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Vascular Pharmacology 38 (2002) 323–337

Vascular Pharmacology

www.elsevier.com/locate/vph

Tight junctions of the blood–brain barrier: Development, composition and regulation

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Received 1 July 2001; accepted 1 February 2002

Abstract

1. The blood–brain barrier is essential for the maintenance and regulation of the neural microenvironment. The main characteristic features of blood–brain barrier endothelial cells are an extremely low rate of transcytotic vesicles and a restrictive paracellular diffusion barrier. 2. Endothelial blood–brain barrier tight junctions differ from epithelial tight junctions, not only by distinct morphological and molecular properties, but also by the fact that endothelial tight junctions are more sensitive to microenvironmental than epithelial factors. 3. Many ubiquitous molecular tight junction components have been identified and characterized including claudins, occludin, ZO-1, ZO-2, ZO-3, cingulin and 7H6. Signaling pathways involved in tight junction regulation include G-proteins, serine-, threonine- and tyrosine-kinases, extra and intracellular calcium levels, cAMP levels, proteases and cytokines. Common to most of these pathways is the modulation of cytoskeletal elements and the connection of tight junction transmembrane molecules to the cytoskeleton. Additionally, crosstalk between components of the tight junction- and the cadherin–catenin system of the adherens junction suggests a close functional interdependence of the two cell–cell contact systems. 4. Important new molecular aspects of tight junction regulation were recently elucidated. This review provides an integration of these new results.
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Keywords: Tight junction; Blood–brain barrier; Morphology; Freeze-fracture; Occludin; Claudins; G-proteins

1. Introduction

The blood–brain barrier is responsible for maintaining the neuroparenchymal microenvironment. The barrier protects the neural tissue from toxins, buffers variations in blood composition and maintains the barrier function between blood and brain. The main structures responsible for the barrier properties are the tight junctions (Reese and Karnovsky, 1967; Brightman and Reese, 1969; Nabeshima et al., 1975; Van Deurs and Koehler, 1979; Mollgard and Saunders, 1986; Rascher and Wolburg, 1997; Kniesel and Wolburg, 2000). The cells responsible for the establishment of the barrier are the capillary endothelial cells (blood–brain barrier) and the epithelial (glial) cells (blood–cerebrospinal fluid barrier).

The first approaches to functionally understand tight junctions were theoretical considerations concerning logarithmic relationships between the number of tight junction strands and the transepithelial electrical resistance (Claude, 1978; Claude and Goodenough, 1973). The authors predicted permeability and transepithelial electrical resistance (Marcial et al., 1984). The focus was directed at epithelial cells. The notion that also endothelial cells form an efficient permeability barrier came originally from tracer experiments performed with electron microscopy (Reese and Karnovsky, 1967). Nagy et al. (1984) used freeze-fracture to show that the endothelial cell tight junctions are the most complex junctions anywhere in the cardiovascular system. In rat brain, measurements of transendothelial resistance were only possible in pial vessels. The vessels displayed resistances in the range of 1100–1500 Ω cm^{-2} after birth (Butt et al., 1990).

In the last 10 years, the knowledge of the molecular composition of the tight junctions has exploded (Furuse et al., 1993, 1998a,b; Ando-Akatsuka et al., 1996; Morita

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1537-1891/02/$ – see front matter © 2002 Elsevier Science Inc. All rights reserved.
PII: S1537-1891(02)00200-8

et al., 1999a; Balda and Matter, 2000; Tsukita and Furuse, 1999, 2000; Tsukita et al., 2001; Heiskala et al., 2001; Fig. 1). A host of new tight junction proteins has been identified. Furthermore, there is unequivocal evidence for important new lipid structures, particularly involving the outer membrane leaflets of two tight junction-connected partner cells (Hein et al., 1992; Grebenkämper and Galla, 1994). Kan (1993) demonstrated the presence of phospholipids in cylindrical tight junction strands using freeze-fracture labeling with gold-labeled phospholipase A_2. The tight junction particles in freeze-fracture replicas resemble in size the connexons of gap junctions. These structures are constructed from six single units. Therefore, each particle or strand at the fracture face consists of more than one transmembrane molecule or represents the backbone of a lipid/protein-suprastructure. However, how the lipids and proteins are interconnected within the tight junction structure is as yet undefined. The basic molecular equipment of tight junctions generally appears to be similar in all barrier systems, regardless of some molecular differences between epithelial and endothelial tight junctions. More importantly, components of adherens and tight junctions along the entire intercellular cleft are intermingled in endothelial rather than in epithelial cells (Schulze and Firth, 1993). In this review, we will give an overview about the development of the blood–brain barrier, the composition of its tight junctions in health and disease, and signaling processes regulating tight junction function. In doing so, we will try to compare the new findings in epithelial cells and peripheral endothelial cells with the data available in blood–brain barrier endothelial cells.

2. Blood–brain barrier tight junctions during development

One of the most important landmarks of developing microvasculature in the rodent brain are the disappearance

