of barrier function, respectively (Antonetti et al., 1999; Chen et al., 2000).

The increased knowledge of endothelial tight and adherens junction regulation will be of outstanding benefit for understanding the physiology and pathology of vascular permeability. In particular, angiogenic processes in the brain, namely in brain tumors, are accompanied by a breakdown of the BBB (Long, 1970) and an alteration of junctional protein expression (Liebner et al., 2000). Although these findings need further investigation, they provide a perspective for the understanding of the morphology and regulation of various types of TJs.

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