Journal of Cell Science 104, 773782 (1993)
Printed in Great Britain © The Company of Biologists Limited 1993

Immunohistochemical localization of adherens junction components in blood-brain barrier microvessels of the rat

Charlotte Schulze* and J. Anthony Firth

Department of Anatomy and Cell Biology, St. Mary's Hospital Medical School, Imperial College of Science, Technology and Medicine, Norfolk Place, London W2 1PG, UK

*Author for correspondence at: Eisai London Research Laboratories, Bernard Katz Building, University College London, Gower Street, London WC1E 6BT, UK

SUMMARY

The morphology and molecular composition of intercellular adherens junctions have most frequently been described in epithelial cells and the *fascia adhaerens* of the intercalated disc. A group of cytoplasmic molecules is known to be associated with adherens junctions. The intercellular bond is mediated by cadherins which bridge the cells by homophilic binding. Recently, endothelial cells have also been shown to form intercellular junctions of the adherens-type. However, they are morphologically less distinct and little is known about their molecular components.

In this study we report the localization of some adherens junction components in intact microvessels of the blood-brain barrier in the rat. We used antibodies raised against α-actinin, vinculin, zyxin, cadherin (anti-pan-cadherin antibody) and A-CAM (N-cadherin) in immunohistochemical experiments at light and electron microscopical levels. Microvessel walls reacted positively for all antigens throughout postnatal development. All antigens were localised, though not necessarily exclusively, to interendothelial junctions. At the ultrastructural level, pan-cadherin reactivity was present throughout the entire length of the cleft. These results could mean that in blood-brain barrier endothelial cells the complex tight junction is embedded in an adherens junction which occupies the entire length of the cleft.

Key words: adherens junction, cell-cell adhesion, blood-brain barrier, cadherin, A-CAM (N-cadherin), vinculin, α-actinin, zyxin, tight junction, immunohistochemistry, LM, EM

INTRODUCTION

Adherens junctions (AJs) are cellular contacts formed between either a cell and the surrounding extracellular matrix (ECM) or between two cells. Examples of the former type are focal contacts of cells in culture, myotendinous junctions, dense plaques of smooth muscle and basal adhesions to basement membranes (reviewed by Turner and Burridge, 1991; Geiger and Ginsberg, 1991; Critchley et al., 1991). To the latter group of adherens junctions belong the *zonula adhaerens* of epithelial cells, the *fascia adhaerens* of the myocardial intercalated disc, interendothelial junctions and spot adhesions in other cell types (reviewed by Geiger et al., 1990a; Magee and Buxton, 1991; Geiger and Ginsberg, 1991). All adherens junctions are associated with actin-microfilaments, vinculin and α-actinin (Kabsch and Vandekerckhove, 1992; Otto, 1990; Blanchard et al., 1989). At the intramembranous level, cell-ECM AJs are mediated by members of the integrin family of extracellular receptors (Turner and Burridge, 1991), whereas cadherins connect different cells at intercellular AJs by means of homotypic binding (Magee and Buxton, 1991).

In the recent past, a large number of intracellular components of AJs have been identified (reviewed by Tsukita and Nagafuchi, 1990; Geiger and Ginsberg, 1991). While similarities as well as differences in the molecular composition between cell-ECM and intercellular AJs are emerging, it is likely that cell type-specific distribution of junctional components also exists (Geiger and Ginsberg, 1991).

The intercellular AJ in endothelial cells has been shown to contain plakoglobin, vinculin and α-actinin associated with actin, and is believed to be organised in a belt structure similar to, if less regular than, the epithelial *zonula adhaerens* (Franke et al., 1988). However, the topological relationship of the endothelial AJ and its plaque to the adjacent tight junction is not known. Evidence suggests that several cadherins (Heimark et al., 1990; Liaw et al., 1990; Rubin et al., 1991a; Salomon et al., 1992; Lampugnani et al., 1992), at least one cell adhesion molecule species of the Ig-superfamily (Endo-CAM = CD31 = PECAM-1; Albelda et al., 1990; Newman et al., 1990) and at least two integrins (Lampugnani et al., 1991) are localized to the interendothelial junction in cultured endothelial cells and may therefore also be components of the endothelial AJ in vivo.

To date, none of the above mentioned cell-cell adhesion molecules has been localized at the ultrastructural level in intact vessels. We report here the immunohistochemical localization of AJ-associated components in rat blood-brain barrier capillaries, both at the light and electron microscopical levels, and discuss the implications of our results for understanding endothelial AJs.

MATERIALS AND METHODS

Fixation procedure

Neonatal Wistar rats of ages 1-day (D1), 8-day (D8) and 16-day (D16) were deeply anaesthetized by inhalation of ether. Their brains were fixed by transcardiac perfusion with 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 5-15 min, followed by perfusion with the same fixative containing 10% sucrose for the same length of time. The perfusion rate was 0.4-1.3 ml/min depending upon size and age of the animal. Young adult rats (≥5 weeks old) were anaethetized by intraperitoneal injection of Sagatal (approx. 1 ml/kg body mass) and their brains were perfusion-fixed in the same way. The brains were dissected and pieces of suitable size were cut and immersed in the sucrose-containing fixative for 1 h. They were then embedded in Tissue-tek (Miles, USA), mounted on a piece of cork, frozen in iso-pentane cooled by liquid nitrogen and stored at −40°C. Frozen sections of 5 µm thickness were cut on a cryostat, picked up on subbed coverslips and stored at −40°C for up to 6 weeks.

For immunogold staining of ultrathin frozen tissue, adult Wistar rats were perfused in the same way as described above with 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, for 30 min. Small pieces (approx. 1 mm^3) were cut and immersed in the same fixative for 1 h. They were then immersed in 2.3 M sucrose in 0.05 M cacodylate buffer for 2-3 h, before being mounted on specimen pins and thrown into liquid nitrogen, in which they were stored for periods up to 6 months.

Immunohistochemistry

Avidin-biotin complex method

Sections were indirectly immunolabeled by use of an ABC kit (Vectastain Elite ABC kit, Vector Laboratories, UK) according to the manufacturer's instructions. Sections were incubated in the following way: (1) phosphate-buffered saline (PBS, Dulbecco 'A', Oxoid, UK) containing 0.1% Triton (10 min); (2) PBS (10 min); (3) diluted normal goat serum (20 min); (4) primary antibody, diluted in PBS (16 h, 4°C); (5) PBS (10 min); (6) biotinylated goat anti-mouse or anti-rabbit antibody (2 h); (7) PBS (10 min); (8) ABC solution (1 h); (9) PBS (10 min); (10) freshly prepared peroxidase reaction mixture composed of equal amounts of 0.02% hydrogen peroxide in H_2O and 0.1% diaminobenzidine in PBS; (11) tap water (5 min). Sections were mounted in Uvinert mountant (BDH, UK).

Immunofluorescence

For indirect immunofluorescence, sections were incubated in the following way: (1) PBS containing 0.1% Triton (10 min); (2) PBS (10 min); (3) diluted normal goat serum (20 min); (4) primary antibody diluted in PBS (16 h, 4°C); (5) PBS (10 min); (6) secondary FITC- or TRITC-labelled antibody raised in goat against mouse or rabbit, respectively, (Sigma, UK) at a concentration of 1:30-1:70 (2 h); (7) three changes of PBS. Sections were mounted in Vectashield mountant (Vector Laboratories, UK) and viewed in a Zeiss fluorescence microscope. Photographs were taken using Kodak TMAX Professional Films (400 ASA, Kodak, UK).