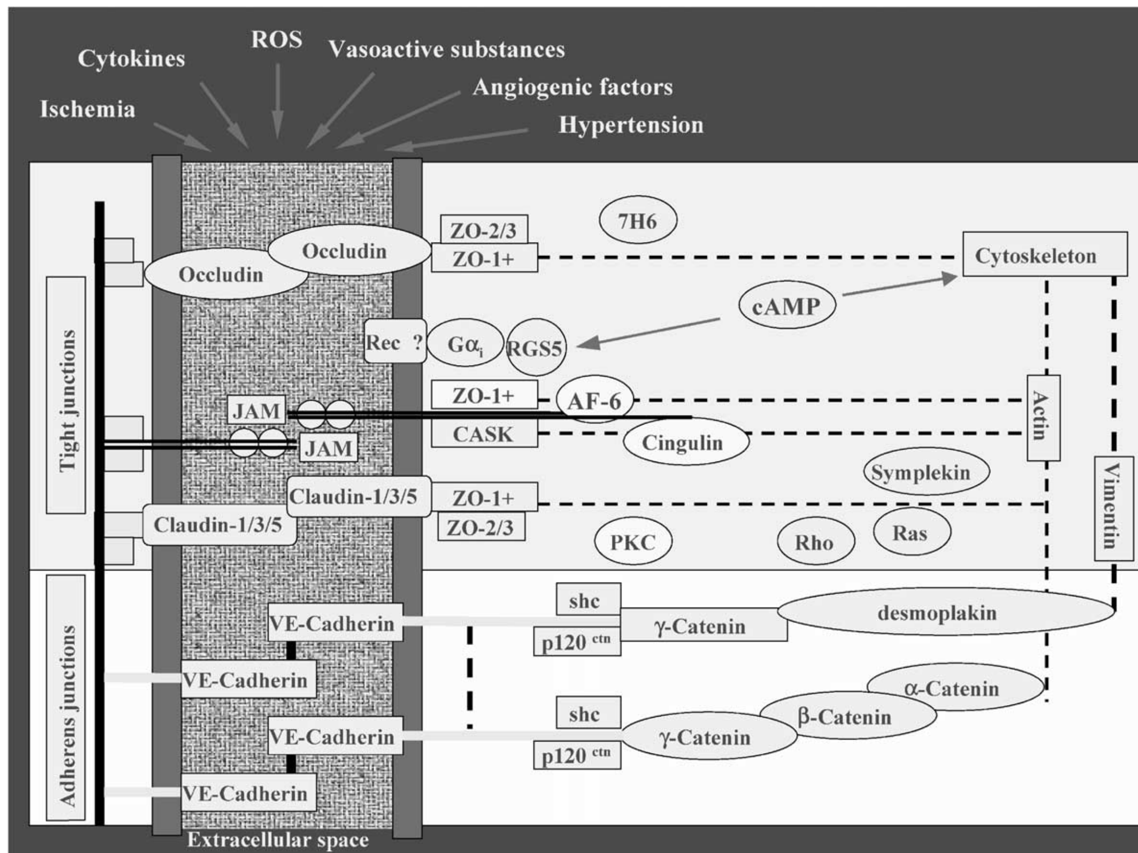

Fig. 1. Molecular composition of tight and adherens junctions. The adherens junctions are necessary for the primary contact between the endothelial cells. This contact is made by cadherins, which are coupled with the cytoskeleton via catenins and/or desmoplakin. Shc-SH2 containing shc protein: target of activated tyrosine kinases; $p120^{ctn}$-armadillo-like protein. The components of the tight junctions are described in detail within the text. The main transmembrane molecules mediating the cellular contact are occludin and the endothelial claudin-1, claudin-3 and claudin-5. These molecules bind via ZO-1/ZO-2/ZO-3 to the cytoskeleton. Molecules such as cingulin, 7H6, symplekin that are important for tight junction structure are described elsewhere (for literature, see text). Another family of transmembrane molecules with functions in leukocyte trafficking is the JAM. Additionally, regulatory proteins have been localized within the tight junction region (G-proteins, e.g., Gαi; RGS5) or are important for tight junction regulation (Rho, Rac, PKC) and cAMP has been demonstrated to directly influence TER and permeability. At top of the figure, some factors are listed, which could influence tight and adherens junction-connected permeability; ROS, reactive oxygen species.

of fenestrations and the appearance of tight junctions in the endothelium between embryonic days (E) 11 and 13 (Bauer et al., 1993; Stewart and Hayakawa, 1994). Early embryonic brain capillaries are permeable to substances that are excluded from the neuronal milieu in the adult brain (Fabian and Hulsebosch, 1989; Johanson, 1980). The external blood–brain barrier of pial vessels in rat embryos shows up to E20 a low transendothelial electrical resistance (Butt et al., 1990) and therefore can be regarded as immature. Also, in the chick cerebellum and spinal cord, a stepwise progression of the endothelial barrier to horseradish peroxidase (HRP) from the superficial to the medullary region has been described between E12 and E15 (Wakai and Hirokawa, 1978). In the developing mouse, Stewart and Hayakawa (1987) demonstrated a gradual decline in both the permeability index and the interendothelial cleft index. The permeability index is defined as the ratio of brain/plasma HRP activity divided by the blood vessel density. The interendothelial cleft index is defined as the proportion of the junctional profile that is composed of junctional clefts. Schulze and Firth (1992) more closely characterized the maturation of the blood–brain barrier in the rat as an increase of the ratio of "narrow zones" to "wide zones" in the interendothelial clefts. Similarly, tight junctional membrane domains of pial microvessels narrowed over developmental periods. However, in a subgroup of pial vessels, endothelial junctions remain separated by a small (2.8 nm) cleft. In the remaining vessels, junctional membranes were fused (Cassella et al., 1997).

At E13, tight junction particle density as evaluated by freeze-fracturing of rat brain endothelial cells was found to be extremely low on both membrane leaflets. During further development, association of tight junction particles is altered from a predominant association with the outer membrane leaflet (E-face) in stages E15 and E18 to a predominant association with the inner membrane leaflet (P-face) in postnatal day (P) 1 and adult (Kniesel et al., 1996). A high degree of E-face association was documented also for human embryos (Mollgard and Saunders, 1986). The predominant P-face association commencing between E18 and P1 is in good agreement with the rapid increase in the transendothelial electrical resistance found in pial vessels of the rat at E21 (Butt et al., 1990).

An interesting correlation exists between astroglial differentiation and blood–brain barrier maturation. The astroglial membranes are characterized by the occurrence of orthogonal arrays of particles (OAPs) with a high density where the glial cells contact the basal lamina at the surface of the brain and around blood vessels (Wolburg, 1995a). In contrast, within the neuropil, the astroglial membranes express only few OAPs. This polarization of astrocytes arises concomitantly with the maturation of the blood–brain barrier and is not maintained by cultured astrocytes (Wolburg, 1995a,b; Nico et al., 2001). The OAPs were described to contain at least the water channel forming protein aquaporin-4 (AQP4; Nielsen et al., 1997; Rash et al., 1998; for a review, see Venero et al., 2001). The role of OAP/AQP4 for the water homeostasis of the brain and the maintenance of the blood–brain barrier has been shown in different approaches (Manley et al., 2000; Frigeri et al., 2001; Ke et al., 2001). Furthermore, the development of the OAP/AQP4-related polarity of astrocytes seems to correlate with the time schedule of agrin expression. Agrin is a heparan sulfate proteoglycan that is present in the subendothelial basal lamina (Barber and Lieth, 1997) and binds to α-dystroglycan (Gee et al., 1994). This in turn is a member of the dystrophin-dystroglycan complex which localizes at endfeet membranes as OAP/AQP4 (Blake and Kröger, 2000). Another member of this complex, α-syntrophin, was shown to be indispensable for the correct perivascular localization of AQP4 (Neely et al., 2001). Under conditions of blood–brain barrier disruption, agrin is lost (Berzin et al., 2000; Rascher et al., 2002), indirectly indicating that agrin is important for functional polarization of astrocytes.

