Cell−Cell Junctions of Dermal Microvascular Endothelial Cells Contain Tight and Adherens Junction Proteins in Spatial Proximity†

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ABSTRACT: Endothelial cell−cell contacts control the vascular permeability, thereby regulating the flow of solutes, macromolecules, and leukocytes between blood vessels and interstitial space. Because of specific needs, the endothelial permeability differs significantly between the tight blood−brain barrier endothelium and the more permeable endothelial lining of the non-brain microvasculature. Most likely, such differences are due to a differing architecture of the respective interendothelial cell contacts. However, while the molecules and junctional complexes of macrovascular endothelial cells and the blood−brain barrier endothelium are fairly well characterized, much less is known about the organization of intercellular contacts of microvascular endothelium. Toward this end, we developed a combined cross-linking and immunoprecipitation protocol which enabled us to map nearest neighbor interactions of junctional proteins in the human dermal microvascular endothelial cell line HMEC-1. We show that proteins typically located in tight or adherens junctions of epithelial cells are in the proximity in HMEC-1 cells. This contrasts with the separation of the different types of junctions observed in polarized epithelial cells and "tight" endothelial layers of the blood−brain barrier and argues for a need of the specific junctional contacts in microvascular endothelium possibly required to support an efficient transendothelial migration of leukocytes.

The endothelial lining of blood vessels plays a pivotal role in regulating the exchange of solutes as well as the transport of macromolecules and cells between the vessel lumen and the subendothelial space. Exchange and transport can be mediated by two principal means, transcytosis through individual endothelial cells and paracellular transport between adjacent cells of the endothelial monolayer. The latter route which is most likely taken by the majority of transmigrating leukocytes requires a regulated opening and closing of the interendothelial contacts (*1*, *2*). Therefore, molecules constituting the endothelial cell−cell contacts, or junctions, have been under intense scrutiny.

Junctions of endothelial cell layers showing a relatively high transendothelial electric resistance (TER),[1] e.g., those of the blood−brain barrier, are organized in a manner similar to that of the prototype junctions known from polarized epithelial cells. They comprise spatially separated tight junctions, adherens junctions, and desmosomes (*3*). Tight junctions form the major barrier to the paracellular diffusion of solutes and the lateral diffusion of membrane lipids and proteins between the apical and basolateral surfaces. Different types of transmembrane proteins are found in tight junctions. Occludin and the claudin family of proteins, which are thought to form a tight seal of the plasma membranes of adjacent cells, contain four transmembrane domains and cytoplasmic C-terminal tails of varying lengths (long in occludin and shorter in the claudins). The associated junctional adhesion molecules (JAMs), on the other hand, are type I transmembrane proteins belonging to the immunoglobulin superfamily. The intracellular domains of occludin, claudins, and JAMs are capable of interacting with the zonula occludens-1 (ZO-1) protein, a PDZ, and guanylate kinase-like (GUK) domain-containing protein. ZO-1 can bind actin filaments, thereby anchoring tight junctions within the cell's cytoskeleton. In addition to ZO-1, other PDZ-containing and in some cases actin-binding proteins assemble into an intracellular protein network at the site of tight junctions, most likely participating in establishing and regulating tight junctions (for reviews, see refs *4*−*7*). The major principal constituents of adherens junctions are the cadherins, of which VE-cadherin is found in endothelial cells. Cadherins are capable of homophilic, Ca^{2+}-regulated interactions responsible for intercellular contacts at the level of adherens junctions. Intracellularly, they are also linked to the actin cytoskeleton in this case via a protein complex involving β- and α-catenin and a number of other actin-binding proteins. α-Catenin can also bind to ZO-1, but such connections appear to be restricted to intercellular contacts lacking well-established tight junctions (for reviews, see refs *8*−*10*).

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[1] Abbreviations: DSP, dithiobissuccinimidylpropionate; JAM, junctional adhesion molecule; RT, room temperature; TER, transendothelial electrical resistance; TfR, transferrin receptor; ZO-1, zonula occludens protein-1.

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While this architecture of spatially separated tight and adherens junctions is relatively well established for cell contacts in polarized epithelia and endothelia of the blood−brain barrier or large arteries (for reviews, see refs *11*−*13*), less is known about the achitecture of junctions in microvascular endothelial cells, the major site of leukocyte trafficking. Electron microscopy analysis of frog mesenteric capillaries revealed the existence of distinct areas of adherens and tight junctions (*14*), although in other systems at least highly organized tight junctions appear to be absent (*15*, *16*). While microvascular adherens junctions and the central role of VE-cadherin in regulating these junctions have been characterized (*17*, *18*), the role of tight junction proteins and the architecture of cell−cell contacts in microvascular endothelium are not understood (for reviews, see refs *12* and *19*).

In an attempt to analyze this architecture and the underlying protein interactions in cell−cell contacts of microvascular endothelial cells, we developed a combined in situ cross-linking and immunoprecipitation approach employing cell layers of the microvascular endothelial cell line HMEC-1. We show that following stabilization by cross-linking junctional complexes can be immunoprecipitated which contain typical components of both tight and adherens junctions. In contrast, co-immunoprecipitation experiments employing cross-linked junctional complexes from polarized epithelial or blood−brain barrier endothelial cells reveal a clear separation of the principal junctional proteins. This indicates that intercellular contacts in HMEC-1 cells exhibit an architecture differing from that of well-separated tight and adherens junctions found in other types of endothelia.

EXPERIMENTAL PROCEDURES

Antibodies. Antibodies employed in immunoprecipitation and immunoblotting experiments were obtained from commercial sources. Polyclonal rabbit anti-occludin, anti-ZO-1, and anti-ZO-2 as well as monoclonal mouse anti-occludin and mouse anti-transferrin receptor antibodies were from Zymed Laboratories Inc. The polyclonal rabbit anti-N-cadherin antibody was from R&D Labarotories. Monoclonal mouse anti-α-catenin, anti-afadin-6, anti-β-catenin, anti-E-cadherin, anti-VE-cadherin, and anti-ZO-1 antibodies were from Transduction Laboratories. Monoclonal mouse anti-β-actin antibodies were from Sigma, and monoclonal mouse anti-α-tubulin antibodies were from Oncogene Research Products. Secondary goat anti-mouse, peroxidase, and cy3-conjugated, as well as goat anti-rabbit, peroxidase, and cy3-conjugated, antibodies were from Jackson Immuno-Research Laboratories. For immunoblotting and immunofluorescence, antibodies were diluted according to the supplier's specifications. Immunoprecipitations employed 2 μg of antibody per reaction.

