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The influence of donepezil and EGb 761 on the innate immunity of human leukocytes Effect on the NF-κB system

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ABSTRACT

Ginkgo biloba special extract EGb 761 and donepezil are drugs used in Alzheimer therapy. The influence of donepezil and EGb 761 on two mechanisms of innate immunity, natural antiviral resistance of human leukocytes *ex vivo* and NF-κB activation, was studied. Correlation between the innate immunity of leukocytes and NF-κB activation was investigated. The effect of the two drugs on resistance of human leukocytes to vesicular stomatitis virus (VSV) infection was also assessed. Two groups of healthy blood donors (n = 30) were distinguished: one with resistant leukocytes (n = 15) and one (n = 15) with leukocytes sensitive to VSV. The degree of natural resistance of human peripheral blood leukocytes (PBLs) was determined by studying the kinetics of VSV replication. NF-κB activation was assayed by immunocytochemical staining. Efficiency of donepezil and EGb 761 was determined by a special regression model. The toxicity of the preparations to PBLs and the cell lines L_{929} and A_{549} and their effect on the different viruses was established. Results showed that donepezil used in concentrations of 10–50 μg/ml and EGb761 of 25–100 μg/ml stimulated resistance of human leukocytes. At the same concentrations both preparations decreased activation of transcriptional factor NF-κB. Correlation between innate immunity of PBLs and NF-κB activation was observed. Comparison of the effects of these two drugs showed that EGb 761 is more effective in stimulating leukocyte resistance. Donepezil and EGb 761 regulated innate immunity of human leukocytes by stimulating resistance and modulating NF-κB activation. The natural drug was more efficient in stimulating innate antiviral immunity of human leukocytes.

1. Introduction

Innate immunity is the first line of defense against invading pathogens. This part of the immune system plays a central role in the pathogenesis of many human infectious and inflammatory diseases [1]. Reactions of innate immunity include phagocytosis, the production and activity of cytokines, chemokines, and other mediators with NF-κB activation, the killing of infected or altered cells by NK cells, complement activated by natural lectins, cytokine-dependent resistance of leukocytes to viral infection, and autophagy to restrict viral replication [2–4].

The innate antiviral immunity of leukocytes *ex vivo* is the main reaction of the innate system and it protects against viral infection. In our earlier studies, leukocyte resistance directed against viruses belonging to different taxonomic groups was found. The differential sensitivity of particular leukocytes to infection by different viruses that we observed suggested the presence of natural innate antiviral immunity [5]. Innate immunity was measured by using the direct method of infection of leukocytes with vesicular stomatitis virus (VSV), which was selected as the indicatory virus for detecting immunity. This virus does not cause natural infection in the European human population. A lack of VSV replication by infected leukocytes (0–1 log TCID50-tissue culture infectious dose) was taken as an indicator of complete immunity, a low level of VSV (2–3 log) of partial immunity, and a high VSV titer (>4 log) of low or deficient innate immunity [6]. Furthermore the resistance/innate immunity of whole PBLs and subpopulations such as: adherent cells, fractions enriched in lymphocytes T and B, NK(+) and NK(−) differ from each other. The separated fractions exhibited higher resistance/innate immunity than the whole PBLs [7].

The level of innate immunity is the most important determinant of body condition and viral susceptibility. Dysregulation of the innate system, i.e. deficiency or over-activation, is associated with many diseases. A deficiency in the innate immunity of leukocytes is usually accompanied by remarkable sensitivity to viral and other infections

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and cancer diseases. We found that the intensity of innate immunity is a prognostic factor for acute leukemia. Our results showed that the course of acute leukemia and effective therapy depend on the resistance of PBLs [5]. Leukocytes of patients with frequent infections of the upper respiratory system or frequent incidences of herpes labialis showed deficiency in innate immunity [8]. Over-activation of innate immunity causes neurodegenerative, autoimmune, and inflammatory diseases. Many studies clearly indicate the participation of innate immunity Toll-like receptors (TLRs) in the immune response in autoimmune diseases [9,10]. An over-activated innate immune reactions are also important in the pathogenesis of neurodegenerative disorders. Neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease, multiple sclerosis (MS), and AIDS dementia could result from over-activation of microglia and neuroinflammation in the brain [11]. Therefore innate immune reactions must occur under strict control [12].

The NF-κB family of pleiotropic transcriptional factors constitutes the universal and evolutionarily conservative transcription of a series genes involved in the innate and adaptive immune response in immunocompetent cells. NF-κB is present in the cytoplasm in a form bound to inhibitor of B (IB), which prevents NF-κB from entering the nuclei. A wide range of stimuli activates NF-κB, including cytokines, activators of protein kinase C, viruses, and reactive oxidative species The activation and nuclear translocation of NF-κB from cytoplasm to nuclei have been associated with increased transcription of a number of different genes, including those encoding chemokines (IL-8) and cytokines (IL-1, IL-6, TNFα, IL-12), adhesion molecules (e.g. intercellular molecule-1, vascular cell adhesion molecule, E-selectin), acute-phase proteins, and inducible effector enzymes (e.g. inducible nitric oxide synthase and cyclooxygenase-2) [13,14]. NF-κB is highly activated at sites of inflammation in such diverse diseases as multiple sclerosis, inflammatory bowel diseases, psoriasis, and asthma [15,16].

