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A MODEL FOR *DE NOVO* SYNTHESIS AND ASSEMBLY OF TIGHT INTERCELLULAR JUNCTIONS. ULTRASTRUCTURAL CORRELATES AND EXPERIMENTAL VERIFICATION OF THE MODEL REVEALED BY FREEZE-FRACTURE

Key words: Tight junctions, freeze-fracture, endothelial cells, intercellular junction assembly, Cytochalasin-D, filipin.

ABSTRACT. The structure and function of intercellular tight (occluding) junctions, which constitute the anatomical basis for highly regulated interfaces between tissue compartments such as the blood–testis and blood–brain barriers, are well known. Details of the synthesis and assembly of tight junctions, however, have been difficult to determine primarily because no model for study of these processes has been recognized. Primary cultures of brain capillary endothelial cells are proposed as a model in which events of the synthesis and assembly of tight junctions can be examined by monitoring morphological features of each step in freeze-fracture replicas of the endothelial cell plasma membrane. Examination of replicas of non-confluent monolayers of endothelial cells reveals the following intramembrane structures proposed as 'markers' for the sequential events of synthesis and assembly of zonulae occludentes: (1) development of surface contours consisting of elongate terraces and furrows (valleys) orientated parallel to the axis of cytoplasmic extensions of spreading endothelial cells, (2) appearance of small circular PF face depressions (or volcano-like protrusions on the EF face) that represent cytoplasmic vesicle–plasma membrane fusion sites, which are positioned in linear arrays along the contour furrows, (3) appearance of 13–15 nm intramembrane particles at the perimeter of the vesicle fusion sites, and (4) alignment of these intramembrane particles into the long, parallel, anastomosed strands characteristic of mature tight junctions. These structural features of brain endothelial cells in monolayer culture constitute the morphological expression of: (1) reshaping the cell surface to align future junction-containing regions with those of adjacent cells, (2) delivery and insertion of newly synthesized junctional intramembrane particles into regions of the plasma membrane where tight junctions will form, and (3) aggregation and alignment of tight junction intramembrane particles into the complex interconnected strands of mature zonulae occludentes. The distribution of filipin–sterol complex-free regions on the PF intramembrane fracture face of junction-forming endothelial plasmalemmae corresponds precisely to the furrows, aligned vesicle fusion sites and anastomosed strands of tight junctional elements.

To test the functional significance of these morphological features of junction-forming cells and to validate the interpretation that they are reliable indicators of the stages of tight junction genesis, primary cultures of bovine brain capillary endothelium were treated with 25 μg/ml of Cytochalasin-D or 0·25 mg/ml of n-ethylmaleimide (Sigma Chemical Co.) in order to prevent cytoskeletal mediation of surface contouring (step 1) or to inhibit vesicle fusion with the plasmalemma (step 2) and thereby prevent junction formation as a consequence of failure of the vesicle fusions to insert tight junctional intramembrane particles into the plasma membrane. Examination of platinum replicas of freeze-fractured control and treated endothelial monolayer cultures confirmed the absence of surface contours in Cytochalasin-D-treated cells, which exhibited no zonulae occludentes, and also clearly showed that n-ethylmaleimide-treated cells, which lacked tight junctions, did not have the rich endowment of vesicle fusion sites (and IMPs) which were conspicuous in control cells. Demonstration of the failure of MDCK cells to form

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tight junctions when cultured in the presence of 5–10 μg/ml of cycloheximide (Griepp *et al.*, 1983) lends further support for the schemata proposed above.

Advantages of this model include: (1) all stages of *de novo* tight junction formation are present in each monolayer culture, and (2) cultures possess vast areas of tight junction-containing membrane which are easily sampled by freeze-fracture. This model will provide the basis for future attempts to identify the signals that regulate tight junction formation, and will facilitate studies to characterize the protein(s) of the endothelial tight junctions, the messages (m-RNA) that code for them, and ultimately, the genes bearing their blueprint.

Introduction

Tight intercellular junctions constitute the anatomical basis of the selective permeability of many types of epithelial cells that partition tissue compartments (Chalcroft and Bullivant, 1970; Staehelin, 1974; Wade and Karnovsky, 1974a, b; Hull and Staehelin, 1976; Van Deurs and Luft, 1979; Pinto da Silva and Kachar, 1982). Among the best known, and most intensively studied, are the zonulae occludentes that contribute to the blood–brain barrier (Brightman and Reese, 1969; Shivers, 1979; Shivers *et al.*, 1984), the blood–testis barrier (Gilula *et al.*, 1976; Pelletier and Friend, 1983), and in epithelia which regulate salt and water traffic in the vertebrate nephron (Peek *et al.*, 1977). Zonulae occludentes are also present between epithelial cells grown in monolayer cultures (Hoi Sang *et al.*, 1979; Cereijido *et al.*, 1980; Bowman *et al.*, 1981, 1983; Griepp *et al.*, 1983) which have been used as models for studies of the cellular events of junction formation (Hoi Sang *et al.*, 1979; Griepp *et al.*, 1983).

The ultrastructural features of the formation of this class of intercellular junction have been reported by numerous investigators (Gilula, 1973; Montesano *et al.*, 1975, 1976; Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Robenek *et al.*, 1979; Suzuki and Nagano, 1979; Griepp *et al.*, 1983; Pelletier and Friend, 1983; Talmon *et al.*, 1984), and can be generally summarized by the following observations: the overall process of tight junction formation can be temporally separated into a synthetic (Griepp *et al.*, 1983) or induction (Talmon *et al.*, 1984) phase, and an assembly phase (Robenek *et al.*, 1979; Griepp *et al.*, 1983). Most of the research efforts have dealt primarily with the assembly phase of tight junction development (Gilula, 1973; Montesano *et al.*, 1975, 1976; Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Robenek *et al.*, 1979; Suzuki and Nagano, 1979; Pelletier and Friend, 1983) and have established detailed accounts of how the structural components of the junctions (the intramembrane particles revealed by freeze-fracture) aggregate or otherwise become aligned into complex rows to form the characteristic intramembranous ridges (and complementary grooves) of this class of junction. The agreement on this portion of tight junction neogenesis is unanimous. On the other hand, the synthetic or inductive phase of tight junction neogenesis is less well understood. For example, it is not clear whether protein synthesis is required for production of junction components in order for junction formation to proceed (Pinto da Silva and Kachar, 1982; Griepp *et al.*, 1983; Talmon *et al.*, 1984). The other cytoplasmic events of the synthetic phase of junction formation are also not understood although, Suzuki and Nagano (1979) and Luciano *et al.* (1979) proposed that initial signs of junction formation include close apposition of cells and contouring of the tight junction-particle free plasma membrane. Even though synthesis and assembly phases of junction development have been mentioned in other reports (Montesano *et al.*, 1975; Robenek *et al.*, 1979), the suggestion of cell surface molding is novel (Luciano *et al.*, 1979; Suzuki and Nagano, 1979) and does constitute a morphological clue that tight junction genesis is in progress. With this exception, no other ultrastructural parameters have been identified for the synthetic phase of junction formation.

One component of the sequence of events that constitute *de novo* formation of tight junctions is missing from all the published accounts of this process—a mechanism for insertion of newly synthesized junctional components (intramembranous particles) into the plasmalemma. In all the published reports of neogenesis of zonulae occludentes, no consideration of the matter of how the protein components of the junction, iden-

tified in platinum replicas as intramembrane particles, and ridges, are delivered and placed in the plasma membrane of the cells forming the junctions (Gilula, 1973; Montesano *et al.*, 1975, 1976; Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Robenek *et al.*, 1979; Suzuki and Nagano, 1979; Griepp *et al.*, 1983; Pelletier and Friend, 1983; Talmon *et al.*, 1984). Until a possible mechanism for insertion of tight junction proteins into the cell membrane can be identified, a morphologically recognizable sequence of structural features, reflecting the cytoplasmic events of the synthetic phase of junction formation, cannot be created. The present report, therefore, proposes a novel new scenario for the morphological events of synthesis and assembly of interendothelial tight junctions, which is presumed to mirror the physiological and biochemical activities of this process as they occur in the cell interior.

An additional purpose of this paper is to report the results of tests which were applied to the components of this new scenario for tight junction development in order to assure the validity of each step as a reasonable marker and morphological expression of the cytoplasmic events of tight junction neogenesis. Previous studies (Hoi Sang *et al.*, 1979; Griepp *et al.*, 1983) have clearly demonstrated the failure of cultured epithelial cells to form tight junctions when grown in medium containing 5–10 μg/ml of cycloheximide, an inhibitor of protein synthesis, thereby demonstrating the dependence of *de novo* genesis of tight junctions on protein synthesis. Since junction formation in the brain endothelial cultures used in the present study must, *a priori*, require synthesis of new junction components, we did not perform inhibition procedures in this study but instead, rely on results of previous investigations of this part of *de novo* formation of tight junctions by cells in culture (Hoi Sang *et al.*, 1979; Griepp *et al.*, 1983). Results of experimental manipulation of the events of tight junction formation reported here, support our interpretation of the morphological parameters presented in the model and suggest that the model for tight junction neogenesis can serve as a useful and important vehicle for dissection and detailed studies of each of the events of tight junction formation.

