

Cultured Brain Microvessel Endothelial Cells as In Vitro Models of the Blood-Brain Barrier

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INTRODUCTION

A central nervous system (CNS) site of action generally requires that, following entry into the systemic circulation, substances must interact with the blood-brain barrier (BBB) en route to brain tissue targets. Many substances with CNS activity, including drugs of abuse, cross the BBB by simple passive diffusion (Oldendorf 1974; Levin 1980; Cornford et al. 1982). Accordingly, the BBB plays an important role in regulating access of drugs of abuse to the brain.

Recent evidence suggests that some drugs of abuse may alter BBB permeability characteristics. The permeability-altering activity of drugs of abuse at the BBB has been reported in chronic amphetamine intoxication (Rakic et al. 1989), acute ethanol exposure (Gulati et al. 1985), and opiate treatment (Baba et al. 1988). Ethanol addiction also has been reported to alter opiate interactions with the transport systems of the BBB (Banks and Kastin 1989). On the basis of these reports, one might infer that interactions of drugs of abuse with the BBB may have implications for the observed pharmacological and toxicological manifestations of exposure to these substances. These implications remain to be explored and confirmed. Consequently, the development of a fundamental understanding of the permeability and metabolic features of the BBB may contribute to a broader knowledge of the activity of drugs of abuse in the CNS.

The complexity of the whole animal as an experimental model has certain limitations when specific events at the cellular or molecular level are examined. In vitro models (i.e., brain microvessel suspensions or cultured brain endothelial cells) offer a promising alternative for the investigation of cellular- or molecular-level characteristics of the BBB (Audus et al. 1990; Audus and Borchardt 1987). The purpose of this chapter is to describe studies of the transport

and metabolism characteristics of the BBB that employ cultured brain microvessel endothelial cells as in vitro BBB models. This chapter does not describe isolated suspensions of brain microvessel endothelial cells as in vitro models, since this subject has been reviewed by Betz and Goldstein (1984) and more recently by Takakura and coworkers (1991 a). In addition, this chapter does not describe how cultured brain microvessel endothelial cells have been used to study the development and regulation of the BBB and pathological changes in the BBB, since these topics are discussed in other chapters in this monograph and have been reviewed previously by Takakura and coworkers (1991 a).

ESTABLISHMENT AND CHARACTERIZATION OF CULTURED BRAIN MICROVESSEL ENDOTHELIAL CELLS AS IN VITRO BBB MODELS

Since Panula and colleagues (1978) demonstrated that rat brain microvessel endothelial cells could be maintained in tissue culture, various kinds of both primary and passaged cultures of isolated brain microvessel endothelial cells have been established from mouse, rat, bovine, human, canine, and porcine brain (for a review, see Audus et al. 1990).

In general, either enzymatic or mechanical dispersal, or a combination of both techniques, followed by either filtration or centrifugation steps are employed to isolate a homogeneous population of brain microvessel endothelial cells from the extremely heterogeneous population of cells found in brain tissues. For example, isolation of a viable, homogeneous population of brain capillary endothelial cells for establishment of a tissue culture system is accomplished by a two-step enzymatic digestion with dispase and a dispase/collagenase mixture of cerebral gray matter and successive centrifugation over dextran and percoll gradients (Bowman et al. 1983; Audus and Borchardt 1986a, 1987). In the authors' laboratories, primary cultures of bovine brain microvessel endothelial cell monolayers have been shown to retain morphological and biochemical properties typical of the BBB in vivo. These include tight junctions, attenuated pinocytosis, lack of fenestra, and the presence of proteins (e.g., γ -glutamyl transpeptidase, alkaline phosphatase, angiotensin-converting enzyme, factor VIII antigen) enriched in the endothelium of the BBB (Audus and Borchardt 1986a, 1987).

