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

Marta Sochocka a,⁎, Ewa Zaczyńska a, Agnieszka Taboł a, Anna Czarny a, Jerzy Leszek b, Maciej Sobczyński c

a Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, R. Weigla Street 12, 53-114 Wrocław, Poland
b Department of Psychiatry, Medical University of Wrocław, J. Kraszewskiego Street 25, 50-229 Wrocław, Poland
c Faculty of Biotechnology, Department of Genomics, University of Wrocław, Przybyszewskiego Street 63/77, 53-148 Wrocław, Poland

A R T I C L E   I N F O

Article history:
Received 26 May 2010
Received in revised form 12 August 2010
Accepted 30 August 2010

Keywords:
Innate immunity
PBLs
NF-κB
Natural drugs
Synthetic drugs

A B S T R A C T

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 L1210 and A549 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.

© 2010 Elsevier B.V. All rights reserved.

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 activation 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 indicator 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.
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 (IκB), 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 [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 × 10⁶ cells/ml.

2.2. Donepezil

A donepezil (2-{(1-benzyl-4-piperidyl)methyl}-5,6-dimethoxy-2,3-dihydroinden-1-one) 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 bilibilide.

2.4. Virus

Vesicular stomatitis virus (VSV), Indiana strain, Rhadoviridae, was propagated and titrated in L292 cells. The titer of the virus was expressed with reference to the value of TCID₅₀ (tissue culture infectious dose based on the cytopathic effect caused by the virus in about 50% of infected cells).

2.5. Cells lines

L292 cells (ATCC CCL 1), a murine fibroblast-like cell line, were maintained in Eagle’s medium with 10% c.s., antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin), and 2 mM l-glutamine.

A549 cells (ATCC CCL 185), a human epithelial-like cell line, were maintained in Dulbecco medium with 10% c.s., antibiotics (100 μg/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-di methylthiazol-2-yl]-2,5-diphenyltetrazolium bromide] (Sigma Chemicals Co., USA) with formazan. Leukocytes were cultured in 96-well plates (2 × 10⁶ 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 × 10⁶ 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 × 10⁶ cells/ml) with a VSV dose of 100 TCID₅₀. 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 L292 cells. The titer of virus is expressed in TCID₅₀. Based on the replication
of VSV in PBLs, we identified three different levels of natural antiviral immunity: a VSV titer higher than 4 log TCID₅₀ was considered deficiency of resistance/innate immunity, a titer of 2–3 log indicated partial resistance, and a titer of 0–1 log indicated complete resistance/innate antiviral immunity.

2.9. Immunocytochemical staining of PBLs for NF-κB

PBLs were placed on poly-1-lysine-coated microscope slides using cytocentrifugation for 5 min at 500 rpm. The slides were fixed in 4% paraformaldehyde solution at room temperature for 15 min. After washing in distilled water, endogenous peroxidase activity was blocked by incubating the slides in a 3% hydrogen peroxide solution in methanol for 10 min and washed in 10 mM phosphate-buffered saline (PBS, pH = 7.5). The PBLs were treated with universal blocking serum for 20 min at room temperature. Then the cells were incubated at room temperature for 2 h in a wet chamber with a polyclonal rabbit anti-NF-κB IgG antibody (p65 subunit; Chemicon International Inc., CA, USA). After washing in PBS, the preparations were incubated with a biotinylated secondary anti-rabbit antibody (Novocastra Laboratories Ltd., UK) at room temperature for 30 min. This was followed by washing in PBS and an application of peroxidase-conjugated avidin (Novocastra Laboratories Ltd., UK) at room temperature for 30 min. After washing in PBS, chromagen fast diaminobenzidine-DAB was used for 5–10 min (Liquid DAB Substrate Kit for Peroxidase; Novocastra Laboratories Ltd., UK). The preparations were counterstained in hematoxilin and finally washed with distilled water. PBLs expressing p65 in the nucleus were labeled as NF-κB(+) cells. Activation of the NF-κB system in PBLs was expressed as the percentage of NF-κB(+) cells of all the quantified PBLs.

2.10. Statistical analysis

Friedman ANOVA and the post hoc Wilcoxon test with Benjamini-Hochberg correction were used for comparisons between groups. Results are presented as the mean, standard error, and non-outlier range.

A General Linear Model with quasi-Poisson errors and the Spearman correlation coefficient r_S was used to describe the relationship between innate immunity and NF-κB activity. The level of innate immunity was the dependent variable (Y) in the regression model. This variable assumed three values: 1—good resistance/innate immunity, 2—partial resistance/innate immunity, and 3—deficient or low resistance/innate immunity. The independent variable was NF-κB activity.