Material and Methods

Isolation of brain microvessels and culture of endothelial cells

Bovine brain capillaries were isolated according to the procedures of Betz *et al.* (1979) and Bowman *et al.* (1983), to yield a final suspension of microvessels which were freed of basal laminae and pericytes. Endothelial cells isolated in this manner exhibited 80–90% viability by trypan blue exclusion and the yield was approximately 2×10^8 viable cells. The cells were seeded onto fibronectin-coated substrates (Bowman *et al.*, 1981) and grown in a culture medium consisting of α-MEM (Gibco, Grand Island Biological Co., Grand Island, New York) containing 29 mM HEPES buffer (pH 7·4) with 10% horse plasma-derived serum (Hyclone, Inc., Logan, Utah). Plasma-derived serum was used to prevent growth of cells not containing FVIII/vWF-AG (Bowman *et al.*, 1981). Cells to be freeze-fractured were seeded onto either fibronectin-coated polystyrene coverslips (Lux Scientific Corp., Newbury, Ca., 25 mm round), or onto fibronectin-coated Thermonox (Lux Scientific Corp., Newbury, Ca., 24×30 mm) plastic coverslips and cultured in the medium described above.

Freeze-fracture electron microscopy

Monolayers of endothelial cells grown on fibronectin-coated plastic coverslips were first washed with one change of phosphate-buffered saline (pH 7·5), and then immediately fixed for 1 hr in 3% glutaraldehyde (Eastman Chemicals, Rochester, New York), buffered to pH 7·35 with 0·1 M sodium cacodylate and containing 0·01% Ruthenium Red (Shivers and McLachlin, 1984) in order to lightly stain cells of the monolayer for identification during subsequent processing. The cultures were washed in three changes of buffer (0·1 M sodium cacodylate, pH 7·35) and then placed in 30% glycerol in 0·1 M sodium cacodylate (pH 7·35) overnight in the refrigerator.

The following day, each plastic coverslip containing the Ruthenium Red-stained, cryoprotected monolayer of endothelial cells, was examined with a dissecting microscope and the areas of the monolayer which exhibited a dense population of cells were located and pieces of the coverslip approximately 2·5×2·5 mm were cut out of the

coverslip with a small scalpel. This yielded a small, rhomboidal piece of coverslip with a population of endothelial cells attached. Each piece of coverslip was inverted, placed on a drop of Elvanol–glycerol mixture (Pauli *et al.*, 1977; Shivers and McLachlin, 1984) situated on a flat gold specimen disc (Balzers, Liechtenstein), and frozen in a slurry of liquid nitrogen-cooled Freon-22. The Elvanol–glycerol medium was prepared according to the method of Pauli *et al.* (1977), for monolayer cell cultures grown on plastic coverslips. The frozen monolayers which had been stored in liquid nitrogen, were fractured on a Balzers BAF 301 freeze-etch unit and replicated with platinum and carbon (Shivers and Brightman, 1976; Shivers and McLachlin, 1984). Platinum replicas were cleaned in household bleach, washed in three changes of filtered (0·1 μM Millipore, Millipore Corp., Bedford, Ma.), autoclaved, distilled water, and picked up on bare 200-mesh copper grids. Replicas were examined and photographed in a Philips 201 electron microscope operating at an accelerating voltage of 60 kV.

Experimental procedures

In order to test the involvement of the cytoskeleton in tight junction formation, some cultures were grown in medium containing 25 μg/ml of Cytochalasin-D (Sigma Chemical Co., St Louis, Mo.) (Knight and Baker, 1982; Meza *et al.*, 1982; Meller and El-Gammal, 1983; Baker and Knight, 1984; Shannon *et al.*, 1984). These cultures were maintained at 37°C for 4 hr and then prepared for freeze-fracture.

Other cultures were grown for 4 hr at 37°C in medium containing 0·25 mg/ml of n-ethylmaleimide (Sigma Chemical Co., St Louis, Mo.) (Knight and Baker, 1982; Baker and Knight, 1984), in order to inhibit infusion of cytoplasmic vesicles with the plasmalemma (exocytosis) and thereby demonstrate their involvement in junction formation. These cultures were also prepared for freeze-fracture electron microscopy.

Demonstration of the distribution of sterol complexes in junction-forming regions of the intramembrane fracture faces of endothelial cells grown on plastic coverslips, was accomplished by fixing the monolayers of cells for 4–12 hr in fixative containing 0·01% filipin (gift of the Upjohn Co., Kalamazoo, Michigan). The filipin solution was prepared according to the method of Pelletier and Friend (1983) in a 3% glutaraldehyde solution buffered to pH 7·35 with 0·1 M sodium cacodylate and containing 0·01% Ruthenium Red. Following filipin treatment, the cell monolayers were prepared for freeze-fracture according to the procedure outlined above.

Fig. 1. 'New' endothelial cell from 2-day-old monolayer culture. Presence of numerous cell processes (P) and rounded profile (left side of micrograph) indicate this cell has not completely flattened and spread on the substrate. No surface contours or vesicle fusion sites are apparent on the plasma membrane of this cell. ×32,000.

Fig. 2. Flattened endothelial cells from 3-day-old cultures exhibit elementary contours of the 'apical' cell surface. In this view, elevations or terraces of the intramembrane surface are indicated by black asterisks, while the furrows or valleys of the surface between the terraces are marked with white squares. Compare this view with that of similar cells treated with filipin (Figs. 16, 17). No cytoplasmic vesicle fusion sites are present on the intramembrane surface. The adjacent cell shown here is less flattened and possesses numerous cytoplasmic processes (P). S, substrate. ×25,000.

Fig. 3. Concomitant with appearance of surface contours, flattened endothelial cells begin to display structures interpreted as sites where cytoplasmic vesicles have fused with the plasmalemma. The sites occur as depressions or protrusions on the intramembrane surfaces exposed by freeze-fracture. The fusion sites often occur in clusters (arrows) and exhibit no conspicuous positional organization. ×8,000.

Note: Arrow in the lower right corner of each micrograph indicates the direction of platinum shadowing on the replica.

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P
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Results

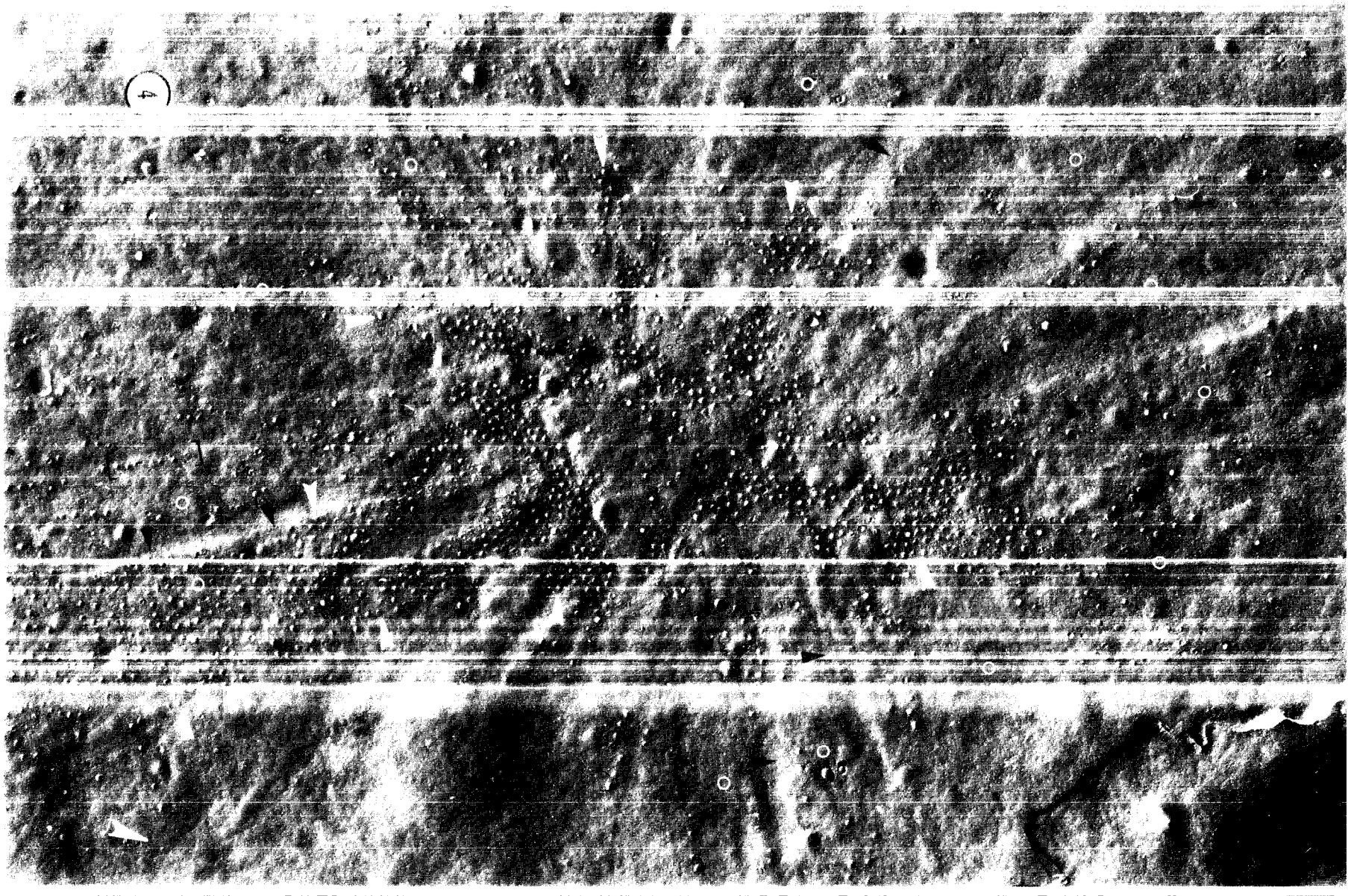
Monolayer cultures of brain endothelial cells contain cells of various age. Although it is impossible to assign a precise age to each cell examined, since no morphological markers indicative of age-related characteristics have been determined for these cells, it is possible to approximate the age of some cells on the basis of their position (location) within the culture. For example, cells situated at the center of colonies are likely those from which the rest of the colony arose, and are therefore older than those cells found at the edge of the colony. Cells at the perimeter of a colony have in all probability, recently completed division and, in many cases, are not flattened and spread out. Instead, the peripheral cells are rounded with conspicuous irregular microvilli (Fig. 1). Conversely, most of the members of a colony of cells display a flattened profile (Fig. 2) with no cytoplasmic extensions. Therefore, if cells are systematically sampled in a radially orientated trajectory from the margin to the centre of a colony, they should exhibit a progressively increasing amount of specialization and differentiation concomitant with their increasing age.