Double-labelling experiments were conducted by incubating the sections with a mixture of both primary antibodies raised in different species. The conjugated secondary antibody was also applied as a mixture.

For localization of F-actin the sections were permeabilized and washed as described above, before being incubated with rhodamine phalloidin (Sigma, UK) for 1.5 h. They were then washed and mounted.

Immunogold labeling on ultrathin frozen sections

Ultrathin sections were cut at −100°C on a Reichert Jung Ultramicrotome (FC-4E, Leica, UK) using tungsten-coated glass knives. They were picked up on droplets of sucrose solution and transferred to formvar-coated nickel grids. Indirect immunocytochemistry was performed as follows. Grids were transferred to droplets of the following solutions: (1) PBS (4 × 5 min); (2) 0.02 M glycine in PBS (10 min); (3) PBS containing 0.1% bovine serum albumin (BSA, Sigma, UK; 3 × 2 min); (4) 5% normal goat serum in PBS (15 min); grids were then carefully dried with filter paper, followed by (5) primary antibody diluted in PBS containing 0.1% BSA (2 h, or 16 h at 4°C); (6) PBS containing 0.1% BSA (2 × 5 min); (7) 5 or 10 nm gold-labeled goat anti-rabbit or anti-mouse secondary antibody (Amersham International, UK; 1 h); (8) PBS (4 × 5 min); (9) 2.5% glutaraldehyde in PBS (2 min); (10) distilled H_2O (4 × 3 min); (11) 2% basic uranyl acetate (pH 7.2-7.4, 10 min); (12) distilled H_2O (3 × briefly); (13) 4% aqueous uranyl acetate (10 min).

A 1.3% solution of methyl cellulose (Tylose, Fluka, Switzerland) in water was prepared as follows: a 2% solution of methyl cellulose was made by adding the powder to water at 95°C, stirring the solution for a few minutes and putting it on ice. Then it was mixed for at least 4-8 h at 0-4°C and left for a further 3-4 days at that temperature, before being centrifuged at high speed (60,000 revs/min in a Beckman 60Ti) for 90 min at 4°C. Methyl cellulose was stored for up to several months at −40°C. The solution was carefully diluted with water before each experiment.

The grids were picked up with forceps and allowed to touch the surface of two drops of ice-cold methyl cellulose solution briefly, before being placed on a third drop for no longer than 20 sec. They were picked up with a thin wire loop and excess methyl cellulose was dried off quickly with filter paper. Before being viewed in a JEOL 100CX electron microscope, the grids were air-dried for a few hours.

Double-labelling experiments were conducted according to the same principles described above.

Primary antibodies and controls

Polyclonal anti-α-actinin antibody raised against purified α-actinin from chicken gizzard was purchased from Sigma (UK). Monoclonal antibodies against affinity-purified chicken heart A-CAM, which reacts with the N-terminal half of the extracellular domain of the molecule, and against purified vinculin from chicken gizzard smooth muscle, were also purchased from Sigma (UK).

The polyclonal antibody developed in rabbit against a synthetic peptide consisting of the C-terminal 24 residues of chicken N-cadherin (anti-pan-cadherin antibody) was a gift from Dr T. Volberg (Dept of Chemical Immunology, The Weizmann Institute of Science, Rehovot, Israel). The C terminus is a highly conserved region between different members of the cadherin family of cell adhesion molecules and this antibody is thought to react with all known cadherins which possess a cytoplasmic tail (Geiger et al., 1990b).

Polyclonal antiserum developed in rabbit against zyxin purified from avian gizzard was kindly donated by Dr A. Crawford (Dept of Biology, University of Utah, Utah, USA). Zyxin is a cytoplasmic component of focal adhesions and epithelial intercellular AJs

(Beckerle, 1986; Crawford and Beckerle, 1991; Crawford et al., 1992).

All antibodies were found to cross-react with rat tissue. As a positive control they were tested on the *fasciae adherentes* of the intercalated disc of rat myocardial tissue, employing the immunogold technique. Pan-cadherin, vinculin and α-actinin give a strong signal at the cytoplasmic aspect of these AJs, and the anti-α-actinin antibody also reacts with the Z-bands within the tissue. Anti-pan-cadherin antibody recognises an epitope about 15-20 nm away from the plasma membrane, deduced from the measurement of over 1000 gold particles, probably binding to the cytoplasmic tail of A-CAM (N-cadherin). Anti-A-CAM antibody recognises an extracellular epitope close to the cell surface. However, the avidity of this antibody is considerably lower than that of anti-pan-cadherin. Anti-zyxin antibody reacts weakly with the cytoplasmic aspect of the junctions, confirming reports about its low concentration in this tissue (Beckerle, 1986).

Negative controls were conducted by exchange of primary antibody for PBS. Blood-brain barrier microvessels were identified by use of an antibody purchased from Affinity (UK), which is specific for the rat blood-brain barrier.

RESULTS

Light microscopy

Employing the avidin-biotin complex method, antibodies against α-actinin, vinculin, zyxin, cadherin (anti-pan-cadherin antibody) and A-CAM (N-cadherin) were tested on frozen sections of adult rat cerebral cortex. Reaction product was clearly seen at the level of the microvessel walls with all antibodies (Figs 1A-C, 2A). The immunolabel after staining with antibodies against α-actinin (Fig. 1A), zyxin (Fig. 1B), cadherin (not shown) and A-CAM (Fig. 2A) is largely limited to the vessels. However, a fairly high level is also found in the surrounding tissue in sections which had been incubated with anti-vinculin antibody (Fig. 1C). No reaction product was seen in negative controls (Fig. 1D).

Testing the same antibodies on brain sections of 16-, 8- and 1-day-old rats revealed that microvessel walls reacted positively at all ages investigated. Fig. 2 shows this for anti-A-CAM antibody. Although the immunolabel appeared to be weaker in younger animals for some antibodies, it was not possible to quantify reliably these potential differences in intensity.

Direct immunofluorescence performed to visualise actin filaments stained large sections of all microvessel walls in adult animals (Fig. 3). F-actin is also present at D16, D8 and D1 (not shown).

Indirect immunofluorescence by use of antibodies against α-actinin, vinculin and zyxin resulted in a punctate staining pattern at the level of the microvessel wall, showing a high frequency of "double dots" (Figs 4, 5, 7, 8). The staining pattern obtained in microvessels which had been incubated with antibodies against cadherin (anti-pan-cadherin antibody) or against A-CAM (N-cadherin) was also punctate (Figs 6, 8). However, "double dots" were very rarely observed and some reaction product seemed to be more diffusely distributed along the vessel wall (Fig. 6).

Double-labelling experiments revealed that α-actinin and vinculin co-localise (Fig. 7). Immunolabel for vinculin and cadherin, as well as for α-actinin and A-CAM (Fig. 8), is also largely restricted to the same points within the vessel wall. However, owing to the different avidity of the antibodies used and the limited resolution of this method, lower quantities of antigen might not be detected. Therefore, small levels of non-overlapping signal may have escaped detection.

