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Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme

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Abstract The aim of the study was to characterize the interendothelial junctions in tumor microvessels of five cases of human glioblastoma multiforme. In addition to morphological analysis, tumors were screened for the expression of junctional proteins, such as occludin, claudin-1, ZO-1 and catenins. The expression of the tight junction protein claudin-1 was lost in the majority of tumor microvessels, whereas claudin-5 and occludin were significantly down-regulated only in hyperplastic vessels. As shown by freeze-fracture analysis, under the conditions of tumor growth tight junction particles of endothelial cells were almost exclusively associated with the exocytoplasmic fracture face, providing evidence for a switch of the particles from the protoplasmic to the external leaflet of the endothelial membrane. These results suggest a relationship between claudin-1 suppression and the alteration of tight junction morphology, which is likely to correlate with the increase of endothelial permeability. Underlining the undifferentiated state of tumor microvessels, plakoglobin, a crucial protein for mature endothelial junctions, was not detectable in most microvessels, whereas β-catenin was abundantly labeled. In this context, it is of particular interest that the majority of microvascular pericytes were negative for alpha-smooth muscle actin, which is a marker of differentiated pericytes, although pericytes were frequently found in electron micrographs. In conclusion, the data suggest that the increase in microvascular permeability in human gliomas, contributing to the clinically severe symptoms of brain edema, is a result of a dysregulation of junctional proteins.

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Introduction

The blood-brain barrier protects the brain from a changing composition of the blood and creates a stable microenvironment in the neural parenchyma. It is located in the endothelial cells, and restricts the paracellular diffusion of hydrophilic molecules by complex tight junctions and a low degree of transcytosis. On the other hand, brain endothelial cells are equipped with various specific transporters for compounds essential for the brain metabolism [22, 35, 36]. In brain tumors, blood vessels characteristically lose their blood-brain barrier properties, a process, which results in a dramatic increase of vascular permeability, leading to the most serious clinical sign of brain edema [26, 33]. Glioblastoma multiforme is the most malignant brain tumor and is characterized by pronounced hypercellularity, pleomorphism, numerous mitoses, foci of necrosis and palisading, and excessive vascularization. The morphological alterations of the blood vessels as described by a large body of literature [6, 7, 14] are related mainly to the formation of fenestrations, to the increase/decrease of the number of caveolae/mitochondria, respectively, to the thickness of the subendothelial basal lamina and to the pericytes. Little is known about the barrier-related molecular alterations, and this require further investigation.

The elucidation of the molecular composition of tight junctions has advanced considerably in recent times. Since 1993 it is known that the four-transmembrane domain protein occludin is a constituent of tight junctions [9], including those of blood-brain barrier endothelial cells [15]. The finding that occludin-deficient stem cells form morphologically normal tight junctions [37] was followed by the discovery of the claudins, a new gene family of tight junction-related proteins which also have four transmembrane domains but no homology to occludin

[10, 11, 28]. The finding that claudins are able to form tight junctions in the absence of occludin when transfected into fibroblasts was complemented by the fact that transfection with claudin-1 leads to the association of tight junction particles with the protoplasmic fracture face (P-face), whereas claudin-2 transfected fibroblast show particles associated with the exocytoplasmic fracture face (E-face) [11]. However, since claudin-1, claudin-5 and claudin-11 were described as being expressed in the brain [10, 28] and claudin-11 by oligodendrocytes only [29], it was suggested that occludin together with claudin-1 and claudin-5 are the most important constituents of blood-brain barrier tight junctions. Recently, this was confirmed by Morita et al. [30], who demonstrated that claudin-5 is specifically expressed in endothelial cells and forms E-face associated tight junctions. Therefore, the particle association with the P-/E-face in endothelial cells is believed to be a consequence of the combination of claudin-1 and claudin-5.

Functional tight junctions are also characterized by the linkage to the cytoskeleton that is accomplished by proteins which belong to the family of membrane-associated guanylate kinase homologues (MAGUKs) [13, 19, 45]. ZO-1, the best known member of this family, binds to the most conserved region of the C-terminal domain of occludin. Additionally, ZO-1 is known to play a role in signal transduction [2, 48] and to have the potential to interact with components of the adherens junction system, e.g., β-catenin [1, 16, 17].