Cell Culture. HMEC-1 cells were cultivated at 37 °C and 3% CO_2 in MCDB 131 medium (Biochrom AG) supplemented with 10% FCS Gold (PAA), 20 mM L-glutamine, 50 μg/mL gentamycin, 10 ng/mL epidermal growth factor, and 10 μg/mL hydrocortisone. HBMEC cells were cultivated at 37 °C and 5% CO_2 in 1680 RPMI (Biochrom AG) supplemented with 10% inactivated FCS (PAA), 10% inactivated Nu-serum (BD Biosciences), 2 mM L-glutamine, 1 mM sodium pyruvate, 1% MEM nonessentiel amino acids and 1% MEM vitamins from Sigma, and 100 units/mL penicillin/streptomycin (PAA). MDCK-C11 cells (*20*) kindly provided by H. Oberleithner (University of Muenster) were cultivated at 37 °C and 5% CO_2 in MEM (Biochrom AG) supplemented with 10% inactivated FCS and 100 units/mL penicillin/streptomycin.

Prior to immunofluorescence inspection, 3×10^6 cells were grown to confluency in 100 mm cell culture dishes. For in vivo cross-linking experiments, epithelial or endothelial cell monolayers were polarized by cultivation on fibronectin-coated 75 mm filters with a pore size of 5 μm (Costar). After the cell monolayer reached confluency, cells were further cultivated for an additional 2 days and then subjected to immunofluorescence or in vivo cross-linking.

Immunofluorescence. HMEC-1 monolayers grown on fibronectin-coated glass coverslips, or HMEC-1 or MDCK-C11 monolayers polarized over 3 days on fibronectin-coated microporous filter units (Costar) were fixed for 20 min with 3% paraformaldehyde in PBS (pH 7.4) and permeabilized for 5 min with 0.2% Triton X-100 (TX-100) in PBS. Immunostaining of HMEC-1 monolayers with anti-occludin antibodies was carried out using HMEC-1 monolayers fixed with methanol for 10 min at −20 °C. Cells were washed three times with PBS, blocked with 5% BSA in PBS for 20 min, and incubated with the specific primary antibodies listed above which were diluted in 5% BSA in PBS. After being washed three times in PBS, samples were incubated with the secondary antibodies for 30 min at 37 °C. After being washed five times in PBS and five times in distilled water, coverslips were mounted in Moviol containing 1% *n*-propyl gallate and analyzed by epifluorescence (DM-RXA, Leica) or confocal microscopy (LSM 510 meta, Zeiss).

In Vivo Cross-Linking. HMEC-1, HBMEC, and MDCK-C11 monolayers ($\sim 1 \times 10^7$ cells per 100 mm dish or 5×10^6 cells per 75 mm filter) were subjected to in vivo cross-linking by overlaying the cells of one 100 mm dish or 75 mm filter with 1.3 mL of Krebs solution (37 °C) containing 400 μg/mL DSP (dithiobissuccinimidyl propionate) and incubating them at room temperature (RT) for 15 min. The cross-linking reaction was stopped by adding 5 mL of a 50 mM glycine/Krebs solution (37 °C) for 5 min. Before and after cross-linking, the different cell monolayers were rinsed three times with Krebs solution (37 °C). Following cross-linking, the HMEC-1, HBMEC, and MDCK monolayers were transferred to an ice-cold metal plate and allowed to cool for 5 min.

Cross-linked or nontreated control cells were extracted with 5 mL of extraction buffer [100 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% TX-100, 200 mM sucrose, and 20 mM Hepes (pH 7.5)] supplemented with a protease inhibitor cocktail of 1 μM pepstatin, 1 μM leupeptin, 1 mM benzamidine, and 1 mM PMSF on a rocking platform for 5 min on an ice-cold metal plate. After the extracted proteins had been removed, any remaining soluble cell proteins were washed away with extraction buffer without TX-100 for 2 × 2.5 min on a rocking platform. Subsequently, the TX-100 insoluble material was scraped into 700 μL of buffer U [150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 30 mM Hepes (pH 7.5), 1% TX-100, and 6 M urea] supplemented with the protease inhibitor cocktail and homogenized by being passed five times through a 22 gauge needle. The urea homogenate was subjected to centrifugation at 16000*g* for 15 min and then at 100000*g* for 30 min. The high-speed

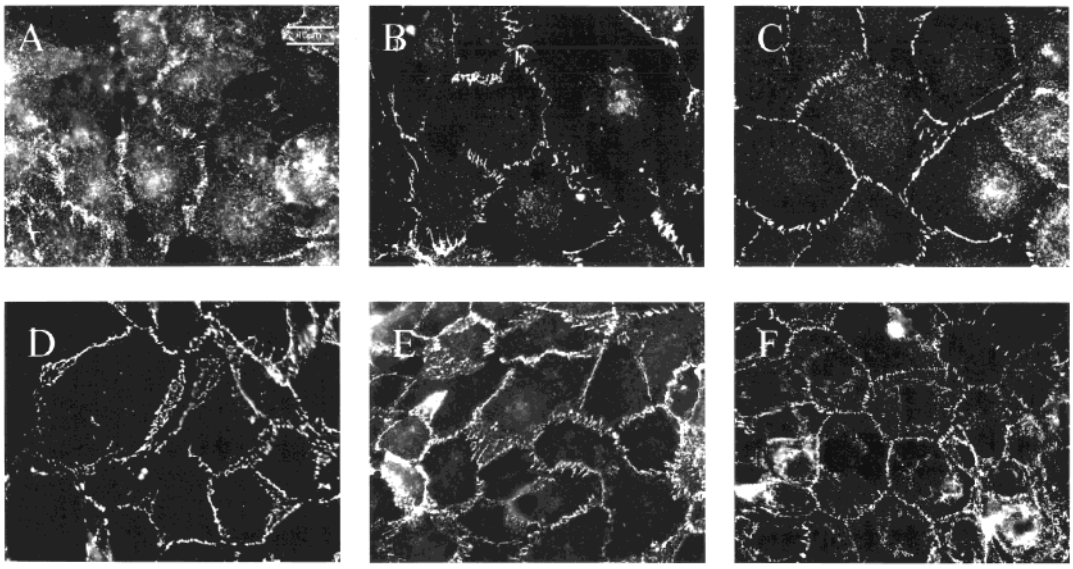

FIGURE 1: Tight and adherens junction proteins are present at cell−cell contacts of HMEC-1 cells. Cells grown to confluency on fibronectin-coated coverslips were subjected to immunofluorescence analysis by employing as primary antibodies those directed against occludin (A), ZO-1 (B), ZO-2 (C), VE-cadherin (D), β-catenin (E), and afadin-6 (F). Note the staining of cell−cell contacts with all antibodies. In the case of anti-occludin immunofluorescence (A), some larger intracellular structures are also labeled, most likely due to some nonspecific antibody reaction. The scale bar represents 10 μm.

supernatant, i.e., the urea soluble fraction, was subjected to a three-step dialysis protocol against buffer U containing, instead of 6 M urea, 1, 0.5, and 0.1 M urea to allow extracted proteins to refold prior to immunoprecipitation. Following the last dialysis, nonrenatured proteins were removed by centrifugation (16000*g* for 15 min).