In the case of brain endothelial cells, which when fully developed *in vivo* or *in vitro*, possess elaborate intercellular tight junctions, cells at the periphery of colonies grown in monolayer cultures possess no tight junctions (Fig. 1) whereas those closer to the colony centre, display tight junctions of varying complexity. The most complex zonulae occludentes occur between cells at the centre of the colony (Figs. 12, 15). These observations serve as a useful prelude to the results of this study which will be presented here, and support the assumption that monolayer cultures of brain endothelial cells possess cells that exhibit a broad spectrum of age-related developmental stages which can be detected morphologically with little sampling difficulty. Since no evidence was obtained in this study for preferential fracturing of junctions or junction-forming endothelial plasma membranes, the fracture of all stages of junction development is presumed to have been random.

New or recently divided cells possess no intramembrane components of tight junctions (Figs. 1, 2). The PF fracture face of the 'apical' membrane of these cells is covered with a randomly dispersed population of intramembrane particles and a few cytoplasmic protrusions or microvilli. In addition, no evidence can be found for the

Fig. 4. Survey replica of a very large surface expanse of a fractured endothelial cell. Surface contours are well developed and generally exhibit a radial pattern of alternating terraces (elevations) and adjacent valleys (white circles) or furrows. Clusters of small circular depressions (white arrowheads) are located in the corridor-like valleys between the elevated terraces of the surface. The circular depressions, which are the result of fusion of cytoplasmic vesicles with the plasma membrane of this cell, area aligned in rows (brackets) generally orientated parallel to the radially situated valleys. Short chains of large intramembrane particles (black arrowheads) are present adjacent to and between the vesicle fusion sites (white arrowheads). Compare the pattern of surface specialization of contours and vesicle fusion sites with that of filipin-treated cells shown in Figs. 16–18. ×19,000.

Figs. 5–7. The pattern of surface contours exhibited by flattened endothelial cells is essentially radial but may be less conspicuous and organized than that seen in Fig. 4. Careful examination of replicas of newly flattened cells such as that in Fig. 5, reveals early molding of the cell surface into terraces. White broken lines accent this surface specialization. Further development of the cell surface produces more extensive ridges (white triangles, Fig. 6, 7) and valleys (white circles, Figs. 6, 7). Following the initial surface reshaping, small circular vesicle fusion sites (white arrowheads) appear in the valleys between adjacent terraces. At first, these structures display a random orientation (Fig. 5), but soon become aligned into rows or elongate clusters parallel to the long axis of the valley in which they are situated (Figs. 6, 7, 4). Compare these figures with those that illustrate the distribution of sterol–filipin complexes on similar endothelial cells in monolayer culture (Figs. 16–18). Fig. 5, ×29,000; Fig. 6, ×18,000; Fig. 7, ×21,000.



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small, circular PF face depressions (or volcano-like protrusions on the EF fracture face) associated with sites of fusion of cytoplasmic vesicles with the plasma membrane (Fig. 1). Cells adjacent to that shown in Fig. 1, which have begun to flatten and spread on the fibronectin-coated substrate (Fig. 2), display few if any cytoplasmic projections and possess expanses of flattened 'apical' surface. Careful examination of the apical intramembrane surfaces of these cells, which will become juxtaposed to similar surfaces of other cells as the culture continues to grow (i.e. the cells tend to overlap and form layers of more than one cell), exposed by freeze-fracture, reveals a slight contouring of the surface. These gentle contours occur as terraces or ridges (asterisks, Fig. 2) orientated parallel to the long axis of the spreading flattened cell processes, and valleys or corridors (white squares, Fig. 2) situated between them. This arrangement can be easily seen in Fig. 2 by visually aligning either the white squares or the asterisks along a line from the lower left corner of the micrograph to the upper right corner.

Cells which are situated approximately five or six cells centripetal with respect to the center of the colony, are assumed to be older than those at the immediate periphery, and possess structures not found in the more peripherally located cells. These intramembrane structures appear as small, circular PF face depressions (Figs. 3, 5) and are often found as clusters on the intramembrane surface. Numerous large intramembrane particles are found intimately associated with these circular depressions (or volcano-like projections on EF fracture faces). In many instances, these intramembrane particles appear as chains of four to six particles orientated in radial fashion from the center of each depression (Fig. 3.)

The surface contouring and appearance of small circular vesicle fusion sites, become more elaborate and complex in cells judged to be older and, consequently, more differentiated. For example, the cell surface illustrated in Fig. 4 is that of a cell situated about halfway between the center of a colony and the periphery. The radially orientated system of surface contours is conspicuous and well developed. Concomitant with the pronounced system of alternating terraces and valleys (furrows) is an obvious increase in the number of small circular depressions that result from fusions of cytoplasmic vesicles with the plasma membrane (Fig. 4). These fusion sites are clearly situated in the valleys between the surface terraces (Figs. 4–7) and, consequently, display the same radial pattern of alignment as that of the surface contours. In fact, the vesicle fusion sites are frequently found as chains of several dozen depressions positioned parallel to the surface terraces (Fig. 4).

The general arrangement of the intramembrane surface of differentiating endothelial cells is not always visualized in so much detail and beauty as shown in Fig. 4, but is nevertheless unmistably present (Figs. 5–7) in most of the cells that occupy the middle position in the colony (middle=midway between the center and periphery). Most of the cells sampled from a particular region within a colony exhibit similar development of surface specialization. Finally, sampling cells from various locations within a colony, which are in various stages of development of surface molding and vesicle fusion site alignment, permits construction of a morphological sequence of development of these structures.

The small, circular PF face depressions (or EF face volcano-like protrusions) are clearly associated with a conspicuous endowment of intramembrane particles (Figs. 8–10). High magnification views of the clusters and chains of the fusion sites reveals these particles in varying degrees of intimacy with the circular fusion sites. The profiles exhibited by the circular sites range from those in which only a few particles are scattered around the rim or margin of the site (Figs. 8, 9), to others where chains of the particles (circled areas, Figs. 8, 9) appear to emanate from the center of the fusion site. In addition, fusion site-associated intramembrane particles frequently occur as small aggregates (Fig. 10, circled) immediately juxtaposed to the circular depressions (or protrusions). In all of these instances, it is tempting to interpret these observations as indicative of a dynamic process in which the tight junction-like intramembrane particles originate from the fusion sites!

Alignment of the tight junction intramembrane particles into short rows and chains can be noted in platinum replicas of

plasma membrane of those endothelial cells whose surface has begun to differentiate. Careful examination of the membrane surface of the cell shown in Fig. 4 reveals short chains of particles which are orientated parallel to the radially arranged terraces and valleys. Alignment of the junctional intramembrane particles becomes increasingly pronounced when older cells, sampled from regions near the center of the colonies, are studied (Figs. 11, 13, 14). In these instances, many rows of aligned particles are present and are seen to describe a wide variety of configurations and interconnections (Figs. 13–15). Frequently, these rows of particles appear isolated, unconnected to similar arrays, or to form highly branched interconnected networks within a

Figs. 8–10. High magnification of platinum replicas of the vesicle fusion sites reveals many large intramembrane particles closely associated with these sites (circled areas). In these examples, the particles are positioned at the rim or edge of the fusion sites (seen best in circled areas) and in the immediate vicinity of the fusion sites—an arrangement which reinforces the notion that the particles are 'flowing' from the fusion sites on to the adjacent intramembrane surface. In addition to fusion sites in the circled areas, the white arrowheads point to single crater-like vesicle fusion sites from which intramembrane particles appear to emanate. The intramembrane particles which are so closely associated with the fusion sites correspond in size and location to those which comprise the forming tight junction intramembrane ridges in older regions of endothelial cell cultures. Fig. 8, ×60,000; Fig. 9, ×56,000; Fig. 10, ×63,000.

