Characterization of the ZO-1 protein in endothelial and other cell lines

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Summary

A high molecular weight tight junction-associated protein, ZO-1, has been demonstrated in liver (hepatocytes) and in both epithelium and endothelium. We carried out studies to examine the presence of the protein in vascular endothelial cell cultures and several other types of cultured cells, and the relationship between the ZO-1 protein content and confluency of endothelial cell monlayers. Immunofluorescence labelling of endothelial monolayers and two types of epithelial monolayers, IEC-6 and MDCK, with monoclonal antibody against ZO-1 protein localized the protein to the cell peripheries. Its association with the cell periphery only occurred when cells had contact with one another as demonstrated in endothelial cells. We have been able to show a positive correlation between the ZO-1 content of the cells and the extent of monolayer confluency in the endothelial cells by immunoblotting. The protein is much less expressed in nonconfluent endothelial cell monolayers and totally absent from mouse myeloma cultures. The presence and confluence-related expression of the protein in endothelium give support to the hypothesis that tight junctions exist in confluent endothelial cells and that the ZO-1 protein is expressed under the conditions where tight junction interactions occur.

Key words: ZO-1 protein, endothelial monolayer, confluency, tight junction.

Introduction

The *zonula occludens* (ZO) or tight junction is apparently reponsible for establishing high-resistance junctions and sealing the intercellular spaces between cells in continuous monolayer both *in vivo* and in tissue culture. It has been shown conclusively to be present in most epithelia and certain types of endothelia, such as brain vascular endothelium (Rutten *et al.* 1987), that can develop a high transcellular electrical resistance in confluent monolayer form. It has been suggested that tight junctions may also be present in low-resistance generating endothelia such as the aortic endothelium (Albelda *et al.* 1988).

There are several hypotheses regarding the composition of the tight junction. One suggests that the junction is formed with the participation of specific proteins (Griepp *et al.* 1983; Staehelin *et al.* 1969). This hypothesis had not been biochemically substantiated until the demonstration of the ZO-1 protein. Stevenson and Goodenough (1984) isolated a detergent-insoluble tight junction-enriched fraction from mouse liver. This fraction was then used to generate a number of monoclonal antibodies that specifically react with a high molecular weight (225×10^3 and 210×10^3, depending on tissue and cell type) tight junction-associated peptide named ZO-1 (Stevenson *et al.* 1986; Anderson *et al.* 1988). The antibodies cross-react with several tissues (and between species) containing tight junctions and are localized at the junctional complex region of a number of epithelia, including colon, kidney and testis, and to arterial endothelium by immunofluorescence staining of sections of whole tissue (Stevenson *et al.* 1986). The antibodies also stained the junctional complex region in a confluent monolayer culture of MDCK epithelial cells (Stevenson *et al.* 1986; Stevenson *et al.* 1988*b*). The ZO-1 protein was optimally soluble in 6 M urea or at high pH, partially soluble in 0.3 M KCl and essentially insoluble in nonionic detergents, suggesting that the protein is a peripherally associated membrane protein. The protein is an asymmetric monomer in purified form and is phosphorylated at serine residues (Anderson *et al.* 1988). For a more detailed review, see Stevenson *et al.* (1988*a*).

It has been demonstrated that the distribution and content of the protein bear no relationship with the extent of transcellular electrical resistance in two different lines of MDCK cells (Stevenson *et al.* 1988*b*). On the other hand, Anderson and co-workers (1989) have shown that when Caco-2 cells in suspension culture were replated to form confluent monolayers there was a dramatic increase in the expression of the protein ZO-1 with growing time. In this study, we attempted to correlate the content of ZO-1 protein with monolayer confluency of bovine aortic endothelial cell cultures and to examine the presence of the protein in some other types of cell cultures available in the laboratory.

Materials and methods

Monoclonal antibody

The monoclonal antibody produced against a canalicular-enriched membrane fraction from mouse liver (anti-ZO-1) was kindly provided by Dr Stevenson from the Department of Anatomy and Cell Biology of the University of Alberta. Detailed methods for its production have been described previously (Stevenson *et al.* 1986). Several versions of anti-ZO-1 have been reported, including R26.4C, R40.40D3 and R40.76. We were

provided with R40.76. The antibody was contained in culture medium RPMI 1640, which was used directly without further purification.