In contrast to epithelial cells, adherens junctions of endothelial cells, which are basically composed of cadherins and catenins, are known to be intermingled with tight junctions [39]. Catenins belong to the family of armadillo proteins, which mediate the linkage of transmembraneous cadherins to the cytoskeleton [23, 44]. Furthermore, catenins are known to act as transcription factors under developmental and pathological circumstances [5, 8, 27]. In endothelial cells, the expression and localization of β-catenin and γ-catenin (plakoglobin) have been described to be crucial for the functional state of adherens junction [24, 25, 38].

The current work describes the morphology of gliomatous endothelial cells in light and electron microscopy, including the appearance of tight junctions in freeze-fracture preparations. Furthermore, the expression and distribution of tight junction and tight junction-related antigens such as occludin, claudin-1, claudin-5 and ZO-1 [9, 10, 15] and adherens junction proteins such as catenins were investigated.

The results presented here suggest a down-regulation of the main blood-brain barrier component claudin-1 in most microvessels, whereas claudin-5 was frequently found. In parallel, a redistribution of tight junction particles within the junctional membrane of the endothelial cells from predominantly P-face to E-face was observed. Occludin was only down-regulated in hyperplastic gliomatous endothelial cells. With the exception of plakoglobin, other tight junction and adherens junction proteins seemed to be unaffected.

Material and methods

The material was surgically removed from patients suffering from glioblastoma multiforme at the Department of Neurosurgery of Tübingen University Clinics. Immediately after removal (between 5 and 10 min after dissection), tissue was fixed either in 4% buffered paraformaldehyde or 2.5% buffered glutaraldehyde, or embedded directly in Tissue Tek O.C.T. (see below).

All chemicals were purchased from Merck, Darmstadt, Germany, unless specifically mentioned.

Conventional electron microscopy

For conventional electron microscopy, specimens fixed by immersion in 2.5% glutaraldehyde in 0.1 M HEPES (Sigma, Deisenhofen, Germany) containing 0.15 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4 (HMSS) buffer were 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 an EM 10A electron microscope (Zeiss, 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 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.

Antibodies

Production of polyclonal antibody

To obtain a polyclonal antibody against mouse claudin-5/TMVCF, a polypeptide (SAPRRPTANGDYDKKNYV), which corresponds to the C-terminal cytoplasmic domain of mouse claudin-5/TMVCF was synthesized and as a multiple antigenic peptide (MAP) construct on an eight-branched resin [41]. The immunogen was sent to Charles River Laboratory Service System (Kisslegg, Germany) and injected into a rabbit as antigen. 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. The polypeptide was coupled to a modified Fractogel matrix (Merck) [20]. To improve the accessibility for the antibody, three spacer molecules (ε-amino caproic acid, β-alanine, β-alanine) were inserted between the peptide and the matrix. The optimized matrix was used for antibody purification.

Commercial antibodies

The following specific primary antibodies were used for immunohisto-/immunocytochemistry: rabbit polyclonal antiserum against human occludin; rabbit polyclonal antiserum against human claudin-1; rabbit polyclonal antiserum against human ZO-1; mouse monoclonal antibody against human β-catenin (Zymed,

