Analysis of genetic factors influencing the outcome of pneumonia-induced sepsis

PhD thesis

Madách Krisztina MD

Semmelweis University
Doctoral School of Basic Medicine

Supervisor: Dr. Prohászka Zoltán, DSc

Opponents: Dr. Bogár Lajos, professor, DSc
Dr. Zima Endre, PhD

Chairman of the Final Examination Committee:
Dr. Darvas Katalin, professor

Members of the Final Examination Committee:
Dr. Strausz János, professor, DSc
Dr. Rónai Zsolt, PhD

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Introduction

Advances in medical technology, widespread use of immunosuppressive therapies and ageing of the population result in rapidly increasing incidence of sepsis. Despite significant advances both in supportive care and in research on its pathogenesis, sepsis syndrome remains a worldwide problem and the leading cause of death in critically ill patients. The overall mortality rate of sepsis is approximately 30%, whereas the mortality can surpass 50% in patients in septic shock or multiple organ dysfunction syndrome. Patients with apparently similar general condition and severity of infection may present profoundly different survival rates. Individual differences in disease manifestation are influenced by the genetic predisposition of the patient. Single nucleotide polymorphisms and haplotype blocks of chromosomes, taking part in the inflammatory response influencing susceptibility to infection and severity of sepsis, may explain the clinical variability observed during similar infections.

Plasminogen activator inhibitor-1 (PAI-1) - a member of the serine protease inhibitor (serpine) family - is a key element in the inhibition of fibrinolysis. The primary role of PAI-1 ***in vivo*** is fast acting inhibition of tissue- and urokinase-type plasminogen activators. Endothelium- or platelet derived PAI-1 is normally complexed to vitronectin. The PAI-1-vitronectin complex is the most efficient inhibitor of activated protein C and thrombin. PAI-1 competes with thrombomodulin for binding with thrombin. These properties make the PAI-1 a strong local procoagulant, playing a central role in acute phase response provoked during sepsis and trauma. Plasma levels of PAI-1 are influenced by genetic, metabolic, endocrine, dietary, and physical activity factors, and they strongly increase in response to inflammation and injury. In patients with sepsis, the levels of PAI-1 are positively related to poor outcome, increased severity of the disease, and increased levels of various cytokines, acute-phase proteins, and coagulation parameters. The gene coding for PAI-1 has several

polymorphic loci among which the most studied is the 4G/5G insertion/deletion polymorphism (rs1799768) containing either four or five (4G/5G) guanine bases at -675 within the promoter region of the human ***PAI-1*** (***SERPINE1***) gene. Both alleles of this SNP can bind a transcriptional activator, whereas the 5G allele binds a repressor protein at an overlapping site. Therefore homozygosity for the 4G allele renders this negative regulator unable to act, resulting in greater transcription of the ***PAI-1*** gene, while heterozygotes show intermediate phenotype. The transcription and therefore the plasma level of ***PAI-1*** is 25% higher in 4G allele homozygotes, than in 5G allele homozygotes. The prothrombotic activity of the 4G allele has been evaluated in numerous clinical circumstances, significant association between the carrier state of 4G allele with mortality of myocardial infarction has been demonstrated. The 4G allele of the 4G/5G polymorphism has been associated with increased susceptibility to community-acquired pneumonia, and increased mortality in hospitalized patients with severe pneumonia. In addition, the 4G allele was reported to affect the risk of developing severe complications and higher mortality in meningococcal sepsis and trauma.

