Summary of Ph.D. thesis

EFFECTS OF FACTORS INVOLVED IN THE PATHOGENESIS OF HEPATIC ENCEPHALOPATHY AND SOME PHOSPHODIESTERASE INHIBITORS ON NEUROINFLAMMATION-RELATED EVENTS IN PRIMARY RAT ASTROCYTE CULTURES

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SUPERVISORS:

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1. Introduction

The biology of astrocytes has emerged as a rapidly expanding field. The classically accepted paradigm that astrocytes are scaffold and only have supporting role in the central nervous system, has been changed. Astrocytes have many physiological functions, such as removing glutamate from the extracellular space, modulating synaptic transmission by releasing gliotransmitters, participating in the formation of blood brain barrier; they also have role in the brain water homeostasis via aquaporin channels, and regulating blood flow. Accumulating evidence indicates that astrocytes are essential players not only in the physiological events, but also in many pathological conditions, such as Alzheimer's disease, Parkinson's disease, epilepsy, major depressive disorder or hepatic encephalopathy (HE).

HE is a neurocognitive disorder in which brain function is impaired due to either acute or chronic liver failure or porto-systemic shunt.

The precise pathogenesis of HE is not fully understood, however the elevation of plasma and cerebral ammonia (NH_4^+) levels are thought to be the main etiological factor. Besides, increased concentration of manganese (Mn) and oxidative stress in the brain, moreover passage of bacteria or bacterial products from the intestinal tract to the systemic circulation also occurs during HE. Since the blood brain barrier (BBB) is disrupted in acute liver failure, it is assumed that many etiological factors may cross the BBB in HE, thereby the increasing concentration of a given substance in the circulation might lead to increasing concentrations of the that substance in the CNS. In acute HE, cerebral edema and subsequently cerebral hernia leading to death may occur. The main underlying event of brain edema is the astrocyte swelling, the precise mechanism of which is not fully understood, however it is well established that glutamine accumulates in astrocytes, where it is degraded to glutamate and ammonia by glutaminase, causing mitochondrial impairment and cell swelling.

In chronic HE so called Alzheimer Type II astrocytosis could occur, characterized by typical subcellular changes in astrocytes. Astrocytes are not only the main affected cell type in HE, but also have pivotal role in neuroinflammatory events, which has been implicated in the pathogenesis of HE. In neuroinflammation both microglia and astrocytes become activated, they undergo morphological changes and produce a variety of cytokines. Investigations aiming to study the neuroinflammatory role of astrocytes, use a variety of *in vitro* models, either containing microglia, or using different procedures for eliminating microglia from the culture. Reduction of microglia number may be performed by treatment with cytosine β-D-arabinofuranoside hydrochloride (AraC) and L-leucine methyl ester (LME) (chemical microglia elimination) or by shaking (mechanical microglia elimination), and these techniques may be applied in different ways.

Taken together, the potential heterogeneity of the applied *in vitro* models does not allow direct comparison of the results regarding the inflammatory role of astrocytes.

More recently it has been found that glutaminase plays a key role not only in astrocyte swelling, but also in neuroinflammatory processes. Currently, there is no efficient therapy for the suppression of neuroinflammation or to inhibit cerebral glutaminase activity, albeit a growing body of evidence indicates that some phosphodiesterase (PDE) inhibitors may reduce neuroinflammation and inhibit glutaminase activity.

2. Aims of the study

The aims of my study were:

1. to establish the purity of primary rat astrocyte cultures after microglia elimination by the methods of shaking and chemical treatment

2. to assess the cytotoxic effect of some factors involved in the pathogenesis of HE in both primary rat astrocyte cultures purified by shaking and in mixed astrocyte-microglia cultures using three different methods
3. to measure the effect of the same factors on the generation of intracellular reactive oxygen species (ROS) in primary rat astrocyte cultures purified by two different methods and in mixed glial cultures
4. to investigate the pro-and anti-inflammatory cytokine production of primary rat astrocyte cultures purified by two different methods
5. to examine the potential glutaminase-inhibitory effect of some phosphodiesterase inhibitors, such as theophylline and zaprinast in primary rat astrocyte cultures purified by shaking

3. Materials and methods

Primary rat astrocyte cultures were prepared from 2-day-old Sprague-Dawley rat pups. Brains were mechanically dissociated, followed by enzymatic digestion.

Cells were cultured in Petri dishes and after the cultures reached confluency, were transferred to 96-well plates. For further experiments, microglia were eliminated from the cell cultures either with the treatment with AraC and LME or by shaking. In addition, mixed glial cultures without applying purification procedure were also acquired. The purity of astrocyte cultures was determined by immunofluorescence labelling for the astrocyte marker glial fibrillary acidic protein (GFAP) and the microglia marker Ionized Calcium-binding Adapter Molecule-1 (Iba-1).