Basically, two types of experimental systems have been employed for study of transport phenomena using cultured brain microvessel endothelial cells: The first is the uptake study, and the second involves a transcellular transport study. The former system uses microvessel endothelial cell monolayers grown in culture dishes (Scriba and Borchardt 1989a, 1989b). Uptake experiments can be performed also using cerebral microvessel endothelial cells cultured on

microcarriers (e.g., dextran beads) (Bottaro et al. 1986; Kempinski et al. 1987). These systems allow examination of the first step of the transport process, that is, the uptake of the solutes into the brain capillary cells from the luminal side. The most sophisticated in vitro system for transport studies consists of cultured brain microvessel endothelial cell monolayers grown on microporous membranes. Transport studies can then be conducted in side-by-side diffusion cells or the cell-insert system (Audus et al. 1990). These systems afford an opportunity to look at bidirectional transendothelial movement (transfer from brain to blood and that from blood to brain) of solutes across the BBB in vitro since, at least for primary cultures of bovine microvessel endothelial cells, the cells are shown to be morphologically and functionally polarized in terms of ricin recycling (Raub and Audus 1990), transferrin transport (Newton and Raub 1988), and angiotensin II (Ang II) responsiveness (Guillot and Audus 1990, 1991a). The purity of these monolayers is greater than 95 percent (Gurllot et al. 1990). and the transelectrical resistance is $160 \pm 18 \text{ ohm/cm}^2$ (T.J. Raub, unpublished results).

Although bovine brain microvessel endothelial monolayers retain tight junctions, the tight junctions are not identical to those observed in vivo with regard to extent and complexity. This leads to higher leakiness in vitro than in vivo, which is a disadvantage in the use of cultured endothelial cells alone to study transcellular transport.

More impermeable monolayers of cultured brain microvessel endothelial cells may be developed in the future by exploiting the regulatory role of astrocytes in endothelial cell growth and development. It is widely accepted that the brain microvessel endothelial cells in vivo form the structural and functional bases of the BBB; however, some of the BBB functions are known to be regulated by astrocytes, which encircle the microvessel endothelial cells with their foot processes in vivo. Similar regulatory effects of astrocytes on the permeability properties of cultured endothelial cells in vitro have been reported. For example, Cancilla and DeBault (1983) demonstrated that contact with glial cells (a rat line of neoplastic astrocytes designated as C6 glioma cells) or exposure to glial-conditioned media enhances neutral amino acid uptake by passaged mouse cerebral endothelial cells in culture. Conditioned media prepared from astrocytes and C6 glioma cells have been shown to stimulate glucose uptake in passaged mouse cerebral endothelial cells (Maxwell et al. 1989) and in primary cultures of bovine brain microvessel endothelial cells (Takakura et al. 1991 b). Conditioned media from rat astrocytes or C6 glioma cells also have been shown to decrease the permeability of various solutes across monolayers of bovine brain microvessel endothelial cells grown on microporous membranes (Trammel and Borchardt 1989; Raub et al. 1989). Recently, Dehouck and colleagues (1990) observed decreases in permeability

and increases in electrical resistance using an *in vitro* model system consisting of passaged brain microvessel endothelial cells grown on one side of a filter and astrocytes on the other side of the filter.

UPTAKE AND TRANSPORT STUDIES

Nutrients

Since glucose is an important source of energy for the brain, the mechanism of glucose transport across the BBB has been particularly well studied *in vivo* (Pardridge 1983). These studies support the concept that glucose is transported through the cerebral microvessel endothelium mainly by the mechanism of carrier-mediated facilitated diffusion. Glucose transport characteristics also were studied in cultured brain microvessel endothelial cells. For example, Vinters and coworkers (1985) demonstrated that the properties of 3-O-methylglucose (3MG) and 2-deoxyglucose uptake in established lines of cultured mouse cerebral microvessel endothelium are similar to those observed *in vivo*. Recently, characteristics of both uptake and transendothelial transport of 3MG were studied in primary cultures of bovine brain microvessel endothelial cells (Takakura et al. 1991c). The uptake characteristics of 3MG were shown to be identical to those observed *in vivo* and *in vitro* using isolated capillaries. Transport rates from the luminal to abluminal side and from the abluminal to luminal side, measured across the brain microvessel endothelial cells grown onto polycarbonate membranes, were nearly identical, suggesting symmetrical glucose transport across the monolayer of endothelial cells.