Another property of astroglial cells may also have an influence on the blood–brain barrier, namely the expression of the intermediate filament glial fibrillary acidic protein (GFAP). In aged GFAP-deficient mice, the blood–brain barrier was found to be impaired (Liedtke et al., 1996). Moreover, astrocytes of these mice failed to induce a functional blood–brain barrier in aortic endothelial cells in vitro (Pekny et al., 1998). Knock-out mice lacking GFAP and vimentin developed dilated blood vessels in the brain and the spinal cord (Pekny et al., 1999). However, the link between altered astroglial intermediate filament equipment and blood–brain barrier organization or integrity is completely unknown.

3. Structural basis of tight junctions

Tight junctions are domains of occluded intercellular clefts (Farquhar and Palade, 1963; Brightman and Reese, 1969), which in freeze-fracture studies formed intramembrane networks of strands. In ultrathin sections, these strands appeared as a chain of fusion ("kissing") points. As mentioned briefly above, two parameters of freeze-fracture morphology determine the functional quality of tight junctions: the complexity of strands and the association of the particles with the P- or E-face. Concerning the latter, the particles of the tight junction are found to be localized either in the grooves at E-face or at the ridges at the P-face. It is believed that the particle distribution across the replica reflects the strength of the connection to the cytoskeleton. Investigations regarding the particle distribution in situations where the cytoskeletal anchorage has been disturbed have been done intensively in epithelial cells. In principle, epithelial tight junction particles are associated with the P-face forming a network of strands and leaving grooves at the E-face which are occupied by only few particles (e.g., Bentzel et al., 1980; Martinez-Palomo et al., 1980; Griepp et al., 1983; Madara and Dharmsathaphorn, 1985; Noske

and Hirsch, 1986; Kniesel and Wolburg, 1993). Epithelial cells in culture form tight junctions with identical properties as in vivo, namely high P-face association, high electrical resistance and low permeability (Griepp et al., 1983; Madara and Dharmsathaphorn, 1985; Gonzáles-Mariscal et al., 1985; Gumbiner and Simons, 1986). After ATP depletion, MDCK cells suffer from deterioration of the paracellular barrier (“gate”) function, which is accompanied by a reorganization of the actin cytoskeleton (Mandel et al., 1993; Bacallao et al., 1994) and a decreased P-face association of the tight junctions. Additionally, tight junctions of low and high resistance Madin-Darby canine kidney (MDCK) cells differ by their association with the membrane leaflets. Tight junctions of low resistance cells (MDCK II) were discontinuous at the P-face revealing particles on the E-face. Tight junctions of high resistance cells (MDCK I) were highly P-face-associated with almost no particles on the E-face (Zampighi et al., 1991; Furuse et al., 2001). Thus, there is a causal relationship between the degree of particle association to the P-face, the observed transepithelial resistance, and the behavior of the cytoskeleton. However, the relationship between the cytoskeletal anchorage and the association with the one or the other membrane leaflet is imperfectly understood.

Endothelial tight junctions reveal a much lower degree of P-face strand association than epithelial cells. The degree of particle association at the P-face depends on the vascular bed investigated. In peripheral endothelial cells, the particles of the tight junctions are predominantly associated with the E-face (Simionescu et al., 1976; Mühleisen et al., 1989). On the P-face, these tight junctions can be recognized only by particle-poor ridges. These properties are maintained also in culture (Fallier-Becker et al., 1991). In contrast, the tight junctions of the blood–brain barrier endothelial cells of mammalian species reveal the highest P-face association found in any vessels of the body. This particle distribution is not maintained in vitro. Instead, the freeze-fracture morphology of cultured blood–brain barrier endothelial cells is similar to non-blood–brain barrier endothelial cells (Wolburg et al., 1994). The importance of the cytoskeletal integrity for blood–brain barrier maintenance in vivo was demonstrated using cytochalasin B and colchicine treatments, respectively. Whereas cytochalasin infusions led to increased blood–brain barrier permeability, colchicine pretreatment of acutely hypertensive rats attenuated the permeability changes (Nag, 1995).

4. Molecular composition of tight junctions in brain barriers

Several tight junction-associated protein components were identified in cerebral endothelial cell tight junctions recently. Occludin (Furuse et al., 1993; Ando-Akatsuka et al., 1996; Hirase et al., 1997), claudin-1 and claudin-5 were localized in cerebral endothelial cell tight junctions (Morita et al., 1999c; Liebner et al., 2000a,b; Lippoldt et al., 2000a; Fig. 1). The submembranous components ZO-1 (Stevenson et al., 1986; Balda and Anderson, 1993), ZO-2 (Jesaitis and Goodenough, 1994), ZO-3/p130 (Balda et al., 1993; Haskins et al., 1998) and the peripherally tight junction associated proteins 7H6 (Zhong et al., 1994) and cingulin (Citi et al., 1989) were also found to be expressed in the blood–brain barrier (Fig. 1). The features of these molecules and their role in barrier formation are reviewed in detail elsewhere (Rubin and Staddon, 1999; Kniesel and Wolburg, 2000; Balda and Matter, 2000; Tsukita et al., 2001; Huber et al., 2001).