Additionally, the relationship between innate immunity and NF-κB activity was described by the equation:

\[
Y = b_0 \exp(b_1 X)
\]

where Y is the level of innate immunity and X the level of NF-κB activity.

To determine the influence of the two medications on the level of innate immunity depending on time and dose, an experiment based on a central composite design (CCD) was designed. It allowed constructing the equation:

\[
Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_1 X_2 + b_4 X_1^2 + b_5 X_2^2
\]

where Y is the level of innate immunity, X₁ the time measured in hours after drug administer, X₂ the faction of the maximum nontoxic dose of the medication. Response surfaces were the graphical outcomes of such an experimental setup. To check which drug increased the level of innate immunity more effectively, evenness was tested involving both models of the regression in an equation with the type of the drug as an additional variable:

\[
Y = b_0 + b_1 X_1 + b_2 X_2 + b_3 X_1 X_2 + b_4 X_1^2 + b_5 X_2^2 + b_6 X_3
\]

where Y is the level of innate immunity, X₁ the time measured in hours after drug administer, X₂ the faction of the maximum nontoxic dose of the drug, and X₃ the type of the drug (0—Egb 761, 1—donepezil). Results were regarded as statistically significant at
3. Results

The influence of donepezil and EGb 761 extract, both used in Alzheimer therapy, on innate immune reactions were investigated, i.e. the resistance of freshly isolated peripheral blood leukocytes from healthy blood donors to viral infection and NF-κB activation.

3.1. Toxicity and antiviral activity tests for donepezil and EGb 761

First of all the nontoxic concentrations of donepezil and EGb 761 for cells used in the experiments, i.e. PBLs and the cell lines L929 and A549 was studied. The MTT test and trypan blue staining were applied. Fifty μg/ml of donepezil and 100 μg/ml of EGb 761 were the highest nontoxic doses for these cells. Antiviral effects of these concentrations on VSV and HHV-1 (human herpes virus 1) were not observed (results not shown). Both viruses treated with donepezil and EGb 761 replicated in cell lines, whereas replication was inhibited in PBLs, because only leukocytes exhibited resistance/innate immunity.

3.2. Influence of donepezil and EGb 761 on innate antiviral immunity of human PBLs

Early results obtained with donepezil [17] showed that it can inhibit VSV replication in PBLs in a dose-dependent manner (Fig. 1). Our studies presented antiviral activity of donepezil which included stimulation of leukocyte-unspecific resistance as part of the innate immune system. Those results stimulated us to study the effect of a different, natural, drug also used in neurodegenerative disease therapy, i.e. EGb 761, on innate antiviral immunity and determine the influence of these drugs on different mechanisms of innate immunity. The leukocytes of the 30 healthy young blood donors had different susceptibilities to viral infection. The PBLs of 15 donors were completely resistant to VSV infection while 15 were sensitive. EGb 761 inhibited VSV replication in the sensitive leukocytes in a dose-dependent manner and the differences were statistically significant (Fig. 2). Simultaneously it was showed that neither donepezil nor EGb 761, which inhibited VSV replication in sensitive leukocytes, increased the viral titer in resistant leukocytes. Additionally, the influence of donepezil and EGb 761 on leukocyte resistance to VSV infection was measured when the drug was applied 24 h before VSV infection. In this study, no statistically significant influence of donepezil and EGb 761 on leukocytes was observed. PBLs replicated virus similarly with or without drug administration. These results indicate that both drugs have a very strong stimulatory influence on innate immunity of human leukocytes to viral infection.

3.3. Inhibitory effect of donepezil and EGb 761 on NF-κB activation

Improper activation of the NF-κB system is frequently observed in the development of many inflammatory diseases and cancer. In our earlier investigations with NF-κB activation of PBLs from patients with chronic heart failure, over-activation of the NF-κB system was observed [20,21]. Inhibiting NF-κB signaling could be a potential therapeutic strategy. Therefore we cytocentrifuged slides of donepezil- or EGb 761-treated and untreated PBLs stained with the anti-p65 subunit of NF-κB. Both donepezil (Fig. 3A) and EGb 761 (Fig. 3B) statistically significantly reduced the number of NF-κB-positive leukocytes in a dose-dependent manner (n = 15).

3.4. Correlation between NF-κB activation and level of innate immunity of PBLs

To determine the relationship between NF-κB and leukocyte resistance to viral infection, the level of NF-κB active nuclei and level of innate immunity (n = 15) were evaluated using Spearman’s correlation coefficient \( r_S \). The correlation \( r_S = -0.58 \). It was statistically significant (p = 0.0138), which confirmed that the degree of innate immunity was negatively correlated with the activation of NF-κB.

