Endothelial Cell-Astrocyte Interactions

A Cellular Model of the Blood-Brain Barrier

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Recent advances in cell biology open the way for studying aspects of blood-brain barrier (BBB) formation and function that have puzzled investigators since Ehrlich and Goldmann first described the barrier. Although the endothelial cells in the microvessels of all tissues appear to arise from common mesodermal precursors, considerable organ-to-organ variability exists in their ultrastructure and permeability. The most extreme example of restricted microvascular permeability is found in brain, where the endothelial cells are sealed together by continuous complex tight junctions to form a polarized "epithelium" that regulates the passage of small organic molecules and ions between blood and brain.[1]

A constant feature of brain microvessels with barrier properties is an almost total investment with processes from astrocytes.[2] This contact is so extensive that the astrocytes were once thought to physically create the BBB. We now know from the classic tracer studies of Reese, Karnovsky, and Brightman that the anatomic basis of the BBB is provided by the unfenestrated endothelial cells with their continuous and complex tight junctions.[3] Although astrocytic processes encircle the capillaries and attach to a basement membrane shared with the endothelial cells, the foot processes are not sealed to each other and small gaps between the astrocytes allow passage of proteins and other polar molecules in the interstitial fluid up to the abluminal surface of the endothelial cells (FIG. 1).

Even though the astrocytes do not create a physical barrier, their close contact with brain microvessels suggests that they have an important role in the function of the BBB.[4] Evidence for a direct influence of brain cells upon microvascular function is provided by the elegant chimeric experiments of Stewart and Wiley.[5] These investigators implanted embryonic avascular quail brain into the abdominal cavity of a chick embryo. Because of the marked difference in nuclear structure between the quail and the chicken, Stewart and Wily could prove that the blood vessels that formed in the transplanted brain arose from the abdominal vasculature of the chicken (FIG. 2). Despite their systemic origin, the chick microvessels growing into the quail brain exhibited the restricted permeability, special structure, and enzymes characteristic of the BBB. In contrast, microvessels growing into embryonic quail muscle implanted into chick brain were highly permeable and lacked BBB enzymes even though they were derived from chick brain. These studies demonstrate a direct influence of brain upon endothelial cell differentiation.

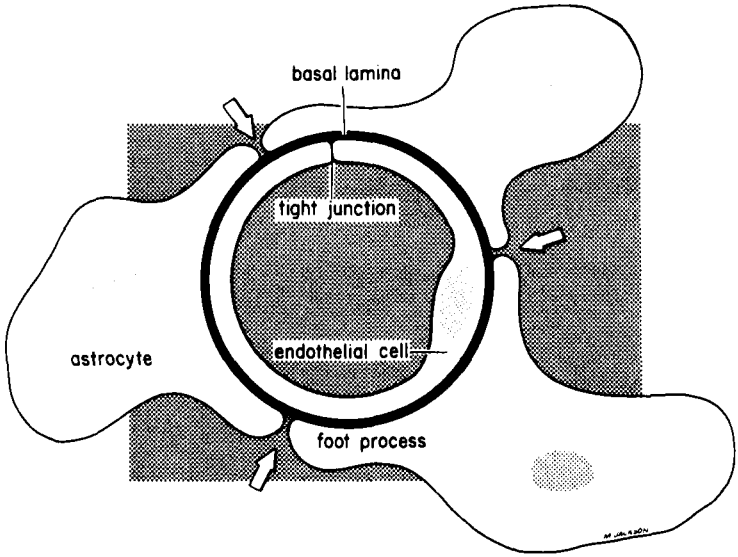

FIGURE 1. Diagram illustrating the close association of endothelial cells in brain capillaries with foot processes extending from astrocytes. The blood-brain barrier is produced by the continuous endothelium. The astrocytes encircle the microvessels, but are not sealed together and interstitial fluid has access (*arrows*) to the basement membrane and abluminal surface of the endothelial cell.

