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REGULAR ARTICLE

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The *complexus adhaerens* of mammalian lymphatic endothelia revisited: a junction even more complex than hitherto thought

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Abstract The significance of a special kind of VE-cadherin-based, desmoplakin- and plakoglobin-containing adhering junction, originally identified in certain endothelial cells of the mammalian lymphatic system (notably the retothelial cells of the lymph node sinus and a subtype of lining endothelial cells of peripheral lymphatic vessels), has been widely confirmed and its importance in the formation of blood and lymph vessels has been demonstrated in vivo and in vitro. We have recently extended the molecular and structural characterization of the *complexus adhaerens* and can now report that it represents a rare and special combination of components known from three other major types of cell junction. It comprises *zonula adhaerens* proteins (VE-cadherin, α- and β-catenin, protein p120^{ctn}, and afadin), desmosomal plaque components (desmoplakin and plakoglobin), and tight-junction proteins (claudin-5 and ZO-1) and forms junctions that vary markedly in size and shape. The special character and the possible biological roles of the *complexus adhaerens* and its unique ensemble of molecules in angiogenesis, immunology, and oncology are discussed. The surprising finding of claudin-5 and protein ZO-1 in substructures of retothelial cell-cell bridges, i.e., structures that do not separate different tissues or cell layer compartments, suggests that such tight-junction molecules are involved in functions other than the "fence" and "barrier" roles of *zonulae occludentes.*

Keywords *Complexus adhaerens* · Junctions · Lymph node · Retothelium · Lymphatic endothelium · Bovine · Rodent · Human

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Introduction

One of the most complex, tenuous, and preparatively fragile cell types of the mammalian body is the stellate cell of the lymph node sinus ("retothelial cells", "sinus-lining cells"). Stellate cells do not form a normal endothelial layer but are characterized by numerous thin filopodia-like processes forming a closely interwoven, labyrinthine meshwork in the luminal space, either as freely suspended processes or coating the collagenous trabecular fibers (for reviews see, e.g., Fujita et al. 1973; Raviola 1975; Weiss 1977; Wacker 1994). Essential elements for the establishment and maintenance of this meshwork of retothelial cell processes are VE-cadherin-containing cell-cell adhering junctions differing markedly in size and shape. Because of their complex morphology and molecular composition (in particular, the occurrence of desmoplakin and plakoglobin but not of other desmosomal components), these junctions have been considered as an adherens junction type in their own right, are termed "*complexus adhaerens*", and are integrated, in the lymph node, into a larger structural system termed a "*syndesmos*" (Schmelz et al. 1990, 1994; Schmelz and Franke 1993; see also Wacker et al. 1992; Wacker 1994). Similar desmoplakin-containing *complexus adhaerentes* have been identified in the lining endothelium cells of a specific subtype of lymphatic capillaries of various organs (Schmelz et al. 1994; Sawa et al. 1999; Ebata et al. 2001a,b; for reviews, see also Lampugnani and Dejana 1997; Sleeman et al. 2001; Jussila and Alitalo 2002; Stacker et al. 2002; Al-Rawi et al. 2005; see, however, also Fedele et al. 2004; Wessells et al. 2004) and in certain endothelial cell cultures (Valiron et al. 1996; Kowalczyk et al. 1998). Importantly,

Gallicano et al. (2001) have noted the absolute requirement of desmoplakin for the formation of the vasculature system in embryogenesis and of microvessel formation in culture (Zhou et al. 2004; see also Cattelino et al. 2003).

As the retothelial jungle of cell processes in the lymph node sinus serves a series of important functions by reducing the flow speed of the percolating cells and particles, thereby facilitating the recognition, monitoring, and further treatment of the incoming components of the lymphatic fluid (including potential metastases), we have felt the need for a comprehensive cell and molecular biological analysis of the retothelial junction system of *complexus adhaerentes* in the lymph node sinus and in certain other parts of the lymphatic system. Here, we present the surprising finding that the *complexus adhaerens* is even more complex than previously shown, as it combines molecular constituents known from all three major categories of adherens junctions, i.e., *zonulae adhaerentes*, desmosomes, and tight junctions.

Materials and methods

Tissues and cell cultures

Freshly removed bovine or rodent tissue samples containing lymph nodes or rich in lymphatic capillaries were snap-frozen or fixed in formaldehyde or glutaraldehyde as described (Schmelz et al. 1994; Langbein et al. 2002, 2003). Human tissue samples obtained during surgery and routine pathology were similarly frozen and fixed. Aldehyde-fixed tissue specimens were dehydrated and embedded in paraffin or Epoxy resins and processed essentially as described (Schmelz et al. 1994; Langbein et al. 2002, 2003).

For comparisons, the following monolayer cell cultures and lines were used: human colon carcinoma CaCo2, glioma U333CG, keratinocytes HaCaT, endothelial HUVEC, breast carcinoma MCF-7, liver carcinoma PLC, bovine endothelial CPAE, and murine 3T3 (for sources and details, see, for example, Peitsch et al. 2001; Straub et al. 2003).

Antibodies and immunolocalization

The primary antibodies applied included diverse monoclonal and polyclonal antibodies against various cadherins (notably N-, VE-, E-cadherin, cadherin-11, desmogleins, and desmocollins), proteins binding to adhering junction plaques and actin, and tight-junction constituents (for reagents and sources, see Table 1). The specific secondary antibodies and protocols used for immunolocalization by light and electron microscopy of cryostat-sectioned material and by confocal laser-scanning immunofluorescence microscopy were as described (e.g., Langbein et al. 2002, 2003; Straub et al. 2003; for conventional electron microscopy, see Schmelz et al. 1994; Langbein et al. 2002). For light-microscopic immunolocalization of antigens in formaldehyde-fixed and paraffin-embedded material, the "antigen retrieval" technique by microwave treatment was used essentially as described (e.g., Shi et al. 2000; Peitsch et al. 2005; Riedel et al. 2005).

The cell cultures mentioned were primarily used for controls of the reactivity and specificity of the reagents used as detailed elsewhere (Mertens et al. 1996, 2001; Peitsch et al. 1999, 2001).