Fig. 11. Survey of very early stage of tight junction assembly. Short chains of junctional intramembrane particles (white arrowheads) are scattered along the interfaced plasmalemma fracture faces of adjacent cells. In the lower half of this micrograph, are regions where intramembrane particles are beginning to assemble and align in linear fashion (lower three white arrowheads). C, cytoplasm of endothelial cell. Compare this stage of tight junction assembly with that illustrated in Fig. 12, which is conspicuously more advanced and complex, and with that of filipin-treated cells in Fig. 19. ×61,000.

Fig. 12. Survey view of a forming tight junction on the intramembrane surface of a 7-day-old endothelial cell. Orientation of the strings of particles into parallel arrays (open white arrows) is clearly seen, as are the numerous instances where the long strands appear to branch (black arrows). A few vesicle fusion sites (circled) remain in this region but their frequency decreases as the complexity of the junction develops. This view is typical of that seen in much of the intramembrane surface exposed by fracture of brain capillary endothelial monolayers and supports the proposal of this *in vitro* system as a useful model for study of the *de novo* synthesis and assembly of tight intercellular junctions. Compare this view of developing junctions with that in filipin-treated cells shown in Fig. 19. ×33,000.

Fig. 13. Incomplete rows of intramembrane particles identical to those near the crater-like cytoplasmic vesicle-plasma membrane fusion sites, are present in older cells of the growing monolayer of endothelial cells. Usually, strands of these particles appear to stream from the fusion sites (circled) and form increasingly long and complex strands (arrows). ×32,000.

Fig. 14. Survey of 7-day-old endothelial cell cultures shows many cells which possess strands of intramembrane particles that appear to extend from the rim of the crater-like vesicle fusion sites (circled) and to have assembled into highly tortuous, branched rows (white arrows). These intramembranous elements of new zonulae occludentes, though highly complex, do not yet form a complete, continuous network of the intricacy seen in mature brain capillary endothelium. ×49,000.

Fig. 15. Endothelial cells which are endowed with zonulae occludentes (short large arrows) identical (if not even more complex) to those of mature brain endothelia, are easily found in 14-day-old endothelial cell cultures. These cultures are about 95% confluent and their cells are flattened to form a sheet-like monolayer. The layer often exhibits extensive overlapping of adjacent cells and occasionally, the layer of cells may be several cells thick. Components of these tight junctions, especially those at the margins of the junctional complex (long arrows) appear to be adding individual intramembrane particles. Compare this micrograph with that of an identical region of endothelial cell plasmalemma treated with the antibiotic filipin (Fig. 19). ×43,000.


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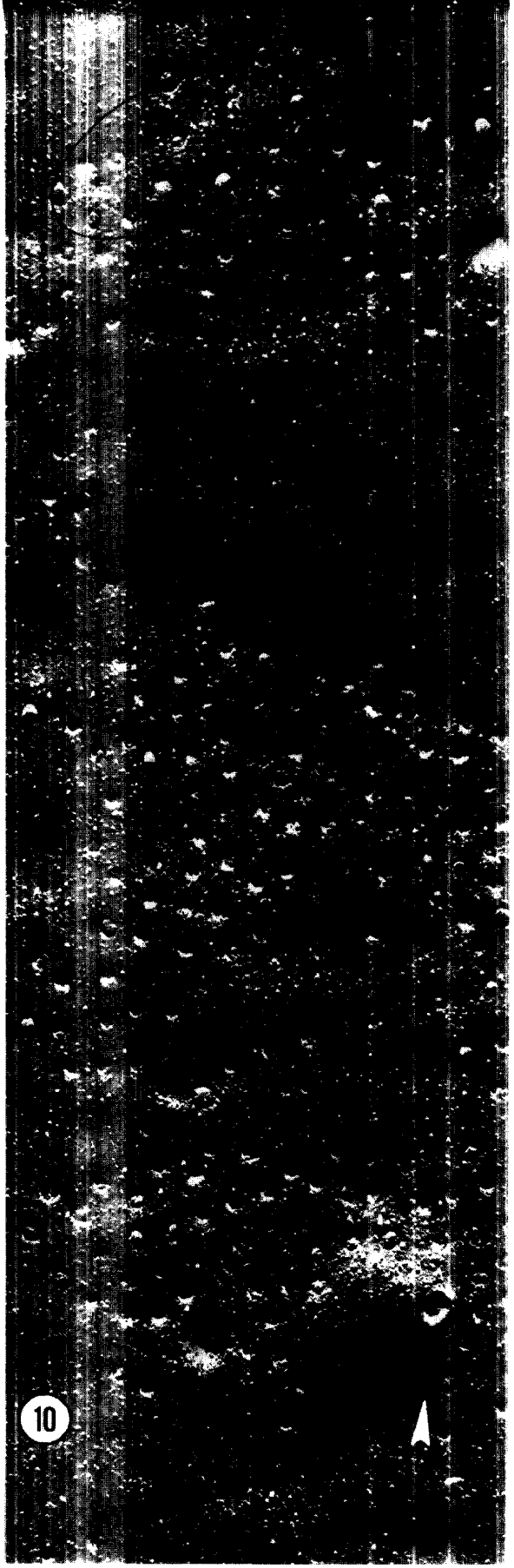
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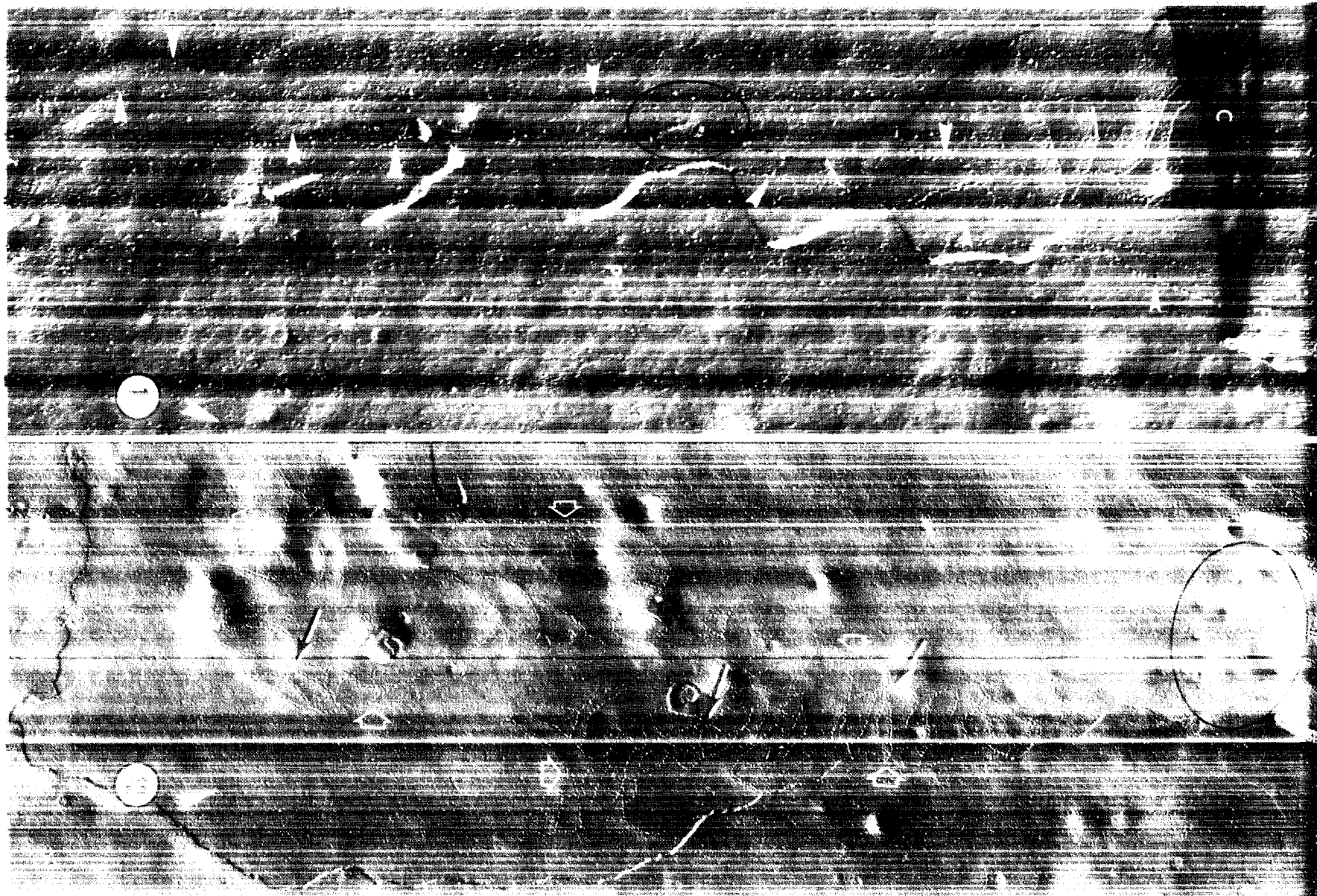

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relatively small area of intramembrane surface (Figs. 13, 14).

