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PROTECTION OF THE BLOOD-BRAIN BARRIER UNDER PATHOLOGICAL CONDITIONS

Ph.D. thesis

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1. INTRODUCTION

1.1. The blood-brain barrier: overview and culture models

The microvessels of the brain constitute the blood-brain barrier (BBB). The anatomical basis of the BBB are the endothelial cells, which have a dynamic interaction with the other neighboring cells, such as astroglia, pericytes, perivascular microglia, and neurons. The cross-talk between these cells induces a unique phenotype in endothelial cells including (i) a morphological barrier based on interendothelial junctions that markedly limit paracellular permeability, (ii) a unique pattern of receptors, transporters and drug efflux pumps, and (iii) enzymatic and metabolic barriers. The three major functions of the BBB are the creation and maintenance of ionic homeostasis for neuronal functions, supply of the central nervous system (CNS) with nutrients, and protection from toxic insults.

The tightness of the paracellular barrier, regulated by adherens and tight junctions (TJs), is a fundamental property of BBB. TJs prevents hydrophilic molecules, ions and water to enter freely the CNS. TJs are multiprotein complexes situated in lipid rafts and composed of integral proteins occludin and claudins, that associate with cytoplasmatic linkers zonula occludens proteins (ZO) and β-catenin. ZOs interact with occludins, claudins and junctional adhesion molecules and also participate in cell signaling. β-catenin attaches junctional protein, cadherins to the actin cytoskeleton. Absence of fenestration and low number of pinocytotic vesicles are also important elements of the physiological barrier. The transendothelial electrical resistance of brain parenchymal microvessels reflecting paracellular tightness *in vivo* exceeds 1000 $\Omega \times cm^2$. Receptors, transporters and enzymes are localized in a polarized way in brain endothelial cells. These cells express a variety of transporters for nutrients, like glucose, amino acids, nucleosides to feed neural cells. Cerebral endothelium protects the CNS from xenobiotics by efflux transporters like P-glycoprotein and multidrug resistance proteins.

Cell culture-based models proved to be one of the most versatile tools in basic BBB research and also in testing CNS drug penetration. All models should show some elements of general endothelial and specific BBB properties. Furthermore, an ideal *in vitro* model should not only show BBB characteristics, it also needs to be simple to use, reproducible, cost-effective and applicable for both research and drug development purposes.

1.2. The blood-brain barrier under pathological conditions

Cerebral endothelial cells forming the BBB are key elements in protection of the brain under pathological conditions, which is increasingly emphasized in the literature. Recent clinical data indicate that vascular changes play an important role early in Alzheimer's disease (AD) pathogenesis. The hallmarks of AD are perivascular and brain parenchimal deposits of amyloid-β peptides (Aβ). At the level of capillaries due to faulty

Aβ clearance microvascular degeneration leads to increased deposition of basement membrane proteins and perivascular amyloid formation, which decrease capillary blood flow. Hypoperfusion increases the dysfunction of efflux pumps and the accumulation of metabolic waste products, alters pH and electrolyte composition and all these changes aggravate neuronal dysfunction.

We have tested amyloid-β 1-42 ($A\beta_{42}$) and methylglyoxal, two pathological factors involved in AD. Methylglyoxal not only induces carbonyl and oxidative stress in cells, but also enhances the formation of Aβ-aggregates.

1.3. The blood-brain barrier as a therapeutical target

The role of the BBB to protect the brain in both physiological and pathological conditions has been unequivocally proved, therefore, protective strategies at the level of BBB are in the focus of new studies. There are several groups of potentially protective molecules acting on brain endothelial cells. A limited number of anti-inflammatory molecules, with proved efficacy on other cell types and in clinical studies, were selected for our experiments. From the three different compounds, pentosan polysulfate (PPS) and edaravone are already used in clinical therapy for other indications, while docosahexaenoic acid (DHA) is widely used as a dietary supplement.

1.4. Aims

We have selected $A\beta_{42}$ and methylglyoxal as toxic agents to study potential protective molecules using *in vitro* BBB models. Based on our previous results and literature data, three compounds, PPS, DHA and edaravone were chosen for our studies.