4.1. Occludin

Occludin was the first tight junctional transmembrane molecule discovered (Furuse et al., 1993). The tight junctions in occludin-deficient mice (Saitou et al., 2000) were not affected morphologically, and transepithelial resistance as measured in small and large intestine epithelial cells was not altered compared to wild-type mice. However, the mice developed chronic inflammation and hyperplasia of the gastric epithelium, calcifications in the brain and around brain vessels, thinning of bones, postnatal growth retardation, testicular atrophy and abnormalities in sexual behavior (Saitou et al., 2000). The authors concluded that the functions of tight junctions or of occludin are more complex than previously expected. Moreover, occludin is not required for the formation of tight junction strands. It has been shown that the cytoplasmic domain of occludin is highly phosphorylated when localized within the tight junction (Sakakibara et al., 1997). Recent data demonstrated that phosphorylation of occludin regulates tight junction permeability in a G-protein-dependent or -independent manner, according to the receptor involved, independently of cytoskeletal changes (Hirase et al., 2001). Both external loops as well as the transmembrane and the C-terminal cytoplasmic domains of occludin are important for the regulation of paracellular permeability (Balda et al., 2000; Huber et al., 2000). The N-terminal cytoplasmic domain of occludin regulates transepithelial migration of neutrophils, which process is independent of the transepithelial resistance and the paracellular permeability (Huber et al., 2000). On the other hand, recent experiments also demonstrated that occludin is responsible for sealing of tight junctions (Lacaz-Vieira et al., 1999). The authors demonstrated that peptide molecules homologous to segments of the first external loop of occludin interfere with the resealing of tight junctions opened by Ca^{2+} removal. Taken together, from these and previous studies (Balda et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997), it seems that mature cells need occludin to regulate rather than establish their barrier properties.

4.2. The claudins

The claudins are the tight junction molecules, which seem to fulfill the task of establishing barrier properties

(Furuse et al., 1998a,b, 1999, 2001; Morita et al., 1999c; Tsukita and Furuse, 1999). These proteins are integral membrane proteins that share the four transmembrane domains of occludin, but do not contain any sequence homology to occludin. The first detected claudins isolated from chicken liver junctional fractions were called claudin-1 and claudin-2 (Furuse et al., 1998a,b). They soon turned out to be members of a growing family of tight junction proteins (Morita et al., 1999a; Mitic et al., 2000). These proteins are now believed to be responsible for permeability restriction (for reviews, see Tsukita and Furuse, 1999, 2000; Tsukita et al., 2001). The claudins are not randomly distributed throughout the organs, but instead follow certain rules that are not completely understood. For example, claudin-5 was originally described to be restricted to endothelial cells (Morita et al., 1999c). However, the protein was recently also found in surface cells of the stomach and of the large and small intestine (Rahner et al., 2001). Claudin-11 was originally found to be expressed as a single claudin in parallel-stranded tight junctions of central myelin sheaths and Sertoli cells of the blood-testis barrier (Morita et al., 1999b). This finding was supported by the absence of any tight junctions in oligodendroglial and Sertoli cells in claudin-11-deficient mice (Gow et al., 1999). However, the plexus epithelial cells express beside claudin-1 and claudin-2 also claudin-11 (Lippoldt et al., 2000b; Wolburg et al., 2001).

Functional investigations support the view that the composition of the claudin species directly determines barrier function (Furuse et al., 2001). Tight junction-negative L-fibroblasts when transfected with claudin-1 or claudin-3 form tight junctions associated with the P-face (Furuse et al., 1999). When transfected with claudin-2 or claudin-5, the cells form tight junctions associated with the E-face (Furuse et al., 1998a,b; Morita et al., 1999c). In contrast, occludin was found to be localized at both fracture faces (Hirase et al., 1997). Whereas occludin induces short strands, the claudin-induced strands are very long and branched resembling endogenous tight junctions (Tsukita and Furuse, 1999; Furuse et al., 1999). Transfection of MDCK cells with claudin-1 increased the transepithelial resistance about 4-fold and reduced the paracellular flux (Inai et al., 1999). Transfection with claudin-2 of high-resistant MDCK I cells that normally express claudin-1 and claudin-4 mimicked both the resistance behavior and the tight junction morphology of low-resistant MDCK II cells (Furuse et al., 2001). These results support the view that the composition of the claudin species directly determines barrier function. Claudin-4 was formerly known as the *Clostridium perfringens* enterotoxin receptor (CPE-R). By treatment of MDCK I cells, CPE selectively removed claudin-4 from the tight junctions. Tight junctions were disintegrated to a simple network with only few anastomosing strands and the TER was decreased. After CPE removal, barrier properties were re-established (Sonoda et al., 1999). Further, it was concluded that the combination and stoichiometry of the claudins might be responsible for the outcome of a given resistance or permeability resulting in the constitution of aqueous pores (Furuse et al., 1999; Tsukita and Furuse, 2000). Indeed, mutations of paracellin-1 (PCLN-1; claudin-16), localized in epithelial tight junctions of the kidney, have been found to cause renal Mg^{2+} wasting. PCLN-1 alone or with other constituents forms an intercellular pore for the passage of Mg^{2+} or that it operates as a sensor that can alter paracellular permeability or both (Simon et al., 1999).

The only claudins detected in endothelial cells thus far are claudin-1 and claudin-5 (Morita et al., 1999c; Liebner et al., 2000a,b; Lippoldt et al., 2000a). Additionally, a novel anti-claudin-3 antibody recognized cerebral capillary endothelial cell tight junctions (Engelhardt and Wolburg, unpublished). The occurrence of claudin-1 in the blood–brain barrier seems to be variable among different species and is not definitively clarified. As mentioned before, claudin-1/3 is associated with the P-face, whereas claudin-5 with the E-face, at least under transfection conditions in vitro (Furuse et al., 1998a,b; Morita et al., 1999c). Blood–brain barrier endothelial cells in vivo reveal a P-face/E-face ratio of about 55/45% (Kniesel et al., 1996), as well, claudin-1/3 and claudin-5 are well expressed. In non-blood–brain barrier endothelial cells, tight junctions are almost completely associated with the E-face and claudin-1/3 is rarely or not expressed. Blood–brain barrier endothelial cells cultured in vitro develop tight junctions, which are associated with the E-face (Wolburg et al., 1994) and express less claudin-1 (Liebner et al., 2000b). Under pathological conditions such as malignant glioma or experimental allergic encephalomyelitis, claudin-1/3 was found to be lost and/or the tight junctions were E-face associated (Liebner et al., 2000a; Hamm et al., in preparation). However, in asymptomatic stroke-prone spontaneously hypertensive rats, we found a decrease of P-face associated tight junctions. However, this finding was not accompanied with a decrease of claudin-1 (Lippoldt et al., 2000a). We speculated that additional mechanisms different from protein insertion, including post-translational signal transduction processes, could also be involved in tight junction regulation (see below). Very new findings point to a promoter function of claudin-5 in pro-matrix metalloproteinase (MMP)-2 activation by membrane-type matrix metalloproteinase (MT-MMP). This new function of claudin molecules may be important in angiogenesis and in disease processes with increased vessel permeability (Miyamori et al., 2001).

