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Genetic Variation in Complement Component 2 of the Classical Complement Pathway is Associated with Increased Mortality and Infection: A Study of 627 Trauma Patients

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Abstract

Trauma is a disease of inflammation. Complement Component 2 (C2) is a protease involved in activation of complement through the classical pathway and has been implicated in a variety of chronic inflammatory diseases. We hypothesized that genetic variation in C2 (E318D) identifies a high-risk subgroup of trauma patients reflecting increased mortality and infection (Ventilator associated pneumonia: VAP). Consequently, genetic variation in C2 may stratify patient risk and illuminate underlying mechanisms for therapeutic intervention.

Methods—DNA samples from 702 trauma patients were genotyped for C2 E318D and linked with covariates (age: mean 42.8 years, gender: 74% male, ethnicity: 80% Caucasian, mechanism: 84% blunt, ISS: mean 25.0, admission lactate: mean 3.13 mEq/L) and outcomes: mortality 9.9% and VAP: 18.5%. VAP was defined by quantitative bronchoalveolar lavage (>104). Multivariate regression determined the relationship of genotype and covariates to risk of death and VAP. However, patients with ISS $\geq$ 45 were excluded from the multivariate analysis, as magnitude of injury overwhelms genetics and covariates in determining outcome.

Results—52 patients (8.3%) had the high-risk heterozygous genotype, associated with a significant increase in mortality and VAP.

Conclusion—In 702 trauma patients, 8.3% had a high-risk genetic variation in C2 associated with increased mortality (OR=2.65) and infection (OR=2.00). This variation: 1) Identifies a previously unknown high risk group for infection and mortality; 2) Can be determined on admission; 3) May provide opportunity for early therapeutic intervention; and 4) Requires validation in a distinct cohort of patients.

Introduction

An individual's stress response following multi-system trauma is characterized by an increase in the body's demand for energy, inflammation, free radical production and high mortality.¹

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² Though we can often predict a patient's clinical course based on demographics, physiology and severity of injury, it is not uncommon for a young, healthy patient with an apparently benign injury pattern to display an overwhelming inflammatory response such as Adult Respiratory Distress Syndrome, Abdominal Compartment Syndrome or Multiple Organ Failure. Because these syndromes often occur without a clear physiologic explanation, we have explored more covert answers – answers that may lie in the genome.

Studying the genome in critical care patients is difficult. Consequently, we have developed the concept of Environmentally Determined Genetic Expression (EDGE).³ Simply, the EDGE Concept states the vast majority of genetic polymorphisms are not pathologic. However, when a patient is exposed to stress such as multi-system trauma, a few polymorphisms in critical pathways alter outcome. Genetically encoded differences in expressed proteins react differently as patient acuity increases. In other words, minor genetic variations, which under normal circumstances are not pathologic, may become pathologic when the patient is stressed by a life-threatening injury.

One application of the EDGE Concept is in the role of inflammation. The genetics of the inflammatory response is complex and potentially a two edged sword. Insufficient inflammation may result in infection; while a hyper-inflammatory response potentially results in the development of complications and increased mortality.

Complement activation is one key component of the acute inflammatory response. The Complement System is a set of more than thirty proteins that serves as an important effector arm in immune defense.⁴ In addition to inducing inflammation, the Complement System plays a major role in protecting against infection and killing diseased cells.

There are three major pathways of complement activation: Classical, Lectin and Alternative. The *Classical pathway* is activated by antigen-bound antibodies. The *Lectin pathway* is activated by mannose containing polysaccharides; and the *Alternative pathway* is activated by microbial substances and other “foreign” surfaces. Complement Component 2 (C2) is believed to be a critical factor in complement activation in the Classical and Lectin pathways.

We hypothesize genetic variation in regulatory protein C2 might alter complement activation, resulting in differences in mortality or infection among trauma patients. Identification of the site of these genetic variations may stratify patient risk, illuminate underlying disease mechanisms and suggest new areas for drug development.