---

Fig. 2. The effect of the Ginkgo biloba extract EGb 761 on VSV replication in human PBLs isolated from healthy blood donors (n = 15). Freshly isolated PBLs (2 × 10⁶/ml) from healthy donors were infected with VSV (10^2 TCID₅₀/ml). After 40 min of virus absorption at room temperature, half of the PBL suspension was treated with EGb 761 and half not. Then the cells were washed 5 times with culture medium and incubated at 37 °C for 3 days. Samples of medium were collected each day and treated in L929 cells. Statistical analysis was performed with ANOVA Friedman’s test. The results are presented as the mean, standard deviation, and non-outlier ranges.
immunity of leukocytes correlated with the level of NF-κB activation. Coefficient of determination $R^2 = 0.34$. It indicated that level of innate immunity of leukocytes in 34% could be explained by the level of NF-κB activation and in 66% by other factors. Thus the correlation was not complete, which showed why the percentage of positive NF-κB in leukocytes of several donors while the degree of innate immunity was very good. To better understand the dependence between NF-κB activation and innate immunity, a regression model was constructed. The model was statistically significant ($p = 0.018$) and correlation between the level of NF-κB activation and the degree of innate immunity is shown in Fig. 4 as the curve of the equation:

$$Y = 2.6833e^{-0.02536x}$$

As presented in Fig. 4, the curve decreases. Previously mentioned that 1—means good resistance/innate immunity, 2—partial resistance/innate immunity, and 3—deficient or low resistance/innate immunity. Therefore, together with increasing NF-κB activation, the level of VSV

---

**Fig. 3.** Changes in the NF-κB activation in uninfected and VSV-infected PBLs from healthy blood donors ($n=15$) exposed in vitro to donepezil (A) and EGb 761 (B). Activation of NF-κB was measured using rabbit anti-NFκB p65 subunit polyclonal antibody. VSV-infected and uninfected PBLs were incubated with different concentrations of donepezil or EGb 761 for 3 days. Each day a preparation of leukocytes was dyed with immunocytochemical staining. DAB is a detection system which labels NF-κB-positive cells dark-brown. Activation of the NF-κB system in PBLs was expressed as the percentage of NF-κB(+) cells from all quantified PBLs. Statistical analysis was performed with ANOVA Friedman’s test. The results are presented as the mean, standard deviation, and non-outlier ranges.
replication decreased, that is the degree of innate immunity increased. The model explained ca. 34% of the variance in the dependent variable and it agreed with the earlier estimated determination factor.

3.5. Influence of the two drugs on innate immunity of human PBLs

Early results showed that donepezil and EGb 761 stimulated the level of innate immunity of human leukocytes. The next step was to test the influence of the two drugs on innate immunity of leukocytes depending on the time of drug administer and its concentration. It was very interesting to see if donepezil and EGb 761 stimulated leukocyte resistance in the same manner or if there were differences. For this purpose an experiment with response surfaces was proposed. It allowed determining a suitable regression equation. On the basis of this experiment, two regression models for donepezil and EGb 761 were determined. Then the significant differences between these models were examined.

Model for donepezil:

\[ Y = 0.8469 + 0.0446X_1 - 2.5229X_2 - 0.0128X_1X_2 - 0.0003X_1^2 + 1.5172X_2^2 \text{ (mat and meth) (2)} \]

Model for EGb 761:

\[ Y = 0.0317X_1 - 2.4168X_2 - 0.0184X_1X_2 + 1.9313X_2^2 \text{ (mat and meth) (2)} \]

The coefficient of determination for donepezil was \( R^2 = 0.981 \) and for EGb 761 it was \( R^2 = 0.974 \). The time of incubation as well as the concentration significantly influenced the degree of leukocyte resistance to VSV infection. The results were shown in Figs. 5 and 6. Both donepezil and EGb 761 inhibited VSV replication in human leukocytes, but the effective concentrations differed. The highest dose of EGb 761 decreased VSV replication more than donepezil did. This suggests that the natural drug was more effective in stimulating leukocyte resistance. To determine the effectiveness of donepezil and EGb 761, two regression models were used; identity of these models, shown as response surfaces, were tested, including an additional variable, i.e. the drug. The effective concentrations of the drugs differed therefore we tested relative concentrations. The factor of the drug variable was statistically significant \( (p = 0.00000012) \) and amounted \( b_n = 1.0466 \) (mat and meth [3]). This means that if donepezil was used, the VSV replication level was fairly higher (by about 1.0466) with concentration and time were on the same level.