4.3. Adhesion molecules

Adhesion molecules such as the junctional adhesion molecule family (JAM) and the newly discovered molecule endothelial cell-selective adhesion molecule (ESAM) are localized at tight junctions as well. JAM-1 (formerly JAM; Martin-Padura et al., 1998) is expressed in endothelial and epithelial cells, whereas JAM-2 (Aurrond-Lions et al., 2001) and JAM-3 (formerly VE-JAM; Palmeri et al., 2000) are

expressed in most vascular endothelial cells. These molecules belong to the immunoglobulin superfamily and mediate homophilic and probably also heterophilic interactions in the tight junctional region. JAM-1 binds to ZO-1, cingulin (Bazzoni et al., 2000), AF-6/Afadin and Cas kinase/lin-2 via a PDZ binding motif at the carboxy terminus (Martinez-Estrada et al., 2001), and to the multi-PDZ domain protein 1 (MUPP1; Hamazaki et al., 2002). JAM-1 is known to increase cellular resistance in cells that do not normally form tight junctions (Martin-Padura et al., 1998). A blocking antibody against JAM-1 inhibits leukocyte extravasation in vitro and in vivo (DelMaschio et al., 1999). JAM-1 transfection reduces paracellular permeability and promotes occludin localization at intercellular junctions (Dejana et al., 2000). These data suggest that these molecules are involved in organizing the tight junctional structure and in leukocyte extravasation. JAM-2 is a molecule highly expressed in embryonic tissue (in contrast to JAM-1), kidney vascular structures, high endothelial venules in lymphoid organs and lymphendothelial cells. The cytoplasmic tail of JAM-2 is necessary for tight junction direction. JAM-2 when transfected into CHO cells, decreased paracellular permeability and improved the sealing of the cells. Overexpression in MDCK cells led to an enrichment of the protein at the tight junctions (Aurrond-Lions et al., 2001). JAM-3 has been found by investigating lymphocyte homing to secondary lymphoid organs. It is expressed in high endothelial venules, in lymphoid organs and also in endothelial cells of blood vessels of different size in rat and human heart, human placenta, lung and foreskin. It is localized at intercellular boundaries like JAM-1 and -2 and can inhibit transmigration of monocytes (Palmeri et al., 2000).

ESAM (endothelial selective adhesion molecule) is also localized within the tight junctional region (Hirata et al., 2001; Nasdala et al., 2002). ESAM encodes a type I transmembrane glycoprotein with a signal peptide, V- and C2-type immunoglobulin domains and a cytoplasmic domain. The cytoplasmic domain of ESAM is longer than that of JAM and is homologous to the coxsackievirus and adenovirus receptor (CAR). The mature protein consists of 361 (human ESAM) and 365 (murine ESAM) amino acids, respectively. It is expressed in endothelial cells, megakaryocytes and platelets. Its role in vascular morphogenesis is currently under investigation.

4.4. Submembranous tight junction-associated proteins

Submembranous tight junction-associated proteins, such as the zonula occludens proteins-1, -2 and -3 (ZO-1, -2 and-3) are members of a family of membrane associated guanylate kinases (MAGUK). They share three defined core regions: a SH3 domain, a guanylate cyclase and a PDZ domain. These domains are important in signal transduction and in anchoring the transmembrane tight junction proteins to the cytoskeleton. SH3-domains commonly bind signaling proteins and cytoskeletal elements. Guanylate kinases catalyse the ATP-dependent transformation of GMP to GDP. But the homologous domains in some MAGUKs are enzymatic inactive, since binding sites for either ATP and/or GMP are lacking. PDZ-domains are known to mediate specific binding to carboxy-terminal cytoplasmic ends of transmembrane proteins. Binding of MAGUKs to the cytoskeleton was demonstrated for p55 and hDlg via the band 4.1-protein (Lue et al., 1994) and for ZO-1 via fodrin (Itoh et al., 1993; for further description of the role of ZO-1 in tight junction regulation, see Gonzáles-Mariscal et al., 2000; Heiskala et al., 2001). The GUK-domain binds to occludin (Furuse et al., 1994; Itoh et al., 1999) and the PDZ domain to the claudins (Itoh et al., 1999). Recently, a multi-PDZ domain protein 1 (MUPP1) was described possibly interacting with the COOH terminus of claudin-1 and JAM (Hamazaki et al., 2002).

5. Regulation of blood–brain barrier tight junctions

5.1. Modulation of tight junction characteristics

In vitro models of the blood–brain barrier have been established to investigate regulatory mechanisms (Méresse et al., 1989; Rubin et al., 1991; Tontsch and Bauer, 1991; Abbott et al., 1992; Wolburg et al., 1994; Pekny et al., 1998; Stanness et al., 1999; Franke et al., 2000; Gaillard et al., 2001). Some studies have shown that astrocytes or related neuroepithelial cells participate in the induction of barrier properties in endothelial cells (Wolburg and Risau, 1995). Arthur et al. (1987) and Wolburg et al. (1994) provided evidence for the release of humoral factors by astrocytes which were suggested to contribute to tight junction formation. In contrast, Tao-Cheng et al. (1987) found the requirement of a direct contact between astrocytes and endothelial cells. From transplantation experiments, it became evident that blood–brain barrier characteristics are determined to a large extent by extrinsic factors (Wolburg and Risau, 1995). Factors released from astrocytes seem to be necessary (Arthur et al., 1987; Tao-Cheng et al., 1987; Tontsch and Bauer, 1991; Dehouck et al., 1994), but are not sufficient to induce and maintain blood–brain barrier characteristics (Rubin et al., 1991; Wolburg et al., 1994). Until now, the factors which are effective in blood–brain barrier induction remain obscure, since the environment of brain capillaries is complex and there are putatively more influences of distinct origins, which might work synergistically in a defined temporal and spatial pattern. At least, the glial cell line-derived neurotrophic factor (GDNF) seems to belong to the cocktail of factors necessary for blood–brain barrier induction (Igarashi et al., 1999; Utsumi et al., 2000; Yagi et al., 2000).