The astrocyte seems a likely candidate for mediating this effect of brain tissue upon capillary structure and function. Ultrastructural studies of regions of the brain without a barrier, astrocytic brain tumors, and microvessels in the retina support this hypothesis. Several small regions of the brain stem and hypothalmus are involved in neuroendocrine feedback and are more permeable than the rest of the brain.[6] Microvascular endothelial cells in these areas are fenestrated and lack the special features found elsewhere in the brain. The characteristic close apposition of astrocytic processes to the endothelial cell is also absent in these regions, leaving open spaces between the basement membrane of the capillary and the nearby astrocytes (FIG. 3). Similarly, the highly permeable microvessels within astrocytic brain tumors also lack close contact with the transformed astrocytes.[7] In contrast, microvascular permeability in the retina, like the brain, is markedly restricted and contributes to the formation of the blood-retinal barrier.[8] Consistent with the proposed role of the astrocyte in brain capillary differentiation, the retinal microvessels are also surrounded by foot processes from astrocytes[9] (FIG. 4).

The constant close association between astrocytes and endothelial cells in areas of the brain and retina with a barrier, and the lack of these contacts in tumors and brain regions without a barrier together with the transplantation studies, provide the *in vivo* evidence for attributing formation of the BBB to an interaction between astrocytes and endothelial cells. The development of methods to isolate endothelial cells and astrocytes from brain and to grow the two cell types separately in culture provides a new approach for studying features of their interaction that may be important to formation of the BBB.

Highly purified suspensions of microvessels can be isolated from brain tissue. These preparations have been particularly useful in characterizing the transporters involved in moving solutes into and out of the endothelial cells. Since the endothelial cell is an obligate part of the pathway for passage of polar molecules across the BBB, the ability to isolate microvessels provided the first direct approach to study the cells controlling exchange of molecules across the BBB. During the process of isolation, the astrocytes are stripped away from the capillary walls. A recent review describing the use of microvessels isolated from brain to study cellular characteristics of the BBB is available elsewhere.[10]

When isolated brain microvessels are treated with collagenase or other preparative enzymes, it is possible to separate and grow the endothelial cells in tissue culture. Work in our laboratory by Bowman *et al.*[11] and Dorovini-Zis *et al.*[12] established that primary cultures of brain endothelial cells retain some barrier features. Using either rat-tail collagen or fibronectin as an attachment substrate, brain microvascular endothelial cells form a continuous monolayer. The cells are connected by tight junctions that restrict the intercellular passage of horseradish peroxidase, a protein similar in size to plasma albumin (FIG. 5). The endothelial cells are not fenestrated and contain few pinocytotic vesicles. The tight junctions can be reversibly separated by exposing the monolayers to hypertonic solutions of arabinose and show a dose-response similar to that which opens the BBB *in vivo* after infusion of the same hypertonic solutions

