Genetic control of the alternative pathway of complement in humans and age-related macular degeneration

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Activation of the alternative pathway of complement is implicated in common neurodegenerative diseases including age-related macular degeneration (AMD). We explored the impact of common variation in genes encoding proteins of the alternative pathway on complement activation in human blood and in AMD. Genetic variation across the genes encoding complement factor H (CFH), factor B (CFB) and component 3 (C3) was determined. The influence of common haplotypes defining transcriptional and translational units on complement activation in blood was determined in a quantitative genomic association study. Individual haplotypes in CFH and CFB were associated with distinct and novel effects on plasma levels of precursors, regulators and activation products of the alternative pathway of complement in human blood. Further, genetic variation in CFH thought to influence cell surface regulation of complement did not alter plasma complement levels in human blood. Plasma markers of chronic activation (split-products Ba and C3d) and an activating enzyme (factor D) were elevated in AMD subjects. Most of the elevation in AMD was accounted for by the genetic variation controlling complement activation in human blood. Activation of the alternative pathway of complement in blood is under genetic control and increases with age. The genetic variation associated with increased activation of complement in human blood also increased the risk of AMD. Our data are consistent with a disease model in which genetic variation in the complement system increases the risk of AMD by a combination of systemic complement activation and abnormal regulation of complement activation in local tissues.

INTRODUCTION

Abnormal activation of the alternative pathway of complement has been implicated in cardiovascular, ischemic, inflammatory and neurodegenerative diseases (1–6). Age-related macular degeneration (AMD) is a neurodegenerative disease and a major cause of untreatable blindness (7). Biochemical and genetic studies have demonstrated excessive deposition of complement in eyes harboring AMD and the involvement of variation in four genes encoding proteins of the alternative pathway of complement (8–14). Thus, AMD is an excellent model for understanding how the genetic control of complement activation alters disease risk.

Early AMD is characterized by the accumulation of inflammatory proteins, lipids and byproducts of the visual cycle in and under the retina (8,15,16). Complications of early AMD can lead to severe vision loss in 7% of Caucasians by age 75 or older and include patchy atrophy of the retina.
(geographic atrophy) and abnormal angiogenesis (exudation) (7). Age, race, family history and genetics are established, non-modifiable risks for AMD (17). Most genetic variation altering AMD risk is found in genes encoding protein components of the alternative pathway of complement. These genes include complement factor H (CFH), complement component 3 (C3) and the complement component 2/complement factor B (C2/CFB) locus (9–14). A region on chromosome 10q26 (age-related maculopathy susceptibility 2, ARMS2) spanning a hypothetical gene (LOC387715) and the promoter of high-temperature requirement A1 (HTRA1) is also a major genetic risk for AMD (18,19). Anti-inflammatory factors such as exercise, healthy body weight, eating fruits and vegetables, and consumption of fatty fish reduce the risk of AMD, while pro-inflammatory factors such as smoking, high-fat diet and obesity increase AMD risk (20,21). Such modifiable risk factors are thought to impact the activation of complement (22–24).

Constant activation of the alternative pathway of complement occurs through a slow spontaneous ‘tickover’ mechanism driven by spontaneous hydrolysis of an internal thioester bond in C3 (25). The C3 convertase (C3bBb) is formed by binding of factor B to C3b and subsequent cleavage of factor B to Ba and Bb by the serine protease factor D. Formation of the C5 convertase (C3bBbC3b) cleaves C5 to form C5b, which initiates assembly of the membrane attack complex (C5b-9) responsible for sublytic attack or cell lysis. Small protein fragments called anaphylatoxins (C3a, C5a) that recruit and activate the cellular immune system are formed during the cleavage of C3 and C5. An extensive regulatory network that includes factor H suppresses activation of the alternative pathway (26).