Cultures were treated with different concentrations of hydrogen peroxide (H_2O_2), lipopolysaccharide (LPS), manganese (Mn) and ammonia (NH_4^+) for various incubation times. Cell viability was measured by three different methods: propidium iodide (PI) and neutral red uptake, and determination of lactate-dehydrogenase (LDH)-activity. Intracellular ROS formation was quantified by a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA).

Inflammatory response of cell cultures were examined by measuring the interleukin (IL)-6, IL-10, IL-1ß and tumor necrosis factor (TNF)-α protein level with ELISA method. IL-6, IL-10 and TNF-α expressions were quantified from the cell culture supernatant, while IL-1ß was detected from cell lysate. Glutamate and glutamine assays were performed in the intra-and extracellular compartment as well, in order to assess the glutaminase activity of both theophylline and zaprinast. Using colorimetric assays, the EC level of glutamate and glutamine were measured in the cell culture medium, while the IC concentrations of these metabolites were detected in the cell lysate.

4. New scientific results

1. Microglia reduction by shaking results in higher purity of astrocyte cultures compared to chemical microglia elimination, thus the two methods are not equivalent
2. Neutral red uptake assay is more appropriate method for the evaluation of NH_4^+-and H_2O_2-induced cytotoxicity than propidium-iodide uptake and LDH activity measurement.
3. Mixed astrocyte-microglia cultures have larger sensitivity to the H_2O_2-evoked oxidative stress than either of the purified astrocyte cultures
4. Both the TNF-α and IL-6 production were different in the astrocyte cultures after AraC+LME- and shaking-induced microglia depletion in the baseline condition: astrocyte cultures after chemical microglia depletion secreted around 10-fold more TNF-α and IL-6 than astrocyte cultures after mechanical microglia reduction

5. The LPS-induced cytokine production is varied between the cultures purified by the two distinct methods: In contrast to AraC+LME-treated cultures, LPS increases the IL-1ß and TNF-α release in the cultures purified by shaking, while IL-10 protein level is increased exclusively in the AraC+LME-treated cultures.
6. Both NH_4^+, Mn^{3+}cause the elevation of IL-1ß in astrocyte cultures purified by shaking
7. Both theophylline and zaprinast increases the extracellular level of glutamate

5. Scientific publications

Bárány, Z. B., Sterczer, Á., Jócsák, G., Frenyó, V. L., & Kiss, D. S. (2017). A hepaticus encephalopathia kóroktana, kórfejlődésének újabb szempontjai. Irodalmi összefoglaló. Magyar Állatorvosok Lapja, 139(3), 157-168.

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Jocsak, G., Ioja, E., Kiss, D. S., Toth, I., Barany, Z., Bartha, T., ... & Zsarnovszky, A. (2019). Endocrine Disruptors Induced Distinct Expression of Thyroid and Estrogen Receptors in Rat versus Mouse Primary Cerebellar Cell Cultures. Brain sciences, 9(12), 359.

Kerek Á., Bárány Z., Sterczer Á. & Jócsák G. (2020). A neuroinflammáció kórfolyamata és egyes terápiás vonatkozásai. Irodalmi összefoglaló. Magyar Álltorvosok Lapja, 142(12), 755-767.

Differential production of interleukin-6 and tumor necrosis factor-α in primary rat astrocyte cultures using two distinct methods of microglia elimination *(Elfogadás alatt, Clinical & Experimental Neuroimmunology)*

6. Conference presentations

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Bárány ZB., Kiss DS, Tóth I., Jócsák G., Bartha T., Frenyó VL., Sterczer Á.: Primer patkány asztroglia sejtek citokin termelése oxidatív stressz hatására, P1.1.3, In: Joint Conference of the Hungarian Physiological Society and the Hungarian Pharmacology, Microcirculation and Physiological Societies/ A Magyar Élettani Társaság, a Magyar Kísérletes és klinikai Farmakológiai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság közös Vándorgyűlése, Debrecen, Hungary, 2017.

Bárány Z., Kiss DS., Tóth I., Jócsák G., Bartha T., Frenyó VL., Sterczer Á.: Cytokine production induced by oxidative stress in primary rat astrocytes, P1-338, In: Meeting of the Hungarian Neuroscience Society & Federation of European Neuroscience Societies Regional Meeting, Pécs, Hungary, 2017.

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Bárány Z, Kiss DS, Tóth I, Jócsák G, Frenyó VL, Bartha T, Zsarnovszky A, Sterczer A: Az oxidatív stressz és a tumor nekrózis faktor alfa (TNF-α)-termelés vizsgálata hepaticus encephalopathiaban. Akadémiai Beszámoló (2019).

Barany Z., Kiss DS., Toth I., Jocsak G., Bartha T., Frenyo VL., Zsarnovszky A., Sterczer A: Examination of the oxidative stress and the tumor necrosis factor-α production in hepatic encephalopathy. GLIA 67:S1 pp. E549-E550. Paper: P1-25 (2019).