The most complex and complete (continuous) networks of tight junctional particle strands are seen in membrane fracture faces from the older, centrally located endothelial cells (Figs. 12, 15). In these cells, which are presumed to be fully differentiated endothelial cells, the zonulae occludentes describe a system of complete interconnected intramembrane ridges and grooves, identical to those displayed by the same cells *in vivo* (Shivers, 1979; Shivers *et al.*, 1984). The complex tight interendothelial junctions are most easily found in replicas of cultured endothelial cells by scanning along regions where the fracture plane has exposed several layers of plasma membrane (Figs. 11, 12, 14). In such replicas, the interface of the contiguous cells almost always contains a well-developed, highly anastomosed zonula occludens.

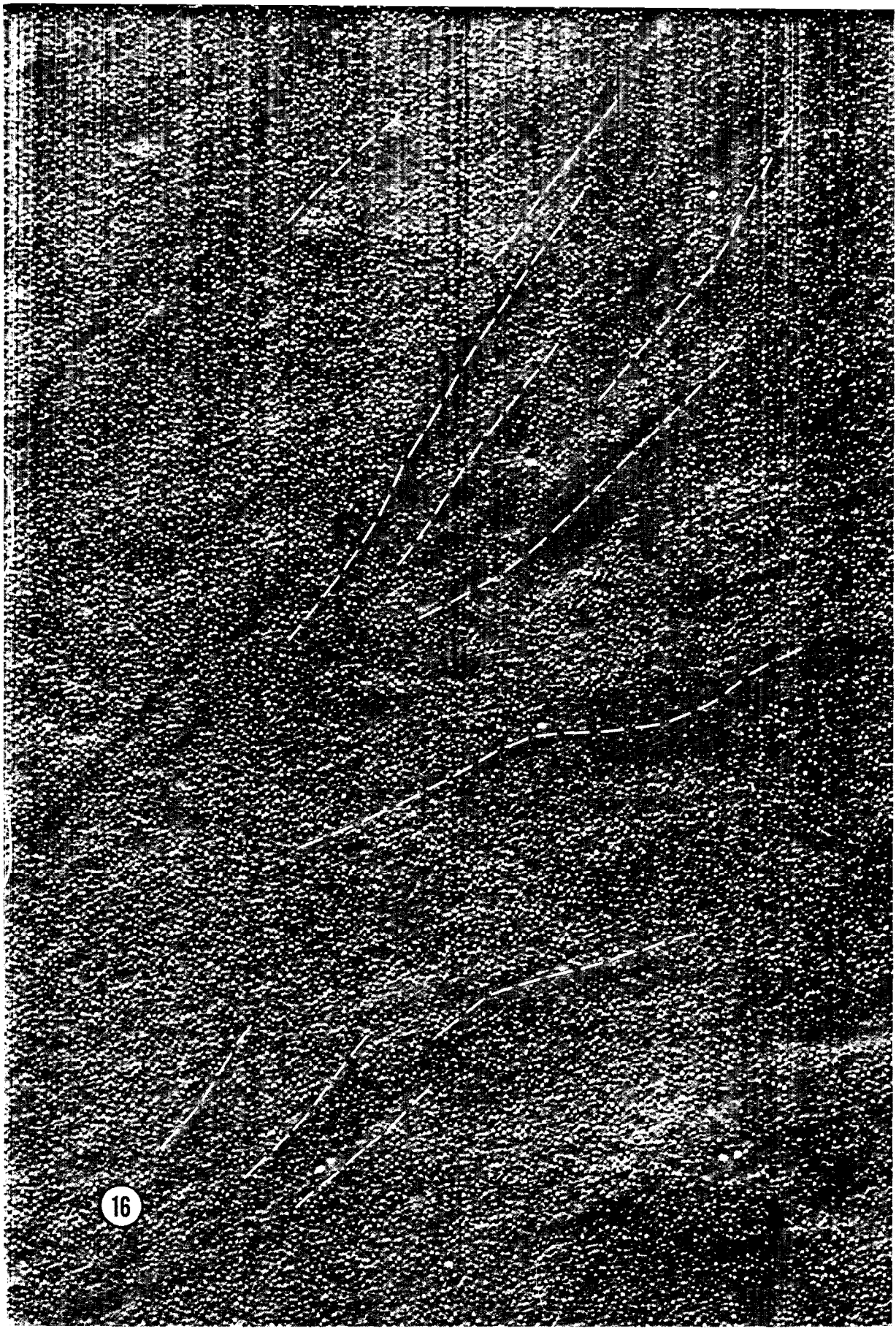
The population of vesicle fusion sites described above, which is most obvious in newer, less-developed cells in the monolayer (Fig. 4), is dramatically reduced in older cells which possess more complex and complete tight junctions. A few vesicle fusion sites can often be seen, however, positioned within the lattice-like network of branched junctional strands (Figs. 13, 14). In such cases, the crater-like fusion sites retain an alignment parallel to the shallow intramembrane surface corridors within which the long strands of tight junction particles are situated. These arrays of assembling tight junctions are assumed incomplete, and in older cells, which are endowed with zonulae occludentes judged to be intact and complete, the circular vesicle fusion sites are infrequent (Figs. 12, 14).

Fixation of brain endothelial cell cultures in glutaraldehyde containing 0·1% filipin serves to map the distribution of sterol complexes in the endothelial plasmalemma (Pelletier and Friend, 1983; Pumplin and Bloch, 1983; Risinger and Larsen, 1983). Newly flattened endothelial cells, situated at the periphery of colonies of cells, exhibit surface contours which appear to lack sterol–filipin complexes (Figs. 16, 17). The parallel furrows that are presumed to represent future regions of membrane where tight junctions will form (Figs. 2, 4–7), are conspicuous in their paucity of filipin-binding sites and, in fact, appear to be identifiable on the basis of their inability to bind the antibiotic (Figs. 16, 17). The similarity between IMP-free furrows that appear in newly flattened brain endothelial cells (Figs. 2, 4–7) and the intramembrane regions that fail to form sterol–filipin complexes (Figs. 16, 17), is remarkable.

Endothelial cells which are located midway between the center of a colony of cells and the periphery of the same colony, are presumed older than those described above, and display vesicle fusion sites aligned along sterol–filipin-free furrows of the intramembrane fracture surface (Fig. 18). These regions are identical to those described above for endothelial cells judged to be in a later stage of development (Figs. 3, 4–7) than the newly divided cells at the periphery.

Endothelial cells located in the central region of a colony exhibit complex zonulae occludentes, all of which reside in sterol–filipin-free intramembrane regions (Fig. 19). The striking correspondence between the tight junction-containing intramembrane surfaces of cultured brain endothelial cells (Figs. 12–15) and the sterol–filipin-free areas, also containing elaborate tight junctions (Fig. 19), is clear evidence in support of the notion that development and residence of zonulae occludentes is restricted to specific

Fig. 16. Survey view of newly flattened endothelial cell treated with the antibiotic filipin. This cell exhibits the early stages of surface contouring (compare this view with that in Figs. 2, 4–7), that herald the onset of junction formation. Sterol–filipin-free regions of membrane correspond exactly to the intramembrane particle-free furrows (white broken lines) situated between surface elevations, and to the same regions shown in Figs. 4–7. ×28,000.



localities within the endothelial plasmalemma.

Brain endothelial cells cultured in the presence of n-ethylmaleimide, an inhibitor of exocytotic vesicle fusion with the plasma membrane (Knight and Baker, 1982; Baker and Knight, 1984), display no evidence for vesicle fusion sites on the intramembrane surfaces exposed by freeze-fracture (Fig. 20). Examination of the exposed fracture face of the cell membrane reveals an almost complete absence of small circular vesicle fusion sites (Fig. 20). Intramembrane particles appear aggregated into loosely packed clumps which are situated adjacent to particle-free areas of intramembrane surface (Fig. 20). The density of intramembrane particles on the PF fracture face of endothelial cells treated with this sulfhydryl reagent appears reduced as compared to the density of particles present on the PF face of normal endothelial cell plasma membranes (compare Fig. 20 with Figs. 3, 9, 10). Cross-fractures of endothelial cytoplasm failed to reveal cytoplasmic vesicles in cells grown in n-ethylmaleimide-containing medium, and since the fracture surfaces of these cells contained no structure which could be identified as belonging to zonulae occludentes (i.e. chains or strands of junctional particles), it is presumed that junction formation was prevented (inhibited)!