A major question in understanding the pathophysiology of neurodegenerative diseases, including AMD, is the extent to which abnormalities in local tissues (e.g. oxidation and toxic byproducts of the visual cycle of the retina) versus systemic blood factors lead to disease. Initial reports have suggested that there is increased activation of the alternative pathway of complement in subjects with AMD (27,28). These studies employed modest sample sizes and the observations were not replicated in independent groups of subjects, an essential step in genetic and proteomic association studies. Here we report a quantitative genomic association study consistent with genetic risk for AMD (18,19). Anti-inflammatory factors such as exercise, healthy body weight, eating fruits and vegetables, and consumption of fatty fish reduce the risk of AMD, while pro-inflammatory factors such as smoking, high-fat diet and obesity increase AMD risk (20,21). Such modifiable risk factors are thought to impact the activation of complement (22–24).

RESULTS

Impact of age, gender and renal function on complement levels

Plasma levels of alternative pathway of complement proteins were measured in 125 subjects with AMD and 149 control subjects without AMD (Table 1). Plasma levels of substrate factor B, two pathway regulators (factors D and H) and three activation split products (Ba, C3d and C5a) were determined.

Age significantly increased plasma levels of factor B, factor D and Ba (Supplementary Material, Table S1) in both cases and controls, demonstrating increased formation of the C3 convertase with aging. Gender had modest impact on plasma complement levels, but decreasing renal function as assessed by measurement of plasma creatinine substantially increased plasma complement levels and activation (Supplementary Material, Table S1). Time to completion of blood processing and smoking history did not affect complement levels. Plasma protein levels were corrected for age, gender and creatinine levels using quantile regression and standardized to facilitate comparisons of all six plasma levels (Supplementary Material, Fig. S1). The standardization of each plasma value was performed by subtracting the mean of the residual and dividing by the standard deviation (SD) as described in the Supplementary Material.

Plasma levels in subjects with and without AMD

Uncorrected plasma levels of factor B, factor D, Ba and C3d were significantly elevated in plasma from AMD subjects (Table 2). After correction and standardization, factor D, Ba and C3d remained significantly associated with AMD (Table 2). Thus, complement substrate and activator levels (factors B and D) and markers of complement activation (Ba, C3d) were increased in the plasma of AMD subjects. We observed a trend for greater increases in plasma protein levels of factor D, factor B, Ba and C3d in advanced subtypes of AMD (Supplementary Material, Fig. S2), suggesting that complement activation in the blood could be associated with progression of AMD.

Genetic control of complement activation in humans

We sought to determine the extent to which polymorphisms and haplotypes in the genes encoding CFH, C2/CFB, C3 and ARMS2 predicted plasma complement levels. Cases and controls gave similar findings, thus combined results on all subjects are presented. Coding variants in CFH, C2 and CFB were associated with altered plasma levels of Ba and C3d (Supplementary Material, Tables S2 and S3). The ARMS2 single nucleotide polymorphism (SNP) showed no
Increased Ba levels also were associated with risk (Glu, and C3d (Leu9His in common (risk) haplotype spanning (Table 3 and Supplementary Material, Table S4). The in 4% of subjects was associated with increased C3d levels and C3d levels (Table 3). An uncommon haplotype present proteases, while the protective 1 (P1) haplotype decreased Ba and possibly C3d levels (Table 3 and Supplementary Material, Table S2). Thus, unexpected and novel haplotype-specific patterns of activation of the alternative pathway of complement were observed.

**Combined protein and genetic models for AMD risk**

We estimated the ability of the six complement proteins, 11 genetic variants, or the combination of protein levels and genetic variants to predict AMD status using stepwise logistic regression and Bayesian model averaging. Using plasma protein levels, both models showed that only Ba and factor B contributed to the final model and were able to correctly classify 67% of case and control subjects (Fig. 1A; Supplementary Materials, Table S6 and Fig. S3). Case and control subjects were discriminated with 70% accuracy using genetic variants in CFB, C2/CFB and C3 (Fig. 1A and Supplementary Material, Table S6). Addition of the ARMS2 SNP to the model resulted in higher predictive accuracy [Fig. 1B; area under the curve (AUC) = 0.77]. Both models required only one SNP from each AMD risk locus for optimal prediction of AMD status (Supplementary Material, Fig. S4).