As mentioned earlier, cerebral endothelial cells lose their barrier characteristics when cultivated and resemble peripheral endothelial cells. However, the elevation of cellular cAMP levels by forskolin and the treatment with condi-

tioned medium derived from astrocytes resulted in an increase of tight junction complexity and of P-face association of tight junctional particles (Wolburg et al., 1994). The combination of astrocyte-conditioned medium and forskolin was most effective in the improvement of barrier properties in cultured endothelial cells (Rubin et al., 1991; Wolburg et al., 1994).

5.2. Second messenger pathways

In vitro studies using epithelial cells indicated that G-proteins play an essential role in maintaining barrier integrity as defined by the transepithelial resistance or permeability. The G-proteins involved in that process are the classical heterotrimeric G-proteins and the small G-proteins/small GTPases (Ras superfamily). Several $G\alpha$ subunits have been localized within the tight junction of cultured epithelial cells, such as $G\alpha_{i2}$ (Denker et al., 1996; Saha et al., 1998), $G\alpha_{12}$ (Dodane and Kachar, 1996) and $G\alpha_{O}$, when transfected into MDCK cells (Denker et al., 1996). Moreover, in immunoprecipitation studies, it has been demonstrated that $G\alpha_{O}$ could be co-precipitated with ZO-1 (Denker et al., 1996). The activation of heterotrimeric G-proteins leads to activation of second messengers like cAMP/cGMP or Ca^{+}. Expression of constitutively activated $G\alpha_{O}$ and $G\alpha_{i2}$ subunits in MDCK cells led to an increased transepithelial resistance of the cell monolayer. Additionally, $G\alpha_{O}$ accelerated tight junction biogenesis, whereas $G\alpha_{i2}$ was important for development and maintenance of the tight junctions (Denker and Nigam, 1998; Saha et al., 1998). However, a receptor coupled to the heterotrimeric G-proteins and the signal transduction pathways modulating the tight junction function has not been found yet. In contrast to the results obtained by Denker and co-workers, Balda et al. (1991) reported on an increased transcellular resistance in epithelial cells after treatment with pertussis toxin (PTX), an inhibitor of G-protein signaling. The mechanism of this action has not been unraveled yet. These contradicting data could reflect different underlying mechanisms. Indeed, it has recently been shown that PTX acts via extracellular signal-regulated kinase (ERK)-activation involving a new protein kinase C dependent signaling pathway (Garcia et al., 2001).

Heterotrimeric G-proteins were detected by Western Blot in cultured rat cerebral endothelial cells including the $G\alpha$ subtypes i1, i2, i3, q/11, O as well as $G\beta$ (Fabian et al., 1998). We could localize $G\alpha_{i3}$ within the tight junction region in rat cerebral endothelial cells in vivo and demonstrated the importance of G-protein signaling for the tight junction morphology (Lippoldt et al., in preparation). We found that G-protein inhibition by treatment with PTX in vivo led to an increased E-face association of tight junction particles in rat blood–brain barrier endothelial cells not influencing tight junction molecule expression or permeability towards albumin. We concluded that G-protein signaling has an impact on the blood–brain barrier structure via intracellular signaling pathways affecting the cytoskeletal anchorage of transmembrane tight junctional molecules. Indeed, recent work by Garcia et al. (2001) demonstrating that PTX induced an ERK-dependent modification of cytoskeletal targets in peripheral endothelial cells supported our hypothesis. Moreover, PTX has been demonstrated to induce barrier dysfunction in pulmonary artery endothelial cells (Patterson et al., 1995).

5.3. Small G-proteins/GTPases

The RhoA and Rac1 small GTPases were shown to play a promoting role in the regulation of tight junction structure and function. In MDCK-cells expressing RhoA and Rac1 mutants, the organization of tight junctions-strands is grossly altered and the permeabilities for inulin, anionic or neutral dextran, as well as the transcellular electrical resistance, are strongly affected (Jou and Nelson, 1998; Jou et al., 1998). The inhibition of the Rho pathway by *Clostridium botulinum* exotoxin resulted in a disorganization of the perijunctional actin ring and ZO-1 in T84 cells, and the transient expression of RhoC resulted in actin concentration at intercellular contacts (Nusrat et al., 1995; Madara, 1998; for a recent review, see Sears, 2000).

Interestingly, the GTPase-dependent pathways regulating the intercellular permeability seem to operate in an opposite way in epithelial and endothelial cells. In cerebral endothelial cells, the activation rather than inhibition of the Rho pathway in vitro by lysophosphatidic acid (LPA) (Moolenaar, 1995) disrupted the paracellular barrier (Schulze et al., 1997). Accordingly, the inhibition of the Rho pathway prevented the lysoposphatidic acid-induced increase in permeability (Hirase et al., 2001). Inflammation as caused by thrombin, histamin and cytokines increases the transendothelial permeability in vitro by affecting tight and adherens junctions via reorganization of the actin cytoskeleton and formation of intercellular gaps (Lum and Malik, 1996; Wojciak-Stothard et al., 1998; Johnson-Léger et al., 2000; Mayhan, 2000; Liu et al., 2001). In human umbilical vein endothelial cells (HUVEC), this affection of the permeability requires the activation of the Cdc42-, rac-, Rho-cascade following stimulation by TNF-α (Wojciak-Stothard et al., 1998). In vitro studies in HUVE cells demonstrated that a Rho/Rho kinase-dependent pathway is a central target for inflammatory agents like bacterial toxins to induce increased vascular permeability (Essler et al., 1998, 2000). Thereby, Rho/Rho kinase inactivates the myosin light chain (MLC) phosphatase, producing enhanced MLC phosphorylation leading to endothelial cell contraction and increased permeability (Essler et al., 2000). Likewise, the zonula occludens toxin (Zot) produced by *Vibrio cholerae*, and its human homologue zonulin was reported to bind to a specific surface receptor, which was purified from the brain. After binding to its receptor, Zot is internalized and triggers signaling processes including phospholipase C- and PKCα-activation and actin polymerization, which lead to

tight junction disruption (Lu et al., 2000). Ras signaling has been investigated in pulmonary artery endothelial cells as well. Activation of protein kinase C by phorbol ester treatment led to barrier dysfunction via sequential activation of Ras/Raf-1and MEK signaling pathways followed by the activation of ERK (Verin et al., 2000).