The human major histocompatibility complex (MHC) is recognized as the most important genetic region regarding common human disorders including inflammatory, infectious and autoimmune diseases as well as organ transplantation. The MHC consists of relatively long stretches of highly conserved DNA sequences derived from a common remote ancestor, called ancestral haplotypes (AHs). The AH8.1 haplotype is very frequent (~10%) in the Caucasian population. The AH8.1 is characterized – among others – by HLA-DQB1*0201 (DQ2), HLA-DRB1*0301 (DR3), AGER (formerly called as RAGE) -429C, C4A*Q0, C4B1, BfS, HSP70-2 (referred as HSPA1B as well) 1267G, TNF -308A, LTA 252G, HLAB* 0801 (B8), HLA-Cw*0701 and HLA-A*0101 (A1) alleles. AH8.1 is associated with numerous immunopathological differences, several immune-mediated diseases, and colorectal

carcinoma. The impressive number of diseases associated with AH8.1 shows that this haplotype may influence several aspects of the immune response. An increased spontaneous apoptosis of blood lymphocytes and an elevated production of various autoantibodies were shown in AH8.1 carriers. One of the most characterizing features of AH8.1 is an increased spontaneous production of TNF-α. The immunopathological alterations described in AH8.1 carriers may also influence the effectiveness of host-defences against various microorganisms.

Objectives

Our aim was to discover which genetic factors may have an influence on the outcome of pneumonia-induced severe sepsis in Caucasian patients.

As imbalance between fibrin generation and dissolution may contribute to progression of sepsis, development of multiple organ dysfunction syndrome and mortality, and the level of PAI-1 inversely correlates with the outcome of sepsis, we investigated the 4G/5G polymorphism of the ***PAI-1*** gene in regard of the severity and outcome of pneumonia-induced sepsis.
According to our first hypothesis, the carriers of the 4G allele of the ***PAI-1*** polymorphism may have higher risk for worse outcome of pneumonia-induced sepsis. Based on the above, we searched for answers to the following questions:

1. Is there any association between the genotype distribution and allele frequency of the ***PAI-1*** 4G/5G polymorphism and higher risk for worse progression of sepsis, development of septic shock and multiple organ dysfunction syndrome?
2. Is there any association between the DIC score at admission and the allele frequency of ***PAI-1*** 4G/5G polymorphism?

3. Is there any association between the genotype distribution and allele frequency of the ***PAI-1*** 4G/5G polymorphism and the intensive care unit mortality?

The human major histocompatibility complex (MHC) is one of the most important genetic regions in regard of disorders including inflammatory and infectious diseases. Formation, survival and spread of the highly conserved blocks characteristic of this region are presumably shaped by evolutionary processes. Haplotypes reflect the sequence of a whole gene (or genes) including coding and non-coding regions, therefore they correspond more directly to the unit of biological function than SNPs. One of the most wide-spread haplotypes is the AH8.1 (~ 10%) in the Caucasian population. The AH8.1 includes several candidate genes studied so far in regard of outcome of sepsis. Therefore we examined the influence of AH8.1 carrier state on the severity and mortality of pneumonia-induced sepsis.
According to our second hypothesis the AH8.1 carriers may have lower risk for worse outcome in pneumonia-induced sepsis. Based on the above, we searched for answers to the following questions:

1. What is the influence of AH8.1 on the severity of pneumonia-induced sepsis, on the development of septic shock?
2. What is the influence of AH8.1 on the mortality of pneumonia-induced sepsis?

When evaluating the effects of SNPs on individual and variable course of sepsis, clinical characteristics – including the aetiology of the infectious process, virulence of the pathogens, undrainable surgical source of sepsis, time between initialization of symptoms and admission to hospital and adequate treatment, presence of comorbidities, differences in ethnic origin and gender distribution – may confound the evaluation of genetic factors on individual differences in sepsis. In order to minimize these pitfalls – frequently

disregarded in previous studies – we carried out our researches in a relatively homogenous cohort of Caucasian patients with severe sepsis due to pneumonia.

Methods

Patients and definitions

The study enrolment was carried out between June 2004 and June 2007. From an original cohort of 301 critically ill patients diagnosed with sepsis, consecutively admitted to the Department of Anaesthesiology and Intensive Therapy of Semmelweis University, 208 patients met the criteria of severe sepsis due to pneumonia and were enrolled in the study within 24 hours of admission to the ICU. The analysis of the association of 4G/5G polymorphism of the ***PAI-1*** gene with multiple organ dysfunction, septic shock and mortality as well as the analysis of the association of the 8.1 ancient haplotype with severity of disease progression and mortality was carried out in the same patient cohort. Exclusion criteria were: primary site of infection other than lungs, undrainable surgical source of sepsis, malignancy and final stage of chronic disease, chronic treatment with steroids or immunosuppressive drugs, AIDS and pregnancy. Written informed consent was obtained from patients or their relatives, and the study was approved by the local ethics committee.