In a combined protein and genetic model, plasma levels and complement SNPs were able to discriminate between AMD and control subjects with 74% accuracy and inclusion of the ARMS2 SNP in the model increased the accuracy to 79% (Fig. 1 and Supplementary Material, Table S6). The final model required only factor D and a SNP from the CFB, CFB and ARMS2 loci for optimal prediction (Supplementary Materials, Table S6 and Fig. S6). Using haplotypes instead of SNPs in the combined protein and genetic model gave similar results (Supplementary Material, Fig. S7). The association of plasma complement levels with AMD risk could be accounted for by genetic variation in complement genes, except for the elevated levels of factor D.

**DISCUSSION**

Our results have important implications for understanding the contribution of the complement system to common complex traits and improving our understanding of the pathophysiology of AMD. We demonstrated for the first time to our knowledge that genetic variants in complement genes are highly predictive of the plasma levels of alternative pathway regulators, substrates, and activated split products in older individuals. We have confirmed using a larger sample size that activation of the alternative pathway of
Table 3. Haplotypes in the CFH, C2/CBF and C3 loci significantly altering plasma levels of complement regulators, precursors and activation products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Haplotype frequency</th>
<th>Protein</th>
<th>Coding variants</th>
<th>Haplotype frequency</th>
<th>Protein</th>
<th>Coding variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFH</td>
<td>AACAAC (P1)</td>
<td>fH</td>
<td>62I, 402Y</td>
<td>0.17</td>
<td>C3d</td>
<td>102G</td>
</tr>
<tr>
<td></td>
<td>GACAAC (P2)</td>
<td>fH</td>
<td>62V, 402Y</td>
<td>0.15</td>
<td>C3d</td>
<td>102G</td>
</tr>
<tr>
<td></td>
<td>GACGGC</td>
<td>fH</td>
<td>62V, 402Y</td>
<td>0.10</td>
<td>C3d</td>
<td>102G</td>
</tr>
<tr>
<td>C2</td>
<td>CACAAC</td>
<td>fB</td>
<td>9L, 32R</td>
<td>0.89</td>
<td>C3d</td>
<td>102G</td>
</tr>
<tr>
<td></td>
<td>CACCGG</td>
<td>fB</td>
<td>9L, 32Q</td>
<td>0.08</td>
<td>C3d</td>
<td>102G</td>
</tr>
<tr>
<td>C3</td>
<td>AGAGAC</td>
<td>fH</td>
<td>402H</td>
<td>0.25</td>
<td>C3d</td>
<td>102G</td>
</tr>
</tbody>
</table>

The corrected and standardized estimates correspond directly with the change in standard deviations; for example, one copy of the P2 haplotype decreases the combined plasma levels of factor H/factor H-related-1 (FH/FHR-1) by 0.52 SDs. The uncorrected estimate reflects the change in the actual concentration measured.

Subjects with the protective P2 haplotype (hH62V, 402Y; CFHR3-Del, CFHR1-Del) carrying the downstream deletion of complement factor H-related genes 3 (CFHR3) and 1 (CFHR1) had decreased combined levels of factor H and factor H-related 1 (FHR-1) as expected. This haplotype is not thought to have a major independent contribution to AMD risk or fluid-phase regulation of complement activation, consistent with our observations (30,31). The absence of an impact on the fluid phase activation of complement by the CFH risk haplotype suggests the substantial contribution to AMD risk tagged by hH62V, 402Y is mediated locally at the cell surface or in extracellular tissues of the eye. This concept is consistent with the demonstrated alteration in binding of fH402H protein to cell surfaces and polyanions (32). Recently reported in vitro studies showed that the non-synonymous variations in factor B (fB320G) that protect against AMD risk and modestly reduced complement activation in blood in our study (Table 3 and Supplementary Material, Table S5) have reduced ability to form the C3 convertase and activate complement (33). The modest increase in complement activation by the C3 SNP is consistent with our understanding of this protein’s function, but functional studies have not been performed (Table 3). Thus, our quantitative genomic observations are consistent with biochemical studies of common factor H and factor B protein variants encoded by haplotypes across these loci.