4. Discussion

In many infectious, inflammatory, autoimmune, and neurodegenerative diseases and cancer, dysregulations in innate immunity are observed. It is still unclear whether these dysfunctions are caused by or are the result of disease development. Therefore, searching for drugs with potential regulatory effects on innate immune reactions seems very important and suitable. Now the question is whether regulation of innate immunity mechanisms can influence on disease difficulty.

The most important results of our study were the amplification of innate antiviral resistance of PBLs by donepezil and EGb 761 and a reduction in NF-kB activation. The antiviral activity of donepezil was presented for the first time earlier [17]. Here we confirmed a similar effect for the \( G. \) \( biloba \) extract EGb 761. Present knowledge about donepezil and EGb 761 in AD therapy showed their protective and stimulating effects on the central nervous system (CNS). The most important are the stimulation of neurogenesis and improvements in nerve conduction and brain circulation. Recently, communication between the nervous and immune systems has become increasingly better understood and described. Changes in the interactions of the two systems are usually connected with the activity of many stress factors and immune deficiency. The possible influence of drugs used in neurodegenerative diseases on the immune system is now of interest in many research and development centers. The results of our studies indicate stimulating and modulating effects of both preparations on the innate immunity of leukocytes and cytokine production. Villasenor-Garcia et al. [22] showed a stimulatory effect of EGb 761 on phagocytosis of macrophages, whereas Ilarlori et al. [23] showed the participation of RANTES and MCP-1 in Alzheimer’s disease pathogenesis; a modulating effect of donepezil on the distributions of these chemokines was also confirmed. Our results showed dose-independent antiviral effects of donepezil and EGb 761 on human leukocytes, and this influence was not accidental. An effect on VSV infection in the L029 and A549 cell lines was not observed. Differences in the antiviral activity of the two drugs in leukocytes and the cell lines resulted from the natural development of innate immunity and its stimulation by leukocytes in contrast to L029 and A549. Until now except of amplify of innate immunity reduction of innate immunity of PBLs by donepezil and EGb 761 was not observed, while this effect was suggested by our previous studies. Reduction and simulation of resistance dependent on the level of cell resistance was also investigated when other immunomodulators, such as proline-rich polypeptide (PRP) [24] or flavones from \( Scutellaria \) \( baikalensis \), were used. The effect was observed when the flavones were added to the PBLs just after or one day before viral infection. The effect depended on leukocyte resistance and the concentration of the flavones [25].

The precise mechanism of activity of donepezil and EGb 761 is still unclear. Suppressor of cytokine signaling proteins (SOCS), adenosine, and members of the Tyro3 family are natural regulators of innate immunity [26]. Perhaps donepezil or the plant preparation showed a regulatory effect by stimulation of these molecules. Yoshimura et al. [27] showed that SOCS1 and SOCS3 inhibited NF-κB-dependent pro-inflammatory cytokine production by degradation of p65 or phosphorylated MAL. SOCS activated by TLR ligands therefore also inhibited Janus kinases (JAKs) and STAT complexes and caused inhibition of the expression of genes for interferons [28,29]. The possible participation of the newly discovered microRNA (miRNA) in innate immune regulation was also postulated. miRNAs are a class of short, non-protein-encoding RNAs that regulate the expression of protein-encoding genes at the posttranscriptional level. Regulating the differentiation of dendritic cells and macrophages via Toll-like receptors were also investigated. Small interfering RNA of viral origin functions as an intracellular mediator in the suppression of viral infection in eukaryotes as diverse as plants, insects, nematodes, and fungi and it is speculated that endogenous mammalian miRNA can
have similar impact. The antiviral function of miRNA probably drives the expression of different subsets of miRNA in different cellular lineages. This can lead to the great number of roles miRNA plays in lineage differentiation and stability [30]. Therefore, follow-up investigations to relate the mechanisms of the reactions of the two drugs are required.

Our results also presented for the first time a statistical analysis of the effectiveness of donepezil and EGb 761 in increasing leukocyte resistance, including the time of administration, the concentration, and the type of drug. The response surfaces for the two drugs showed that the plant preparation was more efficient than the synthetic one in increasing the innate immunity of leukocytes. The effect was probably dependent on differences in the mechanisms of action and the drugs' constitution. Synergy is the basis of effective activity of many drugs. In natural plant preparations, active substances are rarely found separately, but are mostly complexes of different substances which interact, regulate, and intensify. The side effects of natural plant preparations are also better tolerated by the human body than synthetic preparations, of which many doctors are now convinced.