Alternatively, the TX-100 insoluble remnants of cross-linked HMEC-1 monolayers were extracted with 5 mL of buffer U (containing protease inhibitors) per dish or filter on an ice-cold metal plate for 30 min on a rocking platform. The extracted material was pooled and centrifuged at 100000*g* for 30 min to remove the remaining insoluble material. The protein-containing supernatant was then concentrated between 5- and 7.5-fold in a centrifugal filter device (Centriprep with a 30 kDa cutoff, Amicon) and the concentrated protein was finally subjected to the stepwise renaturation dialysis. Both protocols yielded comparable results with respect to the amount of renatured protein.

Immunoprecipitation. Immunoprecipitations employed 1 mL of renatured or TX-100 soluble protein extract and 2 μg of rabbit antibodies coupled to 6.7×10^7 M-280 Dynabeads or 2 μg of monoclonal mouse antibodies coupled to 6.4×10^7 M-450 Dynabeads, respectively. The antibodies coupled to the Dynabeads were directed against the different junction proteins (see above). Coupling of antibody to Dynabeads was performed in urea-free buffer U for 1 h at 4 °C. To remove antigens binding unspecifically to Dynabeads containing the secondary sheep anti-rabbit or sheep anti-mouse antibodies, 3.3×10^7 M-280 or M-450 Dynabeads were first incubated for 1 h at 4 °C with the renatured HMEC-1, HBMEC, or MDCK extracts. The unbound fraction was then incubated with the specific primary antibody-coated beads for 2 h at 4 °C. Subsequently, the beads were washed three times with urea-free buffer U (with the protease inhibitor cocktail), and the beads were treated for 5 min at 95 °C with an equal volume of 2× SDS sample buffer. Proteins released from the beads were analyzed by SDS−PAGE and immunoblotting using in separate experiments a series of antibodies against junction-associated proteins. Immunoblotting employed PVDF membrane and Tris-borat buffer for the electrophoretic transfer. Antibody incubations were performed at dilutions recommended by the suppliers.

RESULTS

Tight and Adherens Junction Proteins Are Present in Cell−Cell Contacts of Microvascular Endothelial Cells. The HMEC-1 cell line is a human endothelial cell line of microvascular origin which has been shown previously to respond to inflammatory activation with an increased level of surface expression of adhesion molecules participating in leukocyte capturing. Moreover, in a two-chamber cell culture assay, the transendothelial passage of monocytes and granulocytes can be reproduced faithfully with monolayers of activated HMEC-1 (*21*, *22*). Therefore, we decided to analyze the cell−cell contacts of HMEC-1 in more detail. Using specific antibodies, we first elucidated whether typical tight and adherens junction proteins are expressed in these cells and whether these proteins are localized to regions of intercellular contacts. Figure 1 reveals that this is indeed the case for the tight junction markers occludin, ZO-1, ZO-2, and afadin-6 as well as for the adherens junction proteins VE-cadherin and β-catenin.

Next, we compared in polarized HMEC-1 monolayers, the distribution of a typical tight junction marker (ZO-1) with that of a protein associated with adherens junctions (β-catenin). Confocal $x-z$ scans of double-labeling experiments reveal that both proteins show a completely overlapping localization along the lateral membranes of HMEC-1 cells. In contrast, the two markers are separated to some extent in the lateral junctions of polarized monolayers of a human brain microvascular endothelial cell line (HBMEC). These specialized endothelial cells whose origin is the blood−brain barrier are known to establish a high transendothelial electric resistance indicative of well-organized tight junctions (*23*). Separation of tight and adherens junction markers is even

