GENETIC POLYMORPHISMS OF RAGE, NOD1 AND ß-DEFENSINS IN MULTIPLE SCLEROSIS, STROKE AND PANCREATITIS

Ph.D. Thesis

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Szeged

2011.

INTRODUCTION

Innate immunity relies on the detection of danger- and pathogen associated molecular patterns and a prompt or quick response to maintain the homeostasis of the host. Several families of secreted or cellular pattern-recognition receptor molecules - including receptor for advanced glycation end products (RAGE), Toll-like receptor family, Nod-like receptors and Rig-I-like receptors - are able to sense endogenous danger signals or invading pathogens. Activation of these receptor molecules leads to a multistep cascade including nuclear factor-κB, mitogen-activated protein kinases, and type I interferon response resulting in production of mediators of inflammation and antimicrobial defense molecules and/or alarmins. Among the latters, different types of defensins can be regarded as the main effector molecules of mucosal immune system.

The receptor for advanced glycation end products (**RAGE**) is a member of immunoglobulin superfamily of cell surface molecules. RAGE binds advanced glycation end products, but also, a wide scale of endogenous alarmin molecules that are emerged from dying host cells into the extracellular spaces upon microorganism-induced or other type of cell damages, such as ribonucleic and deoxyribonucleic acid, amphoterin, ß-sheet fibrils and S100 proteins. The ligand/RAGE interactions and the resulting RAGE upregulation are believed to activate many cell signaling pathways, leading to enhanced production of reactive oxygen species and proinflammatory cytokines. Due to its broad spectrum of sensed molecular patterns, RAGE may contribute to the pathogenesis of several multifactorial diseases. Three single nucleotide polymorphisms (SNPs) have been highlighted including two common functional SNPs (−429T/C [rs1800625] and −374T/A [rs1800624]) in the promoter region and a common coding change of a glycine to serine at amino acid 82 (G82S or Gly82Ser [rs2070600]). The −429T/C and −374T/A SNPs have been revealed to have effects on transcriptional activity. The G82S SNP influences the AGE-binding domain, and the RAGE 82S allele up-regulates the binding of S100/calgranulins. The expression of RAGE is increased in neurodegenerative diseases, therefore we investigated the relevance of SNPs of RAGE promoter in the risk of multiple sclerosis.

The NLR (Nod-like receptor) family has more than 20 members including nucleotide-binding oligomerization domain containing protein 1 and 2 (**NOD1** and NOD2). These cytosolic receptors are essential in sensing intracellular microbial components. NOD1 is activated by peptides that contain D-γ-Glu-meso-diaminopimelic acid that is derived from peptidoglycan present in almost all Gram-negative bacteria, and is produced either during synthesis and/or degradation. NOD1 is ubiquitously expressed in various cell types, including epithelial cells, antigen presenting cells, human gingival fibroblasts, myofibroblasts, astrocytes and microglia. NOD1 participates in the response to *Chlamydia*, *Shigella*, enteroinvasive *Escherichia coli* and *Campylobacter.* The c.796G>A polymorphism of NOD1 (rs2075820) has been described; it encodes a changed protein, altering a glutamic acid residue (E), where the A allele corresponds to the K variation-lysine (E266K). In view of the close relation between NOD1 activation and *Chlamydia* infection, we considered it of interest to investigate the genetic polymorphism of NOD1 from the aspect of the development of stroke. Any impairment in function of NOD receptors may lead to an increased risk of infection, or an inappropriate mucosal defense mechanism.

Antimicrobial peptides (AMPs) are key components of innate immunity. Activation of pathogen associated molecular patterns in various epithelial cells induces anti-bacterial responses. The sufficient defensin production are crucial in maintaining effective antibacterial defense. Human **ß-defensins**, important components of host defense at the mucosal surfaces, display a broad spectrum of activity against bacteria, fungi and viruses, and also exhibit other functions, including potent chemotactic activity toward immature dendritic cells and memory

T cells. Human ß-defensins are apparently expressed in epithelial cells of the skin, gut, respiratory and urogenital tissue. It is noteworthy that HBD-1 mRNA expression has also been detected in pancreatic acinar cells. While human beta-defensin 1 (HBD-1) is produced constitutively, HBD-2 is induced by interleukin-1ß, tumor necrosis factor-α and bacterial lipopolysaccharide, and by contact with Gram-negative and Gram-positive bacteria, and various fungi. Thus, HBD-2 levels are increased in inflammatory disorders, whereas HBD-1, being constitutively expressed, may serve as a defense in the absence of inflammation. Three frequent single nucleotide polymorphisms at positions c.–20G>A (rs11362), c.–44C>G (rs 1800972) and c.–52G>A (rs1799946) in the 5'-untranslated region of the DEFB1 gene were described. The level of β-defensin expression varies from individual to individual and it has been suggested that this variation is due to genetic differences in the genes encoding the HBDs. Two types of genetic polymorphisms have been identified in genes encoding defensins, copy number variations and single nucleotide poymorphisms. Hollox *et al* have described copy number polymorphisms of the DEFB4 gene, which encodes HBD-2, with copy numbers ranging from 2 to 12 per diploid genome. Higher DEFB4 copy numbers correlated with higher levels of DEFB4 mRNA expression. Accordingly, it is presumed that this polymorphism may be an important component of genetic variation in susceptibility to infections.

Associated diseases

The adequate function of the components of the innate immunity is crucial in maintaining the homeostasis of the host. Genetic polymorphisms in the genes encoding PRRs and AMPs altering expressional level or subsequent amino acid sequence may impair their functions, and contribute to the pathogenesis of several multifactorial diseases. Examples of such diseases that were investigated in our studies are highlighted below.