Cell cultures

Most cell cultures were purchased from the American Type Culture Collection, Bethesda, MD, including bovine aortic endothelium, rat intestinal epithelial cell (IEC-6) and MDCK cells. A mouse myeloma cell line was a gift from the Department of Immunology at the University of Alberta. All the cells were grown in minimum essential medium (MEM), with 10% foetal calf serum (FCS) (both from GIBCO) and penicillin–streptomycin (100 i.u. ml^{-1} and 100 $\mu g\ ml^{-1}$, Sigma) added, except for myeloma cells, which were grown in RPMI 1640 (GIBCO) with 10% FCS and without penicillin–streptomycin. The cultures were contained in either 75 cm^2 tissue culture flasks or 500 cm^2 culture trays. In the studies examining the ZO-1 protein as a function of the extent of confluency of endothelial cells, the cells were either seeded at different densities and harvested at the same time, or seeded at the same density and harvested at different times to achieve different degrees of confluency.

Trypsinization (0.04% trypsin in calcium-free Puck's saline with EDTA) was used to collect endothelial, IEC-6 and MDCK (Madin-Darby canine kidney) cells for reseeding. Cells were harvested with a rubber policeman for the experiments.

Immunofluorescence labelling of monolayers

Endothelial, IEC-6 or MDCK cells were grown to confluence in Lab-Tek tissue culture chamber/slides (Nunc Inc.) coated with polylysine (100 $\mu g\ ml^{-1}$, Sigma). The chamber/slides were thoroughly washed with FCS-free MEM before cells were seeded. Confluent cells were fixed for 20 min with fresh 4% paraformaldehyde, made up as follows: 50 ml of a 2-fold concentrated phosphate-buffered solution (192 mM NaOH, 244 mM NaH_2PO_4, pH 7.0–7.2) was added to 40 ml of distilled water and heated to 60°C. A 4 g sample of paraformaldehyde (Sigma) was added with stirring until it was dissolved and then 5 ml of 100 mM $MgCl_2$ was added. The volume was brought to 100 ml, then filtered. Cells were permeabilized with fresh 0.1% Triton X-100 (Fisher) in Dulbecco's phosphate-buffered saline (PBS) for 5 to 10 min. They were washed with the PBS five times both before and after permeabilization. The treated monolayers described above were