Patients were treated according to the Surviving Sepsis Campaign guidelines for the management of severe sepsis and septic shock. Patients received empiric broad-spectrum antibiotic therapy according to the expected susceptibility of the probable pathogen. After receiving positive results (lower respiratory tract or blood culture) we de-escalated antibiotic therapy according to susceptibility of the pathogens. All patients were followed up during their hospital stay until they were discharged from the ICU or died. The diagnosis of pneumonia was made on the basis of appearance of new infiltrate on the chest x-ray in the presence of cough or fever.

All patients met the criteria of the British Thoracic Society for severe pneumonia. Severe sepsis was defined as acute organ dysfunction secondary to infection, and septic shock defined as severe sepsis resulting in hypotension despite adequate fluid resuscitation according to the 2001 SCCM/ESICM/ACCP/ ATS/SIS International Sepsis Definitions Conference. MODS has been defined as "the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention". Clinically, MODS was considered as a sequential or concomitant occurrence of a significant derangement of function in two or more organ systems of the body, against a background of critical illness. Disseminated intravascular coagulation (DIC) score was calculated at admission according to the International Society on Thrombosis and Haemostasis. Patients with chronic obstructive pulmonary disease (COPD) fulfilled the criteria of the American Thoracic Society (ATS) and the European Thoracic Society (ETS) updated in 2004

Materials and methods

Genomic DNA was extracted from white blood cells using the method described by Miller and colleagues.

The ***PAI-1*** -675 locus was amplified using the forward 5'-CACAGAGA GAGTCTGGCCACGT-3' and the reverse 5'-CCAACAGAGGACTCTTGGTCT-3' primers. The amplified DNA was incubated with ***Bsl***I restriction enzyme and the cleaved fragments were analyzed by electrophoresisin a 2% gel with ethidium bromide.

Genotyping of HSP70-2 1267ANG (rs1061581), AGER -429TNC (rs1800625) and LTA 252ANG (rs909253) polymorphisms was carried out by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and the TNF -308GNA SNP (rs1800629) was genotyped by TaqMan® probe (Applied Biosystems) based allele discrimination. Negative (no DNA) and positive (samples of known genotype) controls were used in each experiment. The results were confirmed by random re-genotyping of approximately 10% of the samples. Copy numbers of the C4A

and C4B genes were determined by the method of Szilagyi et al. Each person who carried less than two copies of C4A gene was considered a C4A*Q0 carrier. Simultaneous carriage of C4A*Q0, TNF -308A, AGER -429C, HSP70-2 1267G and LTA 252G alleles was assumed as the carrier state of AH8.1, which was confirmed by HLA determination (patients who had at least one of the HLA-B8, HLA-DR3 and HLA-DQ2 alleleswere considered AH8.1 carriers). Serological typing of HLA-B was performed using the standard microlymphocytotoxicity method (Innotrain Diagnostik GmbH, Kronberg, Germany). HLA-DQB1 and HLA-DRB1 alleles were detected by HLA-DBR1 and HLA-DQB1 kits (Olerup SSP TM DQ kit, Olerup SSP AB, Saltsjöbaden, Sweden) using sequence specific primer (SSP-) PCR method.
Data were collected in MS Excel 2003 (Microsoft, Redmond, WA, USA) and were analyzed with SPSS 13.0 for Windows (SPSS, Chicago, USA) software. Categorical variables were reported as absolute values and percentages, and continuous variables as medians and interquartile ranges. Categorical data were compared by using Pearson's Chi-square test or Fisher's exact test; continuous data were compared with categorical data using the nonparametric Mann–Whitney U and Kruskal-Wallis tests. All reported p values were two-tailed and $p<0.05$ was considered significant. Hardy-Weinberg equilibrium analysis was performed by comparing the detected genotype distribution with the theoretical distribution estimated on the basis of the allele frequencies. Multiple logistic regression analysis was used to evaluate independent predictors for the end-points. Hazard risk of in ICU mortality associated with genotypes and other independent variables was estimated using a Cox proportional hazards regression analysis. ***Post-hoc*** power analysis was performed by Statistica software (Tulsa, OK, USA) for chi-squared test. Kaplan–Meier analysis was performed to estimate survival from the date of first symptoms of pneumonia to the date of last follow-up or the date of death in the AH8.1 study. The log-rank test was applied to compare survival rates.