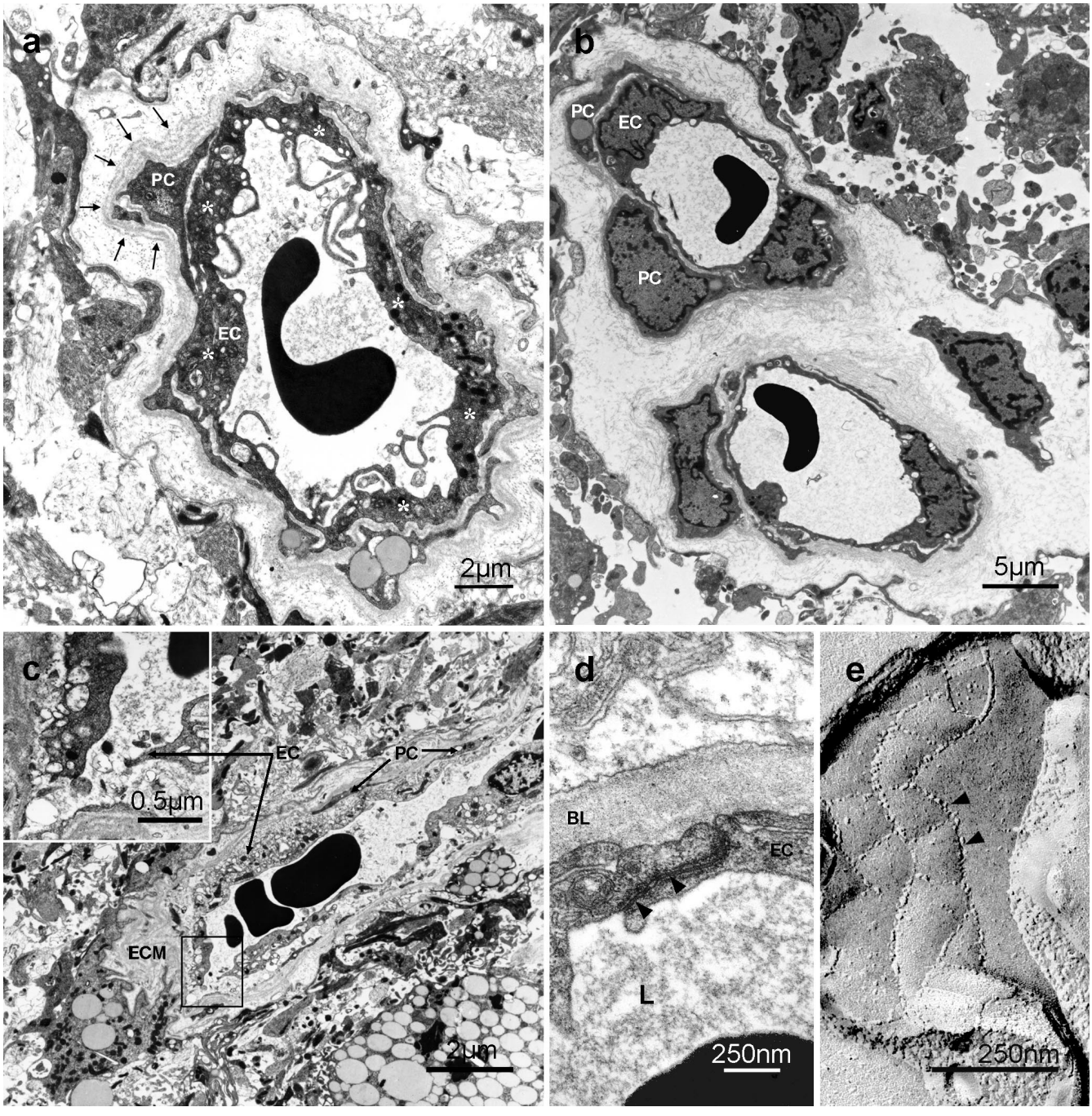


Fig. 1 a–e Electron microscopy of various microvessels in human glioblastoma multiforme. **a** Hyperplastic tumor microvessel. Note that the circumference is formed by more than one EC. The luminal surface of ECs is increased by numerous microfolds. Perivascular space is enlarged and the basal lamina is multilayered (*arrows*). **b** Microvessels from another tumor showing the variability of vascular alterations in different tumors concerning the endothelial surface, the number of ECs per circumference and the coverage by PCs. Note the amount of ECM between the vessels. **c** Microvessel interrupted by a gap in the endothelial lining (see *frame* and *insert*). The ECs are considerably vacuolized but luminal microfolds are lacking. **d** High magnification of interendothelial tight junction of a tumor microvessel. By this method, the morphology of the tight junction appears normal. Note tight junction "kisses" (*arrowheads*). **e** Freeze fracture replica of an endothelial cell tight junction of a tumor microvessel. The tight junction particles are predominantly associated with the E-face (*arrowheads*) (*EC* endothelial cell, *PC* pericyte, *ECM* perivascular extracellular matrix, *BL* basal lamina, *L* lumen)

South San Francisco, USA); mouse monoclonal antibody against human plakoglobin (γ-catenin) (Transduction Lab., Lexington, USA); mouse monoclonal antibody against human von Willebrand factor (Dako, Glostrup, Denmark) and mouse monoclonal antibody against human alpha-smooth muscle actin (Sigma).