Multiple sclerosis (MS), a devastating neuroinflammatory disorder of the central nervous system, can be characterized by multiple demyelization foci of the central nervous system, which, in the course of the disease, can materialize in different time patterns. The main pathological features of the disease are the destruction of the myelin sheaths of the nerve fibers, the relative sparing of the axons, and the infiltration of inflammatory cells in a perivascular distribution. Although the well-defined autoimmune activities of the different types against the central nervous system are of great importance in the course of the disease, the pathomechanism and the direct causative factors have not yet been elucidated. There are data indicating that changes in the level of the ROS may play roles in the pathomechanism of MS. The disease is determined by a combination of exogenous factors and genetic background. Immunohistological studies of spinal cord tissue derived from MS patients have demonstrated enhanced RAGE expression in neurons and inflammatory cells.

Stroke is a major health problem worldwide and there is an urgent need for a better understanding of its causes and for the prevention of its occurrence. Ischemic stroke is a frequent heterogeneous multifactorial disease that is affected by several environmental factors and genetic mutations. Chronic infections with certain pathogens, such as *Chlamydophila pneumoniae*, and genetic parameters that influence inflammatory reactions are suggested to contribute to the disease. Serological evidence of past infection with *C. pneumoniae*, a common respiratory pathogen, has been claimed to be associated with risk of ischemic stroke. The chronic infection of susceptible target cells, especially monocytes and endothelial cells, initiates local inflammation, which is important in plaque formation. *Chlamydiae* induce chronic activation of the immune system, but little is known about the mechanisms of *C. pneumoniae*-induced target cell alterations. The cytosolic NOD proteins are molecules that have been implicated in intracellular pattern recognition of bacteria such as the obligate intracellular *Chlamydia.* NF-kB activation via NOD1 is crucial in maintaining inflammatory

responses and cytokine activation. It is also known that the activation of NOD receptors mediates the induction of human beta-defensins in various epithelial cells. NOD1-mediated endothelial cell activation by *Chlamydophila. pneumoniae* was recently demonstrated; therefore, appears that NOD1 is a potent innate immune receptor for *C. pneumoniae* in endothelial cells and monocytes.

Acute pancreatitis (AP) is a disease with a wide spectrum of clinical courses, ranging from mild to severe forms, with a high rate of complications and a high risk of lethality. Severe AP (SAP) is characterized by various degrees of necrosis of the pancreatic parenchyma and by local and systemic complications, such as the systemic inflammation response syndrome and multiple organ failure. Bacterial contamination of pancreatic necrosis and the ensuing sepsis are the main causes of death in SAP facilitated by gut barrier dysfunction and increased intestinal permeability resulting in bacterial translocation through the gut. Pancreatic stellate cells (PSCs) are able to recognize pathogen associated molecular patterns via their receptors, leading to the activation of signaling pathways and proinflammatory responses. Thus, PSCs might play a role in immune function of the pancreas through the recognition of pathogen associated molecular patterns. Mucosal epithelial cells produce a variety of antimicrobial peptides that protect the mucosal surface against invading microbes. Which is certainly important in preventing the bacterial translocation in the case of mild pancreatitis. There is evidence that host genetic factors can affect the severity of AP. Genes coding for inflammatory cytokines have been reported to act as modifier genes in AP.

Defensin induction by *Chlamydophila pneumoniae*

Chlamydophila pneumoniae, an obligate intracellular parasite and a common cause of acute respiratory infection, has a tendency to cause persistent inflammatory diseases such as atherosclerosis, which may lead to cardiovascular diseases or stroke. Three principle cell types involved in the atherogenic process within the developing atheroma include endothelial cells, vascular smooth muscle cells, and monocytes/macrophages. Recent publications have indicated that *C. pneumoniae* infection induces HBD-2 expression in human mononuclear cells and in gingival fibroblasts. It has been reported that *C. pneumoniae* can infect human endothelial cells and induce the expression of cytokines, adhesion molecules, chemokines and molecules with procoagulant activity. As endothelial cells are related to the physiopathology of stroke, we considered it of interest to investigate the effects of *in vitro C. pneumoniae* infection on the HBD-2 expression in brain capillary endothelial cells.

AIMS

The genetic variations of pattern recognition receptor molecules and defensins may influence the severity and/or the course of multifactorial diseases, such as multiple sclerosis, stroke and pancreatitis. Therefore we investigated:

- Three common genetic polymorphisms of RAGE in patients with multiple sclerosis;
- The c.796G>A SNP of NOD1 gene was investigated from the aspect of the development of ischemic stroke;
- The relevance of three SNPs in the promoter region of DEFB1, that might be related to the development of SAP or to bacterial contamination of pancreatic necrosis, was investigated. Accordingly, the copy number variation of DEFB4 gene, that alters the level of produced HBD-2 peptide, was studied among patients with acute pancreatitis;
- As endothelial cells are related to the physiopathology of stroke, we completed our studies with the investigation of the effects of *in vitro* infection with *Chlamydophila pneumoniae* on the expression of human beta-defensin 2 in human brain capillary endothelial cells.

PATIENTS AND METHODS

Patient group with multiple sclerosis and controls

One-hundred and fifty four relapsing-remitting and 14 secondary progressive type MS patients were included in the study. The clinical inclusion criterion was a diagnosis of clinically definitive MS (Poser criteria). The control group consisted of 136 age- and gender matched healthy blood donors.

Patient group with acute ischemic stroke and controls

A total of 280 patients with acutely developing ischemic stroke, who had never suffered a previous stroke event, were involved in the study. All sera from ischemic stroke patients (from either patients or controls) were tested for *Chlamydophila pneumoniae* IgG. The control group consisted of 150 age- and gender matched healthy blood donors.

Patient group with acute pancreatitis and controls

One-hundred and twenty-four patients with acute pancreatitis were enrolled in our study, 30 of them suffered from the mild and 94 from the severe form of the disease, classified according to the original criteria of Ranson. The control cohort consisted of random, unrelated population of 100 healthy blood donors, who did not have any gastrointestinal and/or liver diseases.