The correct functioning of innate immunity guarantees homeostasis in the organism. Early observations of leukemia and Alzheimer's disease patients [17] stimulated us to investigate drugs which can regulate innate immune reactions. Two drugs used in Alzheimer's therapy were studied: donepezil and an extract from *Ginkgo biloba* (EGb 761). Donepezil is a reversible inhibitor of acetylcholinesterase which delays the breakdown of acetylcholine released into synaptic clefts and thus enhances cholinergic neurotransmission. It is beneficial for people with mild, moderate, and severe dementia due to Alzheimer's disease and is associated with improvements in cognitive function and the activities of daily life [18]. EGb 761 is a standardized extract of *G. biloba* leaves and has antioxidant properties as a free radical scavenger. The standardized extract of *G. biloba* leaves is a well-defined product and contains approximately 24% flavone glycosides (primarily quercetin, kaempferol, and isorhamnetin) and 6% terpene lactones. EGb 761 promotes vasodilation and improves blood flow through arteries, veins, and capillaries. It inhibits platelet aggregation and prolongs bleeding time [19]. In the face of innate immune deficiencies, searching for different drugs with potential innate immunity stimulatory effects seems to be a priority.

In the present study we assessed the effect of donepezil and the *G. biloba* extract EGb 761 on the distribution of NF-κB and the development of innate immunity in human leukocytes. We hypothesized that the plant (*G. biloba*) or the synthetic (donepezil) drug may regulate the innate mechanism by inhibiting NF-κB as a potential therapeutic strategy.

2. Materials and methods

2.1. Isolation of peripheral blood leukocytes (PBLs)

PBLs were isolated from heparinized peripheral blood (10 U/ml) by gradient centrifugation in Gradisol G with a density of 1.115 g/ml (Aqua Medica, Poznań, Poland). Five milliliters of blood was layered onto 3 ml of Gradisol G and centrifuged for 30 min at 2000 rpm. The cells were collected from the interphase, washed two times with Dulbecco medium supplemented with 2% of calf serum (c.s.), and suspended in this medium at a density of 2×10^6 cells/ml.

2.2. Donepezil

A donepezil (2-[(1-benzyl-4-piperidyl)methyl]-5,6-dimethoxy-2,3-dihydroinden-1one) preparation was obtained by dissolving Aricept (5 μg/ml, Pfizer, Switzerland) in deionized water (1 mg/ml).

2.3. EGb 761

The *G. biloba* extract preparation was obtained by dissolving Tanakan (40 μg/ml, IPSEN, France) in <1% DMSO solution (1 mg/ml). EGb 761 is a standardized preparation with 24% flavones and 6% terpenes counting up on bilibalide.

2.4. Virus

Vesicular stomatitis virus (VSV), Indiana strain, *Rhabdoviridae*, was propagated and titrated in L_{929} cells. The titer of the virus was expressed with reference to the value of $TCID_{50}$ (tissue culture infectious dose based on the cytopathic effect caused by the virus in about 50% of infected cells).

2.5. Cells lines

L_{929} cells (ATCC CCL 1), a murine fibroblast-like cell line, were maintained in Eagle's medium with 10% c.s., antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM L-glutamine.

A_{549} cells (ATCC CCL 185), a human epithelial-like cell line, were maintained in Dulbecco medium with 10% c.s., antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM L-glutamine.

2.6. MTT assay for cell viability

Cell respiration, an indicator of cell viability, was determined on the basis of the mitochondria-dependent reduction of MTT (3-[4,5-dimethyltiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma Chemicals Co., USA) with formazan. Leukocytes were cultured in 96-well plates (2×10^6 cells/ml) for 72 h in the presence of various concentrations (5–100 μg/ml) of donepezil. After 3 h of incubation at 37 °C, the formazan blue that formed in the cells was dissolved in SDS (sodium dodecylsulfate) for 2 h at 37 °C. The optical density was measured at 540 nm.

2.7. Trypan blue staining for cell viability

One hundred microliters of cell suspension (2×10^6 cells/ml) was incubated with 100 μl of 0.4% trypan blue. After 15 min of incubation at room temperature, the viability of the cells was measured in a Bürker chamber. Dead cells were labeled with navy-blue and live cells remained unstained.

2.8. Determination of resistance/level of innate immunity of PBLs

Resistance/innate immunity was determined by infection of leukocytes (2×10^6 cells/ml) with a VSV dose of 100 $TCID_{50}$. After 40 min of adsorption, the virus was washed out three times with 5 ml of Dulbecco medium and the cells were suspended in 1 ml of Dulbecco's medium with 2% c.s. A sample of the infected cells was kept at 4 °C and served as a control of the starting level of the virus. The rest of the cells were incubated at 37 °C and samples of medium above the infected cells were collected each day and titrated in L_{929} cells. The titer of virus is expressed in $TCID_{50}$. Based on the replication