Methods

Setting and Study Population

This study was performed at Vanderbilt University Medical Center (VUMC), the only Level I trauma center serving approximately 65,000 square miles. All study procedures were approved by the Vanderbilt University Institutional Review Board. Approximately 4000 trauma admissions occur annually, with 1800 of those admitted to a 31-bed dedicated trauma unit. Fourteen beds in this unit are classified as Trauma intensive care unit (ICU) beds. The study population consisted of 702 consecutive admissions to the Trauma ICU from April 2005 through February 2006. All patients had a single blood sample drawn for DNA extraction within 24 hours of admission to the Trauma ICU. A small number of patients (<5%) were excluded from the genetics registry based on their vulnerable population status: age < 18, known pregnancy, prisoner and death or ICU discharge prior to sample accrual. The primary outcome was in-hospital mortality and infection (Ventilator-Associated Pneumonia, VAP) following admission to the Vanderbilt Trauma ICU.

Data Sources

Data sources for this study included the Vanderbilt Trauma Genetics Registry, the Trauma Registry of the American College of Surgeons (TRACS) and the Electronic Medical Record (EMR). DNA samples in the Trauma Genetics Registry were analyzed to determine the presence of C2 E318D. Demographics (age, gender, ethnicity), Injury Severity Score (ISS), primary mechanism of injury (blunt or penetrating) and discharge status (dead or alive) were extracted from TRACS; the EMR was queried for laboratory data including serum lactate and microbiology culture results. These data were linked and de-identified prior to analysis.

Measurements and Definitions

Patients with VAP were defined as having one or more positive quantitative bronchoalveolar lavage (BAL) cultures with $> 10^4$ colony forming units/ml (cfu/ml) at any time during their hospitalization. Ventilator free days was computed as $\{28 - \text{number of days on mechanical ventilation}\}$ for patients surviving 28 days or longer who were ventilated for 28 days or less, zero for patients surviving less than 28 days, and zero for patients requiring more than 28 days of mechanical ventilation.⁵

Genetics

DNA was isolated from whole blood using PUREGENE (Gentra Systems Inc., Minneapolis, MN, USA). Genotyping was performed with the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems Inc., Foster City, California, USA) and the 5'nuclease allelic discrimination Taqman assay. The C2 E318D polymorphism (rs 9332739) was detected using the ABI sequence detection system. The context sequence for this C/G transversion substitution is ACG ACA ACT CCC GGG ATA TGA CTG A[C/G]G TGA TCA GCA GCC TGG AAA ATG CCA. Genotypic data were analyzed using ABI Sequence Detection System version 2.1 software and confirmed by visual inspection of the plots. Genotypes were classified as undetermined if PCR amplification failed with the specified sets of probes and primers.

Statistical Analysis

C2 E318D single nucleotide polymorphism (SNP) genotype frequencies and other categorical variables were compared between outcome categories using the Pearson chi-squared test or the Fisher exact test. Continuous demographic variables were compared using the Student's t-test or the Mann-Whitney U test if the variables were not normally distributed. Multivariate logistic regression models were constructed to determine the odds ratios and 95% confidence levels for associations between the specific C2 E318D polymorphism, potential confounding variables (such as ISS) and the outcome variables (in-hospital mortality and VAP). Tests for statistical significance were two-sided with an alpha level of 0.05. All statistical analyses were accomplished using the STATA statistical software package (version 10.0; College Station, TX).

Results

The study group consisted of 702 consecutive Trauma ICU patients. 75 patients had an ISS >45 and were excluded from the multivariate analysis (Figure 1). The demographic and clinical characteristics of the study group were stratified by the primary outcome variable, in-hospital mortality (Table 1). The overall in-hospital mortality was 12.3% (86/702). Age (mean, in years) was significantly different between survivors and non-survivors, 41.4 years vs. 48.7 years ($p<0.01$). Males represented 73.4% of this cohort (515/702). While males had a higher rate of in-hospital mortality than females (13.4% vs. 9.1%), the difference was not statistically significant ($p=0.12$). ISS, initial lactate level, highest lactate level within 24 hours and highest lactate level over the entire hospitalization were all significantly elevated in non-survivors as

compared to survivors. There was a modest, but statistically significant, correlation ($r=0.2$, $p<0.01$) between ISS and the highest lactate level over the entire hospitalization. In-hospital mortality also differed by primary mechanism of injury, with blunt trauma having a rate of 12.5% (74/590) compared to 10.7% (12/112) following penetrating trauma ($p=0.58$). As expected, ISS varied significantly between the blunt trauma (mean = 29.0) and penetrating trauma (mean=21.0; $p<0.01$).

