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Genetic polymorphisms in neonatal sepsis

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A B S T R A C T

There is presently a great deal of interest in linking genetic and phenotypic variation in the form of severity of, and susceptibility to, common multifactorial diseases such as sepsis. The genetic background has recently been recognized as an important element in the host response to infection, contributing to the variability in the clinical outcome of critically ill neonates. Host genetic variability in the regulatory and coding region of genes for components of innate immune system may have some bearing on the wide variability existing in the susceptibility to and outcome from sepsis even within similar neonatal intensive care unit populations. The completion of the Human Genome Project has provided insight into human genetic variation, most commonly represented by single-nucleotide polymorphisms, and has generated vast expectations. Polymorphisms in genes coding for proteins involved in the recognition of bacterial pathogens (Toll-like receptor 4, CD14) and the response to bacterial pathogens (tumor necrosis factor (TNF)-α, interleukin (IL)-1, interleukin-1 receptor antagonist (IL-1RA), IL-6, IL-10) can influence the amount or function of the protein produced in response to bacterial stimuli. Combining population genomics, bio-informatics, and clinical data may lead to the discovery of the variations that exist among the genes involved in determining susceptibility to sepsis in neonates. This review provides a background to recent advances in genetics, focusing on the application to neonatal sepsis and the practical difficulties of genetic association studies, as well as underlining the potential impact on clinical practice. Evidence is discussed suggesting that some genetic polymorphisms influence the susceptibility to and outcome from sepsis. Although still in its beginnings in neonatology, genomics will transform also this field but it will take years for the clinical implications to be revealed.

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Despite significant advances in supportive care and in understanding the molecular basis of sepsis and its associated immune response, sepsis remains a major cause of neonatal morbidity and mortality, especially among very low-birthweight (VLBW) infants. The incidence is 1 and 4 cases per 1000 live births for full-term and premature infants, respectively, and the mortality rates range from 15% to 50%.[1,2] This wide range reflects the complex pathophysiology and heterogeneity of sepsis and is conceivably the reason why effective treatment for sepsis has been slow in coming regardless of the multitude of clinical trials.

The susceptibility of neonates to sepsis includes maternal and environmental factors, and host's immune, inflammatory, and coagulation responses, resulting in important interindividual differences with significant clinical implications.[3,4] Sepsis is a complex disease that occurs because of the interaction of a person's genotype with environmental factors. It is evident that sepsis is not a genetic disease and that genetic diseases are not the cause of the development of sepsis in most patients. At the same time, genotype might contribute considerably to outcome of infectious disease, thus genetics may explain the wide variation in the individual responses to infection.[5,6]

Invasion of the host by microbial pathogens causes the extensive activation of the innate immune response, which initiates a chain of events that results in the production and secretion of cytokines and chemokines and the activation of macrophages and monocytes. The activated cytokine network comprises the pro-inflammatory cytokines IL-1, IL-6, and TNF-α, which are responsible for a network of secondary responses, including the release of IL-18, a cytokine that induces the production of interferon (IFN)-γ. The intense pro-inflammatory response that occurs in sepsis is balanced by a variety of counter-regulatory molecules that attempt to restore immunologic equilibrium. Counter-inflammatory cytokines include soluble TNF receptors, IL-1 receptor

antagonist, and anti-inflammatory cytokines such as IL-10.[7-9] Among the host molecules that control the response to sepsis are many examples of genetic variability that affect physiologic activity. Numerous associations between polymorphic genes involved in the immune response and increased susceptibility to or outcome from sepsis have been studied, and in some cases the evidence is persuasive. Complex diseases, such as sepsis, are more amenable to study by gene association studies. Actually, most of the studies regarding the role of genotype in sepsis are gene association studies. In this article, we review all relevant genetic polymorphism studies that may contribute to the pathogenesis of sepsis with emphasis on polymorphisms of the innate immunity, pro- and anti-inflammatory cytokines, and coagulation mediators.