Secondary antibodies for immunofluorescence labeled with dichlortriazinylamino fluorescein (DTAF) and cyanin-derivative dyes Cy2, Cy3 and Cy5 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 to exclude cross-reaction.

Immunohistochemistry

Immunostaining on paraformaldehyde-fixed and unfixed specimens was performed as described [12]. Specimens were immersion fixed for 2 h on ice in 4% freshly depolymerized paraformaldehyde in HMSS. 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), 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), 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*-phenylenediamine (Sigma) as anti-bleaching agent. Fluorescence was visualized with a confocal laser scanning microscope (Zeiss). Images were computer processed using Adobe Photoshop (Adobe, Mountain View, Calif.).

Results

Morphology

The clinical material investigated was restricted to five cases with glioblastoma multiforme. In ultrathin sections, endothelial cells of the blood vessels were often irregularly shaped, and both the luminal and the abluminal membrane were folded. The cytoplasm contained more vacuoles and vesicles in comparison with normal microvessels. The basal lamina of tumor blood vessels was frequently thickened (Fig. 1 a, b) and the basal lamina of pericytes surrounded the cells for a certain distance. Consequently, the topological relationship between endothelial and perivascular cells was disorganized (Fig. 1 a). Frequently, the perivascular space was enlarged and the basal lamina was multilayered (Fig. 1 a). At non-junctional sites, endothelial cell continuity was occasionally interrupted, leading to leakage of erythrocytes (Fig. 1 c). For the most part, the interendothelial junctions looked normal: tight junctions were formed including intercellular "kisses", and the junctional area was osmicated beneath the membrane, indicating cytoskeletal elements (Fig. 1 d).