The main aims of our studies were the following:

1. to examine the effects of $A\beta_{42}$ on morphology and barrier properties using rat co-culture based *in vitro* BBB models and to reveal the underlying mechanisms
2. to test the protective action of PPS on $A\beta_{42}$-induced changes on a BBB model
3. to reveal the protective action of DHA on $A\beta_{42}$-induced changes in brain endothelial cells
4. to study the effects of methylglyoxal on barrier properties of human brain endothelial monolayers
5. to evaluate the barrier protective effects of edaravone on methylglyoxal-induced changes

2. MATERIALS AND METHODS

2.1. Peptides

$A\beta_{42}$, $isoA\beta_{42}$ and scrambled peptides were synthesized at the Department of Medical Chemistry, University of Szeged. Dissolved $A\beta_{42}$ peptide samples contained globulomers or in chain-like or annular assemblies, but not classical protofibrillar aggregates. At physiological conditions (37 °C and pH 7.4) $isoA\beta_{42}$ *in situ* forms oligomeric $A\beta_{42}$. The oligomers applied in 15 µM concentration aggregate slowly *in vitro* after 24 h of incubation.

2.2. Cell cultures

Monocultures, double or triple co-cultures of primary rat brain endothelial cells, and human hCMEC/D3 brain endothelial cell line under passage number 35 were used in the experiments (Fig.1). For primary culture, brain capillary fragments were isolated from the forebrains of 2-week-old Wistar rats and seeded on cell culture inserts coated with collagen type IV and fibronectin as established and described previously on our laboratory. Primary brain microvascular pericytes were prepared from isolated microvessels by using different culture conditions, while glial cells were obtained from brains of newborn rats. To induce BBB characteristics, brain endothelial cells were co-cultured with rat glial cells and pericytes using cell culture inserts. For co-culture, brain endothelial cells were placed on cell culture inserts into multiwells containing astroglia at the bottom of the wells with endothelial culture medium in both compartments. To construct the three cell type BBB model, first pericytes were seeded on the bottom side of the inserts and finally endothelial cells were passaged to the upper side of the inserts. Cultures reached confluency within a week and were used for experiments.

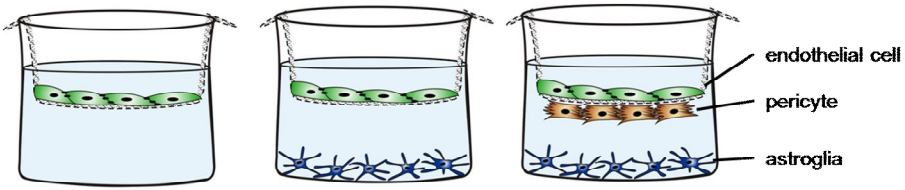

Figure 1. BBB models: monoculture, double and triple co-culture of brain endothelial cells.

2.3. Treatments

$A\beta_{42}$ peptides were tested at 0-200 µg/ml (0-45 µM), while $isoA\beta_{42}$ at 0-30 µM (0-135 µg/ml) concentrations. Methylglyoxal treatment solutions were used in the 100-1000 µM concentration range. The concentration of PPS varied between 1.6 and 166.6 µM (1-100 µg/ml). DHA stock solution (300 mM) was prepared in ethanol and further diluted in culture

medium (1-300 μM) to treat the cells. Edaravone was used in the 600-3000 μM range. Aminoguanidine, a well-known antiglycation agent, was tested at 600-2000 μM concentration and applied as a positive control against the effect of methylglyoxal.