Genetic polymorphism influencing the susceptibility and resistance to infectious diseases

Recent evidence that the genetic background of the host affects the systemic response to infection has stimulated considerable interest in developing approaches to assess risk factors for the development and outcome of sepsis.[10,11] To establish the role of the genetic background in inflammatory responses, it is important to identify genomic markers suitable for clinical use and risk stratification and to understand the effects of genomic variations on gene regulation and protein expression.[12]

Sequencing of the human genome has demonstrated that many genes are polymorphic, including various genes that have been implicated in the development of sepsis. Most of the variations in the human genome occurs in the form of single-nucleotide polymorphisms (SNPs), or single base changes in the DNA sequence. SNPs are allelic variations in a single base pair (insertion, deletion, or substitution), with a frequency >1% compared with the normal allelic variant. There may be one or more polymorphic sites within a gene, and the sites may exist in coding regions of the gene or in noncoding regions that may be involved in the regulation of gene expression. Some of these variations have been shown to influence the level and/or activity of the resulting protein, thereby affecting cell function. The genes most likely to play a role in the variability in susceptibility to, and outcome from, sepsis are polymorphic genes that encode for protein products involved in the pathogenesis of sepsis.[13]

Genetic variability within the genes responsible for the initial recognition step and the subsequent immune response have been implicated in the variability seen in response to infection and could potentially influence the overall susceptibility to and outcome from sepsis. Several genes involved in the regulation of immune responses have been shown to be polymorphic. Thus, the extent of an individual immune response can be variable, depending on the specific homozygous or heterozygous alleles present.[14]

After microbial invasion, cellular receptors that recognize danger, called pattern recognition receptors (PRRs), such as TLR-4 and CD-14, are activated and are expressed on the surface of monocytes and macrophages and are important for lipopolysaccharide (LPS) identification.[15] PAMPs binding to PRRs stimulates signal-transduction pathways that lead to the activation of the nuclear factor-kb family, an important intracellular protein, that translocates to the nucleus causing transcription of pro- and anti-inflammatory cytokine.[7,9]

Examples of inflammatory mediator polymorphisms include genes located on chromosome 6 in the major histocompatibility complex (MHC). Polymorphisms in human leukocyte antigen (HLA) genes correlate with susceptibility to malaria, tuberculosis, HIV, and hepatitis B and C.[16-18] Because the functional role of the HLA is to present antigens to the immune system, it has been postulated that the extraordinary genetic variability of the HLA arose in response to antigenic diversity in infectious organisms. A large number of genes that are known to have immunologic function reside alongside the HLA genes, including TNF-α and various complement and heat-shock proteins.[11]

Various polymorphic genes responsible for the preliminary recognition of bacterial products include receptors, such as TLR and CD14, or Mannose-Binding Lectin (BML), involved with opsonization.

Variability in response to LPS may be due to variability in the gene for TLR-4.[19] A single amino acid change can significantly reduce response to LPS[20,21] and enhance susceptibility to infection. A number of SNPs have been identified in the promoter and coding regions of the TLR4 gene.[22,23] An association between polymorphisms in the TLR-4 and development of gram-negative sepsis and septic shock and mortality in systemic inflammatory response syndrome[24,25] and a link between a TLR-2 polymorphism and severe staphylococcal infections have been described.[26]

Association studies based on the functional plausibility of single SNPs relating to CD14 gene have been conducted. A polymorphic site with a C to T change has been identified 159 nucleotides upstream of the transcription start site.[27-29] Conflicting results in various studies performed to explore whether the -159 polymorphic site is associated with infection or sepsis have been reported. As would be predicted, individuals homozygous for the -159T allele have increased levels of CD14. Undoubtedly, additional accurately controlled studies with increased numbers of patients will be necessary to determine whether the variant is associated with Gram-negative infection, sepsis, or severity of sepsis.

Another molecule involved with opsonization is MBL.[30]

Deficiencies in MBL have been related with increased susceptibility to infections.[31] Variants D, C, and B, have been described as genetic polymorphisms in MBL in amino acids 52, 54, and 57 respectively. These polymorphic sites result in amino acid changes that diminish the ability of the helical tails to polymerize, resulting in an augmented degradation of MBL and reduced serum levels of MBL.[30,32,33] Various studies have demonstrated associations between genetic polymorphisms in the MBL gene and hospitalizations due to infections in children,[34] number of acute respiratory infections in children,[35] increased risk for meningococcal infections.[36]

Recognition of microbial products causes the activation of the innate immune response which includes multiple cytokines, chemokines, and coagulation factors. TNF-α is a pro-inflammatory cytokine and plays a key role in the pathogenesis and the initial activation of the acute inflammatory response. It is known that an exaggerated pro-inflammatory response causing an imbalance between the pro-inflammatory cytokines such as TNF-α and the anti-inflammatory cytokines results in the clinical manifestation of sepsis and septic shock. Genetic polymorphisms within the regulatory regions of the gene coding for TNF-α are perhaps the most extensively studied of all cytokines induced in sepsis. Polymorphisms of TNF-α gene have been linked with increased severity in chronic inflammatory diseases, cerebral malaria, chronic obstructive pulmonary disease, and sepsis.[37,38–41] These include a G to A transition 308 base pairs upstream from the transcriptional start site for the TNF-α gene. A higher frequency of the TNF-α -308 A allele was found in those children with meningococcal disease who died compared with those children who survived.[38] Even those children who were heterozygous at this position (TNF-α -308 GA) were at an increased risk for more severe meningococcal disease and death compared with those children who were homozygous for the wild-type genotype (TNF-α-308 GG).