DNA extraction

For the detection of various genetic polymorphisms genomic DNA was purified from peripheral whole blood. The leukocyte DNA was isolated according to the manufacturer's instructions. And stored at -20°C until further use.

Determination of RAGE SNPs

Restriction fragment length polymorphism (RFLP) assays were used to detect the −429T/C, −374T/A, and the G82S SNPs of RAGE. After the amplification the PCR product was digested with AluI restriction endonuclease for −429T/C and G82S and with TasI for −374T/A. The restriction products were separated by electrophoresis in 3% agarose gels and visualized in UV light after gel red staining.

Determination of c.796G>A polymorphism of NOD1

The 796G-to-A transition in exon 3 was analyzed by PCR-RFLP. To digest the PCR products AvaI restriction enzyme was used, then electrophoresed on 2% agarose gel, visualized under UV illumination and stained with 0.4 mg/l ethidium-bromide. The presence of a AvaI site (G allele) was indicated by cleavage of the 379 bp amplified PCR product to yield fragments of 209 and 170 bp.

Determination of DEFB1 SNPs

Genotyping was performed using Custom TaqMan® SNP Genotyping Assays. Fluorogenic minor groove binder probes were used for each cases using FAM and VIC dyes to determine the genotypes of DEFB1 c.-20G>A, c.-44C>G and c.-52G>A. Thermal cycling was performed on ABI Prism 7000 sequence-detection PCR systems. Initial and post-assay analysis was performed using the Sequence Detection System (Applied Biosystems) as outlined in the TaqMan Allelic Discrimination Guide.

Determination of DEFB4 gene copy number

A TaqMan® real-time PCR assay, specifically for amplification of genomic DEFB4, was established by using a specific set of amplification primers and a VIC-labeled probe. Quantitative DEFB4 amplification data were normalized to FAM-labeled albumin as an internal reference gene, which was co-amplified simultaneously in a single tube biplex assay. Quantification was performed by the comparative threshold cycle (C_T) method.

Cell line and *in vitro* infection with *Chlamydophila pneumoniae*

BB19 cells, human brain capillary endothelial cell line was cultured as monolayer culture and infected *in vitro* with *Chlamydophila pneumoniae* CWL-029 respiratory and CV6 cardiovascular strain at a multiplicity of infection of 5 IFU/cell. Control cells were not infected, or were treated with the same volume of mock prepartions. In addition to infection with viable *C. pneumoniae*, BB19 cells were stimulated with organisms that had been previously heat-inactivated. The plates were incubated at 37°C under 5% CO_2, and infected and control cells were harvested at the times indicated.

RNA isolation from BB-19 cell lysates and PCR amplification

Total RNA was isolated from cell lysates and complementary DNA (cDNA) was generated. After reverse transcription, real-time PCR analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics). Melting curves were generated after each run to confirm amplification of specific transcripts. Relative gene expression is given as a ratio between target gene and GAPDH gene expressions.

Western blot assays

BB-19 cells were homogenized and centrifuged to remove cell debris. Protein concentrations of cell lysates were determined using the Bio-Rad protein assay. To detect beta-defensin, aliquots were resolved by SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes (Bio-Rad). Preblocked blots were reacted for 4 h with specific polyclonal antibodies to human b-defensin 2 (RD Systems, Minneapolis, MN, USA). Blots were then incubated for 2 h with species-specific secondary antibody coupled to peroxidase. Filters were washed five times in PBS-Tween for 5 min after each step and were developed using a chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Human ß-defensin 2 ELISA

For direct testing of the presence of the HBD-2 peptide in the infected and uninfected brain capillary endothelial cells, and for measurement of the secreted HBD-2 protein into the tissue culture medium, a sensitive HBD-2 ELISA (Alpha Diagnostic, San Antonio, TX, USA) was used according to the manufacturer's instructions. The detection limit of this ELISA kit was 0.8 pg/HBD-2 protein/ml.

Immunofluorescent Assay

Expression of HBD-2 peptide by *Chlamydophila pneumoniae* infected and non-infected BB19 cells were investigated by immunofluorescence analysis. Cells were fixed and stained with goat anti-HBD-2 antibody (RD Systems). Bound antibody was detected with FITC-conjugated rabbit anti-goat IgG (Sigma). Fluorescence signals were analyzed via confocal laser scanning microscopy. The immunofluorescence of control and *C. pneumoniae* infected cells was quantitatively analyzed.

Statistical analysis

Statistical analyses for comparison of genotype frequencies between groups were performed by using the $\chi 2$ (chi-square) test, and Fisher's exact test if one cell had $n < 5$ with the probability level of $p<0.05$ indicating statistical significance. The relationship between genotypes and disease severity is presented as the odds ratio (OR), with a 95% confidence interval (CI).The genotype frequencies for each polymorphism were tested for deviation from the Hardy-Weinberg equilibrium by means of the $\chi 2$ test, with one degree of freedom used. Statistical calculations were performed with the Graph Pad Prism 5 (Graph Pad Software Inc., San Diego, CA) statistical program. For the quantification of HBD-2 expression using RT-PCR and ELISA, all values are expressed as mean ± SD. The data were subjected to two-tailed paired Student's test. For all statistical evaluations, $p<0.05$ was considered statistically significant. Data analyses were performed by Graph PadPrism 5 statistical program.

RESULTS

RAGE −374T/A polymorphism in patients with sclerosis multiplex

The distribution of the genotypes was in accordance with the Hardy–Weinberg equilibrium both in the control group and in the MS patients (p=0.975 and 0.999, respectively). There was a significant difference in genotypes between the patients and healthy controls (χ^2 test, p=0.003). It was a lower frequency of the AA genotype among the patients with MS as compared with controls: 14 of the 168 patients (8%) versus 25 of 131 healthy controls (19%); OR=2.75 vs. controls, 95% CI=1.319-5.733, p=0.007. Conversely, a higher frequency of TT genotype was observed among MS patients (84/168=50%) than among in controls (43 of 131=33%; OR=2.22 vs. controls, 95% CI=1.322−3.747, p=0.003). It is noteworthy that none of the 14 patients with secondary progressive type MS carried the AA genotype.