2.4. Viability and cytotoxicity assays

Different methods were applied to determine the effects of treatments on cell viability. MTT dye conversion, lactate dehydrogenase (LDH) release, and real-time cell impedance measurement provided information on cell viability and cytotoxicity. Living cells convert the yellow dye 3-(4,5-dimethyltiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple, insoluble formazan crystals. Decrease in dye conversion rate reflects cellular damage. The control group was considered as 100 % viable. LDH release is the indicator of cell membrane damage, which was determined from culture supernatants by a commercially available kit (Cytotoxicity detection kit LDH). Cell death was calculated as percentage of the total LDH release from cells treated by 10 mg/ml Triton X-100 detergent. Impedance-based cell electronic sensing is a label-free technique for dynamic monitoring of living cells. The Real Time Cell Analyser Single Plate instrument (RTCA-SP, Acea Biosciences) utilizes an automatic and continuous impedance measurement to non-invasively quantify adherent cell proliferation and viability in real-time. The cell index at each time point was defined as $(R_n-R_b)/15$, where R_n is the cell-electrode impedance of the well when it contains cells and R_b is the background impedance of the well with the medium alone. This method has been successfully applied to measure number, adherence, growth and health of cells in control and treatment conditions.

2.5. Investigation of cell morphology

Changes in morphology and cell-cell connections induced by treatments were confirmed by phase contrast, electron, and holographic phase contrast microscopy. For electron microscopy cells were washed and fixed after treatments, then postfixed in OsO_4 and dehydrated. Finally, the membranes of the culture inserts with the cells were removed and embedded in Taab 812. Following polymerization, ultrathin sections were cut by ultramicrotome and examined using a Hitachi 7100 transmission electron microscope. Digital holographic images were taken with a Holo-Monitor M3 instrument (Phiab). Endothelial cells were cultured on collagen coated culture dishes with borosilicate glass bottom. Holographic images of the same culture area were captured before and during treatments. Cell morphological changes were analysed by the Holostudio 2.4 software.

2.6. Evaluation of the barrier integrity

Transendothelial electrical resistance (TEER), representing the permeability of tight junctions was measured by an EVOM resistance meter (WPI). It was expressed relative to the

surface area of endothelial monolayer ($\Omega \times cm^2$). Fluorescein (mw: 376 Da), and fluorescein isothiocyanate labeled dextran (FITC-dextran, mw: 4.4 kDa) were used as markers of paracellular flux, and Evans blue-labeled albumin (EBA, mw: 67 kDa), as a tracer for transendothelial transport. The concentrations of the marker molecules in samples from the upper and lower (donor and acceptor) compartments of the culture inserts were determined and transendothelial permeability coefficient value (P_e), or the apparent coefficient (P_{app}) was calculated.

2.7. Immunohistochemistry

Cell-cell connections and morphology of primary rat brain endothelial cells were confirmed by immunostaining for tight junctional proteins ZO-1, occludin and claudin-5. Human hCMEC/D3 cells were investigated for junctional proteins β-catenin and claudin-5. Staining was examined by NikonEclipse TE2000 fluorescent microscope, Olympus Fluoview FV1000 or Leica SP5 confocal laser scanning microscopes.

2.8. Detection of reactive oxygen species

Chloromethyl-dichloro-dihydro-fluorescein diacetate (DCFDA), a fluorometric detection probe was used to measure reactive oxygen species (ROS). This indicator penetrates the cells by diffusion and becomes deacetylated by intracellular esterases. Oxidation of DCFDA by reactive oxygen species yields a fluorescent molecule, which was measured by Fluostar Optima fluorescent plate reader at 485 nm excitation and 520 nm emission wavelengths. The fluorescent values were presented as percent of the control group after 1 h incubation with DCFDA indicator.

2.9. Functional assay for P-glycoprotein activity

Activity of P-glycoprotein (P-gp) was determined by the measurement of cellular accumulation of rhodamine 123. Following treatments endothelial monolayers were incubated with 10 μM rhodamine 123 for 1 h at 37 ˚C and rhodamine 123 content in cells was determined by a fluorescent plate reader (excitation at 485 nm, emission at 538 nm).

2.10. Protein modification experiment

Bovine serum albumin (50 mg/ml) was incubated with methylglyoxal (2 mM) in the presence or absence of protective agents in PBS (pH 7.4, 37 °C) for 7 days. The formation of modified albumin was assessed by the characteristic fluorescence excitation wavelength at 360 nm and emission wavelength at 460 nm with a fluorescent microplate reader.

