Changes of permeability in response to histamine and tumor necrosis factor-α and their possible mechanism of action on an *in vitro* model of the blood-brain barrier

Summary of Ph.D. thesis

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INTRODUCTION

The most important function of the blood-brain barrier (BBB) is the maintenance of the homeostasis of the central nervous system (CNS) environment by separating the brain from the systemic blood circulation. Under physiological conditions, the BBB not only regulates the entry of endogenous compounds, nutrients and drugs into the brain, but also diminishes cellular infiltration compared to peripheral organs. The barrier function of the BBB can change dramatically during various diseases e.g. hypertension, hyperosmolarity, CNS tumors, cerebral ischemia, seizures, cerebral inflammation, and neurodegenerative diseases. It was demonstrated in electron microscopic studies that the cerebral endothelial cells (CECs) forming the BBB have tight intercellular junctions and a relative lack of pinocytotic vesicles. These structural and functional barrier characteristics of cerebral endothelium are determined by the surrounding central nervous tissue, especially by the astrocytes.

Isolation of cerebral microvessels by the method of Joó and Karnushina (1973) and the observation that endothelial cells could resist damage during isolation and remained viable, led to a new generation of *in vitro* BBB model systems. The tissue culture approach offered an easy, reproducible and mass-production method, where CECs maintain both common endothelial and specific BBB markers in long-term cultures. Since then, a considerable amount of new information has been gained concerning the biochemical and physiological properties of the BBB in cultured CECs. Monolayers of endothelial cells derived from cerebral capillaries, cultured and grown on inserts proved to be excellent objects for studying directly BBB permeability. Since astrocytes have been shown to be able to upregulate and

maintain certain BBB characteristics of CECs, permeability studies were carried out on brain capillary endothelial cells cocultured with astrocytes.

Histamine is a known mediator of increased vascular permeability and oedema formation in peripheral tissues. Histamine regulates the cerebral blood flow and it is also involved in the development of brain oedema. Presence of histamine H_2-receptors linked to adenylate cyclase both on luminal and abluminal membranes of the cerebral endothelium suggested that vasoactive mediators had receptors on CECs and might induce macromolecular transport. In the CNS, three distinct histamine pools exist: in neurons, in perivascular mast cells and in capillaries, and high amount of histamine can be released under pathological conditions. The level of histamine metabolizing enzyme, histamine-N-methyltransferase, is very low in CECs, so we assumed that the cerebral endothelium should possess a histamine uptake and release system.

Cytokines are important messenger molecules which mediate the communication between the immune system and brain through BBB. Tumor necrosis factor-α (TNF-α), a proinflammatory cytokine, is produced in the brain in response to infectious, immune, toxic, traumatic and ischemic stimuli and has been shown to be particularly involved in the pathogenesis of brain injuries during CNS infections and neurodegenerative diseases. Wherever TNF-α is released, either from circulating blood cells or from intracerebrally located microglia, smooth muscle cells or astrocytes, it can reach the CECs. According to previous *in vivo* studies, TNF-α administered into the cerebrospinal fluid, induced the opening of the BBB for sodium fluorescein, but no change was found in the permeability of the BBB for albumin after intravenous administration. It remained an open question what effects, if any,

TNF-α could exert on the cultured CECs if the luminal surface of the BBB was exposed to the cytokine.

Second messenger molecules are important regulators of the BBB functions both under physiological and pathological conditions. The synthesizing and degrading enzymes of 3',5'-cyclic adenosine monophosphate (cAMP) were detected in CECs indicating that cAMP is produced and metabolized locally. A lipid soluble derivative of cAMP, dibutyryl-cAMP, administered into the blood circulation *in vivo* could increase the albumin penetration in brain microvessels. On the other hand, a correlation was reported to exist between the activation of adenylate cyclase in CECs and the induction of transcapillary albumin transport during hypoxia. In contrary, no increase of permeability to trypan blue-albumin complex was found in cultured CECs when the endothelial synthesis of cAMP was stimulated by forskolin. Moreover, an elevated level of cAMP resulted in a rapid increase in transendothelial electrical resistance in the presence of astrocyte-conditioned medium and a significant reduction in sucrose permeability in cultured CEC monolayers. The influence of short term elevation of the intraendothelial cAMP level on the transport of small molecular weight substances has been studied in our experiments.