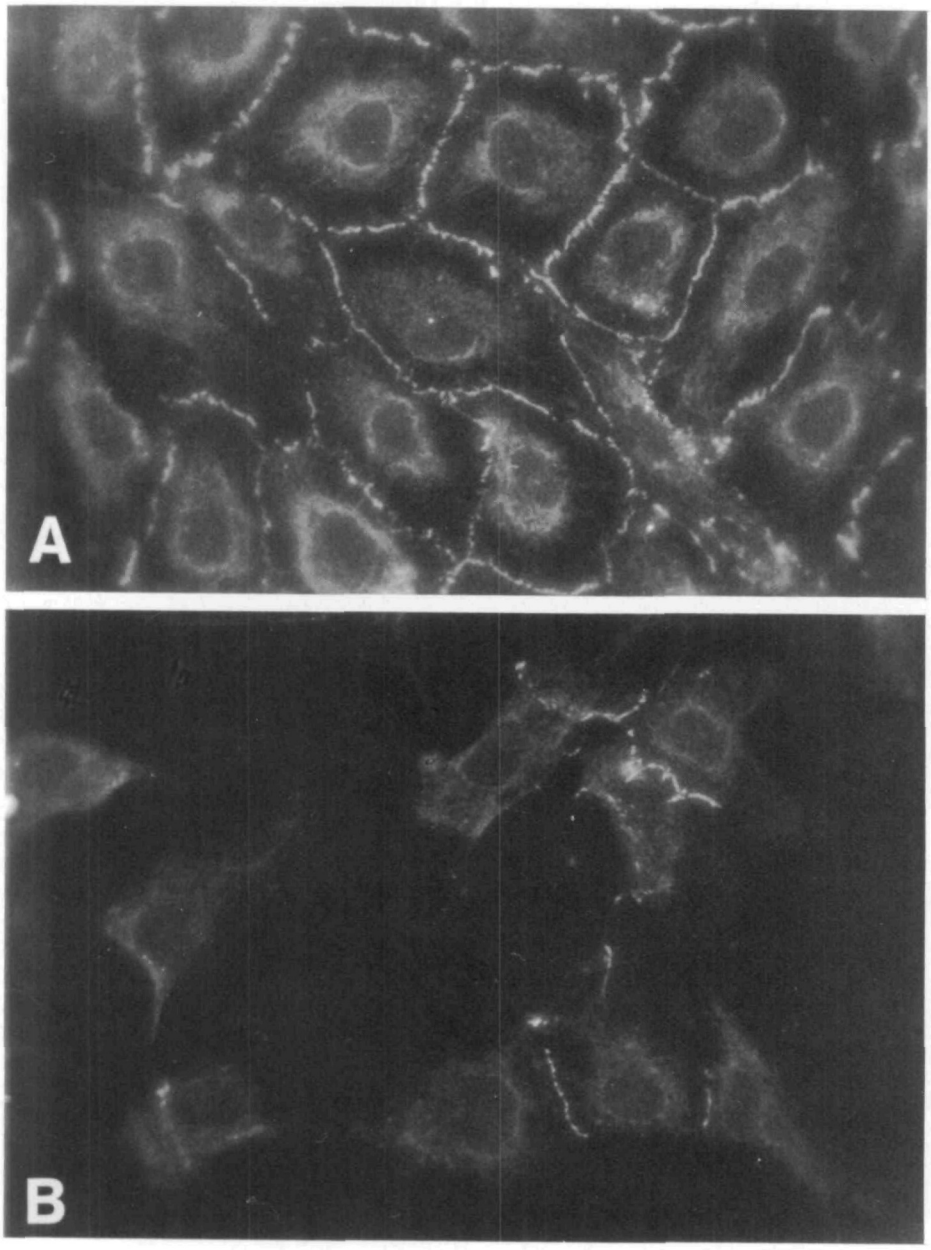


Fig. 1. Indirect immunofluorescence labelling of ZO-1 protein in endothelial monolayers. Confluent (A) and nonconfluent (B) monolayers were prepared by plating the cells at different densities and growing for 1 day before use. Labelling was carried out as described in Materials and methods. Exactly the same experimental and photographic conditions were used for the two monolayer preparations. Cell peripheries were intensively labelled for the protein in the confluent monolayer whereas the peripheries in nonconfluent monolayer lacked labelling, except those in the regions where cell–cell contacts occurred. In the latter case, most of the cell peripheries are difficult to identify on the fluorescence photomicrographs (B) even though the cells as a whole can be seen clearly. Cytoplasmic labelling occurred in both confluent and nonconfluent cells (final magnification, ×2000).

first incubated with medium A (MEM plus 10% FCS) for 20 min. The incubations with antibodies and fluorescent label occurred in the following order: the first incubation using anti-ZO-1 containing RPMI 1640 medium plus 10% FCS was carried out for 2 h. The second incubation used 7.5 $\mu g\,ml^{-1}$ of biotinylated second antibody rabbit anti-rat IgG (Vector) in medium A and was allowed to proceed for an additional 2 h. The third incubation was carried out in the dark with medium A containing 4 $\mu l\,ml^{-1}$ of Texas Red-conjugated streptavidin (Amersham) for 1 h. The cells were washed with MEM plus 5% FCS five times between each of the above incubations. The final wash after streptavidin was with PBS (5 times). All of the above treatments were carried out at room temperature. The plastic chambers were taken off the slides before the latter were mounted. The slides can be stored at 4 °C in the dark for extended periods of time.

SDS–PAGE (SDS–polyacrylamide gel electrophoresis)

The ZO-1 protein was separated by running on 4.5% to 18% gradient SDS–PAGE gels on a Protean II™ slab cell electrophoresis apparatus (Bio-Rad), unless otherwise specified. Cells were harvested with a rubber policeman (for endothelial, IEC-6 and MDCK cells) in PBS or by collecting the culture media directly (for myeloma cells). Cell suspensions were centrifuged at 2000 ***g*** for 15 min to obtain cell pellets. Urea extraction of the ZO-1 protein was done at 4 °C in buffer containing 10 mM Tris–HCl, 1 mM EDTA, 6 M urea and the following protease inhibitors: phenylmethylsulphonyl fluoride (1 mM), aprotinin (5 $\mu g\,ml^{-1}$), leupeptin (4 μM) and trypsin–chymotrypsin inhibitor (20 $\mu g\,ml^{-1}$). All the inhibitors were purchased from Sigma. The pellets in the extraction buffer were first passed through a 22 gauge syringe needle 10 times before sonication with a probe sonicator for 1 min. The syringe passing was repeated. Essentially all the cells were broken by these treatments, as shown by examination by phase-contrast microscopy. The extracts were centrifuged at 90 000 ***g*** for 1 h at 4 °C on a Beckman 55 Ti swinging bucket rotor. The supernatants were transferred to other tubes and mixed with equal volumes of a 2-fold concentrated buffer made of 20% glycerol, 4.6% SDS and 125 mM Tris–base with 100 mM dithiothreitol before loading onto the SDS-containing gel. Protein was measured by the Lowry assay (Lowry *et al.* 1951). SDS–PAGE was also run with whole cells by following the method of Stevenson *et al.* (1986).