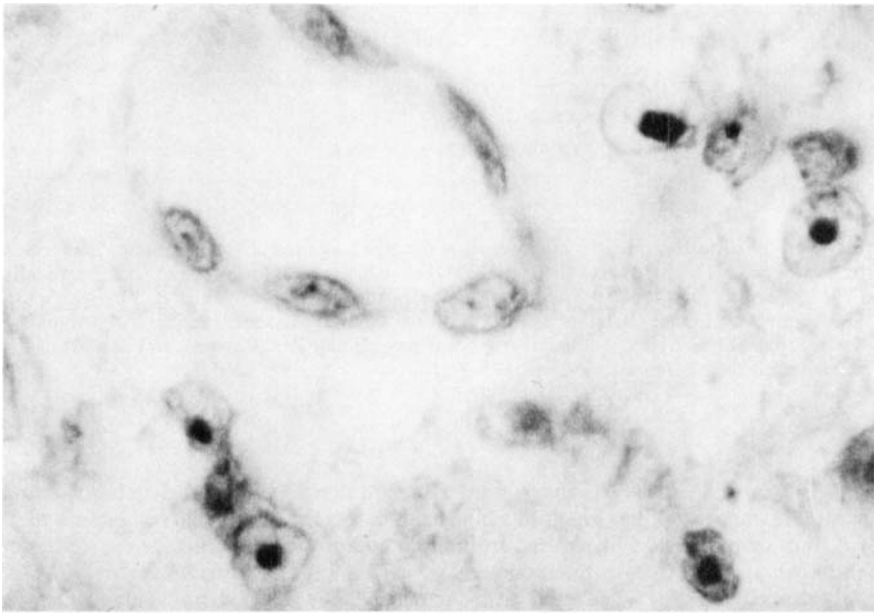

FIGURE 2. Chick endothelial cells vascularizing a graft of quail brain. The nuclei in astrocytes surrounding the microvessel have a distinctive large central nucleolus consistent with their quail origin, whereas the endothelial nuclei have the typical appearance of chick host. Despite the origin of the endothelial cells from the abdominal vasculature, they exhibited the permeability and enzymatic characteristics of the blood-brain barrier.[5] (This photomicrograph was provided by Drs. Stewart and Wiley and is printed with their permission.)

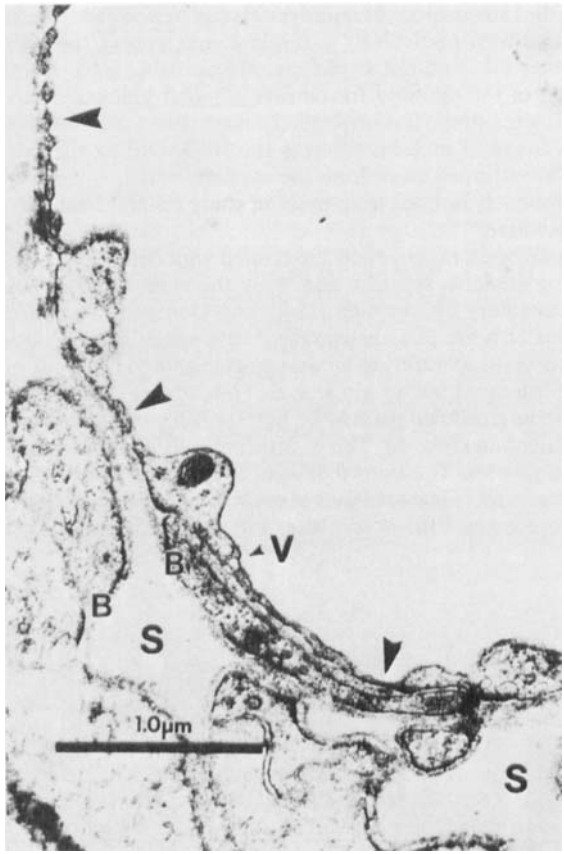

FIGURE 3. Ultrastructure of a microvessel in the area postrema of a mouse brain. This region lacks a blood-brain barrier and the endothelial cell is fenestrated (*arrows*) and contains vesicles (V). The astrocyte in the *lower left corner* is separated from the endothelial cell by spaces (S); the basement membrane (B) on the astrocyte is distinct from the basement membrane surrounding the endothelial cell. (This photomicrograph was provided by Drs. Coomber and Stewart and is printed with their permission.)

into the carotid artery.[13] Using a similar preparation, Audus and Borchardt found saturable transport of large neutral amino acids across the endothelial cell monolayer.[14]

Astrocytes can also be isolated from brain and grown in culture. Some studies of endothelial cell-astrocyte interaction have used a cell line of rat astrocytes (C-6, American Cell Tissue Type) derived from a chemically induced brain tumor. Despite the fact that microvessels growing into implants of C-6 cell tumors *in vivo* are highly permeable and lack BBB characteristics, the C-6 astrocytes *in vitro* were shown to stimulate passaged endothelial cells to exhibit many features typical of normal brain capillaries. In these studies DeBault, Cancilla, and subsequently Beck, and their coworkers isolated an endothelial cell line from mouse brain. These passaged cells when grown as a single cell type did not exhibit barrier features. However, when co-

cultured with C-6 glioma cells, many properties associated with the intact barrier could be identified. These include appearance of γ-glutamyl transpepidase activity in the endothelial cell,[15] evidence for polarity of small neutral amino acid transport,[16] and by cytochemical analysis, induction of sodium-potassium ATPase and alkaline phosphatase.[17] Despite the tumor origin of the glial cells, these studies demonstrate a positive interaction between endothelial cells and astrocytes.

