

Astrocyte-mediated induction of tight junctions in brain capillary endothelium: an efficient in vitro model

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Fourth passage rat brain capillary endothelial cell cultures, which no longer possess the tight junctions characteristic of this highly specialized component of the blood-brain barrier, were used to study induction of zonulae occludentes *in vitro*. These cells, when grown in 50% rat brain astrocyte-conditioned medium and 50% α -MEM on an endothelial cell matrix-coated substrate (Cedarlane Labs, Hornby, Ont.), possessed numerous, elaborately complex, tight junctions which were identical to those displayed *in vivo* by intact brain capillary endothelium. Endothelial cells grown in 50% astrocyte-conditioned medium and 50% α -MEM on bare plastic or fibronectin-coated substrate, possessed no tight junctions. Results of this study clearly demonstrate the local control of tight junction biogenesis in brain capillary endothelial cells depends on: (1) an astrocyte-produced factor(s), and (2) a 'competent' (cell-produced) extracellular matrix.

The basis for the blood-brain barrier in mammals is the selective transport properties of brain capillary endothelium^{5,8}, including the elaborate system of tight intercellular occluding junctions² that occur between apposed membrane faces of these cells. This unique specialization of brain capillary endothelial cells appears late in development and has been postulated to be under the inductive influence of an astrocyte-derived factor(s) produced locally within the central nervous system.

Stewart and Wiley¹⁴ grafted avascular embryonic quail brain tissue fragments into the coelomic cavity of chick embryos. The neural tissue was vascularized by abdominal vessels and these displayed blood-brain barrier characteristics. Conversely, chick brain vessels vascularizing previously avascular quail embryonic mesoderm transplants possessed no such features. Consequently, the authors concluded that some aspect of the neural environment was responsible for the development of the blood-brain barrier characteristics of endothelial cells. Janzer and Raff⁶ injected highly purified astrocyte cell suspensions into the anterior chamber of adult rat eyes; intrave-

nous injections of Evans Blue, after two weeks, demonstrated that the newly formed vessels in the astrocyte aggregates were impermeable to dye. These results strongly suggest that astrocytes are responsible for induction of blood-brain barrier properties in the newly developed blood vessels. Additional support that an astrocyte-derived factor influences brain endothelium specialization was produced by DeBault and Cancilla⁴, who demonstrated that co-culture of brain capillary endothelial cells, previously negative for γ -glutamyl transpeptidase (γ -GTP), with a C₆ rat glioma cell line, caused induction of γ -GTP enzyme activity. In a later study, DeBault³ found that γ -GTP was induced in only those endothelial cells in direct contact with the C₆ cells, thereby showing that induction could not be effected with astrocyte-conditioned medium alone.

Finally, very recent studies¹⁵ in which primary brain capillary endothelial cells were co-cultured with astrocyte-enriched preparations, showed significant enhancement of tight junction length, width, and number, as compared to various controls. Additionally, astrocyte-conditioned medium alone was in-

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sufficient to cause tight junction amplification in the brain endothelial cells.

The present communication reports the use of an in vitro system for studying tight junction induction based upon the premise that the nature of the subjacent substrate is a crucial element in determining the response of brain capillary endothelial cells to soluble astrocyte factors, and that other in vitro models employing co-cultures of endothelial cells and astrocytes to investigate substrate involvement in tight junction induction are pragmatically incapable of resolving the precise nature of the extracellular matrix component of induction.

Rat brain capillary endothelium cultures were established utilizing published techniques¹. Cells were grown in a medium consisting of α -MEM (Gibco, Grand Island Biological Co., Grand Island, NY), 20 mM HEPES buffer (pH 7.4) and 10% plasma-derived horse serum (Hyclone, Logan, UT). Plasma-derived serum was used to prevent growth of cells not containing FVIII/vWF-AG¹. When these cultures reached 4th passage, the cells no longer produced the tight junctions characteristic of brain endothelium, and thus provided an ideal model to study the induction of this structural cell specialization. Enriched astrocyte cultures were obtained utilizing methods similar to those previously published⁷. Briefly, 1–2-day-old rat pups were sacrificed and cerebral tissue was obtained by dissection. Tissue was dissociated by Pasteur pipette, passed through sterile cheese cloth, and cultured in α -MEM with 2 mM HEPES buffer (pH 7.4) and 10% plasma-derived horse serum. Nine to twelve-day-old cultures were placed on an orbital shaker for 24 h; subsequently, cultures were washed with several changes of phosphate-buffered saline.