Results

Retothelial junctions of lymph nodes: immunofluorescence microscopy

Previously, we have shown that the cells of the retothelial meshwork of the lymph node sinus contain desmoplakin, as do the typical small desmosomes of the follicular reticulum cells of the follicles (Fig. 1). In the present study, we have attempted to determine other components of the junctions of the sinus retothelium in direct comparison with the desmosomes that connect the cells of the follicular dendritic reticulum and that are characterized by an orthodox composition, i.e., the presence of desmocollin 2, desmoglein 2, and plakophilin 2, in addition to desmoplakin and plakoglobin (see Figs. 1a, 2a; also Schmelz et al. 1994; Schmidt et al. 1999). These follicular reticulum cells also contain another kind of small junction that shows reactions of typical *puncta adhaerentia* (data not shown).

By contrast, in all three topological categories of sinus (cortical, intermediary, and medullary), the sinus-lining and retothelial cells, including their numerous processes, are connected by junctional structures that are strongly immunopositive not only for plakoglobin (Fig. 2a), but also for desmoplakin (Fig. 2b) and that vary greatly in size and appearance, from small punctate to longer whisker-like and streak-like forms (Fig. 2b; see also Schmelz and Franke 1993; Schmelz et al. 1994). This intense immunolabeling also provides a good illustration of the frequency and the ramifications of these structures, which are among the most abundant and conspicuous elements of the sinus (Figs. 2b, 3a′). Moreover, a direct comparison of the immunostaining on junctions of the follicular reticulum and the sinus retothelial cells provides an excellent control reference for the specificity and reactivity of the antibodies used. For example, among the classical desmosomal components, the significance of the negative retothelial reactions for desmoglein 2 and plakophilin 2 can directly be seen in side-by-side comparisons, with the retothelial desmoplakin reaction on the one hand and the follicular dendritic reticulum reaction on the other (Fig. 3a-b′).

At first glance, our negative plakophilin 2 reaction observed in the retothelium with monoclonal murine (Table 1) and polyclonal guinea pig (not shown) antibodies seems to be in contrast with the observations of Podgrabinska et al. (2002) who have detected plakophilin 2 in immunoblots of electrophoretically separated proteins of cultured lymphatic cells derived from human foreskin vessels but not in blood vessel-derived endothelial cell cultures. However, in these last-mentioned cases, plakophilin 2 is an almost ubiquitous, although minor, constitutent of the nucleus (Mertens et al.

Table 1 Primary antibodies used (*mAb* monoclonal antibody, *As* conventionally prepared antiserum or IgG prepared therefrom, *rb* rabbit, *m* mouse, *g* goat, *gp* guinea pig)

Antigen	Antibody type	Source and references
Cadherins		
N-cadherin	a) mAb, m	Transduction Laboratories, Lexington, Ky., USA
	b) As, rb	R&D Systems, Minneapolis, Minn., USA
	c) mAb, m (A-CAM)	Kind gift of B. Geiger, Weizmann Institute, Rehovot, Israel
VE-cadherin	a) mAb, m (BV9)	Kind gift of E. Dejana, University of Milan, Milan, Italy
	b) mAb, m	Transduction Laboratories
	c) As, rb	Alexis Biochemicals, Gruenberg, Germany
R-cadherin	mAb, m	BD Biosciences, San José, Calif., USA
M-cadherin	mAb, g	Research Diagnostics, Flanders, N.J., USA
Cadherin-11	a) mAb, m	Zymed Laboratories, South San Francisco, Calif., USA
	b) As, rb	
E-cadherin	mAb, m	Transduction Laboratories
Desmogleins 1 and 2	mAb, m (clone 3.10)	Progen Biotechnik, Heidelberg, Germany; Kurzen et al., 1988
Desmoglein 1	mAb, m (clones P23, P124)	
Desmoglein 2	mAb, m (clones G129, G96)	
Desmocollin 1	a) mAb, m (clone U100)	Progen Nuber et al. 1996
	b) As, rb	Natutec, Frankfurt am Main, Germany
Desmocollin 2	a) mAb, m (clone 7G6)	Kind gift of M. J. Wheelock, University of Nebraska, Omaha, Neb., USA
	b) As, rb	Natutec; Progen
	c) As, g	Progen
Desmocollin 3	a) mAb, m (clone U114)	Progen
	b) As, rb	Natutec
Arm-repeat plaque proteins		
Plakoglobin	a) mAb, m (11E4)	Kind gift of M. J. Wheelock
	b) mAb, m (PG 5.1)	Progen Cowin et al. 1986
β-Catenin	a) As, rb	Sigma, St. Louis, Mo., USA
	b) mAb, m	Transduction Laboratories
Protein $p120^{ctn}$	mAb, m	
Plakophilin 1	mAb, m (5C2)	Progen Heid et al. 1994
Plakophilin 2	mAb, m (CM150)	Mertens et al. 1996
Plakophilin 3	mAb, m (270.6.2)	Schmidt et al. 1999
Neurojungin	mAb, m (J19)	Paffenholz et al. 1999
Protein p0071	mAb, m	Kind gift of M. Hatzfeld, University of Halle/Saale, Halle, Germany
Other plaque proteins		
α-Catenin	a) As, rb	Sigma
	b) mAb, m	Transduction Laboratories
Desmoplakin (DP)	a) As, rb	Natutec
	b) As, gp	Progen
	c) mAb, m, DP I and II (2.15, 2.17, 2.20)	Progen Cowin et al. 1985
Vinculin	mAb, m (vin 11–5)	Sigma
α-Actinin	mAb, m	
Tight-junction proteins		
Claudins-1–5	As, rb	Zymed
Claudin-4	mAb	
Occludin	a) mAb	
	b) As, rb	
JAM-A	mAb, m	Transduction Laboratories
Protein ZO-1	As, rb	Zymed
Cingulin	mAb, m (139.3.4.)	Progen Langbein et al. 2002

Table 1 (continued)

Antigen	Antibody type	Source and references
Others		
Afadin	a) mAb, m	Kind gift of Y. Takai, University of Osaka, Osaka, Japan
	b) As, rb	Sigma
Drebrin	mAb, m (clone M2F6)	MoBiTec, Göttingen, Germany
Factor-VIII-related antigen	As, rb	Natutec
LYVE-1 (24–232)	As, rb	Acris Antibodies, Hiddenhausen, Germany

1996, 2001). Moreover, the results might reflect the re-expression of this protein upon cell culturing (for the absence of plakophilin 2 in blood and lymph vessel endothelia in situ, see also Mertens et al. 1999). Correspondingly, the frequent colocalization of desmoplakin with VE-cadherin on retothelial cells is in direct contrast to the separate reactions of VE-cadherin on the endothelial cells of blood capillaries and of desmoplakin on follicular dendritic reticulum cells (Fig. 4a-a″,b).