5.4. Regulatory proteins of G-protein signaling

Recently, a family of G-protein regulating proteins has been identified. These so called RGS proteins (regulators of G-protein signaling) interact with the Gα subunit of heterotrimeric G-proteins. They are key elements inducing receptor desensitization by inactivating the Gα subunit via their ability to accelerate GTP hydrolysis (De Vries and Farquahr, 1999). Interestingly, in *Drosophila* a gene has been identified called *loco* that is important for glial cell differentiation (Granderath et al., 1999). *Loco* encodes a member of RGS proteins that interacts specifically with the Gα subunit of *Drosophila* heterotrimeric G-proteins. This results in a disruption of the blood–brain barrier (Granderath et al., 1999) underlining the importance of astrocytes for blood–brain barrier integrity. A genomic suppression subtractive hybridization (SSH) approach has been used to identify specific genes expressed at the blood–brain barrier (Li et al., 2001). Among other genes RGS5 was found to be expressed at the blood–brain barrier but also in other tissues. By using the same approach, comparing blood–brain barrier capillaries from stroke-prone (SHRSP) versus stroke-resistant hypertensive rats (SHR), we recently reported on the differential expression of RGS5 and found the mRNA localized in the blood–brain barrier endothelial cells (Kirsch et al., 2001). Immunocytochemical detection of RGS5 at the light and electron microscope level revealed its occurrence in tight junctions of blood–brain barrier endothelial cells, and freeze-fracture labeling finally proved that RGS5 is a tight junction molecule. In in vitro experiments and during induction of blood–brain barrier properties, RGS5 was targeted to the tight junctional region (Lippoldt et al., submitted). These data again indicate the complexity of signaling processes at the blood–brain barrier.

5.5. Extracellular matrix and blood–brain barrier

The blood–brain barrier is composed of neuronal and glial cells, pericytes, and the extracellular matrix, in particular the subendothelial basal lamina. The basal lamina of cerebral endothelial cells is very complex and consists of a number of collagens, laminin, fibronectin, entactin, thrombospondin, heparan sulfate proteoglycans such as agrin, and chondroitin sulfate proteoglycans such as 250-kDa CSPG and versican (Abrahamson, 1986; Yurchenko and Schittny, 1990; Vorbrodt et al., 1988; Barber and Lieth, 1997; Gladson, 1999; Savettieri et al., 2000). Cerebral and aortic endothelial cells, when cultured in astrocyte-conditioned medium developed complex tight junctions when grown on endothelial cell matrix. However, this was not the case when fibronectin was used as the matrix protein (Arthur et al., 1987; Shivers et al., 1988). By co-culturing aortic endothelial cells with astrocytes on collagen type I and IV demonstrated that only collagen type IV was able to induce a decrease in the number of vesicles in the endothelial cells and tight junctions which were impermeable to HRP (Tagami et al., 1992). Collagen type IV directly influences the expression of occludin (Savettieri et al., 2000). Thus, the loss of laminin, collagen type IV and fibronectin in focal cerebral ischemia/reperfusion can add to further worsening of endothelial cell function leading to, e.g., hemorrhagic complications in stroke (Hamann et al., 1995). An isoform of the heparan sulfate proteoglycan agrin originally characterized as the essential molecule for clustering acetylcholine receptors at the motor endplate (McMahon, 1990) was described as important for the integrity of the blood–brain barrier (Barber and Lieth, 1997; Berzin et al., 2000) and possibly for the polarity of astrocytes (see above).

5.6. Transmigration of blood cells through the blood–brain barrier

Leukocytes/lymphocytes can infiltrate the brain parenchyma via the blood–brain barrier during inflammation like multiple sclerosis (MS). The mode by which inflammatory cells invade the brain, whether disrupted intercellular junctions or the endothelial cells are penetrated, has been discussed controversially (for a recent overview, see Burns et al., 2000). The endothelial cell adhesion molecule ICAM-1, expressed on endothelial cells during inflammatory diseases, is known to act as a binding molecule for circulating lymphocytes (see, for example, Stanimirovic and Satoh, 2000). The binding of lymphocytes, as mimicked by ICAM-1 cross-linking in RBE4 cells, resulted in actin stress fiber formation. ICAM-1 participates in this process not only as a passive adhesion site for activated circulating lymphocytes, but also is actively involved in signal transduction processes that mediate lymphocyte extravasation through the blood–brain barrier. These include the stimulation of tyrosine phosphorylation of focal adhesion kinase (FAK), paxillin and p130^{cas} (Cas) as well as their association in a multimolecular complex (Etienne et al., 1998) and cortactin (Adamson et al., 1999). ICAM-1 cross-linking also caused the association of tyrosine phosphorylated Cas with the adaptor protein Crk and the GTP exchange factor C3G and Rho activation (Etienne et al., 1998; Adamson et al., 1999) and induced c-Jun-N-terminal kinase (JNK) activation via a Rho-dependent pathway (Etienne et al., 1998). The results suggest the induction of genes that increase cerebral endothelial cell permeability via JNK dependent transcription factor phosphorylation. Moreover, since JNK activation has been shown to affect interendothelial junctional complexes, it can contribute via multiple actions to ICAM-1 induced blood–brain barrier breakdown (Etienne et al., 1998; Adamson et al., 1999).

Finally, as already mentioned above, other mechanisms seem to be involved in inflammation. In HIV-1 associated dementia, blood–brain barrier endothelial cells were shown to lose ZO-1 during monocyte infiltration (Boven et al., 2000). Occludin has recently been shown to be actively involved in transmigration processes (Huber et al., 2000) as well as adhesion molecules localized at tight junctions like JAM-1 to -3 (DelMaschio et al., 1999; Palmeri et al., 2000; Aurrond-Lions et al., 2001) and ESAM (Hirata et al., 2001). This state-of-affairs led to the suggestion of a close interaction of endothelial cell adhesive properties with tight junction associated transmigration regulating molecules.