2.11. Statistical Analysis

All data presented are means ± SD or SEM as indicated in the text. The values were compared using the analysis of variance followed by Dunnett, Bonferroni posthoc tests using GraphPad Prism 5.0 software. Changes were considered statistically significant at $p < 0.05$. All experiments were repeated at least three times, the number of parallel wells or inserts for each treatment and time point varied between 3 and 16.

3. RESULTS AND DISCUSSION

3.1 Effect of pathological factors on brain endothelial cells

3.1.1. Amyloid-β induced changes in brain endothelial cells

In our study, $A\beta_{42}$ (25 μM, 24h) peptide induced remarkable ultrastructural alterations in primary rat brain endothelial cells, which have not been described in the literature earlier. On electron and phase contrast microscopy pictures dark cytoplasm, pronounced vacuolization, decreased number of caveolae and Golgi, and shrunken mitochondria could be observed after peptide treatment. Irregular interendothelial contacts and weak junctions were especially notable, indicating an increased paracellular pathway. These toxic effects were also confirmed by reduced MTT dye reduction, and increased LDH release indicating metabolic alterations and plasma membrane damage. Pretreatment of $isoA\beta_{42}$ elevated ROS production and inhibited the activity P-gp in brain endothelial cells (Fig. 2A).

The effect of $A\beta_{42}$ was also studied on several aspects of the barrier function of rat primary brain endothelial cells. The treatment has decreased the resistance and increased the permeability for markers fluorescein and albumin, respectively. Furthermore, it led to fragmentation and loss of junctional immunostaining for occludin, claudin-5 and ZO-1 in our model (Fig. 2A).

Cell-free aggregation experiments and a culture study on neurons indicated, that methylglyoxal can conribute to the harmful effect of Aβ peptides. Co-treatment with methylglyoxal and Aβ has not been investigated on endothelial cells previously. We found, that brain endothelial damage induced by $isoA\beta_{42}$ (10 μM, 24h) was more pronounced in the presence of methylglyoxal (600 μM). Our data confirm the link between methylglyoxal and AD.

3.1.2. Methylglyoxal induced changes in brain endothelial cells

In the present study we further supported the fact that methylglyoxal alone could induce damage to brain endothelial cells (Fig. 3A). Methylglyoxal exerted a time- and concentration-dependent toxicity on cultured human brain endothelial cells; it significantly reduced the integrity of the barrier measured by both functional and morphological experiments. This is the

first observation on the kinetics of methylglyoxal toxicity by impedance-based cell electronic sensing. Damage by methylglyoxal is mediated not only via carbonyl, but also by oxidative stress. In the present study we confirmed that methylglyoxal (600 μM) treatment promotes oxidative stress in brain endothelial cells. The kinetics of ROS production helped to determine the optimal time point for protection assays and other experiments: the 4 h time point, where ROS formation was still elevated, was purposefully selected.

In good agreement with data from toxicity measurements methylglyoxal increased the permeability of human and rat brain endothelial monolayers. The effect was concentration-dependent, with only high concentrations of methylglyoxal causing significant damage to barrier integrity. We found the redistribution of two junctional proteins important for the regulation of brain endothelial permeability, namely the TJ protein claudin-5 and junction associated protein β-catenin in methylglyoxal treated brain endothelial cells. Decreased expression, delocalization or redistribution of claudin-5 and β-catenin in brain endothelial cells are linked to permeability increase in many pathologies. We demonstrated for the first time, that treatment with methylglyoxal resulted in fragmentation and loss of the continuous cortical pattern of β-catenin in brain endothelial cells, confirming its importance in the regulation of barrier tightness. Moreover, methylglyoxal changed drastically the shape of brain endothelial cells by time: the area of cells decreased, their optical thickness significantly increased indicating cell-cell and cell-basal lamina detachment (Fig. 3A), which are crucial for the maintenance of the integrity of BBB and low passive permeability.

3.2. Protection against brain endothelial dysfunction

Brain endothelial dysfunction initiates and contributes to the disease process in AD. Therefore in addition to neurons, brain endothelial cells are also considered as therapeutical targets in CNS and systemic pathologies. The prevention of methylglyoxal-induced endothelial injury is also in the focus of current research.