Cultures of brain endothelial cells maintained in medium containing Cytochalasin-D, lacked the system of surface contours associated with tight junction formation that is always present in control cell cultures (compare Fig. 21 with Figs. 5–7). Careful examination of replicas of Cytochalasin D-treated endothelia revealed intramembrane surfaces which were generally flat and uniform (Fig. 21). Furthermore, these intramembrane surfaces conspicuously lacked the small circular

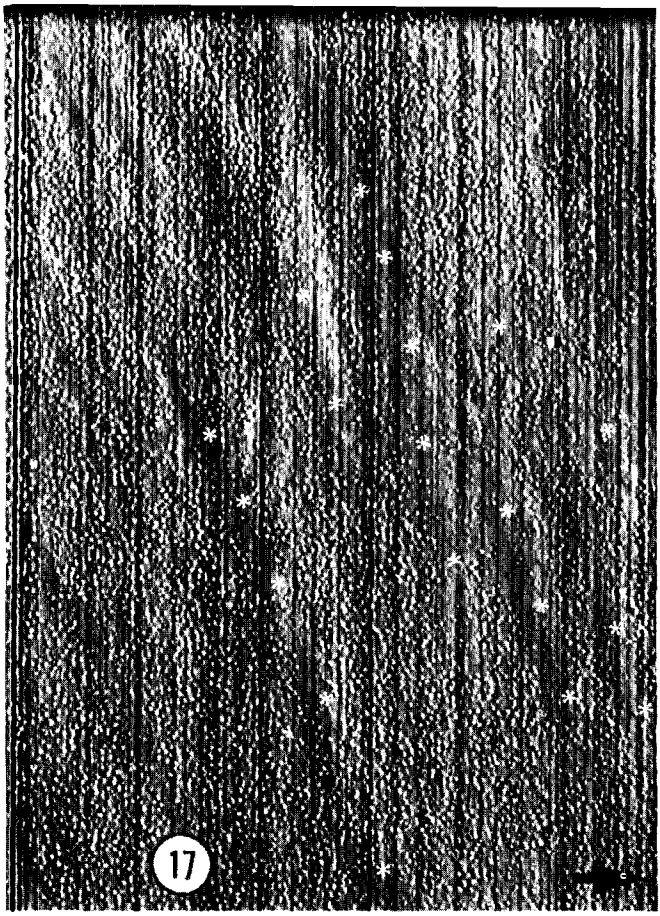
Fig. 17. Low magnification of parallel furrows of intramembrane surface which lack sterol–filipin complexes (white asterisks). This view of the development of surface contours of brain endothelial cells corresponds precisely to that shown in Figs. 2 and 5–7. ×23,000.

Fig. 18. Sterol–filipin complexes are absent from furrows (white asterisks) of intramembrane surface that contain small circular vesicle fusion sites (arrows). This view of the early stage of junction formation in brain endothelial cell culture can be easily compared to identical regions shown in Figs. 5–9. ×16,000.

Fig. 19. Regions of brain endothelial cell plasmalemma that contain the elaborately anastomosed ridges and rows of intramembrane particles—the tight junctions (black arrows)—are devoid of sterol–filipin complexes. Compare this view of the tight junction and its immediate environment, with an identical region shown in Figs. 14 and 15. ×35,000.

Fig. 20. When the fracture plane travels in a stepwise fashion through several layers of endothelial cells, regions of tight junction-containing intramembrane surface are usually revealed (i.e. at white arrows). In this micrograph of n-ethylmaleimide-treated cells, the juxtaposed plasma membranes of two flattened endothelial cells display no evidence of tight junctions (white arrows). No smaller circular depressions resulting from vesicle fusions with the plasmalemma are seen (compare this view with that of untreated endothelial cells in Figs. 8–10). However, several sites on the intramembrane surface (black arrows) may represent forming fusion sites. The surface contains an apparently reduced population of intramembrane particles, which are slightly clumped (solid triangles) and patches of particle-free surface (open triangles). ×63,000.

Fig. 21. The plasma membrane of endothelial cells incubated in medium containing Cytochalasin-D, exhibits no morphological feature associated with tight junction synthesis and assembly. The intramembrane surface is not contoured and the small circular depressions associated with vesicle fusion sites are absent. Compare this view of the Cytochalasin-D-treated plasmalemma with that of untreated cells in Figs. 4, 11–15. Intramembrane particles are often clumped (arrows) and small areas of particle-free intramembrane surface are common. S, substrate. ×46,000.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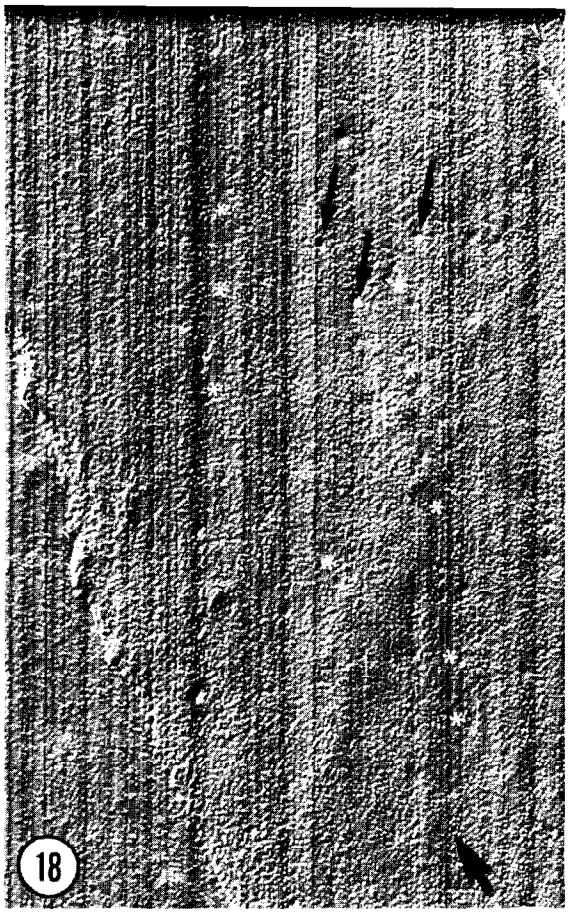
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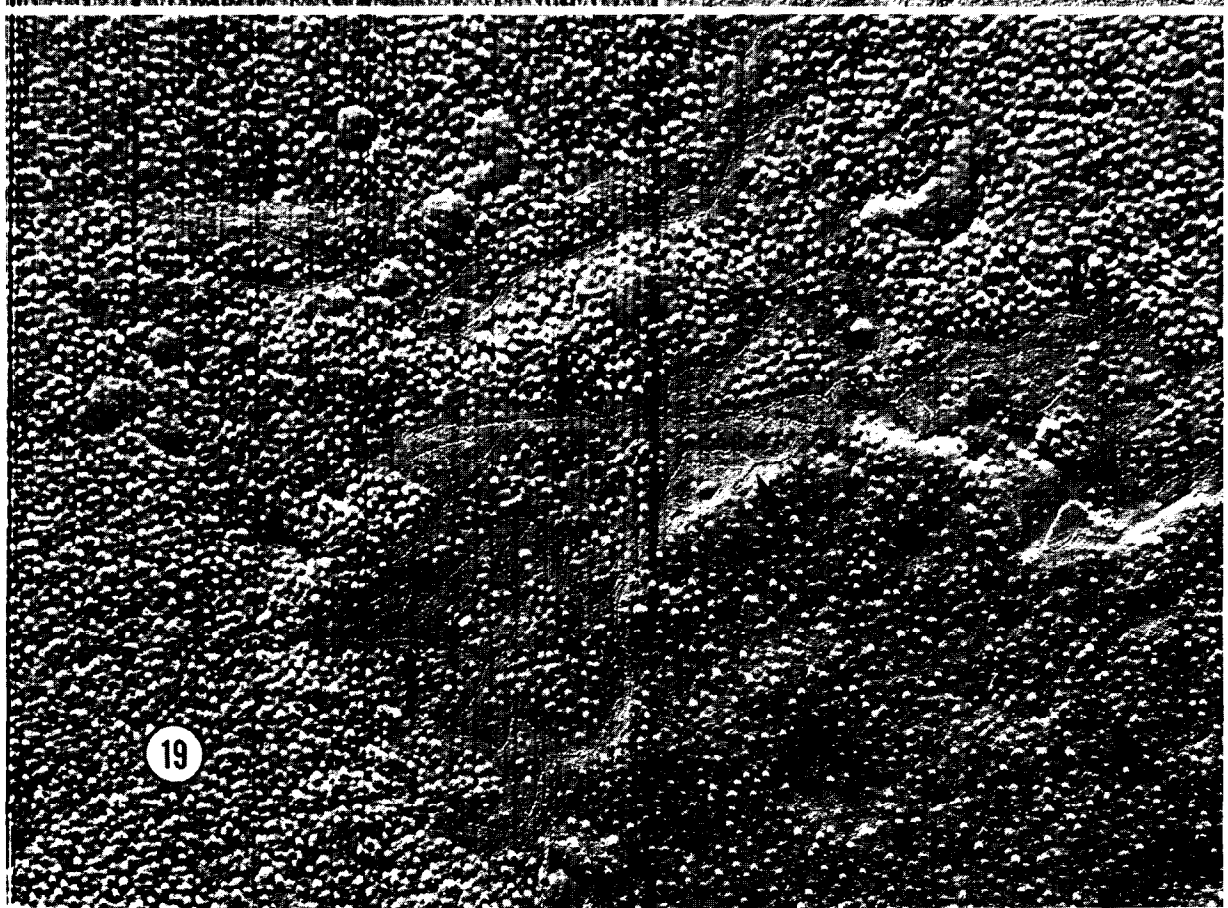
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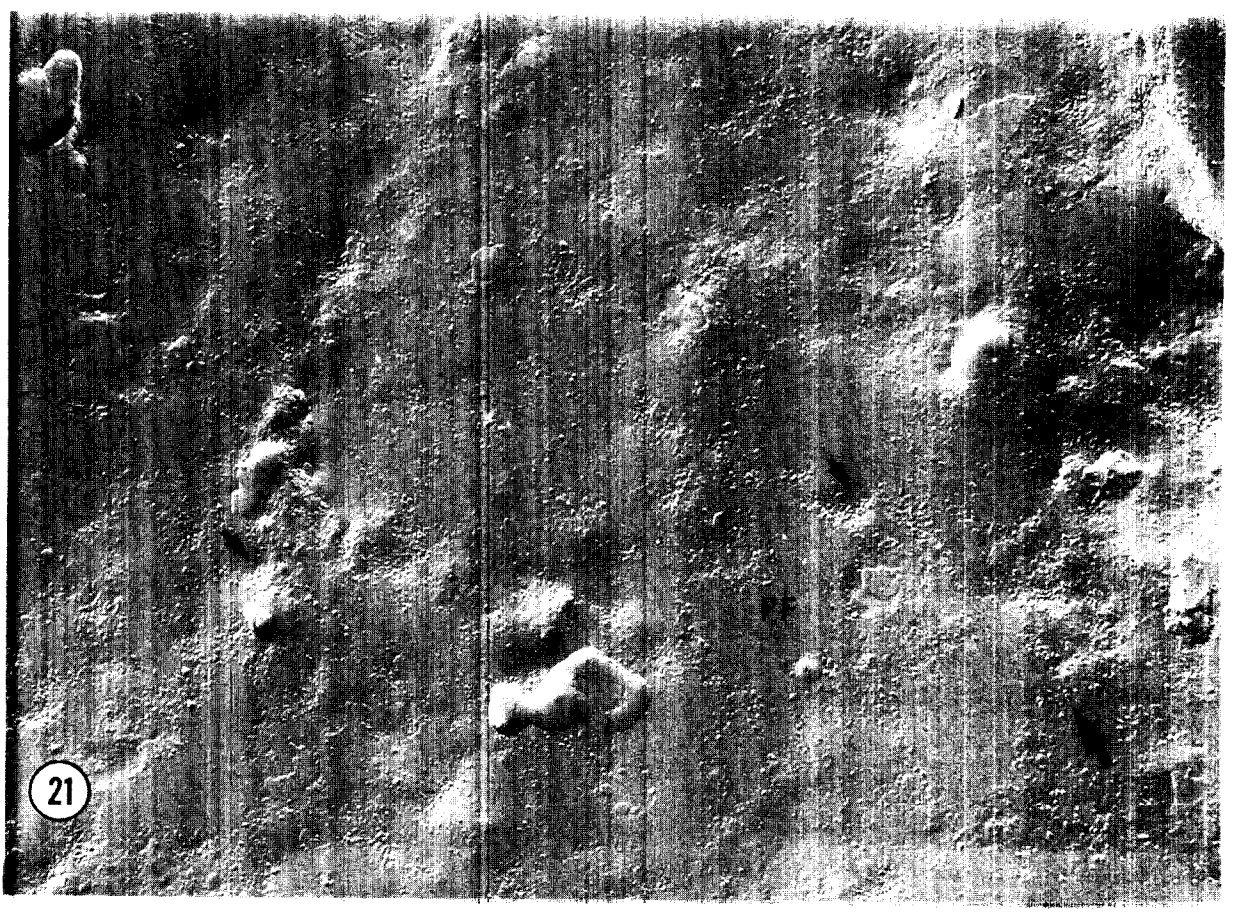