Electrical protein transfer and immunoblot

Proteins were transferred from the gels onto nitrocellulose membranes (Trans-blot Transfer Medium, 0.45 μm, Bio-Rad) (Howe and Hershey, 1981) by using a Bio-Rad Trans-blot apparatus in buffer containing 20% methanol, 150 mM glycine and 20 mM Tris–base. The transfer was done at 60 volts, overnight at room temperature. Immunoblotting of the membranes was done at room temperature. After incubation with the anti-ZO-1 containing medium (undiluted), the nitrocellulose membranes were treated with a horseradish peroxidase-conjugated second antibody goat anti-rat IgG (Sigma, used as 1:300 dilution) in Buffer A (50 mM Tris–HCl, 2 mM $CaCl_2$, 100 mM NaCl, pH 8.0). All the above incubation buffers contained 5% fat-free milk powder (Carnation, Inc.). Colour development was with 5-chloro-1-naphthol (Sigma, 0.5 $mg\,ml^{-1}$ in milk-free Buffer A containing 20% methanol and 0.05% H_2O_2).

Results

Immunofluorescence labelling of ZO-1 in endothelial, IEC-6 and MDCK cells

Immunofluorescence labelling of the protein ZO-1 in endothelial cells was conducted on two types of monolayers: confluent and nonconfluent. As shown in Fig. 1 the protein is extensively expressed at the cell peripheries in the confluent monolayer (A). The label outlined each cell clearly. In contrast, the cell peripheries in the nonconfluent monolayer (B) generally lacked label except in the regions where cell–cell contacts occurred. In both types of cell preparations there was weak labelling of the protein in the cytoplasm of the cells, which enabled us to visualize each of them. By comparing the cytoplasmic labelling of the two types of cells, it seems that confluent cells had more ZO-1 not only in the peripheries but also in cytoplasm than nonconfluent cells. The pattern of labelling in endothelial cells is somewhat different from that in epithelial cells as shown in Fig. 2 and by others (Stevenson *et al.* 1988*b*). The junctional regions where shown by the ZO-1 label as an intercalated line in endothelial cells whereas the label in epithelial cells is shown by a straight line. The

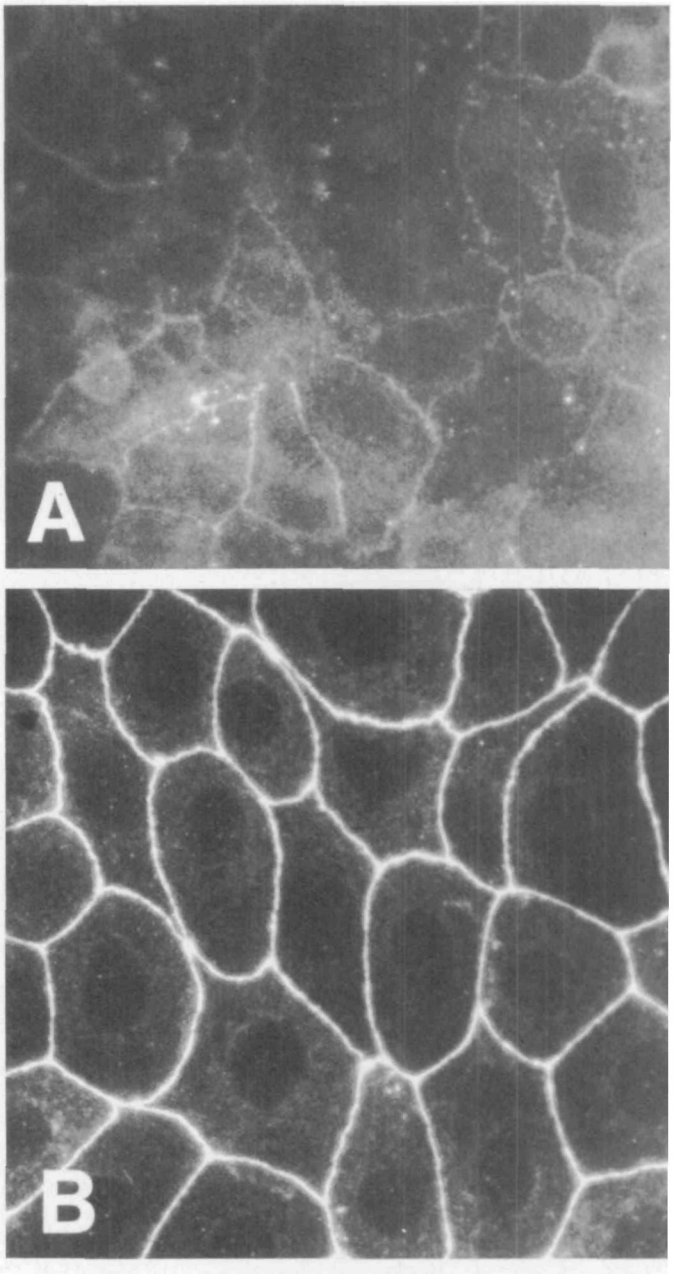


Fig. 2. The same as in Fig. 1, except for confluent IEC-6 and MDCK monolayers. Confluent monolayers were obtained in the same way as described in the legend to Fig. 1. Some of the peripheries in IEC-6 cells (A) were outlined by the ZO-1 labelling. Background fluorescence is also seen in the cell. The labelling in MDCK cells (B) was much stronger than that in both IEC-6 and endothelial cells.