When we systematically examined the reactions of other adhering junction protein candidates on retothelial cells, e.g., by double immunostaining with respect to desmopla-

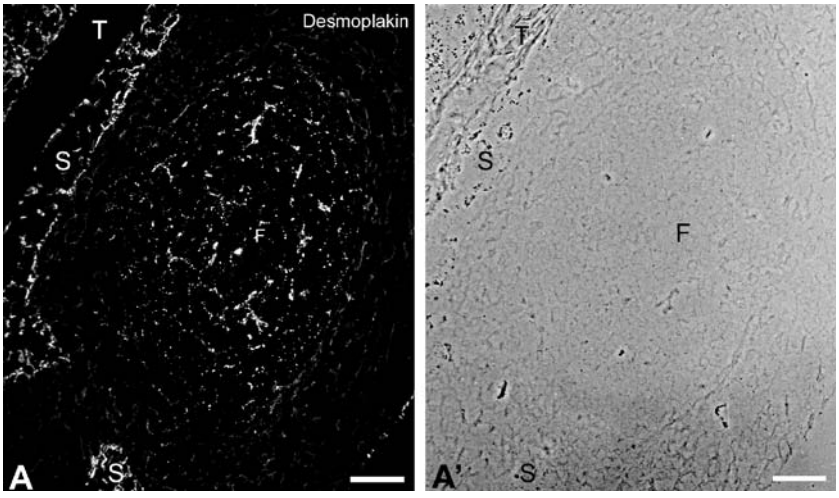

Fig. 1 **a** Immunofluorescence microscopy of a cryostat section through a human lymph node (*T* trabecula) showing the localization of desmoplakin on the retothelial cells of the sinus (*S*) and the dendritic reticulum cells of the follicles (*F*). **a′** Phase-contrast image. *Bars* 50 μm

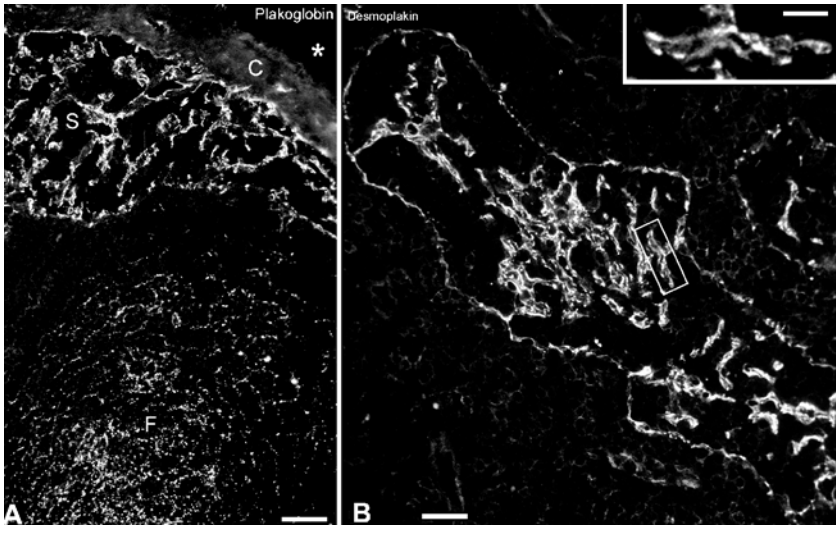

Fig. 2 Immunofluorescence microscopy of cryostat sections through a human lymph node showing the localization of plakoglobin (**a**) and desmoplakin (**b**). **a** Plakoglobin on desmosomes connecting dendritic reticulum cells of follicles (*F*) and those connecting the retothelial cells in the *syndesmos* system of the sinus (*S*). *C* Lymph node capsule, *asterisk* extracapsular space. **b** Higher magnification survey of a near-longitudinal section of a sinus. Note the cross-sections of retothelial cells with punctate or comma-shaped immunopositive structures, and the rod-like or curvilinear immunopositive structures in oblique or near-longitudinal sections. *Inset* Details of the region demarcated by the *rectangle* in **b**. *Bars* 50 μm (**a**), 17 μm (**b**)