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depressions characteristic of cytoplasmic vesicle fusions with the plasmalemma. The vesicle fusion sites are a common feature of control endothelial plasma membrane, where they display an alignment parallel to the system of surface contours (Figs. 9, 10). Finally, the intramembrane particles in Cytochalasin-D-treated endothelial plasmalemmae appeared slightly clumped into loose aggregates situated near small particle-free areas. Since no evidence was seen in the drug-treated endothelial plasma membrane of development of surface contours that signal the onset of tight junction formation, it is presumed that tight junction formation was prevented!

Discussion

The *de novo* synthesis and assembly of intercellular tight junctions must proceed in stepwise fashion through a series of very precise biochemical and structural events in cells. This logical presumption has been suggested by several investigators (Montesano *et al.*, 1975; Robenek *et al.*, 1979; Suzuki and Nagano, 1979; Griepp *et al.*, 1983; Talmon *et al.*, 1984), who have generally subdivided tight junction neogenesis into a synthetic phase and an assembly phase. Each phase of tight junction development must have morphological features associated with it, which reflect the cytoplasmic activities in progress, and can therefore be used as indicators or markers of each phase of junction assembly and synthesis.

The complete process of junction morphogenesis would be most easily studied if a model cell system could be identified in which: (a) *de novo* tight junction formation must occur between most cells in the model, (b) the cell population consists of cells of varying age and degree of tight junction development, (c) distinct morphological features associated with each phase of *de novo* junction formation are easily recognized in cells displaying junction formation, and (d) possess cells whose tight junctions are extensive and highly complex so that fully developed zonulae occludentes are not mistaken as intermediate stages of the process. Unfortunately, no model has yet been proposed for study of junction formation which exhibits the features mentioned above. Therefore, the purpose of this paper is to present a model *in vitro* cell system which enjoys all of the important features noted above, and which can serve as a basis for future investigation of tight junction biogenesis.

Brain capillary endothelial cultures contain cells of varying age depending on their location in the monolayer. With very little difficulty, cells from a broad spectrum of ages can be located and identified within a few centimeters of each other. Cultured brain capillary endothelial cells display the same extensive, complex, and elaborate zonulae occludentes exhibited by brain capillaries *in vivo* (Shivers, 1979; Bowman *et al.*, 1983; Shivers *et al.*, 1984), and thus serve as a very valuable model for junction development in which all stages of junction synthesis and assembly can be identified.

Recognition of the several biochemical and accompanying morphological steps of tight junction neogenesis is facilitated by the brain endothelial cell model proposed here, which exhibits the morphological correlates of the states of junction development. For example, the appearance of a system of surface contours on cells which are beginning to flatten at the periphery of a colony, heralds the onset of tight junction formation and represents the first morphological clue that the development of junctions is about to begin. A similar observation has been made by Suzuki and Nagano (1979), Montesano *et al.* (1975, 1976), Robenek *et al.* (1979), Griepp *et al.* (1983) and by Luciano *et al.* (1979), who termed these structures 'formation folds'.

Formation of this system of surface terraces and intervening furrows or valleys on the surface of cultured brain endothelial cells may be effected by components of the cytoskeleton. Similar reshaping of the surface of a variety of cell types has been suggested to be an event mediated by the cytoskeleton, and substantial experimental evidence in support of this view has been accumulated (Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Cereijido *et al.*, 1980; Hoi Sang *et al.*, 1980; Meza *et al.*, 1982; Geiger, 1983; Robenek and Gebhardt, 1983) for endothelial cells as well as MDCK monolayers. Results of such studies (Gotow and Hashimoto, 1983) support the present interpretation that: (a) surface reshaping is likely controlled by microfilaments of the

cytoskeleton, and (b) junction-containing regions of the plasma membrane are different from non-junctional regions.

Perhaps the strongest evidence that supports the role of cytoskeletal elements in reshaping the cell surface during tight junction formation has been provided by Gotow and Hashimoto (1983) who treated tight junction-rich ependymal cells with Cytochalasin-B and found a reduction in cytoskeletal microfilaments situated adjacent to the junctions, along with a concomitant increase in filipin binding by the junction-containing regions of the plasma membrane. These authors concluded that stability of the junctional regions of the plasma membrane may be in part a result of the presence of underlying cytoplasmic microfilaments and not exclusively dependent on the cholesterol content of the membrane.