Methods are now available to isolate and culture astrocytes from normal newborn rat brain.[18] These nontransformed cells can be propagated for at least several passages and contain glial fibrillary acidic protein, which provides a useful marker of their astrocytic origin. Using astrocytes from neonatal brain, Tao-Cheng, Nagy, and Brightman examined the effect of co-culture upon the freeze-fracture appearance of tight junctions between brain endothelial cells.[19] They found that endothelial cells prepared from bovine brain no longer formed tight junctions after several cell passages, even though they retained other markers of their endothelial cell origin. However, when the passaged endothelial cells were co-cultured with astrocytes, tight junctions appeared that resembled the complex junctions seen *in situ*. These *in vitro* studies demonstrate the ability of C-6 glioma and neonatal astrocytes to influence the morphology and metabolic activity of cultured brain endothelial cells. The biochemical basis for this interaction is not yet understood.

Brain tissue contains several growth factors as well as insoluble matrix material that may act as signals for the endothelial cell-astrocyte interactions important for formation and maintenance of the BBB. For example, extracts of both immature and mature rat brain have a potent mitogenic effect upon brain endothelial cells in culture.[20]

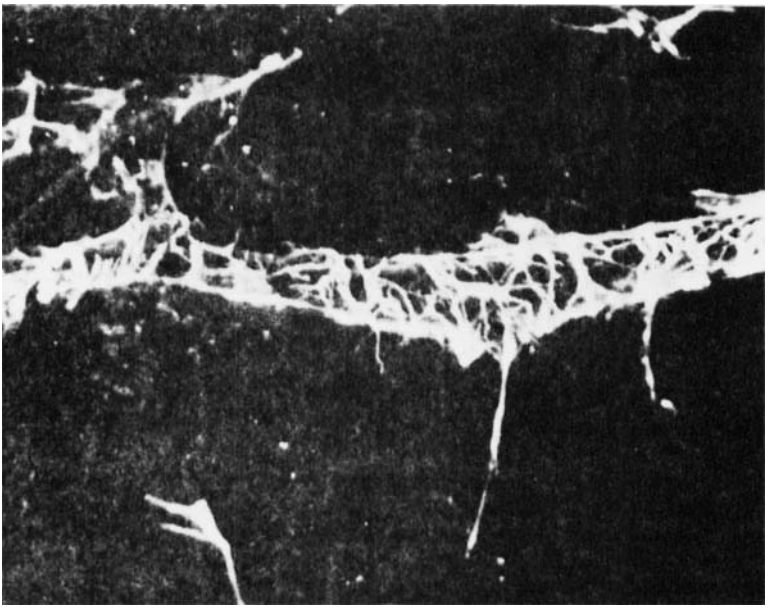

FIGURE 4. Retinal microvessel surrounded by glial processes. Consistent with their barrier features, endothelial cells in the retina have close contact with astrocytes. In the micrograph the astrocytic processes are stained with antibody against glial fibrillary acidic protein. (From Bjorklund and Dahl.[9] Reprinted by permission from the *Journal of Neuroimmunology*.)