Conditioned medium from astrocytes was obtained by incubating the enriched astrocyte cultures

in medium for 36–48 h; medium was then collected and sterilized by passing it through a 0.22 μm syringe filter (Nalge, Rochester, NY) and stored in liquid nitrogen. Experimental substrates used were either bare plastic coverslips (Lux Scientific, Newbury, CA, 25 \times 30 mm)¹, or plastic coverslips coated with commercial endothelial-derived substrate (Extra-Cell, Cedarlane Labs., Hornby, Ont.). Cultures of experimental and control endothelial cells, cultured upon either of the experimental substrates, were prepared for freeze-fracture and electron microscopic analysis according to the procedures of Shivers and McLachlin¹³, and Shivers and Brightman¹².

Brain capillary endothelium cultured in α -MEM/10% horse serum on either plastic coverslips or endothelial substrate, demonstrated no tight junction formation when freeze-fracture replicas of the endothelial cultures were examined (Fig. 1). Consequently, tight junctions were not induced by control medium regardless of substrate.

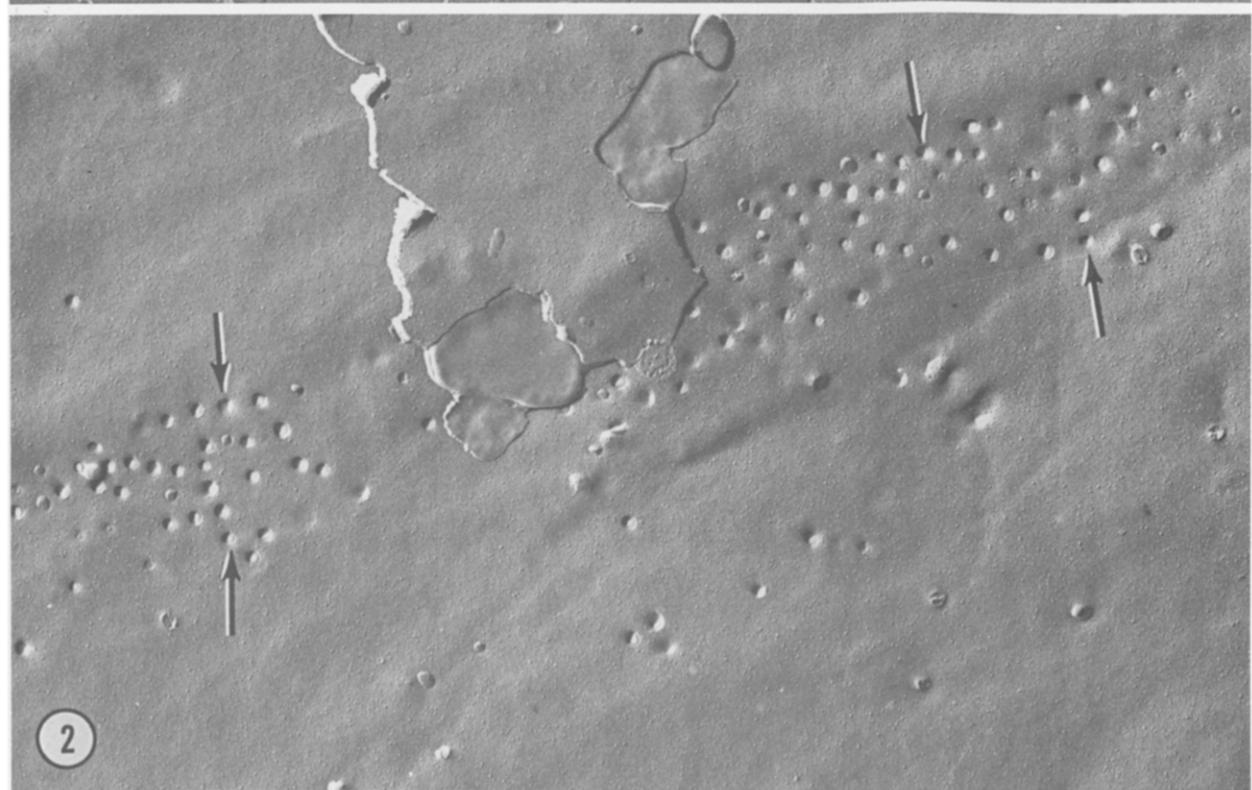
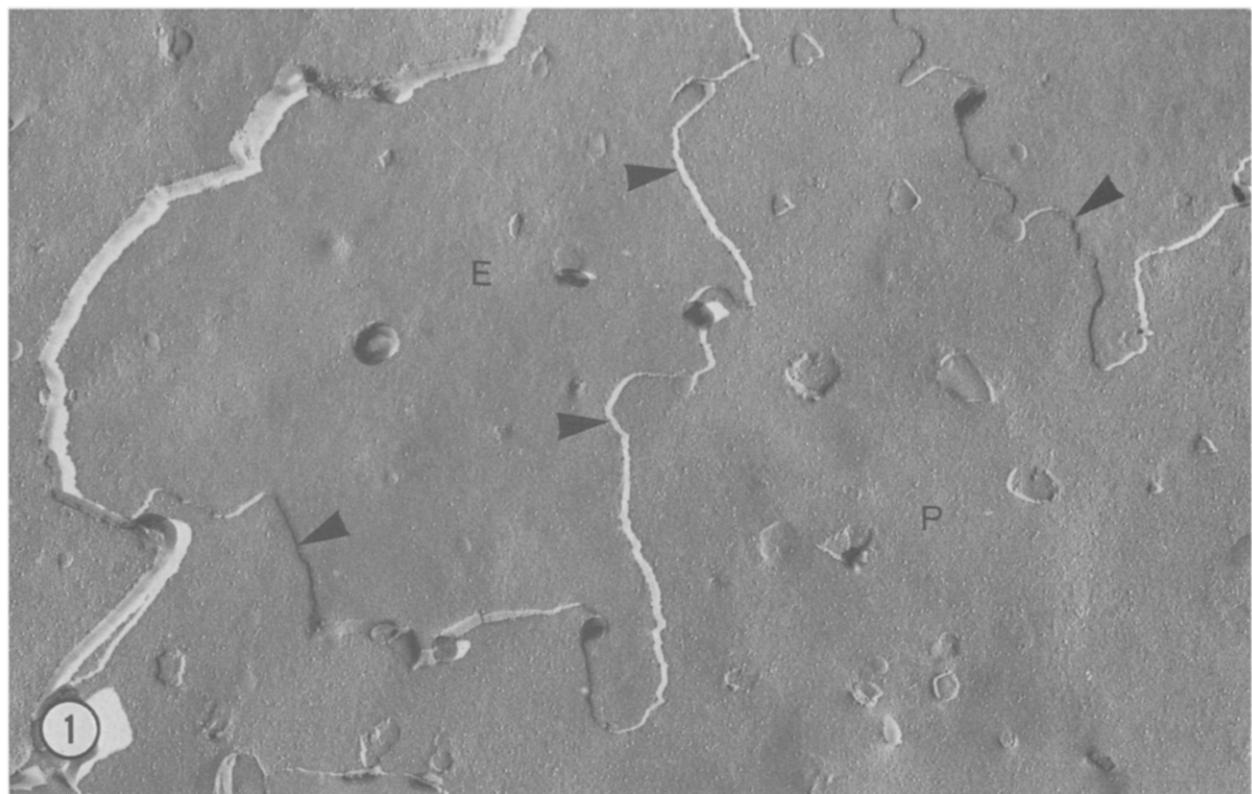
When brain capillary endothelial cells were grown for 3–7 days (confluence) in 50% astrocyte-conditioned medium and 50% α -MEM/10% serum, using plastic coverslips as substrates, no tight junctions were noted. However, long linear arrays of vesicle fusion sites were noted in regions of cellular abutment (Fig. 2). Shivers et al.¹¹ have proposed a model of tight junction formation wherein similar fusion sites are suggested to be where junction components are added to the endothelial plasma membrane and where tight junctions will ultimately form.