6. Blood–brain barrier in disease

Blood–brain barrier dysfunction is a dramatic change that leads to damages in the neuropil and disturbed brain function. Despite of the growing knowledge in blood–brain barrier composition, tight junction structure and factors influencing barrier characteristics in vitro and in experimental animals, our knowledge regarding the molecular mechanisms in disease processes is very limited. The reasons are the limited availability of proper animal models that mimic human brain pathologies, and the inability of the detection of specific cellular signaling mechanisms in vivo, due to a multitude of intrinsic and extrinsic influences that cannot be easily separated. Diseases such as encephalitis, MS, hypertension, stroke or tumors induce deterioration of the blood–brain barrier with devastating influences on neuronal function. There are two main types of blood–brain barrier injuries: (i) the complete breakdown of barrier function and (ii) subtle barrier impairment without manifest end-organ damage.

Brain tumors cause a complete breakdown of the blood–brain barrier that leads to peritumoral edema (Coomber et al., 1987; Long, 1970). In our investigations, we showed that the loss of endothelial tight junction properties in tumors is accompanied by a dramatic decrease of the P-face association of the tight junction particles connected with a massive downregulation of claudin-1. Even in the outer zone of the tumors claudin-1 is already downregulated leading to the hypothesis that claudin-1 downregulation is an early marker of barrier dysfunction (Liebner et al., 2000a). The changes appear with the angiopoetic switch in tumors due to their demand in nutrient supply (Maisonpierre et al., 1997; Zagzag et al., 1999; Holash et al., 1999). The mechanisms include the secretion of growth factors, expression and activation of MMPs (serine proteases) and an increase in specific matrix components like tenascin-C (Conant et al., 1999; Westermark and Kahari, 1999; Jones et al., 1997; Fischer et al., 1999; Plate et al., 1992; Hermansson et al., 1988; Jones and Jones, 2000; Rascher et al., 2002). The ongoing synthesis and secretion of these factors then leads to the dedifferentiation of the endothelial cells from polar cells with morphological and functional well developed tight junctions into angiogenic, proliferating and migrating cells. Barrier breakdown also occurs in MS (Long, 1970; Lassmann, 1983; Engelhardt, 1997) and stroke (Dirnagl et al., 1999). In the experimental allergic encephalomyelitis, as an animal MS model, claudin-1 and claudin-3, but not claudin-5 was specifically downregulated in sections of the vasculature, which were surrounded by inflammatory cells (Hamm et al., in preparation). The implication of this downregulation for the transendothelial migration of inflammatory cells is not understood, because transmigration does not occur through intercellular contacts (Wolburg-Buchholz, Wolburg and Engelhardt, unpublished observations; Burns et al., 2000).

In contrast, in spontaneously hypertensive stroke-prone rats (SHRSP) that have not yet developed stroke, we subtle barrier impairments in the blood–brain barrier (Lippoldt et al., 2000a). We found an increased ratio of the E-face/P-face association of the tight junction particles. This increased E-face association was accompanied by a changed glucose transporter-related polarity of the blood–brain barrier endothelial cells underlining the importance of the tight junction integrity for the fence function. However, we detected no alterations in the immunoreactivity of tight junction proteins or any increased capillary permeability for lanthanum nitrate (Lippoldt et al., 2000a). We hypothesized a rearrangement of the cytoskeletal coupling of the tight junction proteins being responsible for the changed E-face/P-face association. One reason for these alterations were early changes in G-protein signaling in SHRSP cerebral capillaries that could be modulated by pertussis toxin-evoked Gi-protein inhibition in normotensive rats (Kirsch et al., 2001; Lippoldt et al., in preparation). Thus, subtle barrier regulations are not necessarily dependent on the differential expression of tight junction proteins but are also induced by signaling events, leading to a changed tight junction phenotype. Those changes might be an early step on the way to further barrier damages finally leading to stroke. Especially in hypertension induced stroke, laminar shear stress could play a role in the disruption of the blood–brain barrier. It has been described that tight junctional compounds are strongly expressed in endothelial cells that are exposed to high laminar shear stress, but less in areas with low shear stress (Yoshida et al., 1995; DeMaio et al., 2001). Indeed, in SHRSP the cerebral blood flow, as well as cerebral protein synthesis, declines before the onset of stroke (Mies et al., 1999). These events can act in concert and finally lead to barrier breakdown as observed in stroke. It will be of great importance to unravel the connections between tight junction structure, protein expression, signaling processes and physiological parameters like shear stress in health and disease.

7. Blood–brain barrier genomics: new tools

The availability of new molecular methods allowing the investigation of differences in gene expression (i.e., SSH or gene arrays), e.g., among different organs, specific cell types

in different tissue environments and healthy and pathological changed organs gives the opportunity to unravel new molecules differentially regulating cell functions. These studies will open up new avenues to find the factors necessary for the induction of barrier properties in cerebral endothelial cells that could not fully be unraveled until today as well as specific molecules responsible for barrier breakdown in different disease conditions. There are few reports on gene expression approaches. Li et al. (2001) reported on 50 differentially expressed clones isolated by comparing cerebral microvessels with kidney and liver tissue. Over 80% of these clones were selectively expressed at the blood–brain barrier. In another approach, Dombrowski et al. (2001) used cDNA microarrays and protein analysis to study the responses of human brain endothelial cells exposed to flow by contacting astrocytes and neuronal cells (Stanness et al., 1999). The authors found, e.g., differentially expressed genes responsible for mitotic arrest, glycolytic pathways and cytoskeletal genes. We recently reported on the differential gene expression between stroke-resistant and stroke prone spontaneously hypertensive rats using SSH. We found receptor genes, genes involved in G-protein signaling, matrix regulating genes and transcription factors that differ in expression strength in blood–brain barrier capillaries between these pathological models (Kirsch et al., 2001). These methods give an immense amount of data and it will need concerted efforts from molecular biologists, cell biologists, morphologists and physiologists to examine the importance of the gene products that show differences in their expression levels at the blood–brain barrier in development, health and disease.

Acknowledgements

We thank Gesa Rascher and Friedrich Luft for careful reading the manuscript and valuable comments.

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