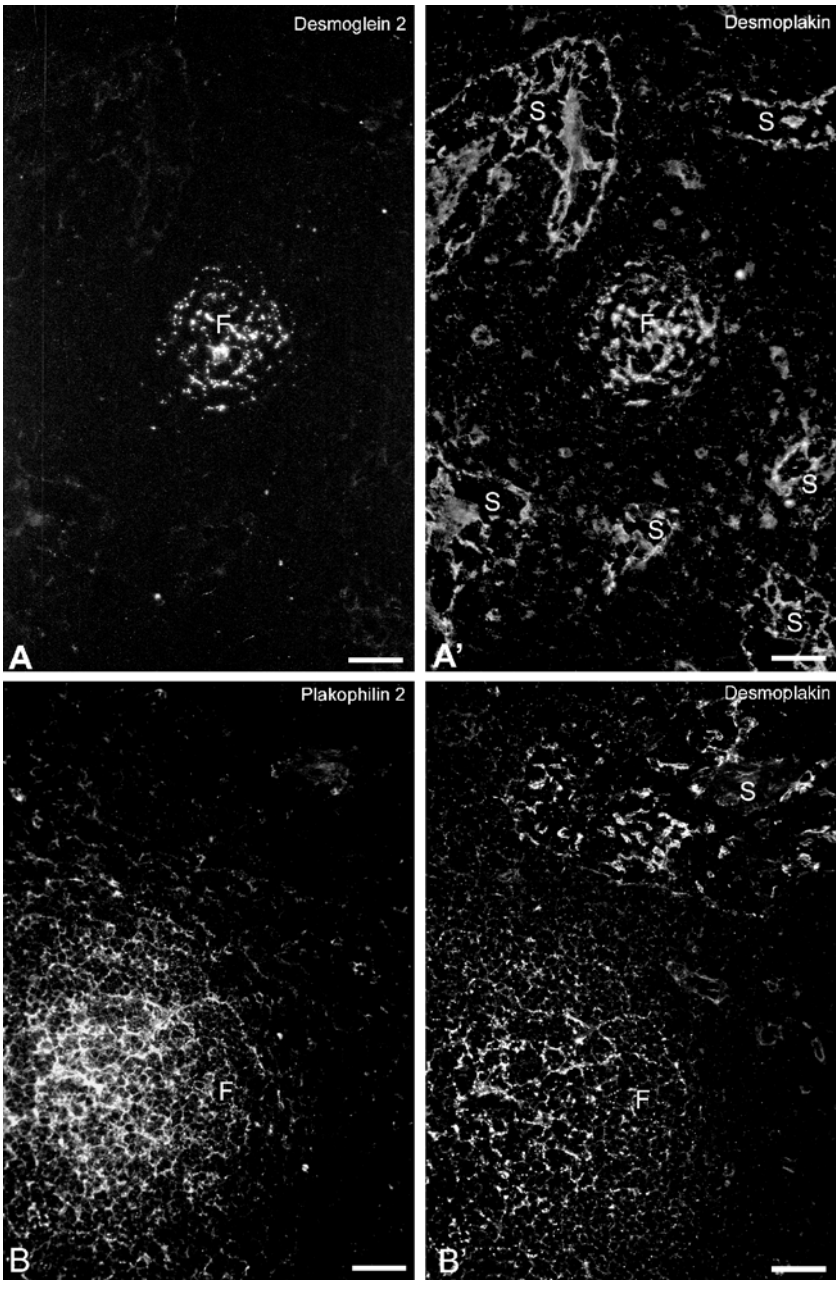

Fig. 3 Double-label immunofluorescence microscopy of cryostat sections through human lymph nodes, comparing the immunolocalization of desmoglein 2 (**a**) and plakophilin 2 (**b**) with that of desmoplakin (**a′**,**b′**). Whereas desmoglein 2 and plakophilin 2 are detected only in the small desmosomes of the dendritic reticulum cells of the follicles (*F*), desmoplakin is present in both the follicular dendritic reticulum (*F*) and the *complexus adhaerentes* retothelial cell system of the sinus (*S*). *Bars* 50 μm

kin and plakoglobin, we noticed far-reaching but not complete colocalization with the adherens junction proteins α-catenin (Fig. 4c), β-catenin, protein $p120^{ctn}$ (not shown; cf. Hämmerling 2004), and afadin (Fig. 5), whereas all other cadherins and plaque components tested (compare Table 1) gave negative results. Much to our surprise, we obtained distinct and intensely positive retothelial junction reactions, showing frequent site of colocalization with plakoglobin and desmoplakin, for claudin-5 (Fig. 6a-b′) and, to a lesser and rather variable degree, claudin-1, which were, as expected, both totally negative on other desmoplakin-positive cells, such as the dendritic reticulum cells of follicles (Fig. 6a,a′). Claudin-5 was (also as expected) also positive on the endothelia of normal blood and lymphatic vessels (e.g., Fig. 6a). We must however emphasize that the claudin-5 reaction extended over most of the retothelial junction structures, as seen, for example, by the far-reaching co-localization with desmoplakin (Fig. 6b,b′), whereas regions with disparate reactions were also frequently seen in which either claudin-5 or desmoplakin was apparent (Fig. 6). Similarly, we noted positive immunostaining for the tight-junction proteins JAM-A and ZO-1 (not shown; cf. Hämmerling 2004) on some of the *complexus adhaerens* structures but negative reactions for other claudins and occludin (not shown). Throughout the study, the endothelial character of the retothelial sinus and of other lymphatic structures was verified by positive reaction for the factor-VIII-related antigen and for LYVE-1, an extracellular domain of the corresponding hyaluronan receptor (not shown).