RAGE −429T/C polymorphism in patients with sclerosis multiplex

The distribution of the genotypes was in accordance with the Hardy–Weinberg equilibrium both in the control group and in the MS patients (p=0.646 and p=0.323, respectively).There was no significant difference in the −429T/C genotypic distribution between the patients with MS and the healthy controls.

RAGE G82S polymorphism in patients with sclerosis multiplex

One hundred eight DNA samples from the control group and 103 samples from the MS patients were further available for investigation of the G82S SNP. The distribution of the genotypes was in accordance with the Hardy–Weinberg equilibrium both in the control population (p=0.976) and in the patients with MS (p=0.970). There was no significant difference in the G82S genotypic distribution between the patients with MS and the healthy controls We could not detect the SS homozygote genotype in either the controls or the MS group. The frequencies of the GG and GS genotypes were similar in the two groups.

NOD1 c.796G>A polymorphism among *Chlamydophila pneumoniae* seropositive stroke patients

The distribution of the NOD1 genotypes was in accordance with the Hardy–Weinberg equilibrium both amongst the control population (χ^2=0.98, p=0. 322) and amongst the patient group (χ^2=1.55, p=0.213). There was no significant difference in the genotype distribution between the patients overall and the healthy controls (χ^2=4.2, p=0.121). When the *Chlamydophila pneumoniae* seropositivity was taken into account, a considerable difference in frequency of the NOD1 polymorphism was observed between the overall group of *C.*

pneumoniae-positive patients and *C. pneumoniae* positive controls (χ^2 =21.813, p<0.001). No significant difference was observed; however, between the NOD1 genotypes of controls and patients amongst the *C. pneumoniae*-negative groups. The AA homozygote and GA heterozygote mutant variants were detected in 16% (25 of 152) and in 51% (77 of 152) of the *C. pneumoniae*-positive stroke patients, as compared with 7% (6 of 84), and 29% (24 of 84), respectively, in the *C. pneumoniae*-positive healthy controls. (OR=2.559; 95% CI=1.105–6.517, p=0.04, and OR=2.56795% CI=1.451–4.540; p<0.001, respectively). The frequency of A allele was 42% in *C. pneumoniae* positive stroke patients, whereas it was only 21% in *C. pneumoniae* positive controls. The difference in genotype amongst *C. pneumoniae* positive subjects was more striking, when the patients were stratified into the small vessel and large vessel groups. Those in large vessel group displayed the highest frequency of the mutated allele A (51%). The frequency of the GA genotype was 56% (37 of 66) amongst the *Chlamydophila*-positive stroke patients with large vessel disease, which means an OR of 3.19, 95% CI: 1.618–6.288, p=0.0008, as compared with the *Chlamydophila*-positive control subjects (24 of 84; 29%). The difference in the frequency of AA homozygotes was even higher amongst these groups, with an OR of 3.824; 95% CI: 1.362–10.50, p=0.008. Conversely, the prevalence of the GG, wild type genotype was significantly lower in the group of patients with the large vessel pathology, than in the controls and those with small vessel disease; 21% (14 of 66) vs. 64% (54 of 84) and 42% (36 of 86), respectively. (OR=0.162, 95% CI: 0.076–0.341, p<0.001; and OR=0.405, 95% CI: 0.194–0.843, p=0.021, respectively.) In contrast, the difference in the genotype frequencies between the stroke patients with small vessel disease and the controls was less marked; considering the GA genotype, there was an OR of 2.177; 95% CI: 1.510– 4.104, p=0.01; and there was no significant difference in the frequency of the AA genotype, i.e., 11% (10 of 86) vs. 8% (6/84), p=0.43. Therefore, we conclude that the SNP of the NOD1 c.796G>A gene is significantly higher amongst *Chlamydophila* positive stroke patients, and the highest frequencies of the GA and AA genotypes are to be observed amongst those patients, with large vessel disease. In other words, *Chlamydophila* positivity with polymorphism of the NOD1 c.796G>A gene is a higher risk factor for the development of stroke with large vessel disease than with small vessel disease. Interestingly, amongst the *C. pneumoniae* negative patients, no significant difference in genotype frequency was observed between the patients with large vessel disease and those with small vessel disease.

DEFB1 c.-20G>A polymorphism in patients with acute pancreatitis

The distribution of the DEFB1 c.-20G>A genotypes was in accordance with the Hardy-Weinberg equilibrium in the control population (p=0.996, χ^2=0.001) and also in the patient group (p=0.665, χ^2=0.199). There was a significant difference in genotype distribution between the pancreatitis patients overall and healthy controls (p=0.03). When the patients were stratified according the disease severity, a more significant difference was observed only between the controls and the patients with severe pancreatitis (p=0.009), but not between the controls and patients with mild pancreatitis (p=0.44), when the genotypes were taken into consideration. Thereafter we compared the numbers of AA homozygotes among the patients with severe pancreatitis and the controls. There was a higher frequency (38%) of the AA genotype among the patients with severe disease as compared with the controls (20%); OR=2.48, 95% CI: 1.305-4.722, p=0.006. The highest frequency of AA genotype was observed among patients with infected pancreatic necrosis (61%). The carriage of the A allele was also significantly different between the patients with SAP (63%) and the healthy blood donor group (44%). Conversely, the prevalence of the DEFB1 c.-20 GG wild type genotype was significantly lower (11%) in the group of patients with severe acute pancreatitis than in the healthy control group (31%).