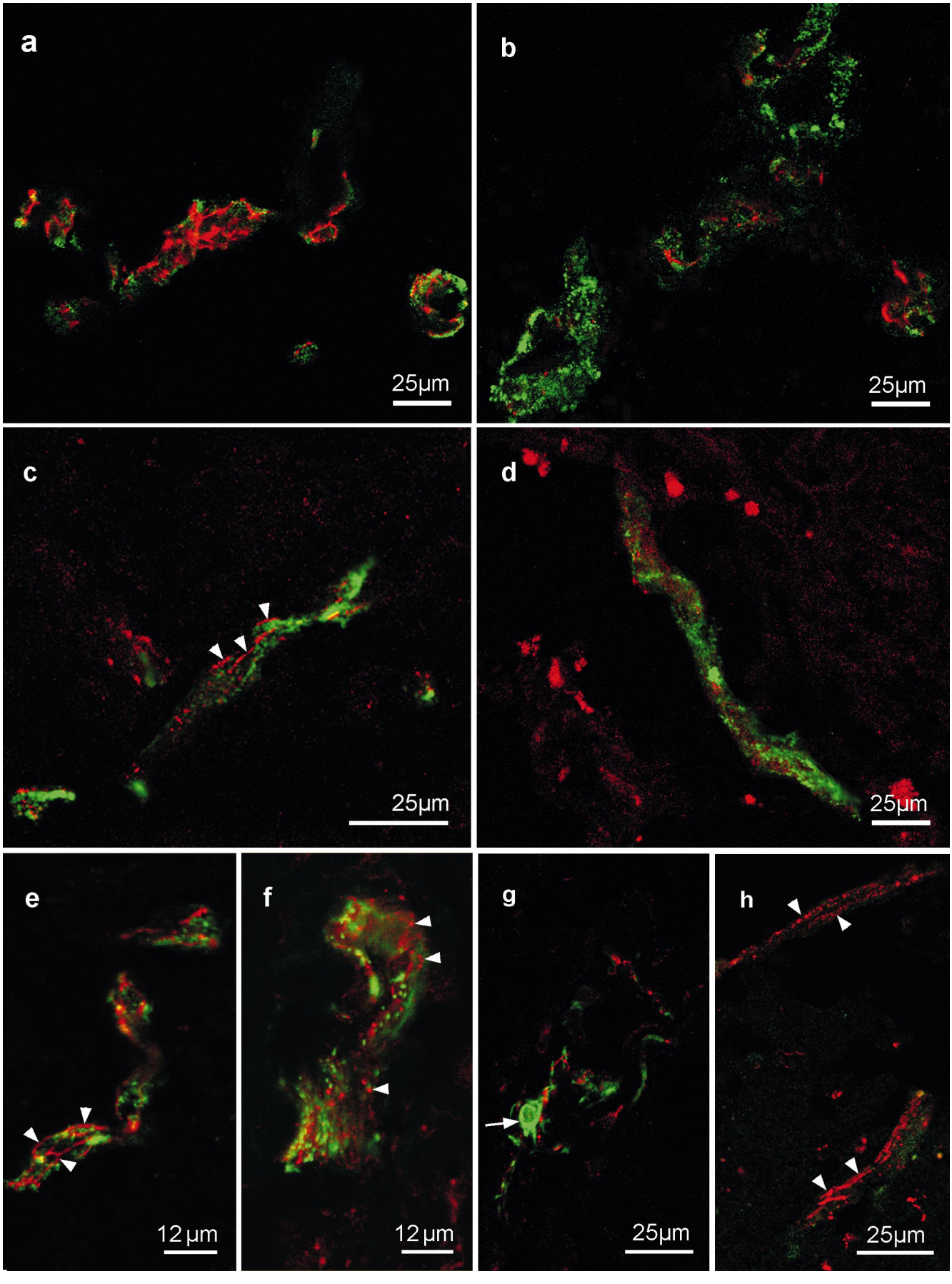
Since ultrathin-section electron microscopy has proved not to be the appropriate method for evaluating tight junction integrity, we additionally inspected the freeze-fracture morphology of tight junctions. Because oligodendrocytes and endothelial cells are the only cells in the mammalian central nervous system carrying tight junctions, and since oligodendrocytic tight junctions differ clearly from their endothelial counterparts, we were able unequivocally to identify endothelial tight junctions. As is known from brain microvessels, they are characterized by a highly complex network of strands predominantly localized on the P-face [31]. Surprisingly, most of the tight junctions showed particles almost completely associated with the external leaflet (E-face) of the junctional membrane, whereas at the P-face ridges, particles were only sparsely distributed (Fig. 1 e). This is in contrast to tight junction morphology of normal brain microvessel endothelial cells, which are associated predominantly with the P-face [21].

Expression of junctional antigens

In a further step, the expression patterns of two different junctional markers, occludin and claudin-1 were investigated by means of immunofluorescence and confocal laser scanning microscopy. The majority of tumor microvessels showed a distribution of occludin (Fig. 2 a) which was identical with that in mature rat brain microvessels (data not shown). A direct comparison between non-gliomatous and gliomatous vessels in the human brain was not possible owing to the difficulty in accessing healthy human brain tissue. As opposed to the majority of microvessels, hyperplastic vessels revealed down-regulated occludin, which was no longer restricted to the junctions (Fig. 2 b). Surprisingly, double-labeling experiments for claudin-1 and von Willebrand factor revealed a complete loss of claudin-1 in nearly all tumor microvessels inspected (Fig. 2 c, d). In contrast, in most tumor microvessels claudin-5 was abundant at interendothelial junctions (Fig. 2 e), whereas in hyperplastic vessels only a faint intracellular spot-like labeling was found (Fig. 2 f).