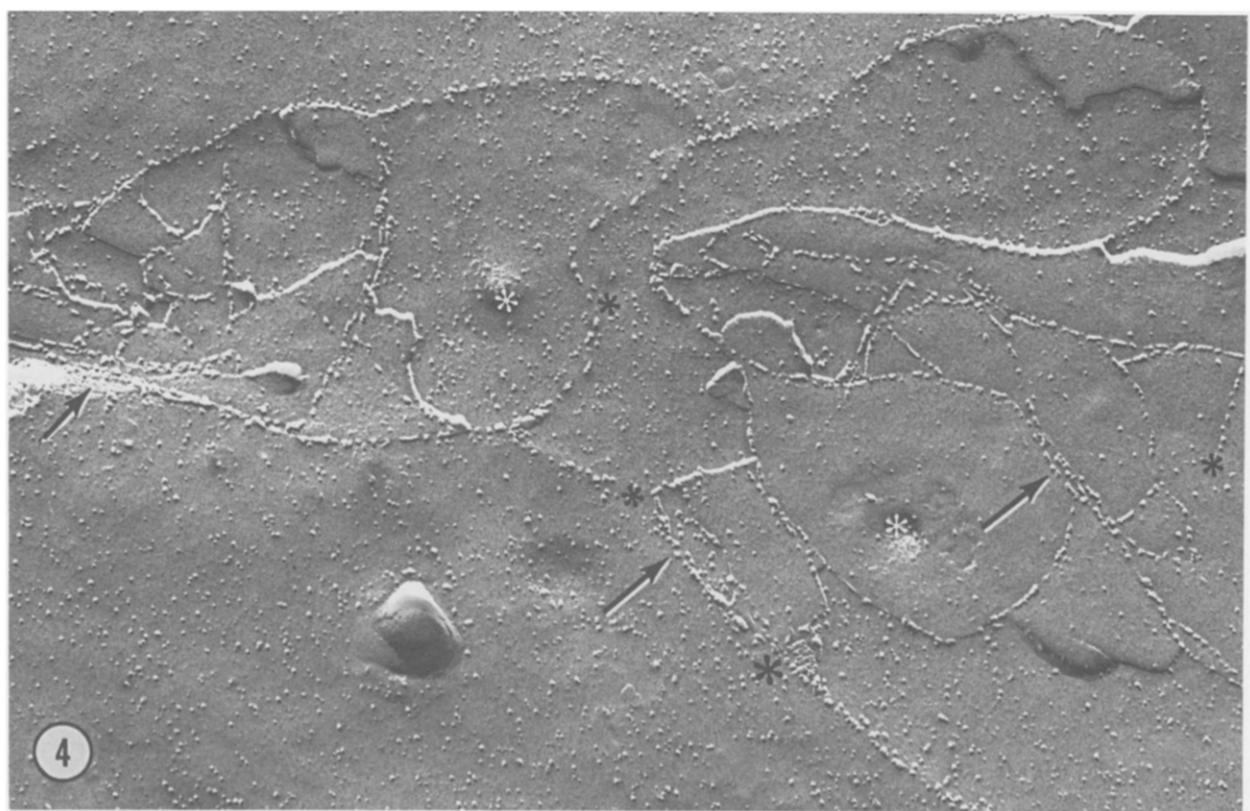
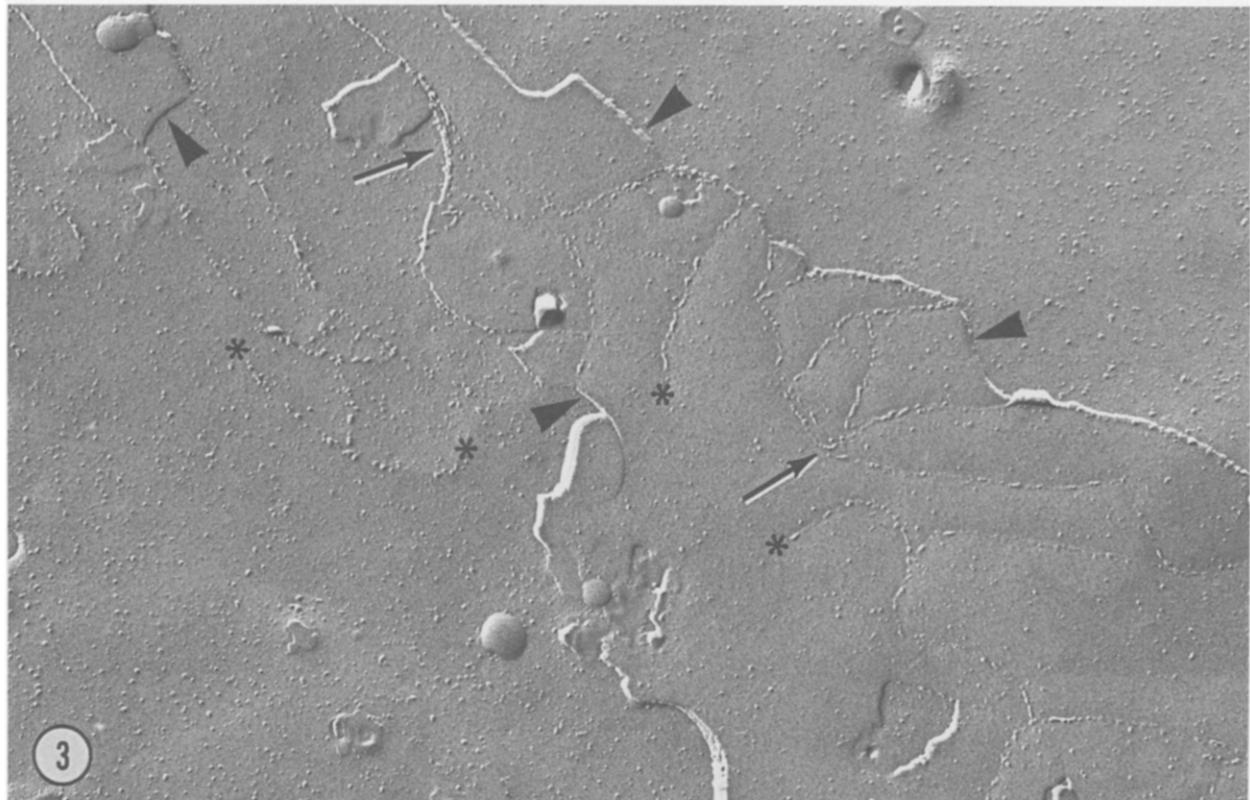
Brain capillary endothelial cells grown in a combined medium of 50% astrocyte-conditioned medium and 50% α -MEM/10% horse serum, on plastic coverslips coated with endothelium-derived extracellular matrix (Extra-cell), displayed extensive and elaborate formation of tight junctions (Figs. 3 and 4).