DEFB1 c.-44C>G polymorphism in patients with acute pancreatitis

The distribution of DEFB1 c.-44C>G genotypes was in accordance with the Hardy-Weinberg equilibrium among the patients with acute pancreatitis (p=0.610, χ^2=0.260) and also in the control population (p=0.597, χ^2=0.279). There was a significant difference in genotype distribution between the patients overall and the healthy controls (χ^2 test, p=0.02). When the patients were stratified according the disease severity, a significant difference was observed only between the controls and patients with severe pancreatitis (p=0.03) and not between the controls and patients with mild pancreatitis, (p=0.84). Interestingly, there was a lower frequency of the GG genotype among the patients with pancreatitis as compared with the controls: 2 of the 124 patients with acute pancreatitis (1%) and none of patients with severe acute pancreatitis (0%) vs. 9 of the 100 healthy controls (9%). Conversely, the prevalence of the DEFB1 CC genotype was 72 % in the group of patients with severe acute pancreatitis vs. 56 % of controls; Fisher test: p=0.02, OR=2.055, 95% CI: 1.27 – 3.745.

DEFB1 c.-52G>A polymorphism in patients with acute pancreatitis

The distribution of the DEFB1 -52 genotypes was in accordance with the Hardy-Weinberg equilibrium in the control population (p=0.502, χ^2=0.250) and also in the patient group (p=0.997, χ^2=0.0001). There was a significant difference in genotype distribution between the pancreatitis patients overall and healthy controls (p=0.004). When the patients were stratified according the disease severity, a more significant difference was observed between the controls and patients with severe pancreatitis. (p=0.001) and not between the controls and patients with mild pancreatitis, (p=0.606) when the genotypes were taken into consideration. There was a higher frequency (41%) of the AA genotype among the patients with severe disease as compared with the controls (18%); OR=3.23, 95% CI: 1.678–6.218, p=0.0005. The carriage of the A allele was also significantly different between the patients with severe pancreatitis (64%) and the healthy blood donor group (39%,). Conversely, the prevalence of the DEFB1 c.-52 GG wild type genotype was significantly lower (14%) in the group of patients with severe acute pancreatitis than in the healthy control group (40%).

Copy number polymorphism of human beta-defensin-2 in patients with acute pancreatitis

In the control group the copy numbers had a range of 2-10 per genome, with a median number of 4 copies. The proportions of control individuals who carry the median (4), less than median (<4) or more than median (>4) number of copies were 24% , 40% and 36% respectively. In patients with acute pancreatitis the frequency distribution of the subgroups was significantly different from that of the control group (p=0.001). 42% of patients with acute pancreatitis overall had a lower copy number <4, while in patients with severe acute pancreatitis the frequency of a low copy number (<4) was 62%, and that of a copy number >4 was only 10%.

Expression of human ß-defensin 2 mRNA in BB19 cells and increase in expression by *Chlamydophila pneumoniae*

To determine whether HBD-2 gene expression is induced in BB19 cells in response to *C. pneumoniae* infection, RT PCR was performed. A time-dependent increase in HBD- 2 mRNA was observed in BB19 cells. The maximum increase in HBD-2 mRNA expression was observed at 24 h at a MOI of 5 IFU. The infection of endothelial cells by CV6, a cardiogenic strain, resulted in a more pronounced expression of HBD-2 mRNA. We compared the effects of heat-inactivated *C. pneumoniae* with those of viable *C. pneumoniae*. In cells stimulated with heat-inactivated *C. pneumoniae*, the expression of HBD-2 was induced, although at a reduced level. Mock preparations did not result in any increase in

HBD-2 mRNA. We tested also the constitutive HBD-1 expression in BB19 cells. In contrast to HBD-2, there was no any increase in expression of HBD-1 mRNA following *C. pneumoniae* infection.

Human ß-defensin 2 protein expression in BB19 cells infected with *C. pneumoniae*

The expression of HBD-2 protein in *C. pneumoniae* infected endothelial cells was determined by immunoblotting. Through use of the anti-HBD-2 antibody which binds to human HBD-2, a single band of about 4 kDa was detected. The signal was strong following the blotting of cell lysates from *C. pneumoniae*-infected cells. The level of HBD-2 was only barely detectable after the processing of the control cell lysate without *C. pneumoniae* infection.

Immunofluorescent staining of BB19 cells for HBD-2

Immunofluorescent staining images of *C. pneumoniae* infected BB19 cells revealed that HBD-2 was diffusely distributed as granules throughout the cytoplasm of the endothelial cells. There was only a weak fluorescence in the uninfected controls. The calculated fluorescence intensity mirrored the ELISA and Western blotting results, indicating that the HBD-2 protein was highly inducible.

Chlamydophila pneumoniae induces HBD-2 secretion by BB19 cells

Experiments were performed to determine the HBD-2 protein secreted by BB19 cells into the tissue culture medium following *C. pneumoniae* infection. The amount of HBD-2 in the supernatants was determined by ELISA. Results of these experiments showed that the concentration of HBD-2 protein was significantly elevated in the supernatant 24 h after *C. pneumoniae* infection. The supernatants from BB19 cells stimulated with heat-inactivated *C. pneumoniae* displayed a moderate release of HBD-2 peptide. The BB19 cells incubated with mock stocks exhibited a similar level of HBD-2 release as that of the noninfected controls (data not shown). In parallel experiments, the concentration of HBD-2 was also determined in the cell lysates. A considerable increasing the amount of HBD-2 was detected in the cell lysates of cultures infected with *C. pneumoniae* CWL-029 or with *C. pneumoniae* CV6 for 24 h (35.2 ± 3.8 pg/ml and 48.4 ± 4.6 pg/ml, respectively, vs 5.5 ± 2.8 pg/ml in the control cell lysates). These results imply that human endothelial cells stimulated by *C. pneumoniae* induce the expression of the HBD-2 gene and the release of HBD-2 protein into the medium.