There has been little work on second messenger modulated kinases in the function of CECs. One important candidate for attention is the calcium/calmodulin-stimulated protein kinase II (CaM PK-II), also called multifunctional calcium/calmodulin-stimulated protein kinase because of its broad substrate specificity. Transcripts for the α- and β-subunits are primarily found in brain, whereas transcripts for the γ- and δ-subunits are present in various tissues, among them in aorta. CaM PK-II is involved in the regulation of the synthesis and release

of neurotransmitters, motility and cell shape, amino acid, lipid and cyclic nucleotide metabolism.

OBJECTIVES

(i) Histamine has been shown in previous *in vivo* studies to increase the permeation of circulating albumin through the BBB, supposedly by the induction of transcytosis in the CECs rather than modifying the permeability properties of the tight junctions. The possible changes in permeability of cultured CECs on the effect of histamine with three tracers of different molecular weight have been investigated.

(ii) A detailed study to clarify the direct effect of TNF-α applied to the luminal membrane of CECs on transendothelial permeability, which may be important in relation to the reactions of BBB during immunological and inflammatory processes, was done.

(iii) There were controversial data reported in the literature on the role of cAMP in the regulation of permeability. So, we re-investigated the issue with direct measurements on the *in vitro* reconstituted BBB model.

(iv) It was not known in the literature if the CaM PK-II, an enzyme being able to respond to elevations of intracellular calcium levels, was present in the CECs.

We decided to check on this apparently important aspect with immunohistochemical detection and with *in situ* hybridization techniques.

MATERIALS AND METHODS

Primary rat cerebral endothelial cell (RCEC) cultures were prepared from the cortical grey matter of the brain of two-week-old Sprague-Dawley CFY rats by enzymic digestions using collagenase and collagenase-dispase. In order to separate microvessels and microvessel fragments from other cells of the brain tissue gradient centrifugations of the homogenate were performed using 25 % (w/v) bovine serum albumin (BSA) solution and a continuous 33 % (v/v) Percoll gradient. Endothelial cell clusters were collected at the end of the procedure, seeded onto rat tail collagen-coated dishes or cell culture inserts and cultured in a medium containing 20% heat inactivated fetal calf serum (FCS). Factor VIII.-related antigen immuno-histochemistry, lectin-binding by peroxidase-conjugated *Bandeiraea simlicifolia* lectin-I B4, alkaline phosphatase histochemistry and transmission electron microscopy were used for the characterization of the cultures.

To obtain bovine brain capillary endothelial cells (BBCECs) individual colonies of primary endothelial cells emerging from cerebral capillaries were subcloned by microtrypsinization. Culture medium for BBCECs contained 10% FCS, 10% horse serum, 1 ng/ml basic fibroblast growth factor. Newborn rat astrocytes, prepared from 2-day-old rat cortex, were seeded on 6-well plates, kept in a medium containing 10 % FCS for 6-8 weeks before starting the coculture. After 4-6 passages

BBCECs were seeded on rat tail collagen coated cell culture inserts, which were placed into the astrocytes-containing wells and cocultured for at least 12 days. Bovine aortic endothelial cell cultures, prepared from aortic arch were also used between passage 4-6 and grown in the same medium as BBCEC.

For the uptake and efflux studies RCECs were incubated with low (0.01-0.50 μM) concentrations of [^{3}H]-histamine. Monolayers on cell culture inserts were used to determine the uptake and efflux rates of histamine through the abluminal membrane of the cells.

To measure transendothelial transport of BBCEC monolayers [^{14}C]-sucrose (m.w. 342 Da), [^{3}H]-inulin (m.w. 5 kDa) and Evans' blue dye-BSA complex (EBA, m.w. 67 kDa) were used. The flux of the markers was expressed as microliters of tracer diffusing from the luminal to the abluminal compartments. Different concentrations of mannitol, histamine, TNF-α and cAMP analogues were administered to the luminal compartment of the inserts during the permeability tests. Statistical analysis was performed using the Student's *t*-tests or Friedman's repeated measure analysis of variance on ranks followed by the Student-Newman-Keuls test.

For F-actin localization, cells were stained by bodipy-phallacidin and viewed for optical sectioning with a Leica confocal laser scanning microscope.

To detect the presence of CaM-PK II in RCECs *in situ* hybridization with specific oligonucleotide probes, immunocytochemistry and Western blot analysis with monoclonal antibodies specific for α- and β-subunits of the kinase and protein phosphorylation experiments were done.