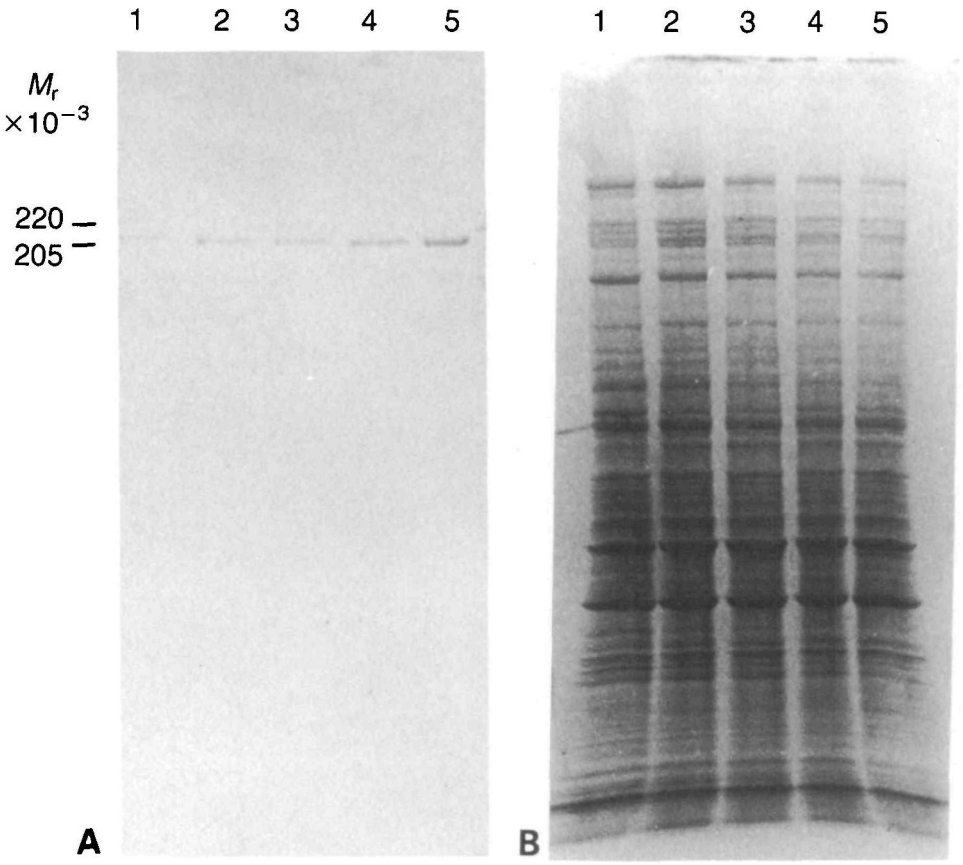
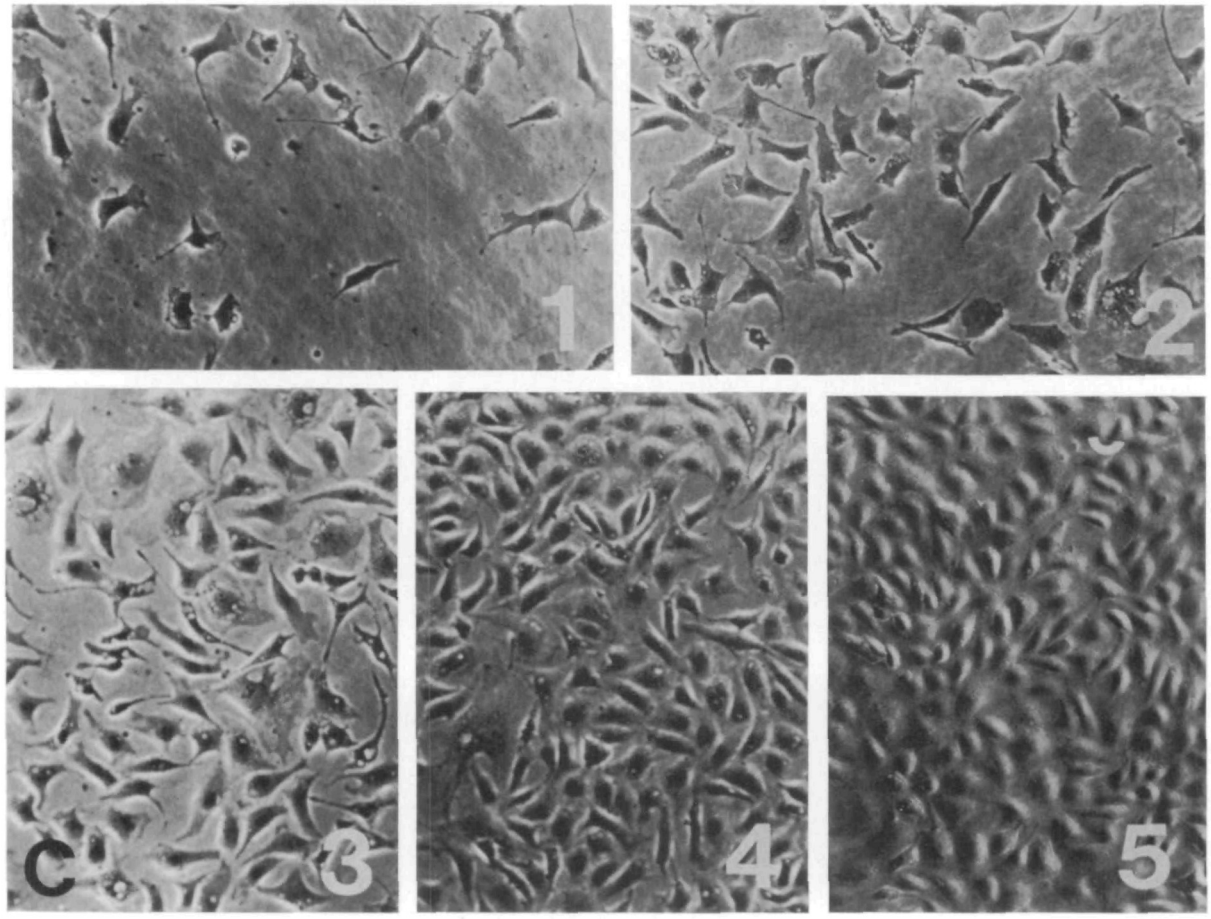