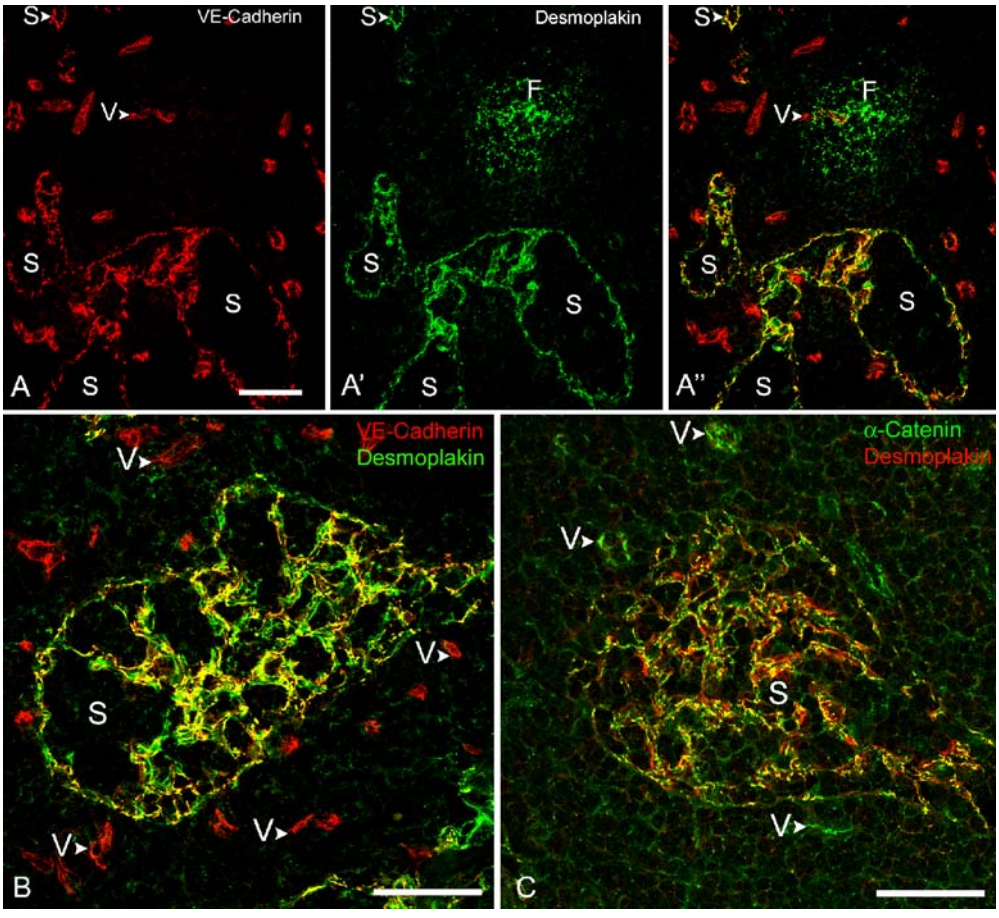

Fig. 4 Double-label immunofluorescence laser scanning microscopy of frozen tissue sections of human lymph nodes, showing the far-reaching colocalization of VE-cadherin (**a**) and desmoplakin (**a′**) in the *complexus adhaerentes* of the retothelial cells of a sinus (*S*), in contrast to the specific localization of VE-cadherin only in the endothelia of the numerous small blood vessels (*V*) and of desmoplakin only in the desmosomes of the dendritic reticulum cells of follicles (*F*) The merged image is presented in **a″**). The abundance and extensions of the *complexus adhaerentes* are illustrated with particular clarity in the merged image of the sinus shown in **b**. Frequent sites of colocalization of catenins and desmoplakin in *complexus adhaerentes* of retothelial cells of the sinus (*S*) are seen in the merged image for α-catenin (**c**). *Arrowheads* denote a subset of endothelia of blood vessels (*V*), showing an exclusive reaction of catenins in the *zonulae adhaerentes*. *Bars* 50 μm

As we have taken special care to control our negative results, e.g., by directly comparing the reactions of the *complexus adhaerens* with those of other nearby structures (i.e., desmosomal components in the retothelial versus the dendritic reticulum follicular cells or in a comparison with typical *puncta adhaerentia* components), we feel entitled to emphasize the experimental significance of most, if not all, of our negative conclusions.

Retothelial junctions: electron microscopy

Electron microscopy confirmed and extended our previous conclusion (Schmelz and Franke 1993) that *complexus adhaerens* junctions markedly vary in size, as their plaque-coated regions could be traced from about 0.3 μm to more than 5 μm and often showed the typical interruptions of the plaque structure (Fig. 7a-c). Bundles of actin microfila-

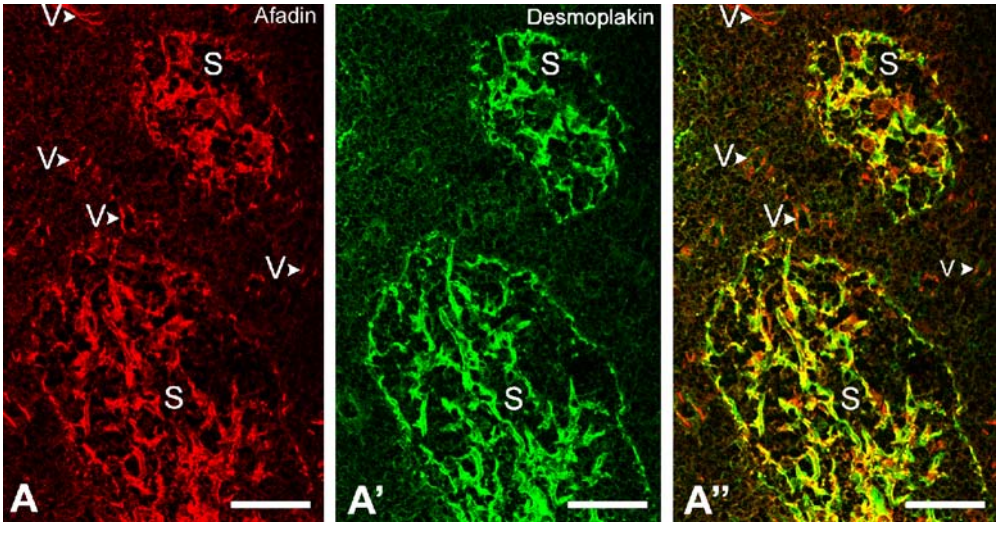

Fig. 5 Double-label immunolocalization laser scanning microscopy of a human lymph node cryostat section showing afadin (**a**) and desmoplakin (**a′**) in an extended colocalization characteristic for the retothelial cells of a sinus (*S*). **a″** Merged image. The adhering junctions of small blood vessels (*V*, *arrowheads*) endothelia are positive only for afadin. *Bars* 50 μm

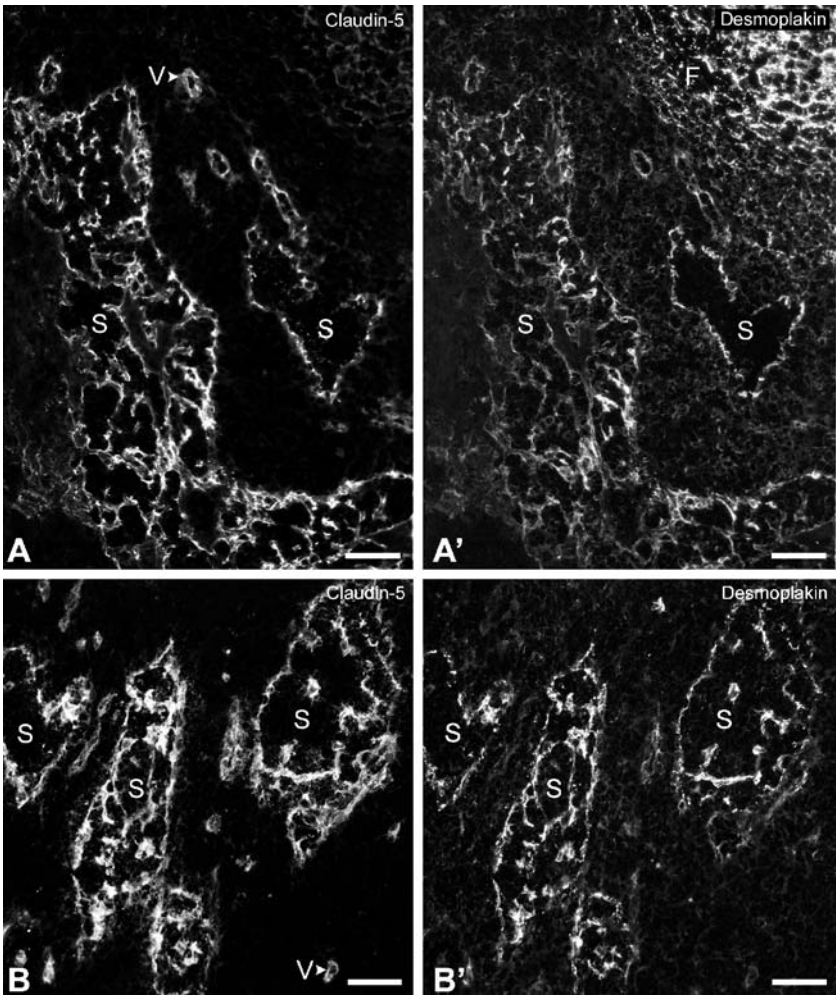

Fig. 6 Double-label immunofluorescence microscopy of frozen sections through human lymph nodes, comparing the localization of claudin-5 (**a**,**b**) and desmoplakin (**a′**,**b′**). Note the extensive colocalization in *complexus adhaerentes* of retothelial cells of a sinus (*S*), whereas an exclusive desmoplakin reaction is seen in the desmosomal structures of the follicular reticulum (*F* in **a′**). Claudin-5 is exclusively present in the adhering junctions of blood vessels (*V*, *arrowhead*). Note also that the intensity of the retothelial claudin-5 immunostaining reaction only partly corresponds to that of desmoplakin. *Bars* 50 μm