NF-κB is a critical activator of genes for inflammation and immunity. It plays a central role in regulating proinflammatory mediators and cytokines important in innate immune reactions. Recently, the cellular and molecular mechanisms of inflammation have been the focus of designing new anti-inflammatory therapies. Inhibition of NF-κB might contribute considerably to the anti-inflammatory as well as anti-cancer and anti-neurodegenerative effects of different drugs [31]. In our study, the effects of donepezil and EGb 761 on NF-κB activation by human leukocytes from healthy blood donors was investigated. It was shown that both drugs strongly inhibited NF-κB activation by inhibiting p65 activation in uninfected and VSV-infected human leukocytes. Donepezil and EGb 761 decreased the level of NF-κB activation in a dose-dependent manner. Similar effects on NF-κB activity for several drugs used to treat inflammatory diseases, from anti-IL-1 and anti-TNF-α therapy to anti-inflammatory drugs such as corticosteroids, acetylosalicylic acid, and non-steroidal anti-inflammatory drugs, were reported. Yin and Gaynor [32] reported that aspirin and salicylates blocked NF-κB activation by blocking the ATP binding site of IKKβ. Therefore it was shown that the inhibition of NF-κB activation by high doses of salicylates in endothelial cells prevents the transendothelial migration of neutrophils [33] and low doses of aspirin reduce vascular inflammation and aortic atherosclerotic lesions in a murine model of...
atherosclerosis. These effect were associated with a reduction in inflammatory cytokine levels and aortic NF-κB activity [34]. Mesalazine, an aminosalicylate derivative with anti-inflammatory properties, prevents IL-1-mediated stimulation of p65 phosphorylation without inhibiting IκBα [35]. Other synthetic drugs were also investigated. Shin and et al. [36] demonstrated that BMD (N1-benzyl-4-methylbenzene-1,2-diamine), a novel synthetic compound, exhibited a dose-dependent inhibitory effect on LPS-mediated NF-κB transcriptional activity in macrophages. The compound inhibited LPS-mediated nuclear translocation of NF-κB p65 and DNA binding activity of NF-κB complex in parallel, but did not affect LPS-mediated degradation of inhibitory κB (IκB). The results indicate that BMD could inhibit the nuclear localization step of NF-κB p65 without affecting IκB degradation. The effect of natural drugs on the activity of the NF-κB system was also reported. Nair et al. [37] showed that flavonoid-queceretin decreased the phosphorylation of IκBα, suggesting that it decreased the activation of NF-κB. This decrease in phosphorylation of IκBα and IκBα may be a direct mechanism by which this substance inhibits the activity of NF-κB. Anti-inflammatory activity was also investigated with procyoinainid extract (PE) from grape seeds. Terra et al. [38] showed that this extract could inhibit NO and PGE 2 production and the translocation of NF-κB p65 to the nucleus. In light of recent investigations, our results seem to be very interesting in the context of different therapeutic strategies, especially those with NF-κB gene expression and the regulation of innate immune mechanisms.

Furthermore, the results of our laboratory showed for the first time correlation between the resistance of human leukocytes to viral infection and NF-κB activation. Statistical analysis showed that a higher level of NF-κB activation was connected with very good innate immunity. However, only in 34% of the level of innate immunity could be explained by NF-κB activation. The results obtained from patients with high leukocyte resistance to viral infection and a low level of NF-κB activation confirmed this effect. Perhaps the level of innate immunity is also highly dependent on other, endogenous transcription factors, for example AP-1 or IRSs.

The influence of donepezil and EGb 761 on innate immune mechanisms is not fully understood. Therefore preventive or target therapy by means of synthetic and natural drugs could be recommended. This treatment aims to balance deficiency or suppression of over-activated innate immune reactions. The recent investigations of many R&D centers are concerned with a better understanding of the role of inflammation in the development of many neurodegenerative, autoimmune, and inflammatory diseases. This is required before we can safely develop therapeutic strategies to prevent neuronal and other cell damage. New therapies must rely on the combination of endogenous anti-inflammatory pathways, the identification of early markers of neuronal deterioration, and treatment with a combination of synthetic and natural drugs involving immune modulation and anti-inflammatory therapies [12].

Conflict of interest

The authors declare that they do not have any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning this submitted work that could inappropriate influence, or be perceived to influence, our work.

Acknowledgements

The authors wish to thank MSc Katarzynie Zwoliśkij (IITD PAN, Poland) for generous gift of anti-NF-κB Ab sample. This work was supported by an Independent Research Grant (No. 2005-0698) from Pfizer Inc.

References