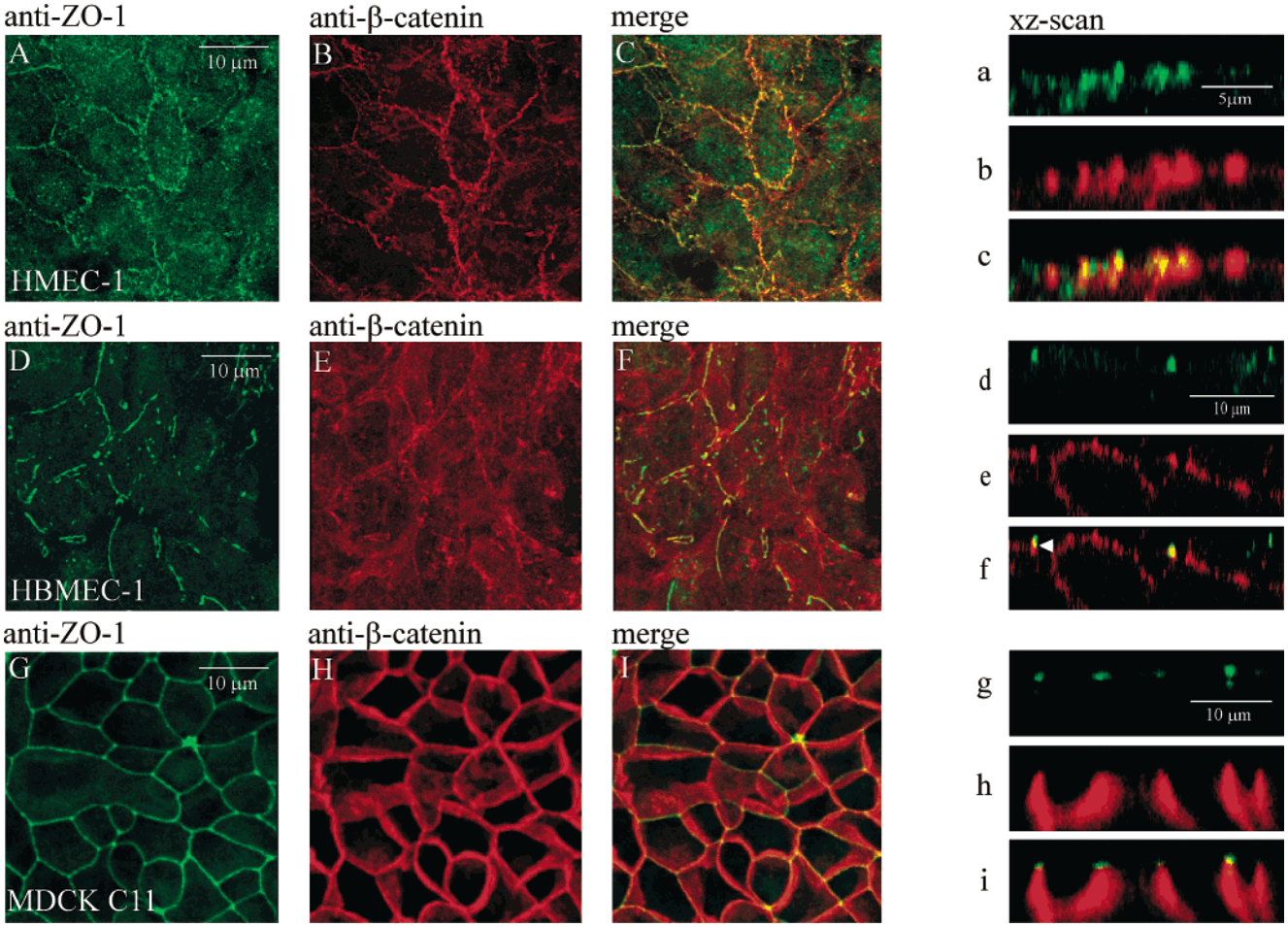

FIGURE 2: Colocalization of tight and adherens junction proteins in HMEC-1 but not MDCK-C11 cells. HMEC-1 (A−C), HBMEC (D−F), or MDCK-C11 (G−I) cells were grown on fibronectin-coated microporous filter supports and allowed to polarize over a period of 3 days. Subsequently, the monolayers were fixed, permeabilized, and stained with anti-ZO-1 (A, D, and G) and anti-β-catenin (B, E, and H) antibodies in colabeling experiments. Stacks of confocal scans are displayed as *xy* (A−I) and *xz* sections (a−i). Note the overlapping localization of the tight (ZO-1) and adherens junction (β-catenin) proteins in HMEC-1 cells. In MDCK-C11 cells and to some extent in HBMEC cells (arrowhead pointing at a lateral junction), ZO-1 is localized apically of β-catenin. In addition to the lateral junction localization, HBMEC cells exhibit some general plasma membrane and/or intracellular localization of β-catenin.

more evident in polarized MDCK monolayers with ZO-1 clearly appearing apically of β-catenin (Figure 2).

In Vivo Cross-Linking Stabilizes Junctional Protein Complexes. The analysis described above established the membrane localization of tight and adherens junction proteins in HMEC-1 and revealed a colocalization of marker proteins of both types of junctions at the light microscope level. However, even the confocal analysis does not rule out the possibility that spatially separated and fully assembled tight and adherens junction also exist in HMEC-1 cells. Thus, we decided to employ a biochemical approach to analyze a possible separation of tight and adherens junctions in HMEC-1 cells. In a first attempt, we used antibodies to tight and adherens junction proteins to immunoprecipitate the respective protein complexes. However, to efficiently extract from HMEC-1 monolayers not only the transmembrane proteins occludin and VE-cadherin but also the cytoskeleton-associated ZO-1 and ZO-2 proteins, we had to employ urea-containing and thus denaturing buffers. This resulted in a disruption of protein−protein interactions and precluded an effective co-immunoprecipitation of any associated protein together with the precipitated antigen (not shown). To circumvent this problem, we stabilized prior to cell lysis protein complexes by in vivo chemical cross-linking. HMEC-1 monolayers or monolayers of other epithelial and endothelial cells were treated with the membrane permeable cross-linker DSP which can be cleaved by reducing agents such as β-mercaptoethanol (*24*). Following the cross-link, cells were subjected to a differential cell lysis which enriched proteins of the junctional complexes in the final urea extract. Subsequently, proteins solubilized by the urea treatment were renatured by dialysis and subjected to immunoprecipitations using antibodies directed against different tight and adherens junction proteins, respectively. Coprecipitated proteins were then identified by immunoblotting. A flowchart of this in situ cross-linking−immunoprecipitation approach is given in Figure 3.

A first series of experiments established the optimal cross-linker concentrations required to induce efficient modification of the typical junction proteins. This turned out to be 400 μg of DSP/mL since at this concentration almost all the junction protein recovered in the urea extract resided in the cross-linked form when analyzed by SDS−PAGE under nonreducing, i.e., cross-link-stabilizing, conditions (an example for ZO-1 is given in Figure 4A). Next we determined by utilizing antibodies to occludin as precipitating antibodies whether cross-link and urea extraction followed by renaturation would preclude the immunoprecipitation of junction

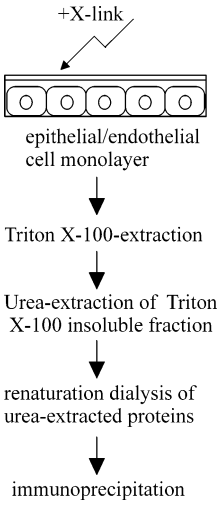

FIGURE 3: Isolation of junctional protein complexes from HMEC-1 monolayers. Flowchart describing the sequential extraction of proteins from cross-linked HMEC-1 monolayers.

proteins. Figure 4B reveals that this membrane protein can be precipitated efficiently from the renatured protein fraction under the conditions that are chosen. Moreover, an immunoblot analysis of the immunoprecipitates revealed the co-isolation of ZO-1, a cytosolic protein known to associate with occludin at tight junctions. The efficient immunoprecipitation and coprecipitation also depended on the cross-linker concentration that was chosen. When 400 μg of DSP/mL was exceeded, the cross-link resulted in the formation of larger complexes that could not be solubilized efficiently. The specificity of the approach was verified by analyzing the behavior of a nonjunctional membrane protein, the transferrin receptor (TfR). Although present in the urea extract and in the soluble protein fraction following renaturation, TfR is not coprecipitated with any of the junctional proteins that have been analyzed when cross-linker concentrations of 400 μg/mL were employed (Figure 4C).

Junctional Complexes of HMEC-1 Cells Harbor Proteins of Tight and Adherens Junctions in Spatial Proximity. The combined in vivo cross-linking−immunopreciptiation approach established a way to analyze the composition of endothelial−epithelial protein complexes assembled at tight and adherens junctions. Therefore, immunoprecipitations following in vivo cross-linking were carried out with antibodies directed against different transmembrane as well as intracellularly associated junction proteins. Subsequently, the immunoprecipitates were probed with a series of anti-junction protein antibodies.