Interleukin-1α and -1β, (IL-1α, IL-1β) are also key pro-inflammatory cytokines produced early in the response to a microbial invasion and play an important role in the pathogenesis of sepsis and septic shock. These molecules stimulate the production of prostaglandins and nitric oxide, two mediators of the vasodilation observed in sepsis.[42] On the contrary, IL-1RA competes with IL-1 for binding to its receptor, and operates as an inhibitor. Elevated serum levels of IL-1 and IL-1RA have been found in patients with meningococcal disease.[43] The genes coding for IL-1α, IL-1β, and IL-1RA are clustered together on chromosome 2, and several polymorphisms have been described in this locus.

Another pro-inflammatory cytokine is IL-6 which activates both B- and T-lymphocytes. Serum levels of IL-6 have been related with the severity and outcome of sepsis.[44-46] The promoter region for IL-6 has been shown to have several SNPs, and polymorphism comprising G to C substitution at position -174 in the promoter region has been extensively studied.[47] Association with sepsis has been found in premature neonates who produce low serum levels of IL-6 associated with the G allele.[48] Furthermore, the promoter polymorphism of the interleukin-6 gene regulates interleukin-6 production in neonates but not in adults.[49]

In addition to activating a pro-inflammatory cytokine cascade, inflammatory stimuli prompt a compensatory systemic anti-inflammatory response, which normally quickly down regulates the initial pro-inflammatory response. These anti-inflammatory cytokines suppress the expression of the genes for IL-1 and TNF-α and inhibit antigen presentation by monocytes and T- and B-lymphocyte function.[9]

IL-10 is mainly produced by monocytes and down-regulates the expression of cytokines such as TNF-α, IL-1α and -β, IL-6, and IL-8 by T-helper cells.[50] It has been proposed that over expression of IL-10 may induce immunosuppression in bacterial sepsis and increase mortality by inhibiting bacterial clearance.[51,52] IL-10 production appears to be regulated primarily at the transcriptional level. Three single nucleotide polymorphisms upstream from the transcriptional start site affect IL-10 expression: at positions -1082 (G to A), –819 (C to T), and -592 (C to A).[53] Conflicting results in various studies performed to explore whether the genetic polymorphisms in the regulatory region of the gene coding for IL-10 are associated with sepsis have been reported.[54]

The pro-inflammatory and anti-inflammatory cytokine pathways are closely linked to other homeostatic pathways, including the coagulation-fibrinolytic system, lipid mediators, acute-phase and heat-shock proteins (HSPs), neutrophil-endothelial cell and hypothalamic-pituitary-adrenal axis activation, immune- and nonimmune-cell apoptosis, increased nitric oxide (NO) production, and the oxidant-antioxidant pathway.[55,56] HSPs are expressed in response to heat, as well as endotoxin and other mediators of severe sepsis.[57] The genes coding for three HSPs reside alongside the HLA genes, in the major histocompatibility complex near the genes coding for TNF-α.[58] Angiotensin I converting enzyme (ACE) is found on endothelial and epithelial cells and is primarily responsible for converting angiotensin I to angiotensin II. Numerous studies have explored the association of the ACE I/D polymorphism with sepsis. A strong association between the D/D genotype and severe meningococcal disease has been reported in children in terms of a higher predicted risk of mortality, greater prevalence of inotropic support and mechanical ventilation, and longer intensive care unit stay.[59]

High plasma concentrations of plasminogen activator inhibitor 1 (PAI-1), an inhibitor of fibrinolysis, have been

reported in sepsis and severe meningococcal disease.[60,61] A single nucleotide insertion/deletion polymorphism is within the promoter region of the gene coding for PAI-1 influencing the amount of PAI-1 production. The major production of PAI-1 has been observed in individuals homozygous for four guanines (4G/4G) rather than in individuals heterozygous (4G/5G) or homozygous for five guanines (5G/5G) , as it was found in a large cohort of children with meningococcal disease.[62]

Candidate genes influencing the intensity of the inflammatory response in neonates

Despite the enormous human and financial costs of infection for neonatal mortality and morbidity worldwide, it remains unclear why neonates are so susceptible. Developmental deficiencies of the host defense system, including a delayed maturation of the specific humoral and cellular immune response of neonatal B and T cells,[63,64] and a decreased competence of neonatal cells to secrete cytokines involved in inflammatory response have been reported. Moreover, a defective activation of the complement cascade[65] and deficiencies of neonatal myelopoiesis[64] as responsible for compromised functions of the innate immune system have been described.