Immunogold labelling

Fig. 9 shows part of a capillary profile of a negative immunogold control experiment. The section is devoid of any traces of labelled, secondary antibody. The interendothelial cleft is characterised by its associated cytoplasmic plaque which appears darker than the remaining cytoplasm and can be seen along the entire cleft. Interendothelial clefts of blood-brain barrier capillaries are tighter and more tortuous compared to those of peripheral microvessels. As a result, the apposing membranes can only be identified in a fraction of the cleft, where they are orientated parallel to the electron beam and therefore appear as two distinct lines. Under our experimental conditions, the endothelium is usually well preserved, with only minor damage due to ice crystal formation. However, ice crystal damage can be considerable further within the tissue.

At the ultrastructural level, α-actinin is located within the cytoplasm along both sides of the entire interendothelial junction (Fig. 10). The distance between antigen and plasma membrane is variable. Both vinculin and zyxin are also located to the cytoplasmic aspect of the junction, mostly to the junction-associated cytoplasmic plaque (Figs 11, 12). However, the intensity of the signals obtained with these antibodies is generally lower. Zyxin is found in small patches along the entire junction. Although Fig. 11 gives the impression that vinculin was restricted to the middle section of the junction, gold particles are occasionally found further towards the luminal and abluminal ends as well.

Incubation with anti-pan-cadherin antibody results in an immunogold signal at the cytoplasmic aspect throughout the entire length of the cleft (Figs 13, 14, 15). Gold particles can be seen in close proximity to tight junctional contacts (Fig. 13) and close to the luminal opening of the cleft (Fig. 15). In addition, label is present at the abluminal wide zone (Fig. 14).

The signal obtained by incubation with anti-A-CAM antibody is generally fairly weak. One or two gold particles are frequently found in interendothelial junctions closely associated with the membranes (Fig. 16A). In addition, some antigen is found associated with the luminal surface or the abluminal plasma membrane, often in areas of cell-cell contact (Fig. 16B).

DISCUSSION

In this study we have used three different immunohistochemical methods to characterise the presence and localization of some AJ components within blood-brain barrier microvessels. The ABC method was employed because of its high sensitivity. It revealed that α-actinin, vinculin, zyxin, cadherin and A-CAM (N-cadherin) are present in microvessel walls of the rat cerebral cortex at as early as

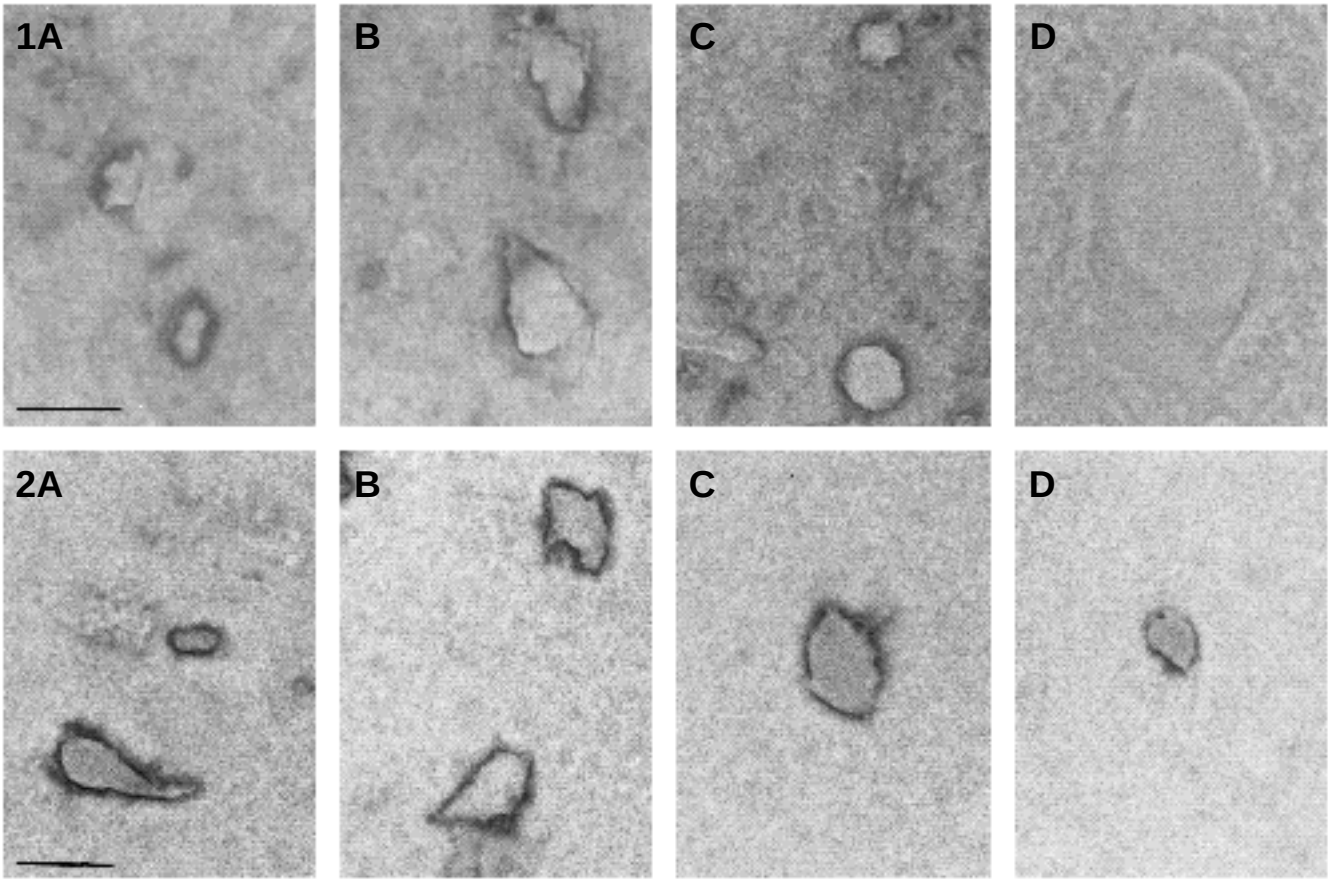


Fig. 1. Immunolabeling of cerebral cortex of adult rats employing the avidin-biotin complex method: α-actinin (A), zyxin (B), vinculin (C), negative control (D). The microvessel walls react positively for all antibodies. Note the high level of vinculin immunolabel in the tissue surrounding the microvessel (C). Bar, 10 μm.