Interestingly, double-labeling experiments revealed that only a subset of tumor microvessels positive for

Fig. 2 a–h Immunofluorescence labeling of tumor microvessels. ▶ **a**,**b** Double labeling against von Willebrand factor (*green*) as a reliable endothelial marker and the tight junction protein occludin (*red*). Both micrographs show vessels from the same tumor. Most vessels are strongly positive for occludin (**a**), whereas some hyperplastic vessels exhibit low level of occludin labeling (**b**). **c**, **d** Double labeling against von Willebrand factor (*green*) and the tight junction protein claudin-1 (*red*). Both micrographs show vessels from the same tumor. Only few vessels are positive for claudin-1 (**c**, *arrowheads*), although the tumor is excessively vascularized as indicated by anti-von Willebrand factor immunoreactivity (**d**). **e**, **f** Double labeling against von Willebrand factor (*green*) and the endothelial-specific tight junction protein claudin-5 (*red*). In the majority of tumor vessels claudin-5 was frequently detected at interendothelial junctions (**e** *arrowheads*). In hyperplastic vessels, claudin-5 is never found at junction sites but intracellularly, in a spot-like manner. **g**, **h** Double labeling against ASMA (*green*) as a pericyte marker and the tight junction protein claudin-1 (*red*). The observation of ASMA-positive pericytes (g, *arrow*) is extremely rare. In contrast most claudin-1 positive (*arrowheads*) microvessels are devoid of ASMA-positive pericytes (**h**)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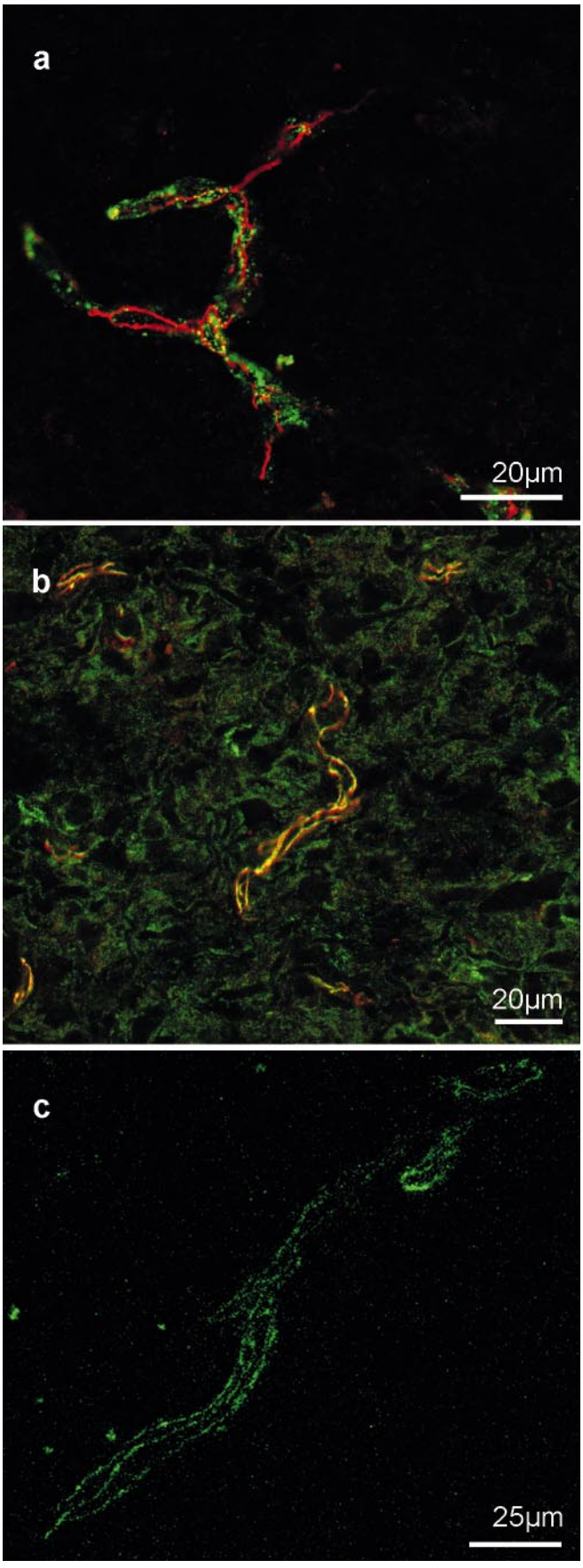