Results

Analysis of the association of the 4G/5G polymorphism of *PAI-1* gene with the severity of sepsis, development of septic shock, multiple organ dysfunction syndrome and mortality

Genotype distribution

In the studied population, the genotype frequencies were: 4G/4G = 30.4% (n = 63); 4G/5G = 50.7% (n = 105); and 5G/5G = 18.8% (n = 39). Genotype frequencies were in Hardy-Weinberg equilibrium (***p*** = 0.92). The calculated allele frequency was 0.56 for 4G and 0.44 for 5G.

Clinical associations of PAI-1 4G/5G polymorphism

The incidence of MODS, septic shock and non-survival were similar and higher in carriers of 4G/4G and 4G/5G genotypes than in patients with 5G/5G genotype. Therefore, these two genotypes were combined in further analyses.

Genotype distribution and allele frequencies of the 4G/4G and 4G/5G genotypes were significantly more frequent in MODS and in septic shock compared with non-MODS and severe sepsis, respectively. Consequently the risk of MODS was 2.74-fold (odds ratio (OR) = 2.74 95% confidence interval (CI) = 1.335 to 5.604; ***p*** = 0.006) and the risk of septic shock was 2.57-fold (OR = 2.57) 95% CI = 1.180 to 5.615; ***p*** = 0.018) higher in carriers of the ***PAI-1*** 4G/4G and 4G/5G genotypes than in individuals bearing the 5G/5G genotype. Accordingly, the frequency of ***PAI-1*** 4G allele in the group of MODS (OR = 1.495; 95% CI = 1.008 to 2.217; ***p*** = 0.045) and septic shock (OR = 1.601; 95% CI = 1.077 to 2.381; ***p*** = 0.019) was significantly different from that of non-MODS and severe sepsis, respectively.

Comparing the genotype distribution between surviving and non-surviving patients, there was a tendency towards higher frequency of the 4G/4G and 4G/5G genotypes (***p*** = 0.085) in non-survivors, and though not in a significant extent but similar result was obtained in regard of the 4G allele frequency as well.

Analyzing the DIC score at admission among carriers of different ***PAI-1*** genotypes, we found that patients bearing the 4G allele had significantly higher DIC scores at the time of admission than 5G/5G homozygotes (2 (0 to 3) vs. 0 (0 to 2), ***p*** < 0.007).

We also evaluated the association of ***PAI-1*** polymorphism with ICU length of stay, invasive ventilation-free days and days without septic shock during the first 28 days of ICU stay. The length of ICU stay did not differ between carriers and non-carriers of the 4G allele (***p*** = 0.858). However, in non-survivors the median ICU length of stay was more than two days lower in patients with the 4G allele than in 5G/5G patients (6 (4 to 11) vs. 8.5 (6 to 18), ***p*** = 0.091). Carriers of the 4G allele had significantly less invasive ventilation free-days during the first 28 days than patients with the 5G/5G genotype (0 (0 to 0) vs. 0 (0 to 6), ***p*** = 0.008). The median of days without septic shock during the first 28 days was lower in patients bearing the 4G/4G and 4G/5G genotypes than in carriers of the 5G/5G genotype (4 (0 to 9) vs. 6 (5 to 9), ***p*** = 0.095).