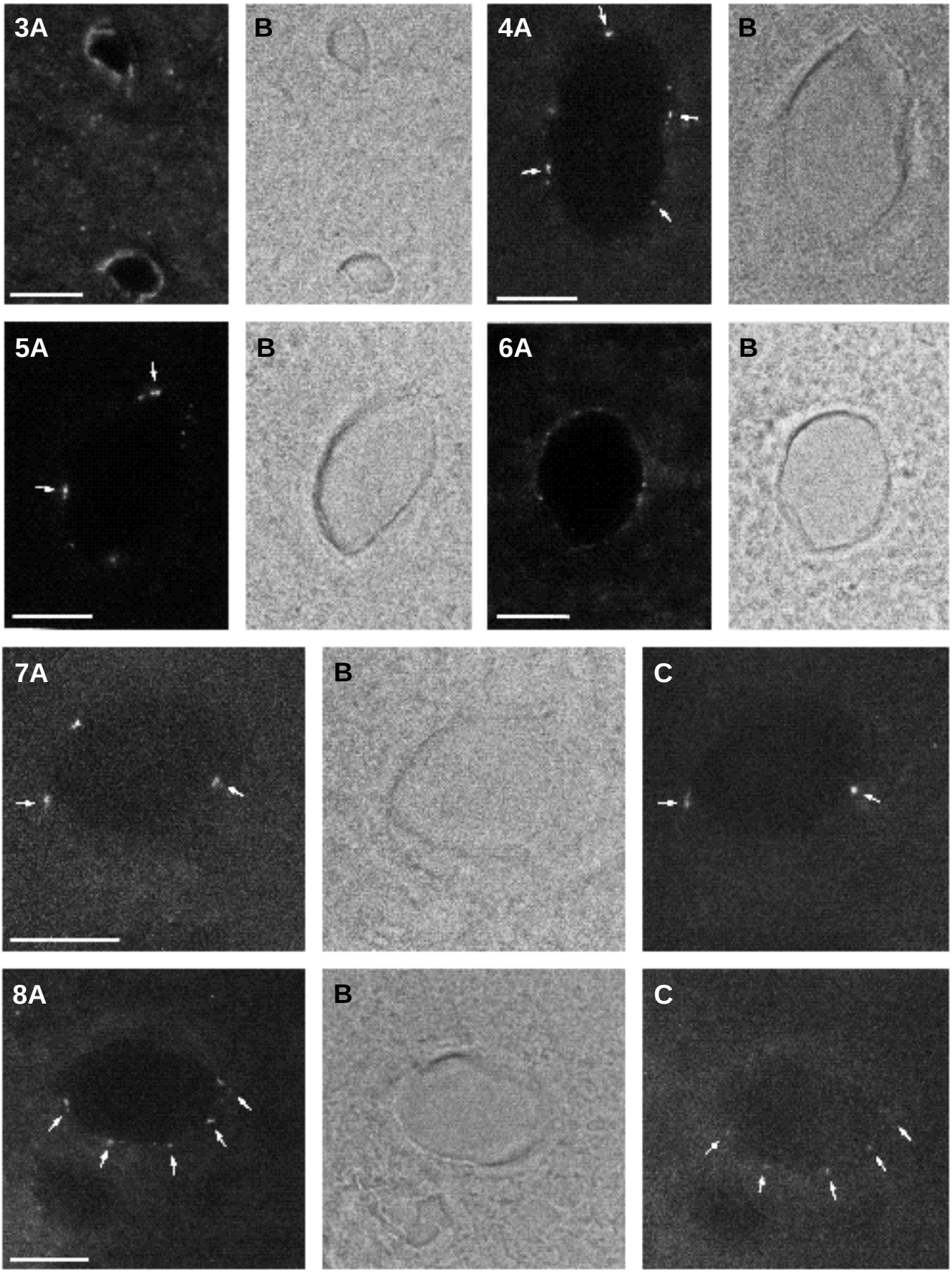
Fig. 2. Immunolabeling of rat cerebral cortex at different ages of the animals with anti-A-CAM antibody using the avidin-biotin complex method: adult animal (A), day-16 (B), day-8 (C), day-1 (D). Microvessel walls react positively at all stages. Bar, 10 μm.

one day of age. Using the immunofluorescence technique on thin cryostat sections allowed antigen localization at the subcellular level. The punctate staining pattern obtained with all antibodies listed above demonstrates that the antigens are enriched in small areas within the vessel wall. The immunogold technique revealed that they are associated with interendothelial clefts.

Cytoplasmic components of adherens junctions

Immunofluorescence staining of vinculin and α-actinin, markers for AJs (Otto, 1990; Blanchard et al., 1989), show a high frequency of "double dots" which are orientated parallel to the luminal surface of the vessels. The microvessels represented in Figs 4-8 are all small venules and arterioles, as deduced from their diameter, in which the interendothelial junctions are usually orientated perpendicular to the luminal surface (unpublished electron microscopical observation). The observed staining pattern of two adjacent dots is therefore consistent with the idea that vinculin and α-actinin are present within the cytoplasmic aspects of interendothelial junctions, at some distance from the cell surface in both cells. This is supported by the evidence provided by the immunogold experiments. However, these experiments were conducted on true capillaries with a diameter of about 5 μm, an attenuated endothelium and intercellular junctions which are characterised by a long overlap of the adjacent cells.

Figs 3-8. Immunofluorescence localization of adherens junction components in rat cerebral cortex. Bars, 10 μm. Fig. 3. F-actin. Almost the entire vessel wall reacts positively for filamentous actin (A). Phase contrast (B). Fig. 4. Vinculin. Staining with this antibody results in a punctate pattern at the level of the vessel wall with a high frequency of "double dots" (A, arrows). Phase contrast (B). Fig. 5. Zyxin. Staining with this antibody also yields a punctate staining pattern at the level of the vessel wall, frequently occuring as "double dots" (A, arrows). Phase contrast (B). Fig. 6. Cadherin (by use of anti-pan-cadherin antibody). The resulting staining pattern is mostly punctate at the level of the vessel wall and possibly diffuse in some areas (A). Phase contrast (B). Fig. 7. Co-localization of α-actinin (A) and vinculin (C). Both antigens co-localise (arrows) at the level of the vessel wall. The unmarked signal in (A) is also present in (C), although in a different focal plane. Note that α-actinin labeling shows a similar "double dot" pattern as that seen for vinculin and zyxin (A). Phase contrast (B). Fig. 8. Co-localization of α-actinin (A) and A-CAM (C). The staining pattern for both components at the level of the vessel wall is punctate and largely overlaps (arrows). Phase contrast (B).