Fig. 1. Brain endothelial cells cultured on either bare plastic or endothelial cell matrix-coated substrate in medium consisting of only α -MEM/10% serum, display no evidence of tight interendothelial junctions. Examination of vast expanses of intramembrane surface exposed by freeze-fracture of the endothelial culture failed to demonstrate occluding junctions in regions of cell abutment or overlap where tight junctions normally exist (arrows). $\times 33,000$.

Fig. 2. Brain endothelial cells cultured on bare plastic coverslips in medium consisting of 50% astrocyte-conditioned medium and 50% α -MEM/10% serum, display no structural evidence for tight intercellular junctions. Regions of intramembrane surface where endothelial cells are abutted or overlapped possess numerous small circular vesicle fusion sites (arrows) situated in areas of intramembrane surface precisely where elements of tight junctions are normally located. These intramembrane profiles of vesicle fusion are identical to those described in the model for tight junction neogenesis¹¹. $\times 23,100$.





Figs. 3,4. Fourth passage brain capillary endothelial cells cultured on extracellular matrix-coated coverslips in medium composed of 50% astrocyte-conditioned medium and 50% α -MEM/10% serum, display extensive profiles of complex interendothelial tight junctions (arrows). The parallel ridges situated at sites of overlapped endothelial cells (arrowheads) are highly branched and interconnected (arrows), creating an elaborate network of tight junctional constituents. Many of the intramembrane ridges are continuous with long strands of intramembrane particles. These strands of particles display free ends (black asterisks) and are presumed to be sites where junctional particles are being added to the forming tight junction. A few small circular vesicle fusion sites (white asterisks, Fig. 4) are situated within the network of anastomosed junctional elements. These tight junctional profiles are identical to those of normal (in vivo) endothelial zonulae occludentes^{9,10}. Fig. 3: $\times 40,500$; Fig. 4: $\times 54,000$.

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Our results indicate that enriched astrocyte cultures are able to produce a soluble factor(s) that can induce tight junction formation in cultures of brain capillary endothelium. Interestingly, the nature of the subjacent substrate was crucially important in determining the nature of the response of the brain endothelial cells to the astrocyte factor(s) contained in the astrocyte-conditioned medium. When grown upon a non-biologic substrate, such as bare coverslips, the brain capillary endothelial cells formed arrays of vesicles on their plasma membranes, indicating that they were engaged in the early stages of tight junction neogenesis¹¹, but were unable to assemble proper tight junctions. However, when an endothelial-derived substrate was substituted for bare plastic, a dramatic response was obtained with the formation

of tight junctions comparable to their in vivo counterparts^{9,10}. In terms of tight junction neogenesis, physical contact between astrocyte and brain capillary endothelial cells is not necessary if an endothelium-derived substrate is utilized.

The study of tight junction synthesis and assembly has been limited by the lack of an easily testable model. Our present system presents a model that will allow the necessary resolution of macromolecular events to facilitate future studies of the genetic and metabolic control of brain endothelial cell specialization.

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