In the univariate analysis of the C2 E318D genotype in the study group, individuals with the GA genotype had a higher mortality than those with the GG genotype [20% vs 11.4%; $p = 0.05$ (Table 2)]. Similarly, the GA genotype appeared to have a higher incidence of VAP than the GG genotype category [33% vs 20.9%; $p = 0.06$ (Table 3)]. Only one patient had the AA genotype, so this genotype was excluded from these analyses.

The multivariate logistic regression model excluded the highest decile of ISS (>45), in whom injury severity posed a potential confounding influence that would impair detection of a genetic signal. The resulting cohort was 627 patients. Both mortality and VAP were significantly more common in patients with severe injury (mortality: 25% vs. 11.2%, $p=0.007$; VAP: 50% vs. 19.6%, $p<0.0001$). The GA genotype was associated with an increase in mortality [Odds Ratio (OR): 2.65, GA vs. GG genotype, 95% CI 1.18-5.96, $p=0.02$; Table 4]. When compared with the GG genotype, the GA genotype was also associated with an increased incidence of VAP (OR: 2.0, 95% CI 1.03-3.88, $p=0.04$; Table 5). This model controlled for demographic covariates known to influence trauma survival (age, gender), as well as injury severity (ISS) and acuity (highest lactate value over the hospital stay). After adjustment for these confounding variables, the GA genotype was an independent predictor for both mortality and VAP in our study population.

Discussion

As early as the 1918 flu pandemic, clinicians have documented the hyper-inflammatory response and its contribution to unexpected mortality in young, previously healthy patients. Trauma surgeons today continue to encounter hyper-inflammatory mediated syndromes: Adult Respiratory Distress Syndrome², Abdominal Compartment Syndrome⁶ and Multi-Organ Failure⁷. It is well documented that C2 polymorphisms and deficiencies have been linked to several chronic inflammatory conditions: age-related macular degeneration (AMD)^{8, 9} and systemic lupus erythematosus (SLE),¹⁰ respectively. Although functional differences between the C2 variants have not been well elucidated, it is possible that each influences the level of complement activation differently. In the rare cases of C2 deficiency, the effect is clearer. Individuals lacking C2 have an impaired ability to activate complement through the lectin and classical pathways. It has been shown that C2 deficient patients have higher levels of circulating immune complexes. The impaired ability of these individuals to clear circulating immune complexes may predispose them to autoimmune disease.⁴ Consequently, we chose to look at the Complement System in general, and C2 specifically, because we believe an understanding of complement activation may lead to understanding inflammation and identify new diagnoses, new ways to stratify patient risk, and new therapy.

In this manuscript we describe preliminary results of an association study which suggest an association between C2 E318D and increased mortality and infection. In the univariate model, the presence of E318D appears to double the mortality rate. In the multivariate model, which controlled for confounders (age, injury severity, gender and lactate), the effect is equally pronounced. Patients with the C2 E318D polymorphism have increased mortality (OR: 2.65; $p = .02$) and increased probability of VAP (OR: 2.00; $p = .04$)

The pathway

The genetics of the inflammatory response is complex. Complement activation is one key component of the acute inflammatory response. Complement protects against infection, kills diseased cells, introduces inflammation, eliminates damaged tissue and promotes wound healing.⁴

There are three major pathways of complement activation: The *Classical pathway* is activated by antigen bound antibodies. The *Lectin pathway* is activated by polysaccharides, particularly those containing mannose. The *Alternative pathway* is activated by many microbial substances and other foreign surfaces.⁴

The three pathways for complement activation converge with the proteolytic activation of Complement Component 3 (C3) through the formation of enzymatic complexes termed C3 convertases. There are two types of C3 convertase. The Alternative pathway C3 convertase consists of activated forms of C3 and Factor B. The Classical and Lectin pathways share the Classical pathway C3 convertase which consists of activated forms of C2 and C4.⁴