DISCUSSION

RAGE Gene Polymorphisms in Patients with Multiple Sclerosis

The up-regulation and pathogenic effects of RAGE in MS highlight the RAGE gene as a candidate for involvement in the pathogenesis of the disease. Our results have demonstrated an association between the −374T/A polymorphism of the RAGE promoter and MS; the frequency of the AA genotype was significantly higher in the control group than in MS patients, suggesting that the AA genotype may have a protective role against the development of MS. There was no significant difference in genotype between men and women nor in genotype distribution if the patients were stratified overall according to the severity of the disease. However, it is noteworthy that none of the patients with the SP type of MS exhibited the AA genotype, though the number of these patients in our study was low (14). Therefore, we presume that the −374T/A polymorphism of RAGE is a risk factor for MS in general and influences the development of the secondary relapsing form of the disease rather than simply the severity. The −374T/A polymorphism of RAGE gene located in the promoter region of the gene has previously been shown to exert significant effects on transcriptional activity. Our

results raise the question of how the altered transcription of the RAGE promoter region can affect the risk of MS. We presume that altered transcription may lead to a reduced degree of expression of the RAGE, which could limit the activation of multiple RAGE-mediated signaling pathways, leading to a different level of susceptibility to MS. There was no difference in frequency of −479T/C genotype between the MS patients and the controls. In accordance with this result, the study of Hudson *et al.* did not reveal any clear differences in actual protein–DNA interactions in the case of the −429T/C polymorphism, but a very distinct difference in transcription factor binding was observed between the −374T and A alleles. The T to A substitution appeared to prevent the binding of nuclear factor, which suggested that a disruption of nuclear protein is involved in repression of RAGE transcription. Transcription factor binding assays revealed the abolition of a nuclear protein binding site with the introduction of the −374A allele, supporting the role rather of this polymorphisms and not of −429T/C in affecting RAGE transcription repression. As concerns the Gly82→Ser polymorphism, Ser82 enhances receptor signaling through mitogen activated protein kinases and nuclear factor of kappa B. However, the RAGE Gly82→Ser polymorphism showed no association with MS in our study. Hence, we assume that inhibition of the expression of RAGE itself and not the modulation of signaling may be a definitive factor in MS. In conclusion, we suppose that the altered transcription induced by the −374T/A SNP could lead to a reduced level of RAGE, which might be a factor protecting against the development of MS. Our findings support the view that RAGE plays a role in the development of MS. Further studies will elucidate whether blockade of RAGE has a therapeutic potential for the prevention of brain tissue injury and neurodegeneration in MS.

Relevance of the genetic polymorphism of NOD1 in *Chlamydophila pneumoniae* seropositive stroke patients

The causation of ischemic stroke is multifactorial a combination of genetic risk factors and environmental factors. Inflammatory parameters and chronic and acute infectious diseases have been considered to modify stroke risk independently of conventional risk factors. Sero-epidemiological studies are limited by the high prevalence of antibodies in adults. The risk due to a prior infection may be influenced by individual inflammatory response of the host. Toll-like receptors and NOD proteins are pattern recognition receptors, which are key elements in the regulation of immune response. TLR4 is the transmembrane lipopolysaccharide receptor, which initiates the innate immune response to Gram-negative bacteria, including *Chlamydophila pneumoniae*. To check the importance of the difference in NOD1 genotype, we investigated the NOD1 SNP in stroke patients. Several SNPs in NOD1 has been described The G796A SNP was chosen in the coding sequence of the gene in exon 3, as it was earlier reported to encode a changed protein, (E266K) in the nucleotide binding domain altering a glutamic acid residue, suggesting a potential functional effect of the mutation. It was recently found, that the NOD1 A allele is associated with reduced expression of NOD1 protein, leading to diminished NF-kB activation in response to *Propionibacterium acnes*. It is suggested that the E266K substitution reduces the stability of NOD1. In addition to the induction of cytokines, NOD activation may be involved in signaling cascade-mediated defensin expression. Any failure in the production of this natural antibacterial protein could lead to an imbalance between invading bacteria and the host innate immunity. Thus, an impaired defensin synthesis might be linked to a higher rate of multiplication of invading bacteria. In our study, the NOD1 genetic polymorphism appeared to be a potential risk factor in ischemic stroke only in *C. pneumoniae*-infected patients. In other words, it may be speculated, that in the case of *C. pneumoniae* infection, a functional deficiency of the NOD1 signaling system could lead to a higher rate of multiplication of *Chlamydophila* and to a more intensive local inflammatory response. Conversely, *C. pneumoniae* infection alone, with an

appropriate immune response is not enough for a definitive inflammatory induction, and plaque formation in the blood vessels. This seems to be supported by the fact, that the proportion of *Chlamydophila* positivity was not significantly higher in our stroke patients. The results of the serological examinations are in contrast with the observation underlining the association with *C. pneumoniae* seropositivity in stroke. In this sense, our results are in agreement with the finding of no association of *C. pneumoniae* antibodies with ischemic stroke. However, we did detect a significant association between the SNP of NOD1 receptor (which also detects *C. pneumoniae*) and stroke only in *C. pneumoniae*-positive patients. To the best of our knowledge, this is the first report concerning the relevance of NOD1 polymorphism in the risk of ischemic stroke amongst *Chlamydophila*-positive patients. There were significant differences in NOD1 G796A genotype distribution between the controls and the stroke patients with *C. pneumoniae* infection. In the interpretation of our data, some limitations should be considered. The small number of subjects in our preliminary study is an indication of a need for caution. For a stronger power, the number of patients in each group should be increased appreciably, which requires a multicenter approach. There is certainly no specific link between any chronic infectious disease and stroke, but it appears much more probably that the chronic infections are risk factors that act in cooperation with conventional risk factors and genetic predisposition, which are neither necessary nor sufficient for disease generation alone. Polymorphism in NOD1 E266K is not a risk factor for stroke in general, but in association with *C. pneumoniae* infection it appeared to be accompanied by an increased risk of the development of stroke; therefore, it could prove to be a valuable marker for an assessment of the risk of stroke in combination with *C. pneumoniae* infection.