To further verify the feasibility of this new approach, we applied it to the well-characterized epithelial MDCK cell line, known to establish separated tight and adherens junctions in its polarized state. Following in vivo cross-linking and immunoprecipitation with antibodies directed against the tight junction transmembrane protein occludin, both occludin and ZO-1 are detected in the precipitate. In contrast, the transmembrane protein of epithelial adherens junctions, E-cadherin, and the intracellularly associated adherens junction marker α-catenin are not recovered in the anti-occludin precipitate (Figure 5A). Vice versa, the anti-E-cadherin immunoprecipitation leads to a coprecipitation of α-catenin but not of tight junction proteins occludin and ZO-1 (Figure 5A). Thus, the well-known morphological separation of tight and adherens junctions in polarized MDCK cells is reflected itself in the in vivo cross-linking−immunoprecipitation approach.

Next and as a further control, we subjected the human brain microvascular endothelial cells (HBMEC) to the cross-linking−immunoprecipitation regime. The co-immunoprecipitations that were carried out support the notion that these cells can also form organized tight junctions. Like results obtained for MDCK cells, immunoprecipitation with anti-occludin antibodies results in a coprecipitation of the tight junction protein ZO-1, whereas the adherens junction proteins N-cadherin and α-catenin could not be detected (Figure 5B). The same results were obtained when immunoprecipitations with anti-ZO-1 antibodies were analyzed for the presence of tight and adherens junction markers (Figure 5B). Immunoprecipitations with antibodies directed against the adherens junction protein α-catenin, on the other hand, led to the efficient isolation of α-catenin and a coprecipitation of N-cadherin (Figure 5B). A minor fraction of ZO-1 (Figure 5B) and some ZO-2 (not shown) but no occludin are also recovered in the anti-α-catenin immunoprecipitate. N-Cadherin was chosen as an adherens junction marker because the levels of VE-cadherin are low in HBMEC cells under the conditions that were chosen (data not shown) and because it is known that N-cadherin can take over the function of VE-cadherin (*25*). Moreover, we chose anti-α-catenin as a precipitating antibody in these experiments because the anti-N-cadherin antibody did not precipitate well.

Taken together, the control experiments with polarized MDCK and HBME cells reveal a separation of tight and adherens junction complexes which is in line with the establishment of bona fide tight junctions of high electrical resistance. The microvascular dermal endothelial HMEC-1 cell line, on the other hand, shows a greatly reduced electrical resistance and higher permeability (*21*), possibly required to support efficient leukocyte diapedesis. Thus, we next characterized the composition of junctional protein complexes in HMEC-1 cells by subjecting HMEC-1 monolayers to the in vivo cross-linking−immunoprecipitation protocol. Figure 6 shows representative examples of some of the immunoblots of the co-immunoprecipitations. It reveals that antibodies against a typical adherens junction protein (VE-cadherin), in addition to precipitating other adherens junction proteins, coprecipitate tight junction markers such as ZO-1 and ZO-2. On the other hand, typical adherens junction proteins such as α- and β-catenin are precipitated together with the tight junction protein ZO-1 or occludin when antibodies directed against the latter are employed in the immunoprecipitation. To address the question of whether the HMEC-1 junctions containing tight and adherens junction markers in spatial proximity are linked to the cytoskeleton, the different immunoprecipitations were also probed for the presence of actin and tubulin. Since an actin association of microvascular VE-cadherin has already been reported (*26*), only examples for anti-ZO-1 and anti-occludin immunoprecipitations revealing a coprecipitation of actin are shown (Figure 6). Likewise, ZO-1 and occluding antibodies coprecipitate actin following in vivo cross-linking of MDCK monocytes (Figure 5A), whereas tubulin is found neither in the HMEC-1 nor in the MDCK immunoprecipitations (not shown). Thus, the actin cytoskeleton could contribute to establishing a proximity of tight and adherens junction