Both types of C3 convertase proteolytically activate C3 into two peptides, C3a and C3b. C3b possesses a reactive thioester that allows it to covalently attach to a variety of surfaces. Factor B then associates with C3b to form the precursor C3 convertase (C3bB). This complex is subsequently acted upon by another complement protease, Factor D, to form the active C3 convertase (C3bB). All pathways are thus amplified by the alternative pathway through C3 activation. In the shared terminal pathway, C5 is proteolytically activated next, to form C5a and C5b. Assembly of the *membrane attack complex* follows, by sequential addition of components C6 through C9 to C5b.⁴

In addition to triggering target cell lysis, complement activation also induces inflammation. The activation of C3 and C5 produces two small peptides, the anaphylatoxins C3a and C5a which are potent inflammatory mediators. These peptides have partially overlapping effects, including immune cell activation, increased vascular permeability and histamine release.⁴ It is these conflicting effects which make the pathway so interesting in trauma patients.

Because the complement system has both positive and negative effects, it is highly regulated. The cascade possesses at least ten negative regulators. Once the C2 zymogen is activated, it forms a proteolytic subunit of the Classical Pathway C3 convertase. This convertase is shared by the Lectin and Classical pathways of complement activation.⁴ There is evidence that circulating self-reactive antibodies can activate complement in the model of ischemia/reperfusion injury.¹¹ Individuals genetically lacking C2 are prone to autoimmune disease and possess circulating auto-antibodies.¹⁰

Relevancy

Rapid complement activation has been shown to occur following trauma, both in animal models and in man. The level of complement activation in the hours immediately after injury correlates well with the risk of mortality. It is therefore conceivable that variants in complement proteins may influence the level of complement activation following injury, particularly for the proteolytic subunit of the Classical pathway C3 convertase enzyme complex.

Strengths and Limitations

This is a genetics association study. Such association studies have two potential limitations that we attempted to avoid: selection bias and clinical variability. To minimize selection bias, we employed a population-based, prospectively collected, cohort of consecutive patients to a single trauma ICU from a large catchment area.

Clinical variability is minimized by aggregating all trauma patients in the hands of a small number of physicians practicing under standardized evidence-based protocols. This structure was designed to minimize variation in clinical care and maximize the strength of the genetic signal. Additionally, the exclusion of patients with an ISS of > 45 was designed to magnify the genetics signal by excluding patients with a low probability of survival. We believe that in the highest decile of acuity, the genetic signal contributing to mortality risk is overwhelmed by the magnitude of injury.

Finally, the C2 polymorphisms were selected because of prior epidemiological evidence suggesting associations with specific phenotypes.^{10, 11} This lends biological plausibility to their potential involvement. It remains possible the C2 E318D polymorphism is in linkage disequilibrium with the causative polymorphism. Additional future research into the functional status of this well-known polymorphism is warranted.

Clinical studies and the ICU environment are difficult. Genetic studies are especially difficult as both the phenotype and the environment are difficult control, the regulatory environment is rigorous and the polymorphisms are highly complex and involve many potential confounding variables. Additionally, there are many polymorphisms worthy of investigation.

Consequently, interpretation of this study requires perspective. This is an association study. We have only demonstrated an association between the specific C2 polymorphism E318D and the outcome criteria: death and infection. This work requires scientific validation in an entirely new population, in thousands of patients, prospectively collected under similar conditions present in this study. This project is currently underway.

The Future

The emerging era of personalized medicine mandates defining the relationship between individual genetic variation and outcome in critical care patients. To understand the interaction between the genome, the ICU environment and inflammation will require: thousands of patients, powerful informatics tools and DNA sequencing.

In the future, advancements will be made in chip technology and the cost will decline. This will allow us to move from pathway analysis to genome wide association studies. Genome wide association studies now can analyze one million single nucleotide polymorphisms (SNP) that begin to cover a significant proportion of an individual's genetic variation. These studies probe a wide variety of biochemical pathways and can generate new hypotheses based on unanticipated associations.

The simultaneous analysis of millions of genes will challenge our computational and analytical skills. We will need to develop new methods of computing, such as parallel processing and quantum computing. We are preparing for the day when trauma patients routinely have DNA genotyping upon admission. Ideally select populations, such as the military, will be genotyped prior to injury. We envision the use of microarray chips containing numerous polymorphisms, all validated, which stratify patients by risk of disease or complication and predict response to therapy. We expect regulatory proteins associated with complement activation to be part of that chip.