Multivariate analysis of factors associated with endpoints

By multivariate logistic regression analysis, three factors were independently associated with MODS and sepsis severity: age, incidence of nosocomial pneumonia and positive microbiological culture. Therefore, these three parameters were introduced simultaneously as adjusting variables in logistic regression models of MODS and severity. The adjusted model indicated an independent association of ***PAI-I*** 4G/5G and 4G/4G genotypes with MODS (adjusted odds ratio [aOR] = 2.957; 95%CI = 1.306 -6.698; ***p*** = 0.009) and septic shock (aOR = 2.603; 95%CI = 1.137 - 5.959; ***p*** = 0.024).

A possible association between baseline variables and ICU mortality was studied by multivariate regression analysis as well. Because of multicolinearity, only the APACHE II score turned out to be independent predictor for ICU death. Therefore, this single parameter was introduced into the model. A tendency for increased risk of death was observed

for the carriers of 4G/4G and 4G/5G genotypes after adjustment. This result was confirmed by using Cox regression analysis adjusted for APACHE II score as a covariate (4G/4G and 4G/5G hazard ratio = 1.866; 95% CI = 0.897 to 3.882; ***p*** = 0.095).

Analysis of the association of 8.1 ancient haplotype with severity and mortality of pneumonia induced sepsis

Frequency of AGER -429T>C, C4A*Q0, HSP70-2 1267A>G, TNF -308G>A, LTA 252A>G polymorphisms and the 8.1 haplotype

Genotype frequencies were as follows in the studied population: AGER -429T/T= 67.1%, T/C=29.5%, C/C=3.4%; C4A*Q0 (−)=79.2%, (+) = 19.8%; HSP70-2 1267 A/A=37.2%, A/G= 46.4%, G/G= 16.4%; TNF -308G/G= 72.0%, G/A= 25.6%, A/A= 2.4% and LTA 252 A/ A= 46.9%, A/G= 44.9%, G/G= 8.2%. All genotype distributions were in Hardy–Weinberg equilibrium. Based on the carriage of these variants, thirty-two hypothetical 8.1 haplotype carriers were identified. After the determination of HLA alleles in this group of patients, twenty-five individuals were found to carry B8, DR3 and DQ2 alleles in addition, and considered heterozygous for AH8.1 (12.1%) - there were no homozygotes. The calculated haplotype frequency was 6.04%.

Sepsis severity

The distribution of the studied genetic factors between the groups with severe sepsis or septic shock, the genotype frequencies of AGER -429, HSP70-2 1267, TNF -308 and LTA 252 polymorphisms, as well as the incidence of C4A*Q0 were not different between the two severity grades. Additionally, the occurrence of septic shock was not significantly different in carriers and non-carriers of AH8.1.

Subjects were stratified by COPD, as pre-existing pulmonary disease may modify the severity of sepsis interfering with the influence of genetic factors, and 37.2% (n=77) of our patients

had COPD. Subgroup analysis is also supported by the fact that the number of pulmonary hospitalizations in the preceding two years ($p<0.001$), Apache II scores ($p=0.044$), hypertension ($p=0.001$) and smoking ($p<0.001$) were significantly and incidence of ischemic heart disease was tendentiously ($p= 0.056$) higher in the COPD group.

Among patients without COPD, carriers of the 8.1 haplotype had a lower risk for septic shock, compared to non-carriers (OR=0.3383; 95% CI=0.1141–0.995; $p=0.043$). In the group of patients with COPD, the incidence of AH8.1 was not associated with the severity of sepsis.

In the logistic model of sepsis severity, COPD as a concomitant pulmonary disease, APACHE II score and the development of ARDS were identified as independent predictors for the whole patient group. As the carrier state of the 4G allele of PAI-1 4G/5G polymorphism (rs1799768) is a predictor of shock, this property was introduced also into the regression models. In the logistic models for the whole patient group, none of the studied polymorphisms turned out to be an independent predictor of septic shock. In the haplotype analysis, however, a lower risk of septic shock (OR=0.315; 95% CI=0.100–0.992; $p=0.048$) was observed in carriers of the AH8.1 haplotype, after the adjustment. Moreover, this adjusted effect was much stronger in COPD-free patients (OR=0.117; 95% CI=0.025–0.554; $p=0.007$). In the group with COPD, the logistic models did not confirm the protective role of AH8.1 against septic shock.