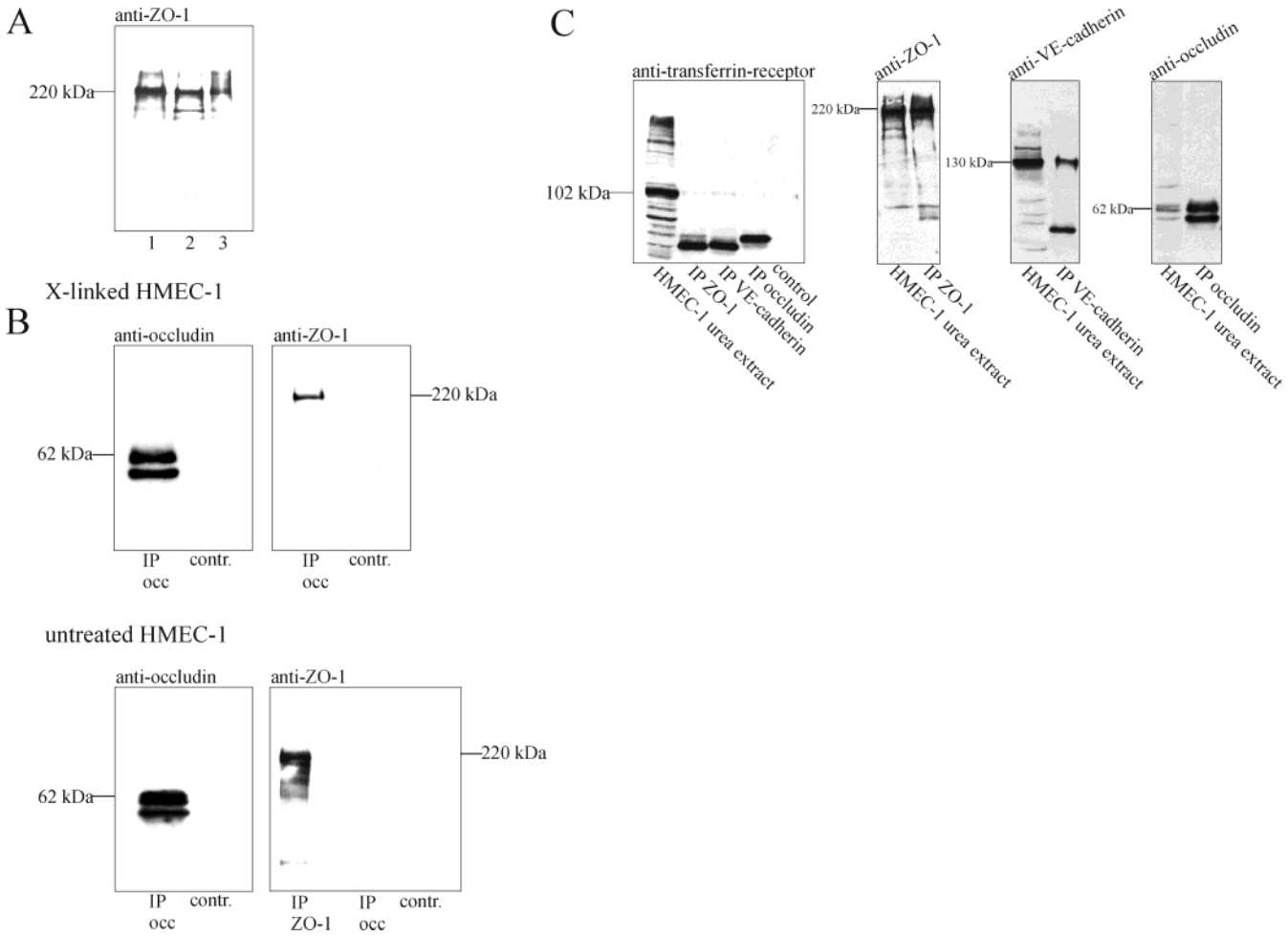

FIGURE 4: In vivo cross-linking and immunoprecipitation of junctional proteins from HMEC-1 cells. (A) HMEC-1 monolayers were subjected to in vivo cross-linking using 400 μg/mL DSP and then to the sequential protein extraction described in Figure 3. Proteins present in the urea extract (lanes 2 and 3) were boiled in SDS sample buffer in the presence (lane 2) or absence (lane 3) of β-mercaptoethanol and then subjected to immunoblotting with anti-ZO-1 antibodies. Note that ZO-1 is efficiently extracted; ~50% of the total ZO-1 protein of HMEC-1 cells (a total lysate is shown in lane 1) are released into the urea extract. The cross-linked protein generates a nondiscrete band in the nonreduced gel sample (lane 3). (B) Proteins present in the urea extract of cross-linked (top panels) or nontreated HMEC-1 cells (bottom panels) were subjected to immunoprecipitation using anti-occludin (IP occ) or nonspecific control antibodies [sheep anti-mouse IgGs coupled to M-450 Dynabeads (contr.)]. The immunoprecipitations were than probed by immunoblotting with antibodies directed against occludin or ZO-1. The lower band of the doublet seen in the IP occ lane stems from the heavy IgG chain of the precipitating antibody decorated by the secondary antibody used in the blot. The control antibody used in the precipitations is a different subtype and thus not stained in the blot. In the case of untreated HMEC-1 cells, an additional immunoprecipitation with anti-ZO-1 antibodies (IP ZO-1) was included in Western blot analysis with anti-ZO-1 antibodies to verify that the protein is present in the extract employed in the immunoprecipitation. (C) Western blot analysis of the different immunoprecipitations indicated using antibodies against a nonjunctional transmembrane protein, the human transferrin receptor. Note that no significant coprecipitation is seen, although the transferrin receptor is present in the urea extract used for the immunoprecipitation (the minor transferrin receptor bands seen in the IP lanes are also visible in the control lane following a slightly longer exposure of the blot, not shown). The bands at the bottom of the blot seen in the immunoprecipitations represent the heavy chains of the precipitating antibodies which are decorated with the secondary antibody used in the blot. Control immunoprecipitations employed sheep anti-mouse IgGs which belong to a different subtype and are not stained in the blot. To verify that the respective antigens were actually precipitated in the different experiments, the immunoprecipitates were also probed by Western blot analysis using antibodies against ZO-1, VE-cadherin and occludin. The respective blots are shown next to a blot of the total urea extract in the right panels.

proteins in HMEC-1 cells. However, actin association cannot be the sole determinant since in MDCK cells it is not sufficient to achieve tight and adherens junction proximity.

Results of all co-immunoprecipitation experiments are summarized in Figure 7. While the amount of coprecipitated proteins varies slightly when different precipitating antibodies are employed, the overall picture clearly shows a general cosegregation of tight and adherens junction proteins. This indicates that intercellular contacts of microvascular HMEC-1 cells lack the clear spatial separation of tight and adherens junctions seen in epithelial and tight endothelial (blood−brain barrier) monolayers and that junctional complexes in HMEC-1 appear to contain co-assemblies of both tight and adherens junction proteins.

DISCUSSION

Intercellular junctions in most epithelia as well as tight endothelial linings of the blood−brain barrier serve important barrier functions, thereby restricting solute, macromolecule, and cell movement between different extracellular spaces. They also provide a framework for the establishment of membrane and thereby cell polarity. Both functions critically depend on the presence of organized tight junctions that restrict the lateral diffusion of membrane lipids and proteins