Relevance of genetic polymorphisms of beta-defensins in severe acute pancreatitis

Maintenance of the integrity of the gut barrier is one of the goals in the treatment of severe acute pancreatitis. For this reason, enteral nutrition has been proposed for restoration and prevention of the morphological changes in the gut associated with fasting. More recently, another method involving the use of probiotics has been proposed to reduce the infection of necrosis by intestinal bacteria. Apart from a direct or indirect action on other bacteria, it is believed, that probiotics act directly on the mucosal immune system, stimulating an increased production of β-defensins. On the basis of these observations, we presumed, that an investigation of the relevance of genetic polymorphisms of beta defensins in acute pancreatitis was plausible. We supposed that any decrease in defensin expression either in the colonic mucosa or locally in the pancreatic tissue could result a higher rate of bacterial translocation from the gut, or even an increased possibility of retrograde infection. The expression of HBD-1 mRNA in the pancreas acinar cells suggest that HBD-1 fulfills physiological functions in the host defense. Our analysis of DEFB1 c.-20G>A genotypes among patients with AP revealed that the AA genotype comprises a higher risk of severe form of disease (OR=2.48). The functional significance of this SNP resides in the formation of a nuclear factor-kappa B transcription factor-binding sequence, and it likely that this mutation might cause a lower hBD1 expression in colonic epithelial cells or in pancreatic acinar cells. Similarly, the G/A SNP at the -52 region of DEFB1 correlates with decreased expression of HBD-1 and this is in accordance with our results demonstrating a higher frequency of the A allele in patients with SAP. It is tempting to speculate that this mutation in the -52 untranslated region might cause a lower HBD-1 expression in epithelial cells with a deficient function of human defensin. It is noteworthy, that 29 of the 94 patients in the SAP group suffered from infected necrosis with multiple organ failure, and 25 of them carried the A allele of c.-20G>A. Similarly, concerning the c.-52G>A SNP, 27 of the 29 patients carried the mutant A allele. We therefore presume that individuals displaying the presence of the polymorphism may be predisposed to the most severe complications of acute necrotizing

pancreatitis. The present study has demonstrated that the distributions of c.-44C>G polymorphism were different in the patients with SAP and the healthy controls, while the frequency of the GG genotype was significantly higher in the controls. This result indicates that the C/G mutation probably leads to strengthened HBD1 antimicrobial activity, which is less frequent among patients with SAP. The SNP at -44C>G generates a putative binding site for nuclear factor-kB, and induces overexpression.These data are consistent with our present observation that the GG phenotype could also be protective in acute pancreatitis. Again, among the 29 patients with infected necrotic pancreatitis only 5 carried the G allele, or protective allele, and none of them was GG homozygote. These observations draw attention to the importance of DEFB1 polymorphism especially as concerns a predisposition to infected pancreatic necrosis. In the present study, acute pancreatitis was characterized by a shift to lower copy numbers of DEFB4 encoding HBD-2. A low beta-defensin gene copy number in Crohn's disease predisposing to colonic localization has been reported, most likely through diminished beta-defensin expression. Similarly, we suppose, that in our study individuals with < 4 copies are at a higher risk of developing severe acute pancreatitis because of .an impaired HBD-2 expression rate. To the best of our knowledge, this is the first time that three DEFB1 SNPs have been tested for a possible association with acute pancreatitis. The DEFB1 G-20A and DEFB1 C-44G and DEFB1 G-52A SNPs differed between the patients with SAP and the healthy controls. Additionally, the low copy number of DEFB4 - the gene of human defensin β-2 - in patients with SAP draws attention to the importance of defensins in development of severe form of AP. Our results should be regarded as preliminary results, indicating the relevance of the SNPs in the genes encoding HBD1 and HBD2 in acute pancreatitis and especially in infected SAP, which should be confirmed on a larger series of patients in a future multicentre study. Although the SNP may be only one of the factors that contribute to the susceptibility to SAP, our data further support the idea, that SAP could be due also to the defective production of beta-defensins It is unlikely that a single modifier gene alone is responsible for SAP. It is more likely, that the combination of a multitude of SNPs, in different genetic loci and genes, contribute to produce the clinical phenotype of SAP. The identification of the variants of the DEFB1 and DEFB4 genes may contribute to a better understanding of the pathogenesis of this disease.

Inducible expression of human ß-defensin 2 by *Chlamydophila pneumoniae* in brain capillary endothelial cells

Expression of defensins in human endothelial cells in response to *C. pneumoniae* has not been studied previously. Sero-epidemiological studies in patients with stroke have provided evidence of an association of risk with prior infection with *C. pneumoniae*. The endothelial dysfunction observed in stroke patients has been related to the physiopathology of stroke, the stroke subtypes, the clinical severity and the outcome. The present study was performed to analyze the expression of HBD-2 and the effects of *C. pneumoniae* on HBD-2 expression in the human brain capillary endothelial cell line BB19. Human ß-defensin 2, an antimicrobial peptide, has so far been found exclusively in a variety of epithelial cells. No data are available on HBD-2 expression in endothelial cells except for the very recent publication by Kawsar *et al*., which reported the expression of HBD-2 in the intratumoral vascular endothelium and HUVECs (human umbilical vein endothelial cells). In our study, the expression of HBD-2 mRNA in brain capillary endothelial cells was first assessed by RT-PCR. As mRNA may not always be translated to protein, we also investigated whether capillary brain endothelial cells express the HBD-2 peptide. The expression of the HBD-2 peptide in endothelial cells was confirmed by immunofluorescence staining for HBD-2 peptide and by Western blotting. The secretion of HBD-2 was confirmed by measuring the HBD-2 in the supernatants by ELISA. In good accordance with the inducible nature of HBD-