Mortality of sepsis

Comparing the distribution of the studied genetic factors between survivors and non-survivors, only the LTA 252 polymorphism showed a significant association ($p=0.036$) with mortality. After stratifying by COPD, the genotype distribution of LTA 252 was significantly different ($p= 0.039$) between survivors and non-survivors only in the group of patients with COPD. According to the multivariate logistic regression analysis, two factors were independently associated with mortality: APACHE II score and the development of

ARDS. Therefore, these two parameters were introduced simultaneously as adjusting variables into the logistic regression models of ICU death. The association with LTA 252 polymorphism was no longer significant after logistic regression analysis adjusted for predictors.

In the haplotype analysis, the incidence of mortality did not differ significantly between carriers and non-carriers of AH8.1 - in the whole cohort and in the stratified groups. The logistic regression models adjusted for predictors did not support a protective role of 8.1 haplotype against mortality. Kaplan–Meier analysis was also performed to study survival pattern of haplotype carriers vs. non-carriers, but no significant difference was found (p=0.715).

Conclusions

Despite significant advances both in supportive care and in understanding the molecular basis of sepsis, the sepsis syndrome remains a worldwide problem associated with a high mortality rate, waiting for solution. The aims of our studies were to better understand the background genetic factors influencing the progression and outcome of pneumonia-induced sepsis.

As imbalance between fibrin generation and dissolution may contribute to progression of sepsis, development of multiple organ dysfunction syndrome and mortality, we investigated effect of the 4G/5G polymorphism of the ***PAI-1*** gene in regard of the severity and outcome of pneumonia-induced sepsis.

- Carriers of the 4G allele of ***PAI-1*** polymorphism have higher risk for MODS and septic shock in Caucasian patients with severe sepsis due to pneumonia according to both adjusted and non-adjusted analyses.

- Disease progression is more fulminant in 4G allele carriers as indicated by the association of ***PAI-1*** genotypes with continuous clinical variables such as ICU length of stay in non-survivors, invasive ventilation-free days, and days without septic shock during the first 28 days.

- Carriers of the 4G allele had higher DIC scores at admission than patients with the 5G/5G genotype, verifying that this polymorphism may influence outcome of sepsis through the disturbance of coagulation.

This observation supports previous studies reporting that the activation of coagulation and the inhibition of fibrinolysis are important in the pathogenesis of sepsis, and support the notion that particular genetic factors may predispose to worse outcome in severe sepsis. Identifying these genetic

factors might, in the future, help to choose the appropriate therapy for patients at different risk.

The aim of our other study was to investigate the influence of AH8.1 carrier state – a wide-spread, highly conserved haplotype in the Caucasian population, located within the MHC, one of the most important genetic regions in regard of disorders including inflammatory and infectious diseases - on the severity and mortality of pneumonia-induced sepsis.

- Septic shock occurred less frequently in AH8.1 carrier Caucasian patients without COPD, suffering of pneumonia-induced sepsis, than in non-carriers.

- According to the multivariate logistic regression analysis, the AH 8.1 had an independent protective role against septic shock in all patients, this protective effect was particularly pronounced in COPD-free patients.

AH8.1 may confer protection against the progression of bacterial infection, and this could explain, at least partially, its high frequency in the Caucasian population. Intensive research into the genetic aetiology of sepsis has been carried out for more than a decade, but only a few promising gene associations have been reported. The analysis of informative haplotypes might provide a possibility to establish a true association with the severity and mortality of sepsis.
Knowing the genetic background of sepsis may ease the comprehensive understanding of its pathomechanism, the more careful planning of clinical studies, the refinement of scoring systems of illness severity, and may help us in developing personalized treatment.

Publications

IF: 15,728

Publications related to the theme of the PhD thesis

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