RESULTS AND DISCUSSION

(i) Luminal application of histamine (10^{-5} and 10^{-4} M) increased the permeability of the BBB for albumin, but not for markers with smaller molecular weights, known as markers of paracellular route. Our *in vitro* data may suggest a selective induction of transcellular passage of albumin through the BBB. These observations are in accordance with *in vivo* data indicating increased pinocytotic activity after *intracarotid* injection of histamine. A rapid and high affinity uptake of histamine into rat cerebral endothelium and a rapid efflux of the amine from these cells towards the luminal compartment were found indicating the presence of a histamine-specific carrier in both the luminal and the abluminal membranes of RCECs with an asymmetrical function on the abluminal (brain) side. Recently on a model of asphyxiated newborn pigs a rise of histamine content in the venous blood, an early increase in histamine content of CSF, and a late increase in the histamine content of brain microvessels have been demonstrated. Taken together *in vitro* and *in vivo* data, it may be concluded that in cases of ischemic challenges, CECs can not only accumulate but also release histamine towards the blood circulation and thereby take part actively in the elimination of histamine from the brain extracellular space.

(ii) Exposure of TNF-α to the luminal membrane of BBCEC monolayers caused no significant change in either sucrose or inulin flux up to 4 h, *early phase*, except for a decrease in sucrose transport 2 and 4 h after the beginning of the treatment with 500 U/ml TNF-α. On the other hand, 16 h after the 1-h challenge, *delayed phase*, TNF-α induced a significant increase of permeability for both

markers. TNF-α led to the formation of stress fibers *via* intracytoplasmic reorganization of F-actin filaments without detectable cell retraction.

(iii) Our data confirm the validity of previous findings obtained from *in vitro* culture systems and indicate that cAMP can rapidly reduce sucrose and inulin flux through BBCEC monolayers, i.e. after a 1-h exposure. It was previously shown that a 1 h addition of a water-soluble analogue of cAMP in the presence of phosphodiesterase inhibitor resulted in a two-to-threefold increase in cAMP levels in CECs. In our study, intraendothelial elevation of cAMP was able to reduce the transport of small molecular weight substances even through monolayers not cocultured *in vitro* with astrocytes. This observation indicates that BBCEC monolayers alone can provide a certain permeability barrier to the tracers studied, but astrocytic influences can further strengthen the tightness of the intercellular junctions.

(iv) The presence of the α-subunit of CaM-PK II in RCECs was confirmed by *in situ* hybridization histochemistry, immunohistochemistry and Western blot techniques, while the β-subunit of the enzyme was barely detectable using these methods. The calmodulin stimulated phosphorylation of the α-subunit was also demonstrated. Since an 19 amino acid long synthetic peptide from the N-terminal end of the CaM-PK II inhibiting the enzyme was found to open diffusely the BBB of the newborn pig in a previous study, a role for CaM-PK II in the regulation of BBB permeability in physiological as well as pathological conditions can be hypothesized.

PUBLICATIONS

Full papers in international journals:

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1. **Deli MA**, Dung NTK, Joó F: Isolation and culture of endothelial cells from cortical microvessels of the rat and the piglet. *Drug transport across the blood-brain barrier (BBB): new experimental strategy. An overview of Endothelial Cell Culture and Microdialysis Techniques as Tools to Study BBB transport*, de Boer ABG and Sutanto W (eds), BIOMED-Concerted Action, 1994, Leiden, pp. 175-188

2. **Deli MA**, Szabó ACs, Dung NTK, Joó F: In vitro endothelial cell culture - Rat: (Immuno)Histochemical and Electron Microscopy detections. *Drug transport across the blood-brain barrier: in vivo and in vitro techniques*, de Boer ABG and Sutanto W (eds), Harwood Academic Publishers, in press

3. **Deli MA**, Ábrahám CS, Dung NTK, Joó F: Preparation of primary culture from newborn pigs. *Drug transport across the blood-brain barrier: in vivo and in vitro techniques*, de Boer ABG and Sutanto W (eds), Harwood Academic Publishers, in press

Other publications:

32 abstracts in international journals referenced by Science Citation Index and 17 abstracts in conference proceedings.

ACKNOWLEDGEMENTS

I am greatly indebted to the late Professor Ferenc Joó, former head of the Laboratory of Molecular Neurobiology, for his scientific guidance and for the encouragement and support he gave me through the years during which I had the possibility to work with him. I thank to the members of the Group of Molecular Neurobiology for their help and friendship.

This work was supported in part by the European Science Foundation (ERG N° 66), by "Drug transport across the BBB: new experimental strategies" concerted action of the EEC BIOMED I programme (N° BMH1-CT92-1193), by the Institut National de la Santé et de la Recherche Médicale (INSERM, réseau Est-Ouest No 94E006) and by the Hungarian Research Fund (OTKA grants T5208, F5207, F12722).