Fig. 3 a–c Immunofluorescence labeling of tumor microvessels. **a** Double labeling against von Willebrand factor (*green*) and the tight junction-associated protein ZO-1 (*red*). All tumor microvessels are positive for ZO-1, restricted to interendothelial junctions. **b** Double labeling against β-catenin (*green*) and ZO-1 (*red*). Both antigens are co-distributed in the junctions of tumor microvessels. The overlap of *red* and *green* leads to *yellow* fluorescence. **c** Labeling of plakoglobin is only very faintly detectable at the contact sites of tumor endothelial cells; therefore, the ZO-1 labeling is not superimposed so as not to mask the plakoglobin labeling

claudin-1 co-distributes with pericytes positive for α-smooth muscle actin (ASMA), an antigen considered a marker for differentiated pericytes (Fig. 2 g, h). This is of particular significance, because electron micrographs regularly demonstrate the presence of pericytes (Fig. 1; see discussion).

To determine whether cytoplasmic tight junction proteins also belong to the set of elements altered in glioma endothelial cells, we labeled tumor cryosections for ZO-1 in combination with von Willebrand factor. In the majority of tumor microvessels ZO-1 seemed not to be changed (Fig. 3 a). Only in some hyperplastic vessels was a discontinuity of ZO-1 labeling along the interendothelial contacts observed (data not shown).

To further characterize the interendothelial contacts of tumor microvessels we labeled tumor cryosections for β-catenin and plakoglobin. β-Catenin was regularly found in tumor microvessels as indicated by double labeling with ZO-1 (Fig. 3 b), whereas plakoglobin was only faintly labeled in a small set of microvessels derived from different tumors (Fig. 3 c).

Neoplastic tumor cells did not show any claudin-1/5 labeling. In contrast, occludin was expressed in gliomatous astrocytes in one out of five glioblastoma samples (data not shown). No substantial labeling of other cytoplasmic tight and adherens junction proteins was seen in glioma cells.

Discussion

Our results show for the first time that in most microvessels of high-grade brain astrocytoma (glioblastoma multiforme) the blood-brain barrier is molecularly altered as revealed by changes in the expression of junction proteins. This suggests a correlation between the molecular and the morphological alterations of tight junction. In addition, discontinuous endothelia were regularly found in the tumors investigated. Except for the size of these gaps, this accords with previous reports on fenestrae-like structures lacking a diaphragm [26, 43].

The extent to which the discontinuity of glioblastoma microvessels contributes to the blood-brain barrier breakdown in these tumors is unclear. Nevertheless, this is a phenomenon which requires further investigation.

The junctions as observed by conventional electron microscopy were sound. However, the distribution of tight junctional particles, as judged in freeze-fracture replicas, was altered from predominantly P- to E-face. This is of particular interest since Kniesel et al. [21] have demon-

strated that in the mature blood-brain barrier of the rat the majority of tight junction particles are located on the P-face and only a minor part is located on the E-face. Since it was shown that brain endothelial cells in vitro lose their high impermeability in combination with a particle redistribution from P- to E-face, it is becoming generally accepted that the increase in the E-face/P-face ratio of tight junction particles correlates with the increase in transendothelial permeability [42, 47]. Interestingly, the tight junctions found in glioma blood vessels are strongly reminiscent of the tight junctions formed in cultured endothelial cells. Therefore, the question arises as to whether this particle switch correlates with an altered expression of tight junction proteins.

In epithelial cells, the role of occludin has recently been described as a regulatory rather than a constitutive one [4]. Against this background, the finding that in brain tumors occludin is only down-regulated in hyperplastic vessels, may reflect occludin expression as a late target in the row of alterations resulting in the breakdown of the blood-brain barrier in gliomas. In contrast, it is possible that the loss of claudin-1 correlates with the switch of tight junction particles from P- to E-face. This interpretation is strengthened by the fact that claudin-5 is not down-regulated in the majority of glioma microvessels. Therefore, we suggest that the particle distribution directly depends upon the ratio between the expression of claudin-1/5.