Although few studies address genetic determinants of neonatal infectious disease susceptibility, several variants in genes involved in the innate immune response have been associated with differential risk for neonatal infection.[4] Most studies conducted in neonates to evaluate the systemic inflammatory status aimed to recognize one or more cytokines as markers to identify infected neonates. It has been reported that plasma levels of TNF-α, IL-1b, IL-4, IL-6, IL-8, and IL-10 are elevated in septic neonates,[66-72] but it is difficult to know whether this is of pathogenic importance or is an epiphenomenon of the disease process.[73] Schultz *et al.*[74] reported an increased production of pro-inflammatory cytokines in term and preterm infants, spontaneously and after endotoxin challenge, indicating a well-developed inflammatory response. They ascribed the greater susceptibility of the neonate for sepsis to an imbalance between pro- and anti-inflammatory cytokines in favor of pro-inflammatory cascade.[74] With the explosion of genetic information and the identification of numerous polymorphic genes involved in the immune response to microbial insult, molecular biology is increasingly affecting critical care medicine and is improving our understanding of the molecular and cellular mechanisms that determine clinical outcome from infectious diseases. Despite the numerous genetic epidemiologic studies conducted in adult populations,[75-77] few reports regarding genetic predisposition to sepsis in neonates have been published. It would be of great significance to understand the molecular genetic basis underlying neonatal sepsis and to determine whether a polymorphic gene encoding for a cytokine involved in the inflammatory response plays a crucial role in the development and outcome of the disease. All genes encoding proteins involved in the transduction of inflammatory processes are candidate genes to determine the human genetic background that is responsible for individual differences in systemic inflammatory responses to infection.[75,78-82] The approach of associating a candidate gene with the incidence of and outcome from sepsis is not as promising in neonates as in adults because of the limited number of studies. Candidate genes evaluated in septic neonates have been the TNF-α gene and IL-1β, IL-4 receptor α-chain, IL-6, and IL-10 genes. TNF-α plays a central role in the pathogenesis of sepsis and its sequelae, and in some studies high levels of TNF-α correlate with severity of disease. Genetic variability at the TNF loci within the MHC on chromosome 6 has been well characterized. Biallelic polymorphisms defined by restriction enzymes (NcoI and AspHI) and other single-base changes at position -308 in the promoter region of the TNF-α gene, consisting of a G (TNF-α-308G) (TNF2) in the common allele and an A (TNF-α-308A) in the uncommon (wild-type, TNF1) allele, have been investigated in experimental *in vitro* studies and in various diseases. Weitkamp *et al.*[83] characterized the genomic distribution and allele frequency of the NcoI polymorphism in preterm and term septic neonates and compared it with clinical and laboratory characteristics to assess its prognostic value for disease progression. They concluded that biallelic NcoI polymorphisms within the TNF locus were not a good prognostic marker for disease progression in septic neonates. Treszl *et al.*[84] studied a small population of VLBW infants with sepsis for the polymorphism 308G in TNF-α gene and other genetic variants of IL-1β, IL-4 receptor α-chain, IL-6, and IL-10 genes. They found no association between these polymorphisms and susceptibility to or outcome from sepsis. Harding *et al.* reported that IL-6 -174 GG genotype influences the defense against bacterial pathogens in the very preterm infant being this genetic variation associated with lower IL-6 response to inflammation.[48]

We evaluated the prevalence of TNF-α and IL-10 polymorphisms in preterm neonates with late-onset sepsis. Septic neonates were compared with a noninfected reference group with similar gestational age, birth weight, Apgar scores, and type of delivery. In the septic neonates, 308G-TNF-α and 1082-IL-10 polymorphisms, analyzed by restriction fragment length polymorphism polymerase chain reaction (PCR), resulted, in homozygous and heterozygous forms, more frequent with statistical significance. Homozygosity for 308G TNF-α was 8.9% versus 1.8% ($p < 0.01$), whereas heterozygosity was 39.9% versus 32.2% ($p > 0.05$). For

1082-IL-10, the values were, respectively, 35.9% versus 14.8% in homozygosity ($p < 0.01$) and 62% versus 47.9% in heterozygosity ($p< 0.05$).[85] This study is still insufficiently powered to confirm these data, and the analysis of a larger group of subjects is needed.