Fig. 3. Endothelial monolayer confluency *versus* the content of the ZO-1 protein shown by immunoblotting. Cells were seeded at about 3% confluence (cells collected from 1 unit of surface area of confluent monolayer were seeded on to about 30 units of surface area) and grown for 3 (lane 1), 4 (lane 2), 5 (lane 3), 6 (lane 4) and 7 (lane 5) days. Numbers in A, B and C correspond. A. Immunoblot result with the ZO-1 protein. B. Coomassie Blue-stained SDS-containing gel. C. Photomicrographs of cells of different confluencies. Urea extracts of the cells were used to run the SDS–PAGE. The same amount of protein was added to each sample. ×93. Phase-contrast.



significance of this difference is not known. The results for IEC-6 cells are shown in Fig. 2A. Cells were outlined by the ZO-1 label. Immunofluorescence studies on MDCK cells have been reported (Stevenson *et al.* 1986; Stevenson *et al.* 1988*a*,*b*). We used in our experiments as a positive control (Fig. 2B). Of these three types of culture, MDCK gave the strongest labelling, IEC-6 the weakest, with endothelial cells in between the two. The order is in good agreement with the immunoblotting results shown in Fig. 5 (below).

Monolayer confluency versus *ZO-1 expression*

Studies on the relationship between ZO-1 expression and monolayer confluency were carried out on endothelial cells, in which our laboratory has an interest in terms of the transcellular transport of large macromolecules. Cell monolayers with different confluencies were achieved either by seeding at the same density and growing for different lengths of time (Fig. 3) or by seeding at different densities and growing for the same period of time (Fig. 4). SDS–PAGE and immunoblotting of protein ZO-1 were carried out by using urea extracts of the cells and whole cells, the former allowing a larger sample load on SDS–PAGE as well as giving sharper bands on immunoblot and the latter better reflecting the protein content of the cells. As seen in Fig. 3A, the expression of ZO-1 protein by immunoblotting is correlated with monolayer confluency, which is shown by phase-contrast photomicrographs of the cells in C. A Coomassie Blue-stained gel is also presented to show the profile of total protein loaded onto the SDS-containing gel (Fig. 3B). Fig. 4 shows the relationship between the ZO-1 expression and the degrees of monolayer confluency, which were achieved by seeding the cells at the same density and growing them for different lengths of time. A similar pattern of confluence-related expression of the ZO-1 protein is observed in both the immunoblot transferred from the SDS–PAGE of urea extracts (A) and the immunoblot transferred from that of the whole cells (B). The amount of sample loaded onto the SDS-containing gel was based on protein for the former and on cell number for the latter. There is no noticeable difference between the two procedures in terms of demonstrating the ZO-1 expression–monolayer confluency relationship. It implies that the possibility of ZO-1 having different urea extractability under different monolayer confluencies is essentially ruled out.