3.2.1. Protection by pentosan polysulfate

PPS (100 μg/ml, 24h) successfully attenuated the toxic effects of $A\beta_{42}$ peptides (25 μM) and protected the barrier integrity of endothelial monolayers. Importantly, PPS alone did not change BBB parameters of TEER, permeability, or morphology in cultured brain endothelial cells. As PPS does not cross the BBB, endothelial cells of the blood-nerve and blood-brain barriers may be among the cellular targets of PPS. Surface plasmon resonance and atomic force microscopy data in our study indicate that PPS can directly interact with $A\beta_{42}$, and may have a physicochemical effect resulting in fewer adherences to the examined surfaces. Pentosan may exert multiple effects; some of these could be cellular while others could be related to its direct

interaction with Aβ (Fig. 2B). The present results support pentosan's dual mode of action and endothelial protective properties.

3.2.2. Protection by docosahexaenoic acid

Our main results indicate for the first time that DHA can protect not only neurons, but also the other elements of the neurovascular unit, like brain endothelial cells, from the toxic effects of Aβ (Fig. 2B). DHA (30 μM, 24h) was effective in toxicity tests, improved the morphological changes and permeability increase after isoAβ_{42} treatment (15 μM). Decrease in P-gp activity induced by isoAβ_{42} treatment was also prevented. Since decreased P-gp function is involved in the pathogenesis of AD, our novel finding strengthens the therapeutic potential of DHA.

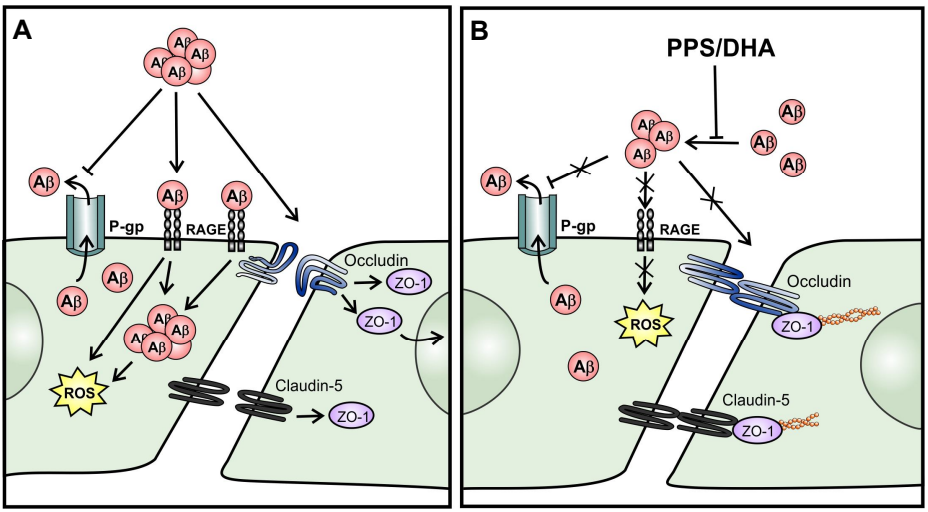

Figure 2. Effect of amyloid-β 1-42 peptide and pentosan or docosahexaenoic acid on brain endothelial cells. Abbreviations: Aβ: amyloid-β 1-42 peptide; DHA: docosahexaenoic acid; P-gp: P-glycoprotein; PPS: pentosan polysulfate; RAGE: receptor for advanced glycated endproducts; ROS: reactive oxygen species; ZO-1: zonula occludens protein-1.

In AD the accumulation of Aβ_{42} induces the production of ROS, resulting in lipid peroxidation. Similarly to cytotoxicity and BBB dysfunction, ROS production could be also inhibited by DHA in primary brain endothelial cells. DHA attenuated the combined toxic effect of Aβ_{42} and methylglyoxal; however, no complete protection was achieved. These observations suggest that DHA enhance the antioxidant defense in the brain, including the BBB, and its mode of action may be related to direct scavenging of ROS or to the induction of antioxidant enzymes. Based on these data, DHA could be a promising molecule for the prevention of BBB

dysfunction. However, to fully prevent the damages caused by carbonyl stress in AD in addition to DHA other agents, like edaravone may be needed.