The passage of amino acids across the BBB was found to be saturable and stereospecific *in vivo* (Oldendorf 1971). Cancilla and DeBault (1983) showed the presence of A- and L-systems for uptake of neutral amino acids in cultured mouse cerebral endothelial cells. Using primary cultures of bovine brain microvessel endothelial cell monolayers grown onto microporous membranes, transport of a large neutral amino acid, leucine, was shown to be saturable, bidirectional, competitive with other amino acids, and energy independent (Audus and Borchartd 1986b). The kinetic parameters for leucine transport appear to be in good agreement with true kinetic parameters of the *in vivo* BBB. The transport of several amino acid drugs-including baclofen (van Bree et al. 1988), α -methyl dopa (Chastain and Borchartd 1989), and acivicin (Chastain and Borchartd 1990)-by the amino acid carrier also has been explored in this system.

Because of a limited capability for *de novo* synthesis of choline, an important precursor to acetylcholine and phospholipid, the brain must depend on the

blood for its supply of choline. Cornford and colleagues (1978) demonstrated the saturability of brain uptake of choline after intracarotid injection in rats. Recently, Estrada and associates (1990) studied choline uptake by bovine cerebral capillary endothelial cells in culture, demonstrating that these cells were able to incorporate choline by a carrier-mediated mechanism. The choline uptake was temperature dependent and was inhibited by choline analogs but was not affected by ouabain or dinitrophenol. Transendothelial transport of choline in cultured bovine brain microvessel endothelial cells has been characterized (Trammel and Borchardt 1987); it has been shown that the transport is saturable and is insensitive to ouabain and sodium azide, suggesting a facilitated diffusion mechanism.

Since the brain is one of the most active tissues for carrying out nucleotide and nucleic acid synthesis, the transport of nucleosides and purine bases across the BBB has been of interest. By employing the intracarotid injection technique in rats, Cornford and Oldendorf (1975) demonstrated the presence of two independent carrier systems for nucleic precursors: a nucleoside carrier and a purine base carrier. Beck and coworkers (1983) have described a carrier-mediated uptake of adenosine into mouse cerebral capillary endothelial cells in tissue culture. Characterization of the nucleoside uptake (e.g., adenosine, thymidine) into monolayers of cultured bovine brain endothelial cells also was studied, and the results suggested the presence of a carrier-mediated uptake of adenosine and thymidine (Shah and Borchardt 1989). Adenosine uptake is primarily via the carrier-mediated pathway, whereas thymidine enters by both a carrier-mediated and a passive pathway. Both nucleosides are extensively metabolized (e.g., phosphorylated) in the cultured bovine endothelial cells.

Peptides and Proteins

Although the physiological role of insulin in the regulation of brain functions remains to be elucidated, van Houten and Posner (1979) revealed that blood vessels throughout the CNS of the rat bind plasma insulin rapidly and with considerable specificity *in vivo*. The binding and receptor-mediated endocytosis of insulin and insulin-like growth factor I (IGF-I) also were studied using cultured bovine brain microvessel endothelial cells (Keller and Borchardt 1987; Keller et al. 1988). Rosenfeld and colleagues (1987) showed the similarity between the characteristics of the specific receptors for IGF-I and insulin-like growth factor II in the cultured bovine brain microvessel endothelial cells and in isolated rat brain microvessels.

Using monoclonal antibodies to the transferrin receptors, Jefferies and coworkers (1984) first reported that rat and human brain capillary endothelia

have receptors for transferrin, an iron-transport protein in the circulation. Newton and Raub (1988) characterized the transferrin receptor in primary cultures of brain capillary endothelial cells, indicating saturable binding and internalization. They also demonstrated the transcytosis and the polarized efflux of transferrin using brain microvessel endothelial cells grown onto polycarbonate filters; these findings are in good agreement with the *in vivo* observations of Fishman and coworkers (1987) and Banks and coworkers (1988).