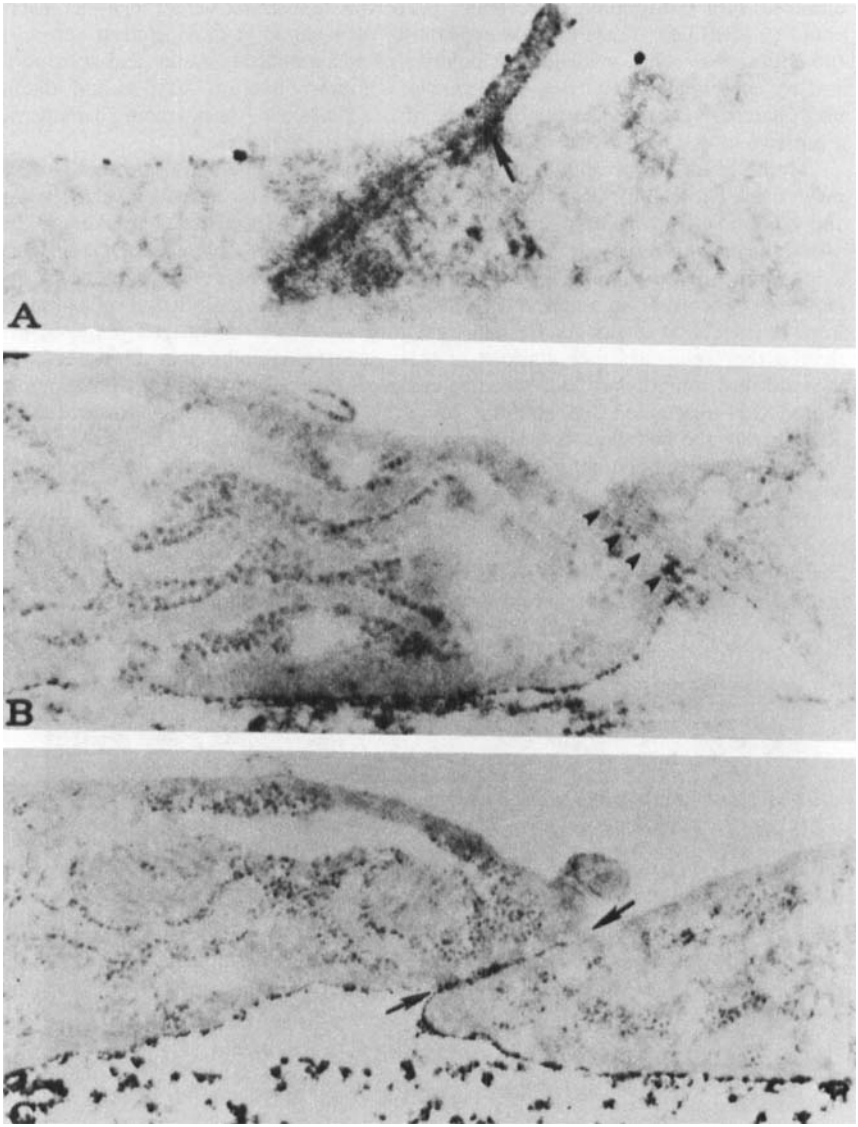

FIGURE 5. Formation of an *in vitro* barrier by brain endothelial cells: effect of hypertonic arabinose. Transcellular movement of the protein tracer horseradish peroxidase (HRP) was monitored by appearance of its reaction product. (**A:**) Control; 5 minutes after exposure to HRP, the cleft between two adjacent endothelial cells contains no tracer. The HRP forms small discrete patches on the surface of the cells, but does not penetrate beyond the first tight junction (*arrow*). The basal surface is free of HRP. ×82,500. (**B:**) Two-minute exposure to 1.6 M arabinose. The extracellular spaces between successive tight junctions (*arrowheads*) contain deposits of HRP. The basal surfaces are labeled with small amounts of the tracer. ×66,000. (**C:**) Five-minute exposure to 1.6 M arabinose HRP penetrates throughout the entire length of an interendothelial cleft (*between arrows*) and forms dense deposits on the basal surface of the cells. ×39,000. (From Dorovini-Zis *et al.*[12] Reprinted by permission from *Brain Research.*)

It is possible to purify this activity from brain, and on the basis of affinity for heparin, molecular weight, and isoelectric constants, it has homology with fibroblast growth factor[21] and endothelial cell growth factor.[22] In addition to having a mitogenic effect upon brain endothelial cells, these factors can induce differentiation in cultured astrocytes, producing a transition from a spherical cell to one with multiple extended processes.[23]

The cellular origins of the various brain factors important in signaling endothelial cell and astrocytic growth, differentiation, and interaction are just now being investigated. It seems likely that signals are operating in both directions. For example, endothelial cells can produce platelet-derived growth factor,[24] for which receptors linked to proliferation exist on the astrocyte[25] but not on the endothelial cell, whereas