3.2.3. Protection by edaravone

Edaravone provided a complete protection against the toxic effect of methylglyoxal (Fig. 3B). We could see a concentration- and time-dependent effect based on kinetic data from impedance measurements. Co-treatment with edaravone (3 mM, 4h) restored barrier properties of endothelial cells and protected against methylglyoxal-induced (600 μM) decrease of resistance and increase in permeability for paracellular and transcellular markers. Increased endothelial permeability was coupled with disturbed localization of junctional proteins claudin-5 and β-catenin after incubation with methylglyoxal, while co-treatment with edaravone restored distribution of both proteins along the cell borders. Moreover, we also demonstrated that edaravone treatment alone tightened the brain endothelial barrier, increased the metabolic activity and impedance of the endothelial layers.

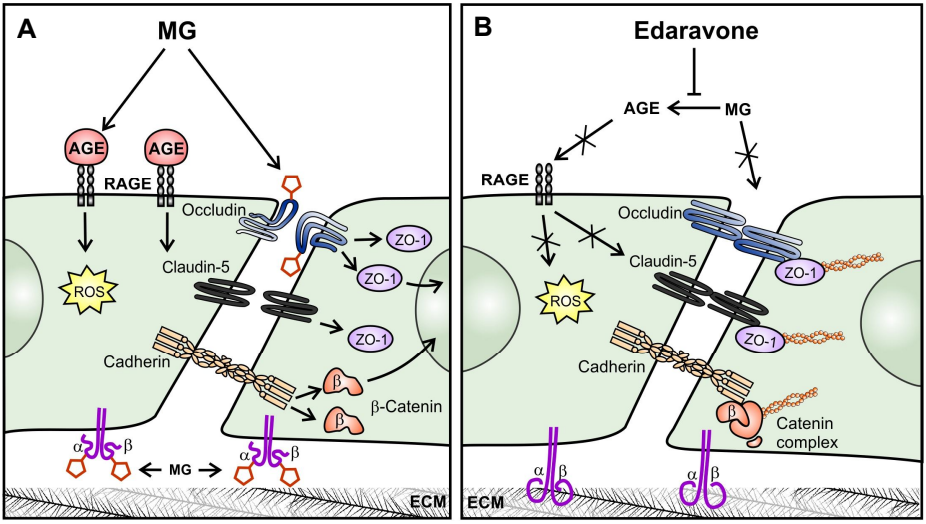

Figure 3. Effect of methylglyoxal and edaravone on brain endothelial cells. Abbreviations: AGE: advanced glycated end-products; ECM: extracellular matrix; MG: methylglyoxal; RAGE: receptor for AGE; ROS: reactive oxygen species; ZO-1: zonula occludens protein-1.

Edaravone is a drug to promote neurorehabilitation after ischemic stroke by protecting against oxidative stress. Its antioxidant effect was observed in our experiments, too. Furthermore, edaravone inhibited protein-modification of BSA by methylglyoxal, therefore it also decreased ROS generated as by-products during protein glycation. Our data expand and further support previous observations on barrier enhancing effect of edaravone at the BBB.

3.3. Conclusion and perspectives

Because of the increasing prevalence of CNS disorders there is a large number of new centrally active drug candidates, especially biomolecules, under development. Reliable BBB models that present physiologically realistic cell architecture and other attributes of *in vivo* BBB phenotype are needed as testing and screening tools. The primary brain endothelial cell-based co-culture models used in our studies involve also other elements of the neurovascular unit, especially glial cells and pericytes, and can be considered as one of the most complex and pertinent BBB models to date. Since species differences exist in the expression and pharmacokinetic parameters of transporters at the BBB, human models are valuable and important research tools. For this reason we also used the human brain endothelial cell line hCMEC/D3 in our experiments. Importantly, the reliability of this model was tested against primary cultures and similar data were obtained on both models.