In the present study, incubation of the endothelial cell monolayer cultures in medium containing the cytoskeletal microfilament disruptor Cytochalasin-D, resulted in failures of the surface contours to form, as well as other structural features of the model. This finding is consistent with those of other investigators who also used drugs such as Cytochalasin-B, colchicine or vinblastin, to disrupt cytoskeletal-mediated cell shape changes (Hoi Sang *et al.*, 1980; Meza *et al.*, 1982; Meller and El-Gammal, 1983). Demonstration here that inhibition of surface remodelling of endothelial cells precludes further development of tight junctions, supports the proposal that the cytoskeletal-mediated surface contouring is an essential first-step in the biogenesis of tight junctions.

Results of this study do not permit speculation as to reasons why the other structures associated with tight junction development failed to appear following Cytochalasin-D treatment. The most likely explanation is that they too may be dependent upon the integrity and mediation of the cytoskeleton for their activities. This notion is supported by results of other studies which have shown that intramembrane particle mobility, receptor site mobility and numerous other activities of cytoplasmic organelles are all mediated by the cytoskeleton (Cereijido *et al.*, 1980; Meza *et al.*, 1982; Plattner *et al.*, 1982; Gabbiani *et al.*, 1983, 1984; Geiger, 1983; Meller and El-Gammal, 1983; Pumplin and Bloch, 1983; Aunis and Perrin, 1984; Shannon *et al.*, 1984).

An important feature of the membrane of the surface contours that appears at the onset of junction formation, is reflected by the use of the cholesterol-specific polyene antibiotic filipin. Results of filipin binding in the present study clearly demonstrate a decreased filipin binding by regions of the plasma membrane where tight junctions will occur. Similar observations have been reported for studies of tight junction formation in other cell types (Chailley, 1981; Gotow and Hashimoto, 1983; Risinger and Larsen, 1983). The nature of filipin binding by surface membrane furrows, along which tight junctions will form, is different from that displayed by the remaining plasma membrane, and supports the notion that regions of the plasma membrane containing tight junctions have a unique identity (Chailley, 1981; Feltkamp and Van der Waerden, 1983; Gotow and Hashimoto, 1983; Risinger and Larsen, 1983), as do regions of plasma membrane that possess other specializations such as acetylcholine receptors (Pumplin and Bloch, 1983). In addition, these observations support the idea that the surface contours which develop in the endothelial cell model of junction formation proposed in this paper, constitute a reliable morphological signal that tight junction formation has begun.

One of the most important features of the model for tight junction development proposed in this study, is the role of small circular PF face depressions (or EF face protrusions) that are situated along the surface contours (junction-competent regions) of the brain endothelial plasma membrane. These structures are identical to the structures formed on the plasmalemma during exo- and endocytosis and are presumed, therefore, to reflect some sort of transmembrane traffic. None of the current studies on tight junction formation deals with the question 'how are the tight junction intramembrane particles delivered and inserted into the plasma membrane?' This is likely due to lack of an appropriate model of tight junction neogenesis in which junction formation occurs *de novo*, instead of simple rearrangements and reformation of pre-existing junctions—systems which do not require addition of new junctional components to the plasma

membrane. Identification of vesicle fusion sites as the device for insertion of tight junction intramembrane particles into the plasma membrane is especially difficult when these structures lack the abundance and conspicuous orientation exhibited at junction formation sites illustrated in this paper. Support for the notion that in the brain endothelial cell model for tight junction development, the newly synthesized components (proteins) are delivered and inserted into the plasmalemma by a cytoplasmic vesicle courier, is provided by studies on the delivery and addition of acetylcholine receptors to the sarcolemma at neuromuscular junctions (Israël *et al.*, 1981; Bridgman *et al.*, 1984; Bursztjan and Fischbach, 1984; Peng and Phelan, 1984). Especially pertinent to this interpretation is the study by Bursztjan and Fischbach (1984) who elegantly showed the role of coated cytoplasmic vesicles in ferrying newly synthesized acetylcholine receptor to the surface of muscle cells, and a recent report by Shivers and Bowman (1985) of a mechanism for delivery and insertion of gap junctional intramembrane particles into the plasma membrane of aorta endothelial cells. The morphological features of vesicle fusion sites presented in this report, which include: (1) alignment of fusion sites along the surface contours (presumptive junction-forming regions of the plasmalemma), and (2) the very intimate association of junction-like intramembrane particles with the fusion sites, strongly suggest a functional relation ship of the fusion sites to junction formation. Further, the apparent 'streaming' of particles from the circular PF face depressions, and occurrence of the particles in short chains orientated radial to the center of the fusion sites, cannot in our opinion be interpreted other than as evidence that junction particles are being inserted into the plasma membrane.

In the present study, experimental evidence in favor of a courier system of cytoplasmic vesicles to carry newly synthesized tight junction protein particles to the surface for insertion into the membrane by fusion of the vesicle with the plasmalemma, is provided by results of culturing endothelial cell monolayers in medium containing n-ethylmaleimide. In such cultures, the vesicle fusion sites appear to be absent and their formation is presumed inhibited by the drug. This reagent has been employed in other studies of exocytotic events of cells, as an inhibitor of exocytosis (Knight and Baker, 1982; Baker and Knight, 1984), and apparently functions in the same manner on brain endothelial cells. Although cultures maintained in the presence of n-ethylmaleimide failed to develop the circular PF face depressions or EF face volcano-like protrusions, which precluded formation of tight junctions, the mode of action and target of this drug are not known. This sulfhydryl agent, used as an inhibitor of calcium-dependent exocytosis (Knight and Baker, 1982), could prevent exocytosis or fusion of the particle-laden vesicles at the level of the plasma membrane, or alternatively, the drug may act through a primary effect on the cytoskeleton and prevent transport of the vesicles to the cell surface. Certainly the latter possibility can be supported by the substantial evidence in the literature involving the cytoskeleton with vesicle (secretory) transport to the cell surface (Plattner *et al.*, 1982; Aunis and Perrin, 1984; Shannon *et al.*, 1984).

The experimental evidence in support of the proposed model for tight junction development presented in this study is circumstantial and, unfortunately, not the result of applying highly specific inhibitors of the events of tight junction neogenesis. Dose levels of the drugs used in this study were within those reported for other systems where cell viability was monitored and are, therefore, presumed to not result in cell death in the brain endothelial cultures. Although vesicle-membrane fusion sites appear to have been inhibited by n-ethylmaleimide and surface contouring prevented by Cytochalasin-D, with subsequent failure of tight junction formation, other cell activities and processes were also undoubtedly affected. Further support for this model must await detailed biochemical and immunological testing of the individual events.

Final stages of tight junction development and maturation essentially involve an ordered aggregation of tight junction intramembrane particles with subsequent alignment into linear arrays along furrows of the contoured surface of the cell. As this process continues, the strands of junctional particles become more interconnected and fewer

are seen as isolated rows or chains of particles. Also, the lateral branching and anastomoses characteristic of the complex zonulae occludentes of brain endothelium (Shivers *et al.*, 1984) occurs in the final steps of assembly. Cells in the monolayer of brain endothelium that possess tight junctions in various stages of assembly, are easily found and studied. There is general agreement between the interpretation of the morphological features of junction assembly presented here and accounts of similar events reported in the literature (Gilula, 1973; Montesano *et al.*, 1975, 1976; Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Robenek *et al.*, 1979; Suzuki and Nagano, 1979; Griepp *et al.*, 1983; Talmon *et al.*, 1984). Recent studies (Miettinen *et al.*, 1978; Hoi Sang *et al.*, 1979; Luciano *et al.*, 1979; Cereijido *et al.*, 1980; Feltkamp and Van der Waerden, 1983; Gotow and Hashimoto, 1983; Green and Severs, 1984; Peng and Phelan, 1984) have provided convincing evidence that the aggregation and alignment of tight junction intramembrane particles is mediated by the cytoskeletal thin filaments. This is an especially exciting prospect which can easily be studied in the model presented here since the stages of junction formation, which describe a temporal sequence, can be identified and consequently, isolated.

The model of *de novo* synthesis and assembly of complex interendothelial zonulae occludentes presented in this report is unique in that it contains the complete spectrum of morphological features that reflect the synthetic and assembly stages of tight junction formation. This feature of the model makes it particularly valuable for studies designed to probe each phase of development since each stage can be unmistakably identified by morphological features exposed by freeze-fracture of brain endothelial monolayers. Two especially important features distinguish this model from others that have been proposed: (1) the pronounced and conspicuous reshaping and partitioning of the cell surface into elevated terraces with intervening valleys—an event that signals the onset of junction formation, and (2) delivery and insertion of tight junctional intramembrane particles into the plasma membrane by cytoplasmic vesicle couriers which fuse with the plasmalemma and can be easily identified in platinum replicas of the cell plasma membrane. This new scenario, unique in its completeness and detail, will provide the foundation for investigation of the role of cytoskeletal elements in junction synthesis and assembly, and perhaps more importantly, the model may be useful for studies to identify the signals that regulate tight junction formation, to characterize the protein(s) of the endothelial tight junctions, and eventually, to understand the instructions that prescribe the synthesis of the junction components.

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