2 in keratinocytes and epithelial cells, we observed a definite increase in the expression of HBD-2 upon exposure to *C. pneumoniae*. In our experiments, heat-inactivated *C. pneumoniae* was still able to induce the expression and secretion of HBD-2, albeit at lower levels. This suggests that, among the potential products of bacteria, one candidate could be a heat-resistant lipopolysaccharide. On the other hand, heat–labile compounds, such as outer membrane proteins or other yet unidentified factors, might also be candidates for HBD-2 activation. Infection of human endothelial cells with *C. pneumoniae* results in the stimulation of a wide variety of cytokines, adhesion molecules, chemokines and proteins with procoagulant activity, which furnishes evidence of the role of infection with this bacterium in the atherogenic process. The role of ß-defensin peptide in endothelial cell biology is not clear. It might be that endothelial cells use defensins to exert antimicrobial activity. Defensins also exert a broad spectrum of immunological effects; human ß-defensins may attract immature dendritic cells, naive T-cells and memory T-cells, which suggests that these defensins are involved in orchestrating an immune response, and it may be hypothesized that HBD-2 contributes to the possible innate immunity involved in atherosclerosis. Further studies are necessary to elucidate the role of HBDs in atherosclerosis or stroke. The result demonstrating that HBD-2 is expressed and produced in the human brain capillary endothelial cells upon infection with *C. pneumoniae* is novel and provides evidence that HBD-2 plays a role in the early immune responses to *C. pneumoniae* and probably in the immunopathogenesis of atherosclerosis. This result might facilitate an understanding of the pathomechanism of the inflammation-mediated events of atherosclerosis or stroke in connection with *C. pneumoniae* infection.

SUMMARY AND NEW RESULTS

1. Our results revealed an association between the -374 T/A polymorphism of the RAGE promoter and multiple sclerosis. The genetic variant -374 AA (which has previously been shown to exert significant effects on transcriptional activity) can be considered a preventive factor as regards the occurrence of MS. Our findings support the view that RAGE plays a role in the development of MS.
2. Polymorphism of NOD1 G796A alone did not prove to be a risk factor for stroke in general, but in association with *Chlamydophila pneumoniae* infection it appeared to be accompanied by an increased risk of the development of stroke.
3. The DEFB1 G-20A and DEFB1 C-44G and DEFB1 G-52A SNPs differed between the patients with SAP and the healthy controls. Additionally, the low copy number of DEFB4 - the gene of human defensin beta-2 - in patients with SAP draws attention to the importance of defensins in development of severe form of AP. The variations in the genes encoding human b-defensin-1 and -2 may be associated with the risk of severe acute pancreatitis and also with infected acute pancreatitis.
4. The results of *in vitro* experiments indicate that human β-defensin-2 is expressed and produced in the human brain capillary endothelial cells upon infection with *Chlamydophila pneumoniae*, and provide evidence that HBD-2 plays a role in the early immune responses to *C. pneumoniae* and probably in the immunopathogenesis of atherosclerosis.

ACKNOWLEDGEMENTS

I would like to express my most sincere gratitude to Professor Yvette Mándi for introducing me to immunobiology and scientific research and supporting me throughout my PhD studies.

I thank all the members of the Department of Medical Microbiology and Immunobiology, Faculty of Medicine, University of Szeged for their restless support during my work. I am thankful for the help and advice I received from Dr. Ferenc Somogyvári, Dr.

Ágnes Katalin Kocsis, Dr. Péter Hofner, Dr. Balázs Németh, Dr. Valéria Endrész, Dr. Klára Megyeri, Dr. László Orosz, and Dr. Zsófia Gyulai, and for the excellent technical assistance from Györgyi Müller, and for Zsuzsanna Rosztóczy for skilful administration.

I thank my research partners, particularly, Dr. Mária Sasvári-Székely, Dr. Réka Kovács-Nagy and Dr. Eszter Szántai, Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest; Dr. Imre Ocsovszky, Department of Biochemistry, University of Szeged; Dr. György Seprényi, Department of Medical Biology, University of Szeged for their help.

I thank my clinical partners, particularly Prof. Dr. László Vécsei, Dr. Sztriha K. László and Dr. Krisztina Bencsik, Department of Neurology, University of Szeged; Dr. Zoltán Szolnoki, Department of Cerebrovascular Diseases, Pándy Kálmán County Hospital, Gyula; Prof. Dr. Tamás Takács and Dr. Annamária Szabolcs, First Department of Internal Medicine, University of Szeged; Prof Dr. Gyula Farkas, Department of Surgery, University of Szeged; Dr. Katalin Tápai, Regional Centre of the Hungarian National Blood Transfusion Service, Szeged.

Finally, I thank all the love, patience and continuous encouragement of my family.

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS:

I. Tiszlavicz Z, Szabolcs A, Takács T, Farkas G, Kovács-Nagy R, Szántai E, Sasvári-Székely M, Mándi Y. Polymorphisms of beta defensins are associated with the risk of severe acute pancreatitis. *Pancreatology*. 2010;10(4):483-90. IF: 2.195 (2009)

II. Tiszlavicz Z, Endrész V, Németh B, Megyeri K, Orosz L, Seprényi G, Mándi Y. Inducible expression of human {beta}-defensin 2 by *Chlamydophila pneumoniae* in brain capillary endothelial cells. *Innate Immun*. 2010 Jul 20. [Epub ahead of print] IF: 2.206 (2009)

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Total impact factor: 9.631

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Total impact factor: 6.301

Cumulative impact factor: 15.932