Wolburg et al. [47] demonstrated that the alteration of the particle association with tight junction strands correlates with the transendothelial electrical resistance (TER) in vitro. Treatments with astrocyte-conditioned medium, which increases the P-face association, results in an increased TER. Therefore, the P-face association and the expression of claudin-1/5 are parameters which may be used to estimate integrity and permeability of brain microvessels. Interestingly, claudin-5 was not detectable at interendothelial junctions of hyperplastic vessels and, as a consequence, these endothelia lack any known transmembrane tight junction protein. Presumably, hyperplastic vessels do not have any barrier function and, therefore, may be of particular importance for the development of brain edema. The surprising finding of occludin expression in astrocytic glioma cells, which was not regularly found, resembles conditions found during ontogeny or in lower vertebrates [46]. Due to their ectodermal derivation, glial cells have the potential for forming tight and/or adherens junctions, a property, which may be reinduced during generalized degeneration of these cells.

To elucidate the expression of the tight junction undercoat proteins in tumor blood vessels we labeled glioma cryosections for ZO-1 by immunohistochemistry. In the glioma material investigated, anti-ZO-1 immunoreactivity was unaltered, regardless of the intensity of anti-occludin immunoreactivity. In agreement with the finding of Kniesel et al. (in preparation) that in development of the rat, ZO-1 is detectable in brain endothelial cells before the tight junctions are fully established, the present results suggest that the expression and membrane localization of ZO-1 in tumor endothelial cells resemble a physiologically immature state of tight junctions.

The expression level and localization of ZO-1 do not correlate with the physiological efficiency of the paracellular barrier function [40]. This may also be confirmed by our data on the expression pattern of junctional components in blood vessels of glioblastoma multiforme. In cellular systems with less elaborate or no tight junctions, ZO-1 is enriched in regions of the adherens junctions [17], where it interacts with components of the cadherin-catenin system [18, 34].

Recently, we demonstrated a correlation between blood-brain barrier maturation and the differential expression of β-catenin and plakoglobin [25]. The latter was found to be the predominant catenin of adherens junction in the mature brain microvessel, whereas β-catenin is expressed mainly in immature and angiogenic vessels. In this context, it was interesting to examine whether glioma microvessels showed an altered expression of adherens junction catenins. We found that β-catenin was abundantly expressed in most tumor microvessels, whereas plakoglobin was only faintly expressed in some vessels. This result accords with those obtained for the tight junction proteins and morphology, indicating the predominance of undifferentiated microvessels in brain tumor in terms of blood-brain barrier characteristics.

The recurrent alterations of blood-brain barrier properties in tumor blood vessels, as described in this study, have to be considered together with the increase of the perivascular space. Our electron microscopy data confirm this increase of the thickness of the perivascular basal lamina and its multiplication. This increment might have consequences for the barrier regulation of endothelial cells (Fischmann et al., in preparation). In this context, it is of particular interest that only a subset of claudin-1-positive tumor microvessels was also positive for the pericyte marker ASMA. However, electron micrographs regularly revealed the presence of pericytes, even though their morphology was altered. Therefore, these results favor the suggestion made by Nehls et al. [32] that ASMA is a marker for differentiated pericytes rather than for pericytes in general. Thus, ASMA may be used as a marker antigen, discriminating resident from angiogenic microvessels in which the intimate relation between pericytes and endothelial cells is altered. The understanding of the spatial arrangement of endothelial cells and pericytes is just at its beginning and should be discussed elsewhere (for review see [3]).

In conclusion, the data presented here suggests that the increase in microvascular permeability in human gliomas is a result of a dysregulation of junctional proteins. Endothelial claudin-1 is down-regulated, which corresponds to characteristic morphological alterations of tight junction fine structure. Furthermore, the extracellular matrix of tumor vessels is massively increased, which affects the spatial relation of perivascular pericytes and endothelial cells. It will be important in future investigations to study the interrelationship between the production and composition of the extracellular matrix, the pericyte/endothelial cross-

talk and the breakdown of the endothelial barrier to understand the basics of glioma-related tight junction disintegration.

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