Conclusions

In summary, trauma patients generate a robust but variable acute inflammatory response, which may protect against infection or may be pathologic. We have demonstrated an association between the C2 polymorphism E318D, a regulator of complement activation, and mortality and VAP in a population of trauma patients. Of the 702 trauma patients in this study group,

8.3% had the high risk genetic variation C2 E318D which was associated with increased mortality (OR: 2.65) and infection risk (OR: 2.00). The C2 E318D variation:

1. Identifies a previously unknown high risk group for infection and mortality;
2. Can potentially be determined on admission;
3. May provide opportunity for early therapeutic intervention; and
4. Requires validation in a new, larger and distinct cohort of patients.

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Figure.

Table 1

Patient demographics, acuity and injury variables by survival.

Characteristic	Survivors	Non-survivors	p-value
Age (years) (mean)	41.4	48.7	<0.01
Ethnicity			
Caucasian	494	68 (12.1%)	
African-American	69	6 (8.0%)	
Hispanic	47	9 (16.1)	
Asian	1	1	
Other	4	0	
Not-specified	1	0	
Gender			0.12
Male	446	69 (13.4%)	
Female	170	17 (9.1%)	
Injury Severity Score (ISS), (mean)	26.7	34.9	<0.01
Initial lactate level (mEq/L), (mean)	3.13	3.84	0.01
Highest lactate level in first 24 hours (mEq/L), (mean)	3.53	4.83	<0.01
Highest lactate level over entire hospitalization, (mEq/L), (mean)	3.60	5.47	<0.01
Main Mechanism, frequency			0.58
Blunt	516	74 (12.5%)	
Penetrating	100	12 (10.7%)	

Table 2

Univariate analysis of C2 E318D genotype with in-hospital mortality. : Individuals with the GA genotype appeared to have a higher mortality rate than the GG genotype category. The p-value for this using Pearson's chi-square test was 0.05. The AA genotype was excluded from the analysis because only a single individual had this genotype

		C2 E318D Genotype		
	AA	GA	GG	Total
Survivors	0	48	568	616
Non-Survivors	1	12 (20.0%)	73 (11.4%)	86 (12.2%)
Total	1	60 (8.5%)	641 (91.3%)	702 (100%)

Table 3

Univariate analysis of C2 E318D genotype with Ventilator-Associated Pneumonia (VAP) Individuals with the GA genotype appeared to have a higher VAP rate than the GG genotype category. The p-value for this using Pearson's chi-square test was 0.06. The AA genotype was excluded from the analysis because only a single individual had this genotype.

		C2 E318D Genotype		
	AA	GA	GG	Total
No VAP	1	40	507	548
VAP	0	20 (33.3%)	134 (20.9%)	154 (21.9%)
Total	0	60	641	702

Table 4

Multivariate logistical regression model of C2 E318D genotype and in-hospital mortality, excluding for ISS>45 (N=627)

	Odds Ratio	95% CI	p-value
Age (years)	1.04	1.02 - 1.06	<0.01
Gender (male)	1.91	0.94 - 3.90	0.07
Highest lactate value over ICU stay (mEq/L)	1.21	1.12 - 1.31	<0.01
ISS	1.04	1.01 - 1.07	<0.01
Main mechanism of injury (blunt)	0.76	0.31 - 1.80	0.53
C2 (E318D) polymorphism, GA genotype vs. GG genotype	2.65	1.18 – 5.96	0.02

Table 5

Multivariate logistical regression model of C2 E318D genotype and Ventilator-Associated Pneumonia (VAP), excluding for ISS>45

Variable	Odds Ratio	95% CI	p-value
Age (years)	1.01	1.00 - 1.03	0.02
Gender (male)	1.46	0.88 - 2.44	0.14
Highest lactate value over course of ICU stay (mEq/L)	1.07	0.98 - 1.15	0.09
ISS	1.07	1.04 - 1.09	<0.01
Main mechanism of trauma (blunt)	1.12	0.57 - 2.21	0.74
C2 (E318D) polymorphism, GA genotype vs. GG genotype	2.00	1.03 - 3.88	0.04