ZO-1 in other types of cells

We examined the presence of the ZO-1 protein in a number of cell cultures available to us. They were two epithelia:

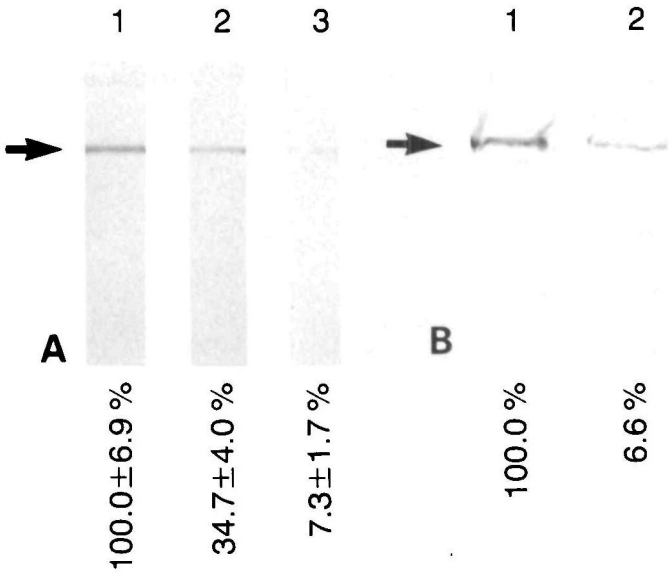


Fig. 4. Endothelial monolayer confluency *versus* the expression of the ZO-1 protein by immunoblotting. Cells were seeded at about 100 % (lane 1 in A and B), 5 % (lane 2 in A) and 1.5 % (lane 3 in A and lane 2 in B) confluence and grown for the same length of time, i.e. 3 days. A shows the immunoblot results from the SDS–PAGE of the urea extracts of the cells, and B shows the results from SDS–PAGE of whole cells. The samples were added to the gels on the basis of protein in A, as in Fig. 3 and Fig. 5, and on the basis of cell number in B (1.8×10^6 cells for each lane). The values below the immunoblots are relative confluence when cell monolayers were used. The monolayer of the largest cell number is designated as having 100 % confluence and the rest are relative to it. In both cases, a clear ZO-1 expression–confluency relationship is observed.

MDCK from canine kidney tubule and IEG-6 from rat intestine; one endothelial line from bovine aorta; and one mouse myeloma cell line. ZO-1 was found to be present in endothelial cells and IEC-6 cells by immunoblotting (Fig. 5, lanes 1 and 2 in A and B), as shown by immunofluorescence labelling of the cells (Fig. 1 and Fig. 2A). MDCK cells also gave a positive immunoblot (Fig. 5, lanes 3 in A and B), which has been reported previously (Stevenson *et al.* 1986; Stevenson *et al.* 1988*a*,*b*; Anderson *et al.* 1988). Fig. 5B is the result of non-gradient SDS–PAGE, instead of a gradient as used in other immunoblotting experiments, to give a more accurate estimation of molecular weight. The molecular weight of the protein in the three types of cells that gave positive immunoblot results appears similar, at 210×10^3 on both gradient (Fig. 5A) and

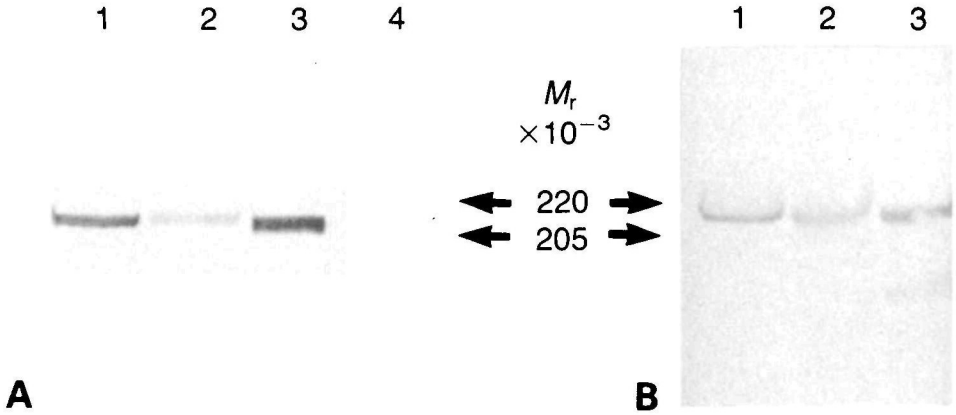


Fig. 5. Immunoblotting of the ZO-1 protein in MDCK (lane 1), IEC-6 (lane 2), endothelial (lane 3) and myeloma (lane 4) cells. Myeloma cells were grown in suspensions, whereas the rest grown in monolayers. Cells were seeded so that they would become confluent for the monolayer-forming cells, and reach steady-state growth for myeloma cells. A. Immunoblot transferred from a gradient (4.5 % to 18 %) SDS–PAGE gel. B. The immunoblot transferred from a nongradient gel (7 %), which gives a more precise estimation of the molecular weight of ZO-1. The same amount of protein was added to each lane of the same gel.