3A
B
4A
B
5A
B
6A
B
7A
B
C
8A
B
C

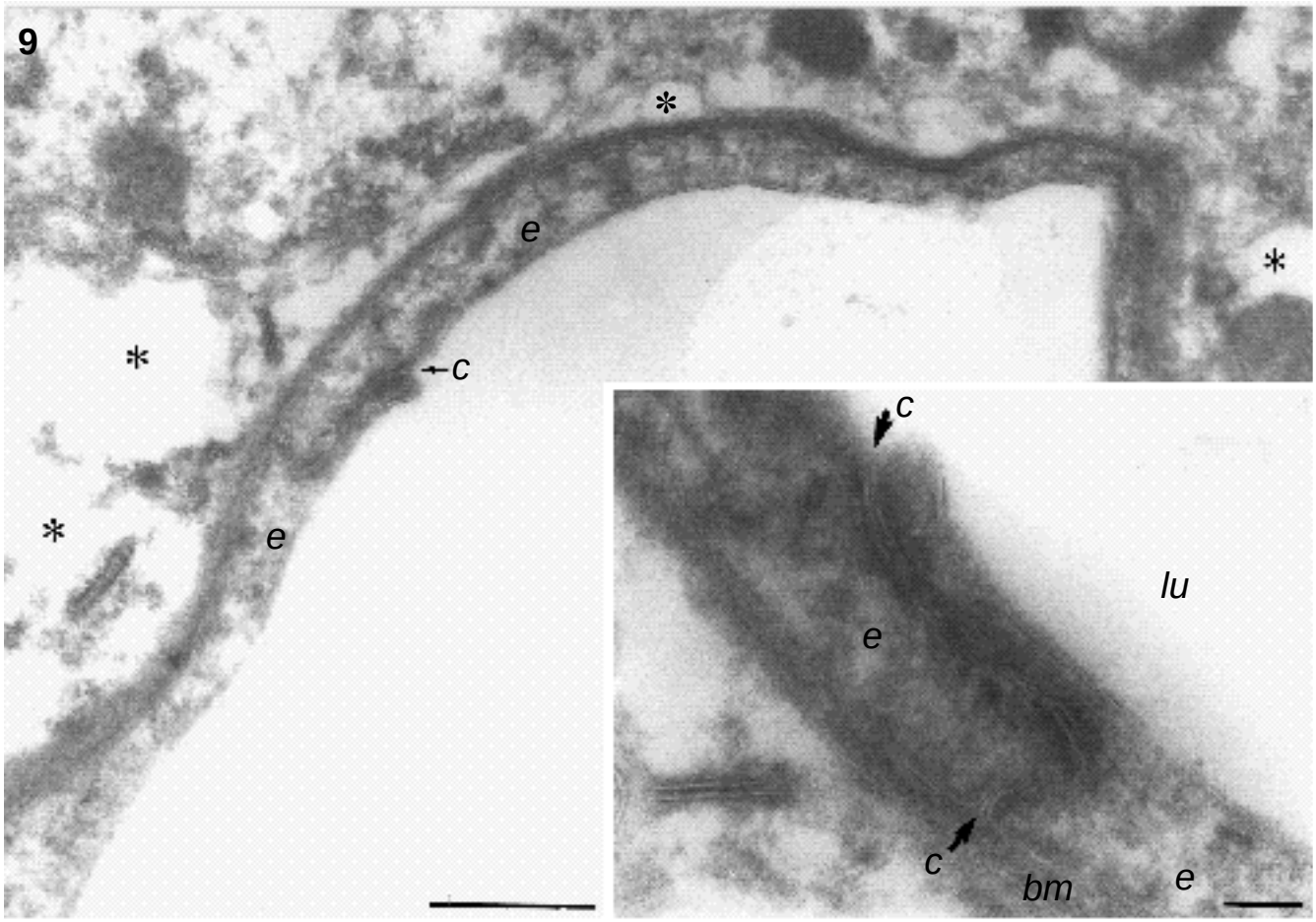


Fig. 9. Negative control of an immunogold experiment in which the primary antibody has been exchanged for buffer. No gold particles are found. The preservation of the endothelium is generally satisfactory. Further within the tissue, tissue damage due to ice crystal formation is considerable (*). The interendothelial cleft (inset) is characterised by its associated, electron-dense cytoplasmic plaque and the fact that the opposing cell membranes can only be seen in a fraction of the cleft. lu, capillary lumen; c, interendothelial cleft; e, endothelium; bm, basement membrane; Bar, 0.5 μm; bar (inset), 100 nm.

Zyxin, an 82 kDa component of AJs, is distributed in the same fashion as α-actinin and vinculin, showing for the first time that this molecule is not only present in focal adhesions and epithelial AJs (Beckerle, 1986; Crawford and Beckerle, 1991), but in endothelial AJs as well. Zyxin has recently been shown to interact with α-actinin in vitro (Crawford et al., 1992). The localization of both proteins at the ultrastructural level to the cytoplasmic aspects of the junctions is consistent with the possibility of such an interaction taking place in vivo. Zyxin was found to be an AJ component of low abundance, with especially low levels in brain tissue (Beckerle, 1986). Our results support this finding.

We did not find any convincing evidence (such as immunogold staining for vinculin, α-actinin or zyxin) for the existence of basal adhesions in the microvessels investigated. However, such cell-ECM AJs might be fairly rare, or the antigen concentration so low, that they have escaped detection in immunogold experiments.

In cultured endothelial cell monolayers and in en face preparations of endothelial cells in situ, filamentous actin has been shown to be mostly organised in dense peripheral bands underlying interendothelial junctions (Gotlieb, 1990; Rogers and Kalnins, 1983). Both tight junctions and AJs are believed to be linked to the microfilament bundles (Schneeberger and Lynch, 1992; Geiger and Ginsberg, 1991). We have demonstrated in this study that filamentous actin is present in microvessels throughout postnatal development. However, the method employed here did not allow us to characterise the specific organization of actin filaments at the subcellular level.

Cadherins

Cadherin, as detected by anti-pan-cadherin antibody, and A-CAM (N-cadherin) both largely co-localise with AJ-markers vinculin and α-actinin in immunofluorescence experiments. This is consistent with the concept of the existence of an epithelial-like intercellular AJ in endothelial cells (Franke et al., 1988), and suggests that A-CAM, and possibly other cadherins, are intramembranous components of this AJ. A-CAM, however, does not seem to be restricted to interendothelial junctions, confirming similar observations made in cultured endothelial cells by use of the same antibody (Salomon et al., 1992). We find some A-CAM