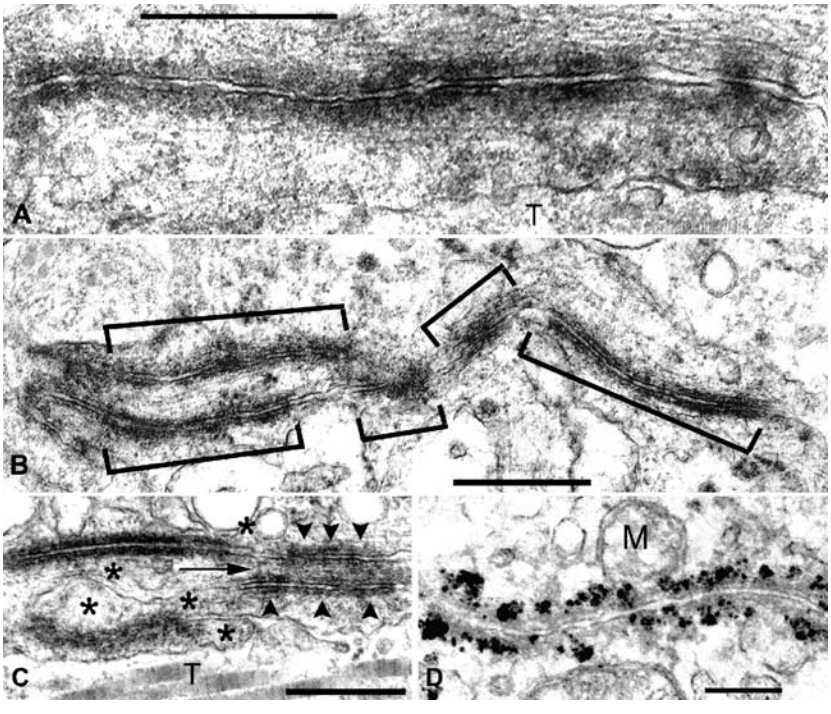

Fig. 7 Electron microscopy of ultrathin sections showing extended *complexus adhaerens* structures connecting retothelial cells of bovine lymph node sinus (*T* collagen-fibril-containing trabeculae, *M* mitochondrion). Note the typical extended plaque-coated region with small interruptions (**a**), a partially folded cell junction region combining several plaque-bearing regions (**b**, *brackets*), a locally intricate interdigitation complex (**c**; *asterisks* various cytoplasmic folds, *arrow* an example of the narrowness that such a cytoplasmic fold can attain), and the immunogold labelling of a *complexus adhaerens* plaque by desmoplakin antibodies (**d**). *Bars* 0.5 μm (**a–c**) and 0.25 μm (**d**)