The BBB culture models enabled us to test the effect of two major pathological factors, $A\beta_{42}$ and methylglyoxal involved in AD. Our observations further support the toxic effects of amyloid peptides and methylglyoxal on brain endothelial cells and strengthen the link between AD and carbonyl stress. Oxidative stress was found as a common pathway induced by both toxic agents in brain endothelial cells, similarly to previous observations on other cellular systems. As a consequence, the BBB is considered as therapeutic target in AD, and anti-inflammatory molecules are among the possible protective pharmacons.

The prospect of reducing the risk of AD by preventative strategies such as diet or lifestyle modification is highly favorable. DHA may be a potent agent to prevent Aβ-induced damages on the cells of the neurovascular unit acting via multiple ways. Based on our new observations DHA might exert a protective effect not only on neurons but also on the BBB and its functions. This effect may be beneficial in the prevention of AD. Pentosan and edaravone can represent a new type of drugs to protect brain endothelial cells in pathological conditions, including Aβ toxicity and carbonyl stress. As a new application these drugs may contribute to the maintenance of BBB functions, brain homeostasis and prevention of neuronal loss in AD. The presented results provide compelling evidence for barrier protective effect of DHA, pentosan and edaravone in cultured brain endothelium. Data from our studies could have therapeutic implication for disorders and diseases that are associated with carbonyl stress and AD.

4. SUMMARY

The role of cerebral endothelial cells forming the blood-brain barrier to protect the brain in pathological conditions is increasingly emphasized in the literature. We have tested amyloid-β 1-42 ($A\beta_{42}$) and methylglyoxal, two major pathological factors, involved in Alzheimer's disease and carbonyl stress. The hallmarks of Alzheimer's disease are perivascular deposits and senile plaques of Aβ peptides in the brain. The formation of these aggregates is enhanced by dicarbonyl compounds such as methylglyoxal. To study potential protective molecules, primary rat and human (hCMEC/D3 cell line) blood-brain barrier models were used. The effect of treatments was tested on cell viability and barrier functions. $A\beta_{42}$ and methylglyoxal induced concentration- and time dependent toxicity and dramatically changed cell morphology. In $A\beta_{42}$-treated brain endothelial cells cytoplasmic vacuolization, disruption of the structure of cytoplasmic organelles and tight junctions were visible. Additionally, methylglyoxal treatment caused detachment of endothelial cells. Further damage of barrier function was demonstrated by decreased resistance and increased para- and transcellular permeability of the endothelial monolayers. The production of reactive oxygen species in brain endothelial cells was also elevated. Pentosan polysulfate, a drug of plant origin, and the polyunsaturated fatty acid docosahexaenoic acid could attenuate the toxic effects of $A\beta_{42}$ peptides. Co-treatment of the cells with $A\beta_{42}$ and the protective agents prevented the morphological changes and cell cultures integrity resembled to the control ones. Furthermore, pentosan and docosahexaenoic acid could significantly protect the barrier from damaging actions of peptides. The barrier function of endothelial monolayers, mirrored by low permeability and high resistance, was restored. At molecular level, pentosan modified the size and decreased the number of amyloid aggregates demonstrated by atomic force microscopy. Docosahexaenoic acid had a different molecular mechanism, it decreased the elevated reactive oxygen species level and restored P-glycoprotein efflux pump activity. These results indicate for the first time that pentosan and docosahexaenoic acid can protect the blood-brain barrier from the toxic effects of $A\beta_{42}$ and these protective molecules may be beneficial in Alzheimer's disease. Edaravone, the active substance of a Japanese medicine, was effective against methylglyoxal induced damages. Co-administration of edaravone restored cell viability, barrier integrity and functions of brain endothelial cells. Edaravone prevented the methylglyoxal-induced cell detachment. The production of reactive oxygen species in human endothelial cells and protein-modification were also blocked. We demonstrated that edaravone is protective in oxidative and carbonyl stress induced barrier damages. Our observations further support the toxic effects of amyloid peptides and methylglyoxal, and show for the first time the protective effect of pentosan, docosahexaenoic acid and edaravone on brain endothelial cells. The present data may contribute to the development of compounds protecting the blood-brain barrier in carbonyl stress related diseases, especially in Alzheimer's disease.

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