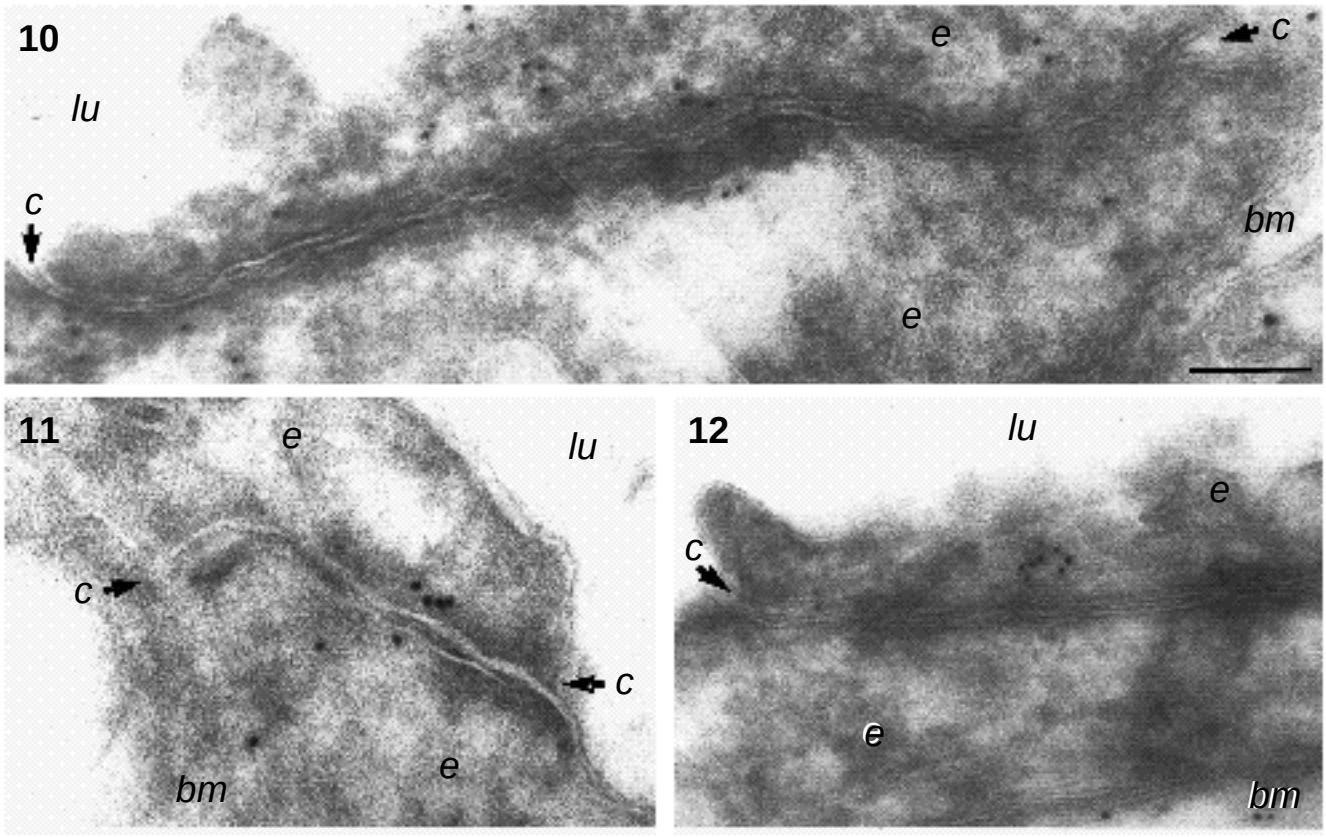


Fig. 10. Immunogold labelling of α-actinin. 5 nm gold particles are found in the cytoplasmic aspects of the interendothelial cleft. The distance between gold particles and the membrane is variable. e, endothelium; lu, lumen; bm, basement membrane; c, interendothelial cleft; bar, 100 nm.

Fig. 11. Immunogold labelling of vinculin. 10 nm gold particles are localised to the cytoplasmic plaque adjacent to the cell-cell contact. e, endothelium; lu, lumen; bm, basement membrane; c, interendothelial cleft; bar, 100 nm.

Fig. 12. Immunogold labelling of zyxin. This micrograph shows the luminal quarter of an exceptionally long interendothelial cleft. 5 nm gold particles are found in infrequent patches in the cytoplasmic aspects of the junction. e, endothelium; c, interendothelial cleft; lu, lumen, bar, 100 nm.

immunoreactivity diffusely distributed throughout the endothelial surface in areas with no apparent cell-cell interaction, and occasionally in places where another cell, possibly a pericyte, is in close contact to the endothelium. Peri-endothelial junctions occur frequently in some tissues, bearing a strong morphological resemblance to interendothelial junctions (Schulze and Firth, 1992a,b). It seems likely that the molecular organizations of both junction types may be similar as well. We are currently studying peri-endothelial relationships in the brain.

The endothelial AJ is believed to differ in depth between subtypes of endothelia, forming either a single belt or a more complex zonular system. However, its topological and functional relationship to the adjacent zonula occludens (tight junction) is not known (Franke et al., 1988).

In thin sections of blood-brain barrier interendothelial clefts, the complex tight junction appears as a succession of individual tight-junctional contacts. We have shown elsewhere (Schulze and Firth, 1992c) that on average the luminal two thirds of the cleft are occupied by this complex tight junction, composed of numerous tight-junctional contacts interspersed with areas in which the membranes are about 15-20 nm apart. The remaining luminal wide zone shows a uniform width also in the order of 15-20 nm. Our immunogold results clearly show that cadherin immunoreactivity is present throughout the entire length of the junction. The associated cytoplasmic components vinculin, α-actinin and zyxin also appear to be broadly distributed along the junction. This could mean that in this type of endothelium the AJ is organised along the entire area of interendothelial cell contact and that a complex network of anastomosing tight junctional strands is embedded within this AJ. Alternatively, different members of the cadherin family might be present, one being specifically associated with the tight junctional domain and another one representing the intramembranous component of an AJ within the abluminal wide zone of the cleft.

Recently, E-cadherin-like immunoreactivity has been localised to interendothelial junctions in cultured brain endothelial cells, and is believed not to be present in non-blood-brain barrier endothelial cells (Rubin et al., 1991a,b). E-cadherin has been shown to play a role in the formation and maintenance of epithelial tight junctions (Gumbiner and Simons, 1987) and as an inducer of cell surface polarity

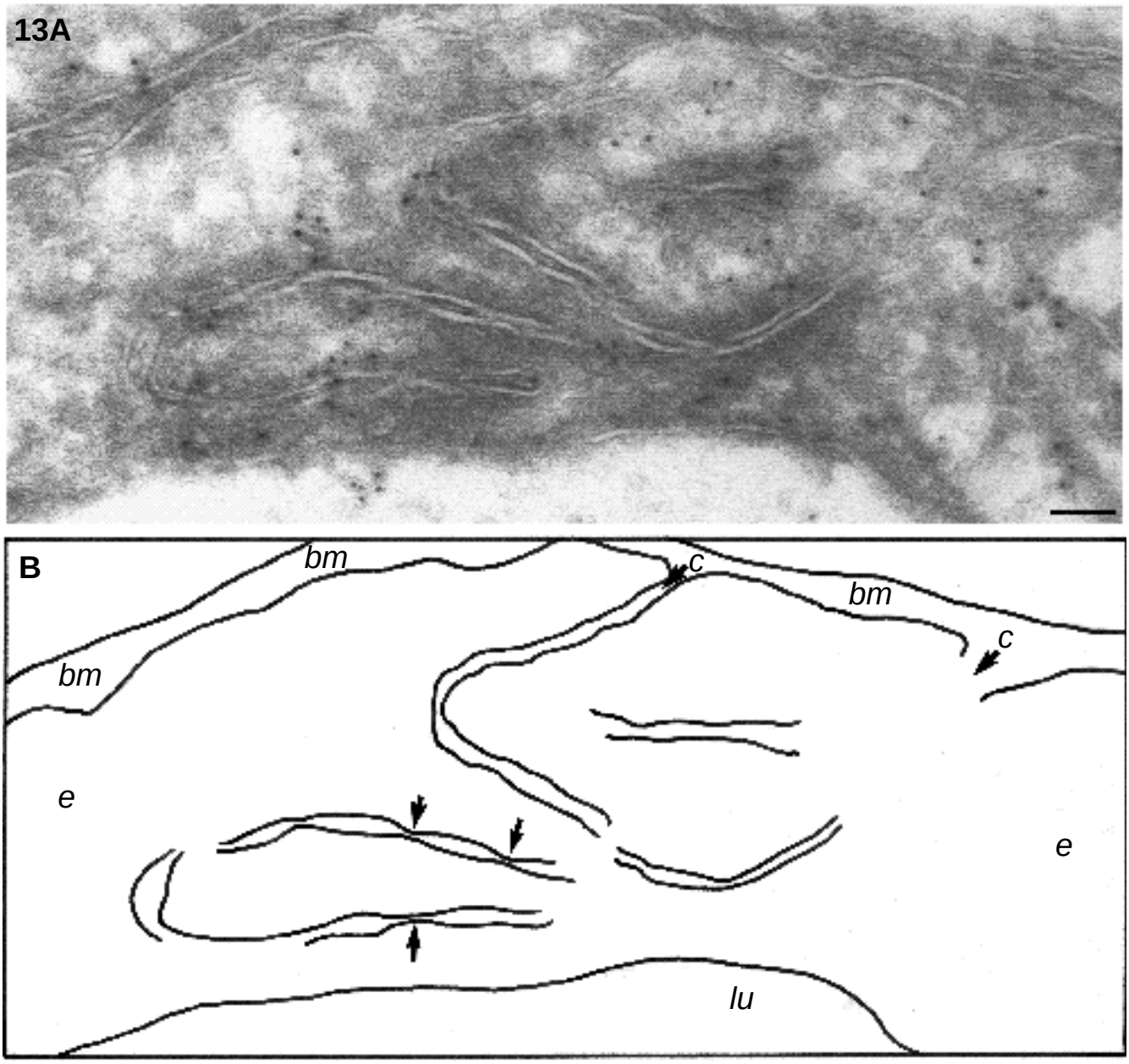
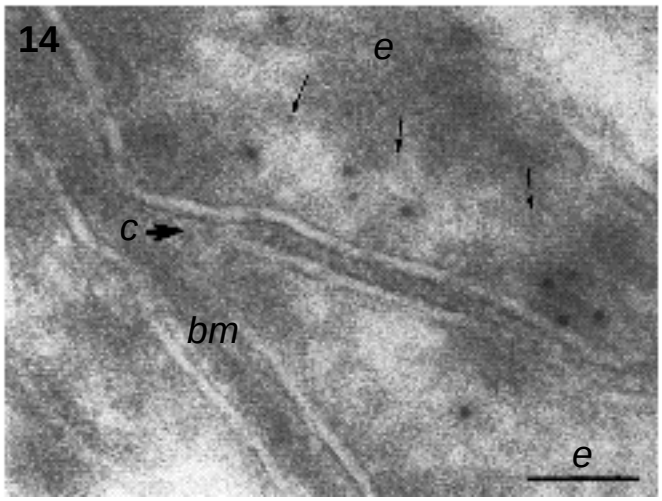