Atrial natriuretic factor (ANF), which is a 28-amino acid peptide produced by cardiac myocytes and released in response to increases in atrial pressure, expresses its natriuretic, diuretic, and hypotensive effects by acting on renal and vascular tissues. A specific receptor for ANF also was identified using primary cultures of bovine brain capillary endothelial cells (Smith et al. 1988). The binding of ANF was specific, saturable, and reversible. ANF also was shown to be rapidly internalized by a temperature-dependent process. A specific receptor for brain natriuretic peptide (BNP), which was found in the brain and has similarity to ANF in its structure and biological activities (Sudoh et al. 1988), was identified recently in primary cultures of bovine brain microvessel endothelial cells (M. Fukuta, M. Nonomura, Y. Takakura, and R.T. Borchardt, unpublished results). These studies suggested that ANF and BNP share the same receptor in bovine brain microvessel endothelium.

Speth and Harik (1985) reported that Ang II binds to microvessels isolated from dog brain in a specific, saturable, and reversible manner and with high affinity. It was suggested that specific Ang II receptor-binding sites are present in brain microvessels and that these receptors may have an important role in regulating the microcirculation of the brain. Work in the authors' laboratories indicates that bovine brain microvessel endothelial cell monolayers retain a high-affinity Ang II binding site that can be competed for by Ang II peptides (Guillot and Audus 1991b).

Recently, the transport of leu-enkephalin across the BBB by a carrier-mediated mechanism has been demonstrated *in vivo* (Zlokovic et al. 1987). In addition, Thompson and Audus (1989) showed that leu-enkephalin transfer across monolayers of brain microvessel endothelial cells occurs at a relatively high rate, which is consistent with a facilitated diffusion mechanism.

Vasopressin transport across the BBB has been examined with primary cultures of brain microvessel endothelial monolayers (Reardon and Audus 1989). Results suggest the existence of facilitated transport of the peptide from the abluminal to the luminal side of the monolayers. This finding is consistent with the *in vivo* characterization of a vasopressin BBB transport system (Banks

et al. 1987). Van Bree and colleagues (1989) also have studied transport of vasopressin using an in vitro system and suggested that no carrier mediation is involved over a higher concentration range. Confirmatory studies are required to clarify the transport mechanism in detail.

Raeissi and Audus (1989) characterized the BBB permeability to delta sleep-inducing peptide (DSIP) in cultured microvessel endothelial cells. The results support in vivo observations indicating that intact DSIP crosses the BBB by simple transmembrane diffusion (Banks and Kastin 1987). Recently, Zlokovic and coworkers (1989) presented evidence in support of a facilitative BBB carrier for DSIP in vivo. Further work is ongoing concerning the solution structure of DSIP, which may help to explain its ability to readily penetrate the BBB (Audus and Manning 1990).

Native albumin, which is an acidic protein in plasma, is considered to pass through the BBB very slowly (Pardridge et al. 1985). However, increased BBB uptake and transport recently has been reported when it is chemically modified. Kumagai and colleagues (1987) demonstrated the enhanced binding and adsorptive-mediated endocytosis of cationized albumin by isolated bovine brain capillaries. The binding was saturable and inhibited by other polycations (e.g., protamine, protamine sulfate, and polylysine). Similar results have been reported for other types of polycationic proteins, including cationized immunoglobulin G (Triguero et al. 1989) and histone (Pardridge et al. 1989). Smith and Borchardt (1989) studied the binding, uptake, and transcellular transport of bovine serum albumin (BSA), cationized BSA (cBSA), and glycosylated BSA (gBSA) in cultured bovine brain microvessel endothelial cells. This study demonstrated that cBSA and gBSA bind to the cells specifically and are transported by an adsorptive-phase endocytotic mechanism. The use of cationized albumin in directed delivery of peptides through the BBB was examined by coupling β -endorphin to cationized albumin via a disulfide linkage (Kumagai et al. 1987).