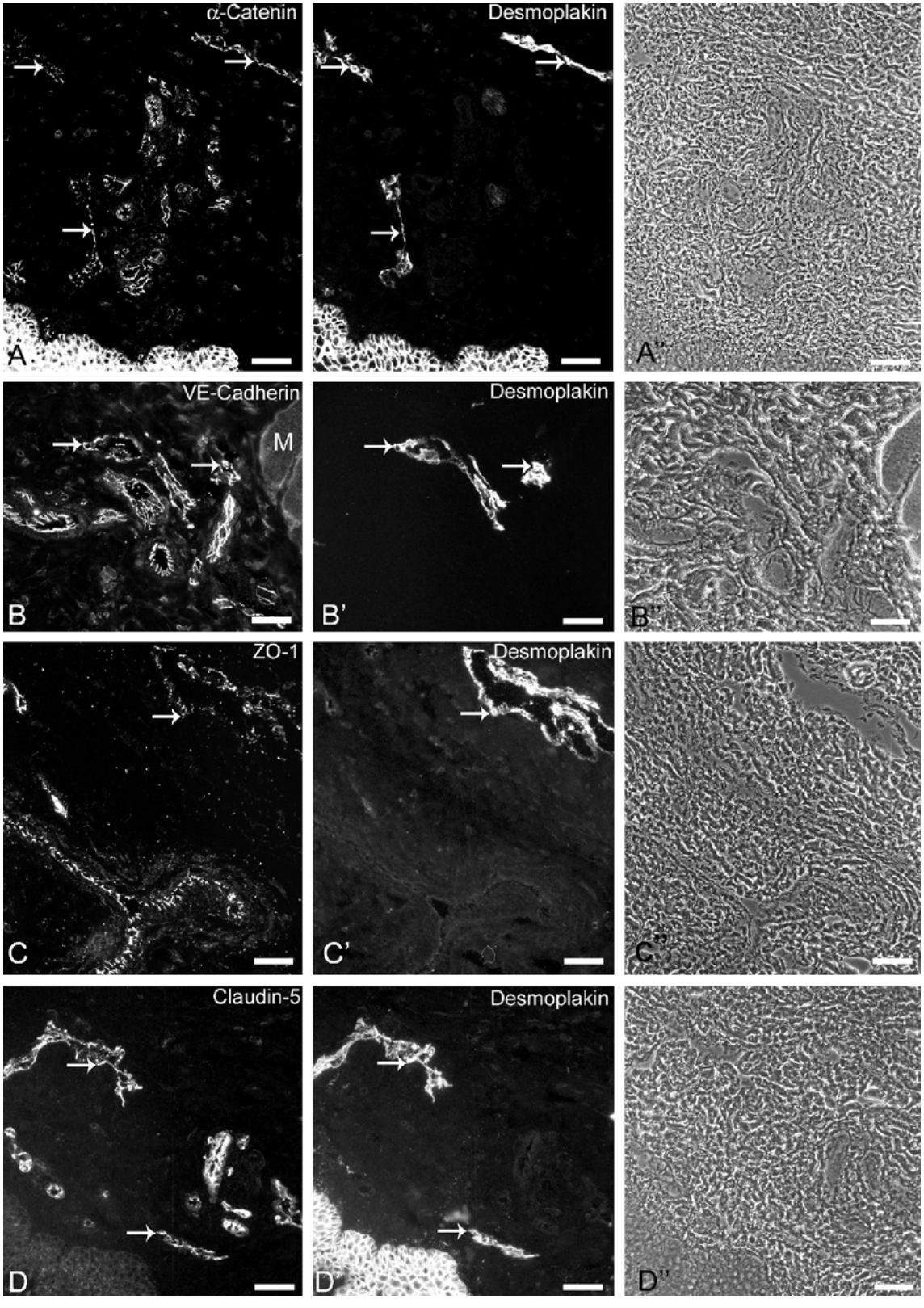

Fig. 8 Double-label immunofluorescence microscopy of cryostat sections through bovine tongue submucosa showing colocalization of α-catenin (**a**), VE-cadherin (**b**), protein ZO-1 (**c**) and clandin-5 (**d**) with desmoplakin (**a′**–**d′**) in adhering junctions of special lymphatic vessels (some are denoted by arrows; **a″**–**d″** present the specific phase-contrast images). Note also the occurrence of α-catenin as well as desmoplakin in the specific *puncta adhaerentia* (**a**) and desmosomes (**a′**), respectively, of the lingual epithelium and the occurrence of other vessels which are positive for the catenins and VE-cadherin but negative for desmoplakin. Bars 50 μm

ments and of intermediate-size filaments were seen to anchor at or laterally associate with these plaques (see, for example, Fig. 7a). However, we also noted that, in cells connected by a *complexus adhaerens* junction, a cell process frequently projected into an invagination of the neighboring cell and that such local filopodia-like processes also formed extended local interdigitations (e.g., Fig. 7b and, in particular, Fig. 7c). Sometimes, more than two cells were interconnected in the same region. Again, the *complexus adhaerens* nature of these endothelium-derived cell processes was generally verified by immunoelectron microscopy, with positive reactions for desmoplakin (Fig. 7d) and VE-cadherin (not shown).

Desmoplakin-positive junctions in endothelia of other lymphatic vessels

The unusual immunostaining reactions described above were not exclusive to lymph node sinus but were also noted in endothelia of certain low-caliber lymphatic vessels, including capillaries in various tissues. Some examples of such reactions in lymphatic vessels of tongue mucosa are presented in Fig. 8a-d″, which shows the positive immunocolocalization of lymphatic endothelial desmoplakin with α-catenin, VE-cadherin, protein ZO-1, and claudin-5 (positive reactions for β-catenin, plakoglobin, protein $p120^{ctn}$, afadin, and protein JAM-A are not shown; see Hämmerling 2004). Electron microscopy revealed that these desmoplakin-positive reactions were generally associated with parts of the *zonula adhaerens*-like junctions encompassing these endothelial cells (not shown; for comparison see, for example, Franke et al. 1988).

Discussion

This report confirms and markedly extends our previous conclusion that the plaque-coated *complexus adhaerens* junctions of a subset of lymphatic endothelia, including certain lumen-lining endothelia and retothelial cells of the lymph node sinus, represent a special kind of intercellular junction in its own right, both by morphological and molecular criteria (Schmelz et al. 1990, 1994; Schmelz and Franke 1993). We have since broadened our molecular analysis and can now state that the *complexus adhaerens* is a junction that combines representatives of all three of the so-called "classical" major junction categories, as it combines typical *zonula adhaerens* proteins (such as a type I cadherin, i.e., VE-cadherin, together with α- and β-catenin, protein $p120^{ctn}$ and afadin) with prominent desmosomal plaque proteins (desmoplakin and plakoglobin), and several tight-junction proteins (such as claudin-5, JAM-A, and plaque component ZO-1). Thus, the *complexus adhaerens* can be regarded as a recombination hybrid of members from three normally separate junction ensembles. Moreover, it can vary greatly in size and shape and, in addition to protrusions and fenestrations (Schmelz and Franke 1993), may also form single and multiple adhering interdigitations (this study).

The cell-type-specific constitutive occurrence of VE-cadherin in adhering junctions of diverse other endothelia and its functional importance for angiogenesis has been amply shown (Lampugnani et al. 1992, 1995; Breviario et al. 1995; Caveda et al. 1996; Dejana 1996; Vittet et al. 1997; Carmeliet et al. 1999; Corada et al. 1999; Gory-Fauré et al. 1999; Hordijk et al. 1999; Vorbrodt and Dobrogowska 2004). Whereas some authors have reported a colocalization with junction-bound N-cadherin (e.g., Schulze and Firth 1993; Luo and Radice 2005), others have claimed that endothelial N-cadherin is transiently or in general diffusely spread on the surface, i.e., is not integrated into the adhering junctions or enriched in the plasma membrane of the abluminal side (Salomon et al. 1992; Lampugnani and Dejana 1997; Navarro et al. 1998; Gerhardt et al. 1999; Liebner et al. 2000a–c; Jaggi et al. 2002; for a recent review, see Dejana 2004). Although occasional immunolocalization experiments have given us the impression that some N-cadherin and even some cadherin-11 may also act as a minor component in certain *complexus-adhaerens*-containing regions, we cannot yet ascribe significance to these weak and variable reactions. Studies of vasculogenesis in gene-deficient animals or gene knock-down siRNA experiments might help to determine whether VE-cadherin and N-cadherin can partly compensate for each other. Of course, we will also have to clarify whether *complexus adhaerentes* contain the corresponding protocadherin 12 (VE-cadherin 2; cf. Telo' et al. 1998; Rampon et al. 2005).