Fig. 13. Immunogold localization of cadherin by use of anti-pan-cadherin antibody. A tortuous interendothelial cleft is seen (A) the course of which has been outlined in (B). Owing to the cutting angle or the oblique orientation of parts of the membranes, the course of the cleft cannot be followed throughout. Numerous 5 nm gold particles are found within the cytoplasmic plaque at 0-40 nm distance from the membranes. They are also found in close proximity to tight junctional contacts (arrows). e, endothelium; c, abluminal openings of the interendothelial cleft, bm, basement membrane; bar, 50 nm.

(McNeill et al., 1990). The close structural relationship of cadherin molecules and tight junctions demonstrated in the present study would support the notion of a similar function for this cadherin in blood-brain barrier microvessels.

Although we know from several light microscopical studies that cadherin immunoreactivity is concentrated along endothelial cell-cell contacts (Heimark et al., 1990; Rubin et al., 1991a,b; Salomon et al., 1992; Lampugnani et al., 1992), little information is available as to which members of this group of cell adhesion molecules are present at this site and nothing has been published about their precise ultrastructural localisation, in particular in relation to tight junctions. Increasing evidence suggests that endothelial cells express several different cadherins (Liaw et al., 1990; Rubin et al., 1991a,b; Salomon et al., 1992) and first experimental results suggest a differential distribution of cadherins on the surface of endothelial cells (Salomon et al., 1992). The availability of specific antibodies against individual members of the cadherin family in the future should enable us to investigate their ultrastructural distribution in an attempt to understand their cellular functions.



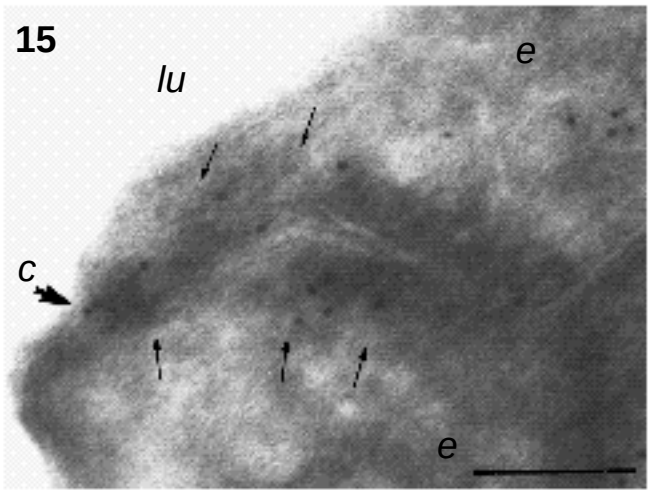
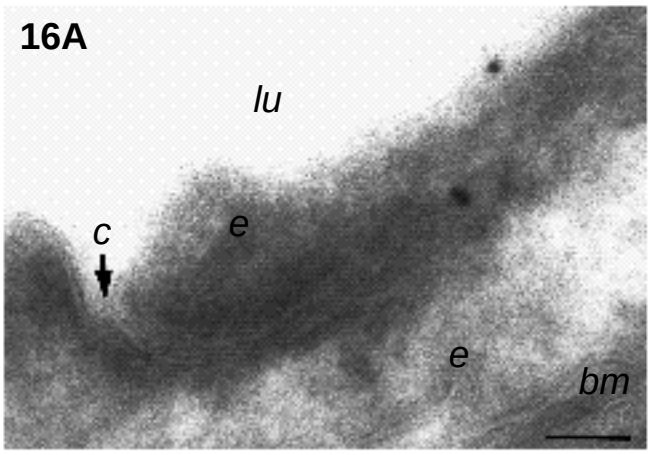


Fig. 14. Immunogold localization of cadherin to the abluminal wide zone of an interendothelial cleft. 5 nm gold particles (arrows) are found within a small distance to the opposing cell membranes. e, endothelium; c, interendothelial cleft; bm, basement membrane; bar, 50 nm.

Fig. 15. Immunogold localization of cadherin to the luminal aspect of an interendothelial cleft. 5 nm gold particles are found within the cytoplasmic plaque right up to the luminal opening of the cleft (arrows). e, endothelium; lu, lumen; c, interendothelial cleft; bar, 100 nm.



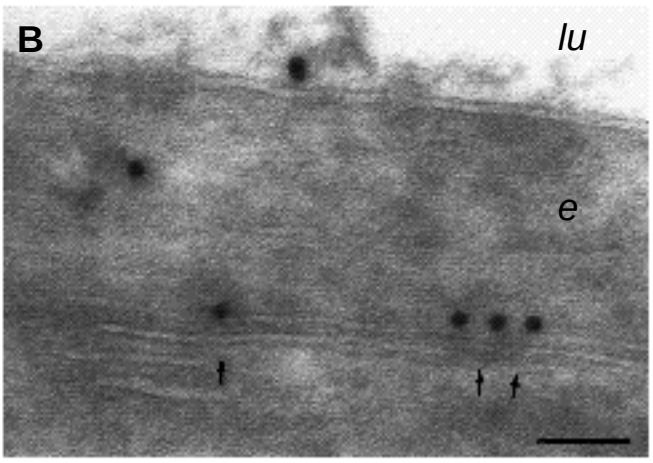


Fig. 16. Immunogold localization of A-CAM. 10 nm gold particles are occasionally found associated with the membranes of interendothelial junctions (A), on the luminal surface (A and B), and associated with the abluminal plasma membrane (B, arrows) in what might be intercellular contacts between the endothelium and underlying pericytes. Bars, 100 nm (A); 50 nm (B).