Drugs

In contrast to water-soluble nutrients and peptides, which are transported by specific carrier- or receptor-mediated systems as mentioned above, most water-soluble solutes, including drugs, pass through the BBB by a passive diffusion mechanism. From in vivo studies, it has been well established that the permeability of these molecules across the BBB depends directly on their lipophilicity and inversely on their molecular size (Oldendorf 1974; Levin 1980; Cornford et al. 1982). Rim and coworkers (1986) and Shah and coworkers (1989) established a positive correlation between lipid solubility of a drug and its permeability across bovine brain microvessel endothelial monolayers grown

onto microporous membranes. As mentioned earlier in this chapter, brain microvessel endothelial monolayers are leakier than the BBB in vivo. Therefore, this leakiness in the monolayers must be corrected for by using impermeant marker molecules (e.g., sucrose, fluorescein, inulin, and dextran) when drug transport studies are conducted (Shah et al. 1989).

IN VITRO STUDIES ON BBB METABOLISM

Several different enzyme systems have been established in primary cultures of bovine brain microvessel endothelial cell monolayers. Both biochemical and histochemical techniques have been used to follow the expression of marker and catecholamine-metabolizing enzymes associated with these monolayers. For instance, enzyme systems considered markers for the BBB, γ -glutamyl transpeptidase and alkaline phosphatase, and for the endothelium, angiotensin-converting enzyme, are retained in the monolayers (Baranczyk-Kuzma et al. 1986). In addition, the brain microvessel endothelial cell monolayers retain the ability to degrade catecholamines through expression of monoamine oxidases A and B, catechol O-methyltransferase, and phenol sulfotransferase (Baranczyk-Kuzma et al. 1986, 1989a; Scriba and Borchardt 1989a, 1989b). Using monolayers of bovine brain microvessel endothelial cells, the metabolism of catecholamine esters (Scriba and Borchardt 1989a) and of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) has been studied (Scriba and Borchardt 1989b).

The lysosomal compartment of endothelia is an important component of the transcytosis of macromolecules (Simionescu 1979). Current interest by the pharmaceutical industry in the delivery of therapeutic biotechnology products to the CNS (Audus et al. 1990) suggests a need for considering the activity of the lysosomal compartment of the BBB. However, little work has focused on lysosomal or acid hydrolase compartments within the BBB in vivo. By both quantitative and qualitative measures, bovine brain microvessel endothelial cells possess a lysosomal compartment that expresses typical acid hydrolases, acid phosphatase, β -galactose, and sulfatases (Baranczyk-Kuzma et al. 1989b). Peptidase activity has not yet been examined in the lysosomal fraction; however, aminopeptidase activity is characteristic of membrane and cytosolic fractions of the cells (Baranczyk-Kuzma and Audus 1987). Corresponding quantitative studies in in vivo enzyme systems and compartments have not been performed and may not be feasible. Therefore, the in vitro model may be a reasonable system in which to initiate further studies on the contributions of the lysosomal compartment to the fate of endocytosed peptides and proteins at the BBB (Baranczyk-Kuzma et al. 1989b).

CONCLUSIONS

The development of in vitro BBB models consisting of cultured brain microvessel endothelial cells has made possible the study of BBB transport and metabolism phenomena at the cellular level. Basic characteristics of BBB transport and metabolism of endogenous and exogenous solutes and their biochemical, pharmacological, ontogenic, and pathological regulation mechanisms have been investigated. This information has led not only to a better understanding of BBB transport but also to the construction of strategies for improving drug delivery to the CNS for diagnosis and therapeutics. To elucidate the complexity of BBB transport, in vivo studies are always necessary at some point; however, in vitro systems can be useful complements to in vivo systems. The tissue culture systems seem to be especially important in the clarification of cellular, biochemical, and molecular features of BBB transport. Appropriate systems should be selected or combined, depending on the purpose of the investigation.

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