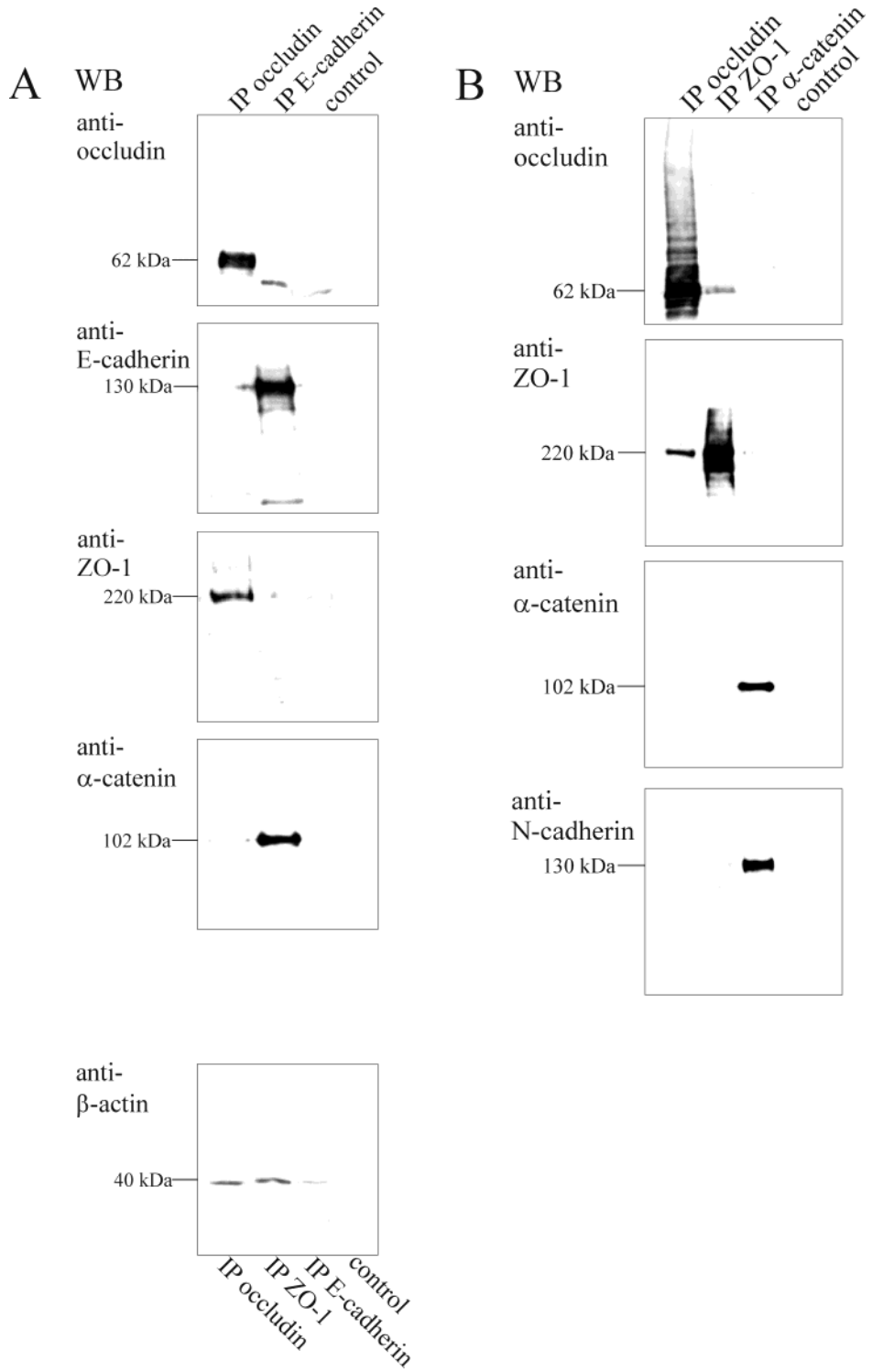

FIGURE 5: In vivo cross-linking and immunoprecipitation of junctional proteins from MDCK and HBMEC monolayers. MDCK (A) and HBMEC (B) monolayers were treated as described in Figure 3. In the case of MDCK cells, immunoprecipitation was carried out with anti-occludin, anti-E-cadherin, and nonspecific mouse IgGs (control). Co-immunoprecipitated proteins were detected by Western blot (WB) analysis with antibodies against occludin, ZO-1, E-cadherin, and α-catenin. Note the lack of co-immunoprecipitation of tight and adherens junction proteins. In addition, immunoprecipitations carried out with anti-occludin, anti-ZO-1, anti-E-cadherin, and nonspecific mouse IgGs (control) were probed with anti-*β*-actin antibodies (bottom panel). HBMEC monolyers were immunoprecipitated with anti-occludin, anti-ZO-1, anti-α-catenin, and nonspecific mouse IgGs (control). Co-immunoprecipitated proteins were detected by Westen blot (WB) analysis with antibodies against occludin, ZO-1, N-cadherin, and α-catenin. With the exception of a minor ZO-1 band in the anti-α-catenin immunoprecipitation, no coprecipitation of tight and adherens junction proteins is observed.

and the paracellular exchange of solutes (for a review, see ref *5*). Endothelia lining the microvasculature of most other tissues, however, are less tight in the sense that they show a much lower transendothelial electric resistance (TER) and a higher permeability toward solutes and macromolecules (for reviews, see refs *7* and *12*). Such differences also reflect themselves in the cell lines used in this study. While the MDCK-C11 and HBMEC monolayers show a relatively high TER of 330 Ω cm^2 (MDCK; *20*) and 360 Ω cm^2 (HBMEC; *27*), polarized HMEC-1 monolayers are characterized by a

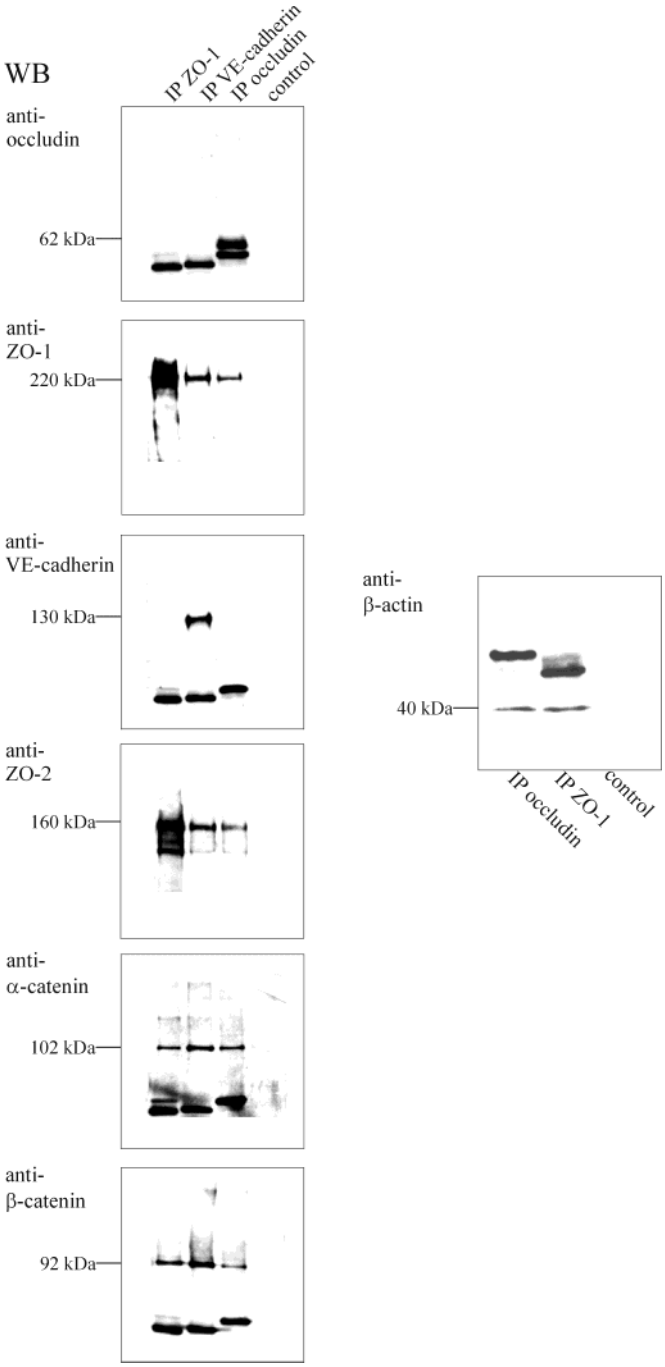

FIGURE 6: Immunoprecipitation of junctional proteins extracted from HMEC-1 monolayers following in vivo cross-linking. HMEC-1 monolayers were subjected to DSP-mediated in vivo cross-linking and the sequential protein extraction described in Figure 3. Following renaturation dialysis, samples were subjected to immunoprecipitation using antibodies directed against ZO-1, VE-cadherin, occludin, or nonspecific mouse IgGs (control). The respective immunoprecipitates were then analyzed by immunoblotting (Western blot, WB) employing antibodies directed against occludin, ZO-1, VE-cadherin, ZO-2, α-catenin, and *β*-catenin. Immunoprecipitations carried out with anti-occludin, anti-ZO-1, and nonspecific mouse IgGs (control) were also probed with anti-*β*-actin antibodies (right panel). Note the efficient coprecipitation of different junctional proteins with VE-cadherin, ZO-1, and occludin antibodies. The lower band detected with anti-occludin antibodies in the anti-occludin immunoprecipitation is due to a reaction of the heavy chain of the precipitating antibody with the secondary antibodies used in the blot.