Limitation of genetic studies

To establish the role of the genetic background in inflammatory responses, it is important to identify genomic markers suitable for clinical use and risk stratification and to understand the effects of genomic variations on gene regulation and protein expression.[86] Therefore, genetic epidemiologic studies must determine whether a septic population contains a distribution of markers that is statistically different from the distribution observed in a control population and whether within the septic population the survivors and non survivors have a significant difference in marker distribution. For those in the field of neonatology, the promise of SNPs and association studies might be an effective advancement for the explanation of the role of genetic variation in sepsis.

However, several limitations to the associations studies should be remarked.[87] A fundamental principle of gene association studies is the measurement of association of a unit of the human genome with a phenotype. To date, the attempt to find the genetic variants that are responsible for susceptibility to severe infection and sepsis mostly consisted of gene association studies in case-control or cohort studies of small sample size.[88] Although it is rational to suppose that polymorphisms associated with augmented disease susceptibility occur in regulatory or coding sequences, this hypothesis appears to be excessively simplistic and underestimates the undetermined potential for other SNPs whose function is currently unknown to alter outcome. For example, the genes coding for TNF-α are situated on chromosome 6 within the major histocompatibility complex near the HLA loci and are in strong linkage disequilibrium with several HLA alleles that may be involved in controlling TNF-α secretion. Thus, the polymorphisms within the promoter region may not directly cause the increased TNF-α secretion but are a marker for some other closely linked genetic factor. Additionally, in large genes, there are frequently exonic SNPs and SNPs in known regulatory regions of the gene, such as the promoter. An association study based on a single SNP gene is optimized when the SNP evaluated is responsible for a change in phenotype or is in high linkage disequilibrium with the causal SNP.[89,90] Otherwise, a SNP may be erroneously characterized as a disease susceptibility allele when it is in linkage disequilibrium with the true causal allele.[90] Our partial knowledge of transcriptional regulation and the structure of linkage disequilibrium, as well as incomplete detection of all polymorphic sites within the human genome, may be considerably responsible for the lack of reproducibility of many gene association studies in sepsis.[91]

An additional concern is that frequency of the polymorphism in the group of patients with sepsis should be compared with the frequency of the polymorphism in a control group of patients with a similar infection who did not develop sepsis, in contrast the comparison is commonly established with the frequency of the polymorphism in a inappropriate control group that is not similarly exposed to the same pathogen.

A further limitation of various studies relates to subjects within the study and control groups who are from different ethnic groups. It is now well known that the frequency of many of these polymorphisms varies between ethnic groups, and consequently the comparisons should only be made within ethnic groups. The use of genomic control SNPs and ethnically homogenous samples helps to avoid spurious associations.[92]

Conclusively it is likely that some SNPs by themselves are not the cause of the increased susceptibility to or outcome from sepsis but rather are markers for an extended haplotype of genetic variations. Considering the number of SNPs in the human genome, and our limited understanding of their function and linkage disequilibrium structure, single-SNP analysis may be less informative than analysis based on haplotypes and haplotype clades.[93] Haplotypes serve as markers of all detected and undetected SNPs within the haplotype and increase the power to associate genotype with phenotype. In both single SNP and haplotype based association studies, it is fundamental to recognize the value of the possible confounding effects of small sample size and population substructure.[94]

Conclusions

The possibility of understanding the genetic contribution to response to microbial pathogens remains one of the most stimulating prospects of the unravelling of the human genome. The identification of strong associations between certain genetic polymorphisms and susceptibility to severe sepsis supports further research using appropriate association studies.

Cytokine gene polymorphisms represent attractive subjects for candidate gene association studies given the significant role of cytokines in the pathogenesis of infectious disease, and this is evident in the increasing number of such reported studies. The results are often limited by limitations of study design such as small sample size and population stratification, the modest magnitude of any individual gene effect, and the inclusion of a limited number of genetic polymorphisms. With careful study design and better understanding of gene function and biological pathways, gene association studies have the potential to revolutionize our clin-

ical activities.

In the near future, patients with critical illness could be genotyped within a few hours of admission to ascertain the genetic basis of their inflammatory response and have their treatment tailored accordingly. Until this time, studies to identify disease susceptibility genes should be in definite disease entities, in ethnically matched populations, and proper sample size to allow multiple comparisons and mapping of multiple markers.

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