With respect to tight-junction proteins, claudin-5 is widely regarded as an established general endothelial marker (Morita et al. 1999; for variations of demonstrability in certain vessels, see also Morita et al. 2003). As we have shown here, this also holds for lymphatic vessel regions characterized by *complexus adhaerentes*. Clearly, however, the studies of claudin-5-deficient mice by Nitta et al. (2003) have revealed that endothelial tight junctions often (perhaps generally) also claudin-12, which apparently is sufficient to maintain the tight-junction structure and most of the barrier functions in endothelia (for some minor functional deficiencies, see Nitta et al. 2003; for claims of the presence of further claudins, see, for example, Haselton and Heimark 1997; Hamm et al. 2004). As several authors have noted, some claudin-1 antibodies may cross-react with other claudins, including claudin-5 (e.g., Liebner et al. 2000a,c; Nico et al. 2003; Nitta et al. 2003), although the occasional reactions of such antibodies on endothelia and retothelia (Hämmerling 2004) have not been considered significant (see, however, for further claudins in certain endothelial tight junctions, Morcos et al. 2001; González-Mariscal et al. 2003; Wolburg et al. 2003). On the other hand, occludin has not been identified in *complexus-adhaerens*-containing lymphatic cells (for the absence of occludin in endothelia of certain small vessels, see also Morita et al. 2003). Localization reactions for the transmembrane proteins of the JAM family of cell-surface molecules have been found in some vessels but not in others, and so it cannot as yet be taken as a general retothelial *complexus adhaerens* marker (cf. Martin-Padura et al. 1998; Liu et al. 2000; Ebnet et al. 2003; for recent

reviews on tight junctions in endothelial cells, see also Martin and Jiang 2001; González-Mariscal et al. 2003; Al-Rawi et al. 2005; Ji 2005).

On its cytoplasmic side, the *complexus adhaerens* plaque contains not only an abundance of plakoglobin and desmoplakin (Schmelz and Franke 1993; Schmelz et al. 1994; Sawa et al. 1999; Ebata et al. 2001a,b; see also Franke et al. 1987, 1988; Valiron et al. 1996; Kowalczyk et al. 1998), but also members of the actin-microfilament-anchoring protein ensemble, such as the two catenins, protein p120^{ctn}, afadin, vinculin, and α-actinin (for blood vessel endothelia, see also Dejana 1996; Lampugnani and Dejana 1997; Navarro et al. 1998; Hamm et al. 2004), and tight-junction proteins, such as ZO-1 (e.g., Stevenson et al. 1986; Li and Poznansky 1997; Tsukita et al. 1999; Wolburg and Lippoldt 2002; Nico et al. 2003; Hamm et al. 2004; Vorbrodt and Dobrogowska 2004). At present, we are also examining the molecular composition of the intercellular junctions of the sinus-lining endothelia of the spleen; these are often characterized by prominent plaques (e.g., Heusermann and Stutte 1974; Kaiserling et al. 1989; Uehara and Miyoshi 1997; see also Schmelz et al. 1994).

This list of *complexus adhaerens* plaque components is probably not complete, and the specific molecular interactions in this plaque system will have to be elucidated by special techniques (for interdependences between the cadherin-catenin and the afadin-containing systems, see also Takai and Nakanishi 2003; Dejana 2004). What is obvious, however, is the essential importance of some of these plaque components for lymph and blood vessel angiogenesis, as has been demonstrated for desmoplakin with striking clarity in vivo and in vitro (Gallicano et al. 2001; Zhou et al. 2004). The demonstration of the importance of desmoplakin in embryonic vessel formation also indicates the fundamental role of the *complexus adhaerens* in development and suggests that the absence of desmoplakin (and thus the *complexus adhaerens*) in most endothelia of later stages, including adult blood vessels, reflects a secondary loss of this junction type, a conclusion that might also present an explanation for its "re-appearance" upon cell culture in vitro (e.g., Valiron et al. 1996). Moreover, the specific regulatory molecules and mechanisms involved in processes of cell recognition, signaling, and transendothelial permeability of lymphatic endothelial cells will have to be determined (for relevant data for blood endothelia and cell cultures derived therefrom, see, for example, Navarro et al. 1995; Del Maschio et al. 1996; Allport et al. 1997; Alexander et al. 1998; Kevil et al. 1998; Corada et al. 1999; Hordijk et al. 1999; Ratcliffe et al. 1999; Burns et al. 2000; Wong et al. 2000; Ferber et al. 2002; Oliver and Detmar 2002; Dejana 2004; Iyer et al. 2004).

Whether the *complexus adhaerens* is a relatively homogeneous molecular continuum or whether it is composed of small mosaic elements with different molecular characteristics remains uncertain. Whereas the major markers, such as desmoplakin, α-catenin, and the three *arm*-repeat proteins (α-catenin, plakoglobin, protein p120^{ctn}), often show far-reaching and, in some places, complete coincidence (indicated by the yellow-orange merged color in double-label immunofluorescence microscopy), local small plaque interruptions have been seen. This extended colocalization provides a particular problem for the tight-junction markers applied. As we have not been able to resolve typical focal tight-junction membrane-membrane contacts ("kisses"), and in view of the lack of relevant electron microscopic studies in the literature, we cannot exclude that the tight-junction elements only occupy a certain proportion of the total *complexus adhaerens*. This is also indicated by the numerous local differences between claudin-5 and desmoplakin immunostaining (Fig. 6). Biochemical studies of the *complexus adhaerens*, e.g., by selective detergent solubilization, followed by immunoprecipitation or size fractionation techniques, directly or after chemical cross-linking, should help to elucidate the relative neighbor relationships of the *complexus adhaerens* molecules.

Our finding of typical tight-junction components, such as claudin-5 and protein ZO-1, at the *complexus adhaerentes* of the intercellular bridges formed by retothelial processes is surprising. Clearly, in these positions, such proteins cannot possibly serve classical tight-junction functions of continuous *zonulae occludentes*, i.e., the "barrier" and "fence" roles in epithelial and endothelial layers. Examination of the lymphatic system of mice deficient in the claudin-5 gene (Nitta et al. 2003) and in genes of other retothelial components will be important for determining possible, as yet overlooked, functions in this system.

Some of our findings should provide valuable cell-type markers in developmental biology and tumor pathology, in cell engineering, and in regenerative medicine. Although this has previously been demonstrated in the case of desmoplakin immunohistochemistry (e.g., Schmelz et al. 1994; Ebata et al. 2001a; see also Gallicano et al. 2001), specific systematic studies will be needed to examine and establish the value of *complexus adhaerens* components for the identification and characterization of certain subtypes of lymphatic endothelia, be it in normal sinus or vessels or in pathologically altered states (for an anthology of changes in endothelial structures in diseases, see Simionescu and Simionescu 1988). An extended study of the pathogenic changes of retothelial and endothelial organization in the lymph system will be published elsewhere.

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