TER of only 50 Ω cm^2 (*21*). Given the different organization of cell−cell junctions shown here for different cell lines, it

A

IP ab / ppt Ag	anti-Occ ms	anti-ZO-1 rabb	anti-ZO-1 ms	anti-ZO-2	anti-VE-Cad	anti-β-Cat
Occ	+++	−	−	−	−	−
ZO-1	++	++	++	++	++	++
ZO-2	++	++	++	+++	++	++
VE-Cad	−	+	−	−	++	+
β-Cat	+	++	++	−	++	++
α-Cat	+	++	++	−	++	++

B

IP ab / ppt Ag	anti-Occ ms	anti-ZO-1 rabb	anti-ZO-1 ms	anti-ZO-2	anti-VE-Cad	anti-β-Cat
Occ	+++	−	−	−	−	−
ZO-1	−	+	+++	−	−	−
ZO-2	−	−	+	+++	−	−
VE-Cad	−	−	−	−	+++	−
β-Cat	++	+	+	−	++	+++
α-Cat	−	−	−	−	+	−

FIGURE 7: Co-immunoprecipitation of junctional proteins extracted from HMEC-1 monolayers. HMEC-1 monolayers were cross-linked in vivo (A) or left untreated (B). Subsequently, the cells were lysed and subjected to the sequential protein extraction (see Figure 3). Immunoprecipitations were then carried out using antibodies directed against the junction proteins that are listed (IP ab), and precipitated proteins (ppt ag) were characterized by immunoblotting using the antibodies indicated in the left panels. Plus signs indicate the relative amounts of junction proteins identified in the immunoprecipitates. Three plus signs indicate maximum values (the amount of antigen immunoprecipitated by the specific antibody). Two plus signs indicate intermediate coprecipitations (∼25−50% of the maximum values). One plus sign indicates a coprecipitation of less than 25% of the maximum values. A minus sign denotes no immunoprecipitation.

appears to be likely that junctional architecture (separation of tight and adherens junctions) affects the permeability barrier functions. Future studies specifically manipulating this junctional architecture of, for example, HBMEC cells and converting it into that of microvascular endothelial cells could address this point.

Since microvascular endothelia perform a crucial function in regulating leukocyte traffic into, for example, inflamed tissue, it is assumed that their intercellular contacts are designed differently to support a dynamic opening and closing. In line with this assumption, it is observed that the number of morphologically well defined tight junctions in endothelia correlates inversely with the need for permeability control (*16*, *19*). While this correlation is based on morphological criteria, it has been shown that some tight junction proteins such as ZO-1 and JAM are expressed in microvascular endothelial cells of, for example, high endothelial venules (*19*, *28*). This could indicate that tight junctions of a different architecture, one presumably required to support transendothelial leukocyte traffic, are present in certain endothelial linings. In contrast to the scarce knowledge of tight junction architecture in microvascular endothelial cells, the VE-cadherin-based adherens junctions of these cells have been characterized in some detail. The crucial importance of such junctions for the control of endothelial permeability was revealed among other things by showing that internalization of VE-cadherin causes a disruption of the intercellular junctions (*17*).

Using a combined in vivo cross-linking−co-immunoprecipitation approach, we show now that the morphological characteristics seen in tissues and cultured cells, i.e., a segregation of tight and adherens junctions in epithelial and tight (blood−brain barrier) endothelial cells, also reflect themselves at the level of protein−protein interactions. It must be noted that the cross-linking approach used here can be carried out with only cultured cells and not with primary tissues but that the results obtained using the control MDCK and HBME cells are in full agreement with what is observed morphologically in tissue sections. Polarized MDCK and HBME cells show well-defined tight junctions separated from their adherens junctions since tight junction markers can be precipitated with only antibodies against tight junction proteins and vice versa. The only exception is a minor interaction of α-catenin with ZO-1 in HBMEC cells, perhaps indicative of an incomplete separation of the two types of junctions in these cells. The scenario is completely different in the microvascular dermal endothelial HMEC-1 cell line which is capable of supporting leukocyte transmigration in a regulated and directional manner (*21*, *22*). HMEC-1 cells express a number of tight junction proteins which are not assembled into protein complexes spatially separated from adherens junctions. Hence, the tight junction proteins of these microvascular endothelial cells reside in the proximity of VE-cadherin-based adherens junctions.

While our results do not allow the distinction between direct and indirect physical interactions between adherens junctions and the tight junction proteins expressed in HMEC-1 cells, the results are reminiscent to some extent of what is observed in early stages of epithelial polarization and in other nonepithelial and nonendothelial cells that lack tight but possess adherens junctions. In both cases, the tight junction protein ZO-1 associates with or is found in the proximity of adherens junctions (*29*, *30*). Thus, it appears that junctional complexes of an intermediate type could exist either temporarily during epithelial differentiation or permanently in certain cell types, including microvascular dermal endothelial cells. Our results reveal that such structures not only contain the intracellularly associated ZO-1 but also harbor the tight junction transmembrane protein occludin.

At present, we can only speculate about the precise architecture of the junctional complexes involving both types of junction proteins. On the basis of what is known for developing epithelia (*8*), we would predict that the principal building blocks of such mixed junctions are cadherin-based adherens junctions. Tight junction proteins could be recruited to such sites, resulting in a specialized protein network possibly required to regulate processes specific to certain microvascular endothelia. The latter are likely to involve paracellular leukocyte trafficking which is known to be affected by the extracellular presence of antibodies directed against tight (JAM) as well as adherens junction (VE-cadherin) proteins (*31*, *32*). Because of the presence of a number of transmembrane tight junction proteins in intercellular contacts of the microvascular dermal endothelial cells, it seems likely that the proteins also fulfill a role in restricting lateral membrane lipid and protein diffusion, thereby establishing membrane polarity. Future studies must reveal whether this is indeed the case and how the proposed functions of these special cell−cell contacts relate to their composition and architecture.

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