non-gradient gels. On the other hand, mouse myeloma cells (lane 4 in Fig. 5A) gave a negative result. Myeloma cells were grown in suspension and did not develop intercellular tight junctions. The absence of the protein in this cell type could provide evidence for the association of the ZO-1 with tight junctions.

Discussion

The main purpose of these studies was to characterize the ZO-1 protein in vascular endothelial cells, with emphasis on the relationship between the protein content and monolayer confluency. On the basis of previous studies (Stevenson and Goodenough, 1984; Stevenson *et al.* 1986; Anderson *et al.* 1988), it appears that this protein is universal among epithelial cells. This has also been demonstrated using immunofluorescence techniques in arterial endothelium of whole tissue sections (Stevenson *et al.* 1986).

Even though epithelial and endothelial tissues have different embryological origins, they share some common properties: the formation of physiological monolayers, the function as a division between two different environments and the development of transcellular electrical resistance in cultured monolayers (Rutten *et al.* 1987; Albelda *et al.* 1988, for endothelium). The presence of tight junctions between epithelial cells has been well established. There is also a general agreement that brain vascular endothelium, which represents a specialized high transcellular resistance barrier, also develops an intercellular tight junction (Dorovini-Zis *et al.* 1987). However, the presence of tight junctions in other types of endothelia, including that from the bovine aorta, remains controversial. Although the exact role of the ZO-1 protein has not been established, it is clear that the protein is associated with tight junctions. The presence of the ZO-1 in cultured cells and in cells of whole tissue sections (Stevenson *et al.* 1986) provides evidence for the existence of tight junctions in endothelium, as suggested (Poznansky and Juliano, 1984). Failure to demonstrate the presence of the protein in myeloma cells (Fig. 5) seems to make this protein unique to epitheliumn and endothelium. It is reasonable to believe that only tight junction-containing cell types should express the protein whereas cells like myeloma that do not have tight junctions should not. However, we cannot draw a firm conclusion until a variety of non-tight-junction-forming cells have been examined to ensure negative results.

We demonstrated a strong correlation between the amount of the ZO-1 protein and monolayer confluency (Figs 1, 3, 4) in the endothelial cell preparation. A similar result was shown in the human intestinal epithelial line Caco-2 cells (Anderson *et al.* 1989). Cells were first grown in suspension for 48 h in Ca^{2+}/Mg^{2+}-free spinner medium, during which time they lost cell–cell contacts. They were then replated at confluent density in Ca^{2+}/Mg^{2+}-containing medium so that fully confluent monolayers formed shortly. When the expression of the protein ZO-1 was examined against monolayer growing time, there was a steady increase up to two weeks. We found that expression of ZO-1 protein in cell periphery in endothelial cells only occurred in the region where cells had contact with each other (Fig. 1). It seems that the same thing happens in MDCK cells (Siliciano and Goodenough, 1988). The difference in monolayer confluency in our experiments and monolayer growing time in theirs may have the same consequence; altered cell–cell contact. All these results support the notion that cell contact is needed for the expression of the protein.

The molecular weight of ZO-1 in mouse liver and kidney was shown to be 225×10^3 and in MDCK cells to be 210×10^3 (Anderson *et al.* 1988). Our study agrees with the 210×10^3 M_r reported in MDCK cells. We also found that ZO-1 in endothelium and ZO-1 in IEC-6 seem to have the same 210×10^3 value.

The reason for using urea extracts to run SDS–PAGE and immunoblots is that larger amounts of ZO-1 protein could be loaded onto SDS-containing gels, which is especially important for examining the presence of the protein in the myeloma cells. Also it resulted in sharper bands. The cells under different degrees of confluency seemed to show no difference in urea extractability of the protein ZO-1, since on the basis of number of cells the protein showed a similar pattern of confluence-related expression (Fig. 4).

In summary, a few points might be concluded from this study: bovine aortic endothelial cells, representative of low-resistance endothelia, do have ZO-1, indicative of the existence of tight junctions. The content of the ZO-1 protein in endothelial cells is positively related to monolayer confluency. It seems that the ZO-1 protein is unique to epithelium and the equivalent endothelium, while being absent from cells like myeloma that grow in suspension culture.

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