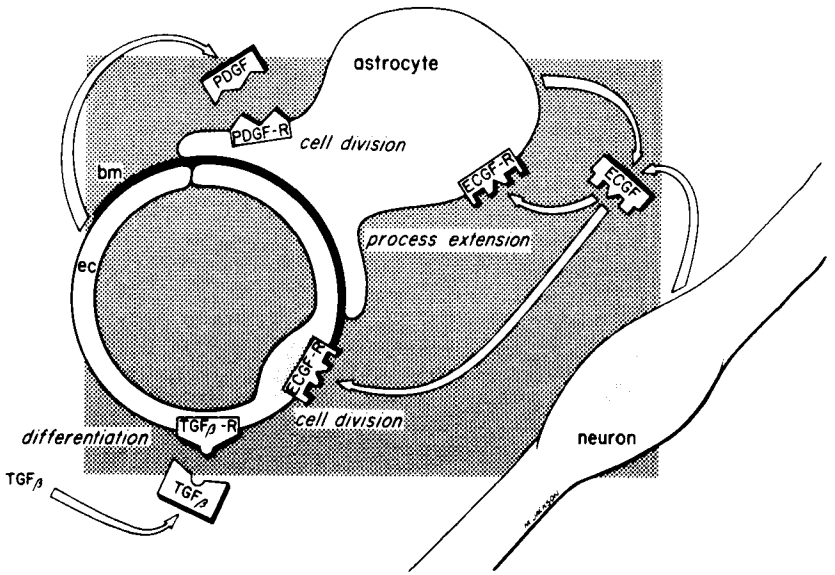

FIGURE 6. A diagram illustrating possible interactions between brain endothelial cells (ec) and astrocytes. Receptors for growth factors important in regulation of proliferation and differentiation are found when these cells are maintained in culture. Platelet-derived growth factor (PDGF) can be synthesized by endothelial cells and has a mitogenic effect upon glial cells. Endothelial cell growth factors (ECGF) are synthesized by neurons or astrocytes and stimulate endothelial cells to proliferate and glial cells to differentiate. Transforming growth factor β (TGFβ) blocks the mitogenic effect of other growth factors and may cause endothelial cells to differentiate. Finally, basement membrane (bm) formed by the endothelial cells and astrocytes may influence the behavior of both cell types.

astrocytes may produce factors such as endothelial cell growth factor, for which receptors exist on both the endothelial cell (linked to proliferation) and the astrocyte itself (linked to differentiation).[23] Transforming growth factor-β, a newly discovered peptide mediator found in many tissues,[26] is able to counter the mitogenic effect of endothelial cell growth factor, presumably by causing differentiation of the endothelial cell and loss of its responsiveness to endothelial cell growth factor.[27] Extracellular matrix (i.e. basement membrane) also appears important in cell interactions, either as a potential signal itself or as a permissive factor for response to the soluble peptides. FIGURE 6 is a hypothetical model showing how some of these signals might coordinate the interaction of endothelial cells and astrocytes to form the BBB.

Considerable information is available concerning the many special anatomic, physiologic, and biochemical features that produce the BBB. The vulnerability of this complex system to injury during different stages of development is also well characterized. Investigations now in progress at a cell and molecular level should extend our understanding of the BBB and encourage development of new techniques to manipulate the barrier for treatment of the neurologic diseases that either damage brain microvessels or require selective bypass to deliver drugs to specific brain regions.

SUMMARY

Microvascular endothelial cells in the brain have a number of special properties that underlie formation of the blood-brain barrier (BBB) and contribute to control of the neuronal microenvironment. Evidence from transplantation experiments indicates that signals arising within brain rather than a programmed commitment of the endothelial cells are responsible for the expression of blood-brain barrier properties. The close anatomic relationship between brain endothelial cells and the foot processes of astrocytes suggests a role for astrocytes as a source of the differentiation signals. It is now possible to isolate and separately culture populations of brain-derived endothelial cells and astrocytes. When the two cell types are grown together, a characteristic morphologic organization occurs that is associated with induction of enzymes and tight junctions similar to those found *in vivo*. Endothelial cells and astrocytes in culture differ in their production of and response to specific polypeptide growth factors. These findings provide the basis for a model of endothelial cell-astrocyte interaction that may explain several aspects of BBB behavior.

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