We are grateful to Dr A. Crawford and Dr T. Volberg for their gifts of antibodies. This study was funded by the British Heart Foundation.

REFERENCES

Albelda, S. M., Oliver, P. D., Romer, L. H. and Buck, C. A. (1990). EndoCAM: A novel endothelial cell-cell adhesion molecule. *J. Cell Biol.* **110**, 1227-1237.

Beckerle, M. C. (1986). Identification of a new protein localized at sites of cell-substrate adhesion. *J. Cell Biol.* **103**, 1679-1687.

Blanchard, A., Ohanian, V. and Critchley, D. (1989). The structure and function of alpha-actinin. *J. Muscle Res. Cell Motil.* **10**, 280-289.

Crawford, A. W. and Beckerle, M. C. (1991). Purification and characterization of zyxin, an 82,000-dalton component of adherens junctions. *J. Biol. Chem.* **266**, 5847-5853.

Crawford, A. W., Michelsen, J. W. and Beckerle, M. C. (1992). An interaction between zyxin and alpha-actinin. *J. Cell Biol.* **116**, 1381-1393.

Critchley, D. R., Gilmore, A., Hemmings, L., Jackson, P., McGregor, A., Ohanian, V., Patel, B., Waites, G. and Wood, C. (1991). Cytoskeletal proteins in adherens-type cell-matrix junctions. *Biochem. Soc. Trans.* **19**, 1028-1033.

Franke, W. W., Cowin, P., Grund, C., Kuhn, C. and Kapprell, H.-P. (1988). The endothelial junction. The plaque and its components. In *Endothelial Cell Biology in Health and Disease* (ed. N. Simionescu and M. Simionescu), pp. 147-166. New York, London: Plenum Press.

Geiger, B. and Ginsberg, D. (1991). The cytoplasmic domain of adherens-type junctions. *Cell Motil. Cytoskel.* **20**, 1-6.

Geiger, B., Ginsberg, D., Salomon, D. and Volberg, T. (1990a). The molecular basis for the assembly and modulation of adherens-type junctions. *Cell Differ. Dev.* **32**, 343-354.

Geiger, B., Volberg, T., Ginsberg, D., Bitzur, S., Sabanay, I. and Hynes, R. O. (1990b). Broad spectrum pan-cadherin antibodies, reactive with the C-terminal 24 amino acid residues of N-cadherin. *J. Cell Sci.* **97**, 607-614.

Gotlieb, A. I. (1990). The endothelial cytoskeleton: organization in normal and regenerating endothelium. *Toxicol. Pathol.* **18**, 603-617.

Gumbiner, B. and Simons, K. (1987). The role of uvomorulin in the formation of epithelial occluding junctions. *Ciba. Found. Symp.* **125**, 168-186.

Heimark, R. L., Degner, M. and Schwartz, S. M. (1990). Identification of a Ca^{2+}-dependent cell-cell adhesion molecule in endothelial cells. *J. Cell Biol.* **110**, 1745-1756.

Kabsch, W. and Vandekerckhove, J. (1992). Structure and function of actin. *Annu. Rev. Biophys. Biophys. Chem.* **21**, 49-76.

Lampugnani, M. G., Resnati, M., Dejana, E. and Marchisio, P. C. (1991). The role of integrins in the maintenance of endothelial monolayer integrity. *J. Cell Biol.* **112**, 479-490.

Lampugnani, M. G., Resnati, M., Raiteri, M., Pigott, R., Pisacane, A., Houen, G., Ruco, L. P. and Dejana, E. (1992). A novel endothelial-specific membrane protein is a marker of cell-cell contacts. *J. Cell Biol.* **118**, 1511-1522.

Liaw, C. W., Cannon, C., Power, M. D., Kiboneka, P. K. and Rubin, L. L. (1990). Identification and cloning of two species of cadherins in bovine endothelial cells. *EMBO J.* **9**, 2701-2708.

Magee, A. I. and Buxton, R. S. (1991). Transmembrane molecular assemblies regulated by the greater cadherin family. *Curr. Opin. Cell Biol.* **3**, 854-861.

McNeill, H., Ozawa, M., Kemler, R. and Nelson, W. J. (1990). Novel function of the cell adhesion molecule uvomorulin as an inducer of cell surface polarity. *Cell* **62**, 309-316.

Newman, P. J., Berndt, M. C., Gorski, J., White, G. C., Lyman, S., Paddock, C. and Muller, W. A. (1990). PECAM-1 (CD31) cloning and relation to adhesion molecules of the immunoglobulin gene superfamily. *Science* **247**, 1219-1222.

Otto, J. J. (1990). Vinculin. *Cell Motil. Cytoskel.* **16**, 1-6.

Rogers, K. A. and Kalnins, V. I. (1983). A method for examining the endothelial cytoskeleton in situ using immunofluorescence. *J. Histochem. Cytochem.* **31**, 1317-1320.

Rubin, L. L., Hall, D. E., Porter, S., Barbu, K., Cannon, C., Horner, H. C., Janatpour, M., Liaw, C. W., Manning, K., Morales, J., Tanner, L. I., Tomaselli, K. F. and Bard, F. (1991a). A cell culture model of the blood-brain barrier. *J. Cell Biol.* **115**, 1725-1735.

Rubin, L. L., Barbu, K., Bard, F., Cannon, C., Hall, D. E., Horner, H., Janatpour, M., Liaw, C., Manning, K., Morales, J., Porter, S., Tanner, L., Tomaselli, K. and Yednock, T. (1991b). Differentiation of brain endothelial cells in cell culture. *Ann. NY Acad. Sci.* **633**, 420-425.

Salomon, D., Ayalon, O., Patel-King, R., Hynes, R. O. and Geiger, B. (1992). Extrajunctional distribution of N-cadherin in cultured human endothelial cells. *J. Cell Sci.* **102**, 7-17.

Schneeberger, E. E. and Lynch, R. D. (1992). Structure, function, and regulation of cellular tight junctions. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **262**, L647-L661.

Schulze, C. and Firth, J. A. (1992a). Junctions between pericytes and the endothelium in rat myocardial capillaries: A morphometric and immunogold study. *Cell Tiss. Res.* (in press).

Schulze, C. and Firth, J. A. (1992b). The interendothelial junction in myocardial capillaries: evidence for the existence of regularly spaced, cleft- spanning structures. *J. Cell Sci.* **101**, 647-655.

Schulze, C. and Firth, J. A. (1992c). Interendothelial junctions during blood-brain barrier development in the rat: morphological changes at the level of individual tight junctional contacts. *Dev. Brain Res.* **69**, 85-95.

Tsukita, S. and Nagafuchi, A. (1990). The undercoat of adherens junctions: a key specialized structure in organogenesis and carcinogenesis. *Cell Struct. Funct.* **15**, 7-12.

Turner, C. E., and Burridge, K. (1991). Transmembrane molecular assemblies in cell-extracellular matrix interactions. *Curr. Opin. Cell Biol.* **3**, 849-853.

(Received 23 October 1992 - Accepted 20 November 1992)