Both plasma protein levels and genetic markers were individually predictive of having AMD (Fig. 1). The combined plasma protein and genetic model was only slightly more predictive of AMD than genetic markers alone, suggesting that the genetic variants explained most of the AMD risk in these subjects. A 1 SD change in the levels of complement substrate (factor B), regulator (factor D) and activation products (Ba and C3d) associated with AMD altered disease risk by approximately 5-fold (Fig. 1C). Increased activation of the alternative pathway of complement in AMD is consistent with the notion that complex traits can arise from subtle changes in pathophysiological pathways (34). Given the importance of the innate immune system, our findings in support of the genetic control of complement activation have important implications for the study of other common complex traits.
Aging and lifestyle choices such as diet are major risks for development of many common complex traits including AMD. Aging was associated with increased activation of the alternative pathway of complement in human blood (Supplementary Material, Table S1). Further, deposition of alternative complement proteins increases with aging in human and animal tissues (35). Although the mechanism through which aging increases activation of complement in blood and tissues is unknown, our observations are consistent with the accumulation of endogenous or exogenous factors that enable intrinsic activation of innate immunity pathways. Dietary choices that increase the risk of AMD also contribute to aging and complement activation (24). It is tempting to speculate that some of the effect of age, diet and lifestyle choices on AMD risk may be mediated through increased activation of the alternative pathway of complement in blood.

Figure 1. Prediction of age-related macular degeneration (AMD) and impact of plasma complement levels on AMD risk. Receiver-operator characteristic curves for the discrimination of AMD status using the proteomic, genetic and combined protein and genetic models with complement gene variants only (A) and combined with genetic variants from the ARMS2 locus (B) are shown. The ARMS2 variants had no impact on complement levels, while variants in the other three loci (CFH, C2/CFB and C3) accounted for activation of the alternative pathway of complement (see text). (A) shows that the majority of the proteomic risk for AMD can be explained by genetic variation. (B) shows that the combined protein and genetic model can distinguish between AMD and controls with nearly 80% accuracy. (C) shows the odds ratio for each combination of a standard deviation (SD) change in plasma levels of factor D, factor B, C3d and Ba on risk of developing AMD relative to a reference group (R) having mean levels of all four proteins. Numbers on the x and y axes represent SD changes above (1), below (−1) and at the mean (0) of the corrected and standardized plasma levels for each protein.
Our observations illustrate the limitations of using modeling studies to predict pathophysiology. For example, an intronic SNP (rs412852) in the CFH locus was the best predictor of AMD (Supplementary Material, Fig. S4), but its minor allele appeared on multiple haplotypes with distinct consequences for plasma complement levels. This SNP appears to be the best statistical predictor of AMD status, because one allele was present on all risk haplotypes, while the other allele was present on all protective haplotypes. Thus, modeling may provide accurate prediction of disease status, but fail to detect the impact of individual haplotypes or variants in a biochemical pathway.

The non-synonymous coding variants in fH, 402Y, fB9L,32Q, fB9L,32R and C3,102G are known to have independent effects on AMD risk and thought to influence complement function as noted above. The variants (factors H and B) protective for AMD risk were consistently associated with decreased complement activation in blood, while the variants increasing AMD risk (C3) increased activation (Table 3). The simplest explanation for these observations is that regulation of complement activation in body fluids (e.g. blood, tissue) may contribute to the development of AMD (Fig. 1C).

In summary, our results suggest genetic control of complement activation in humans. Further, they support a disease model in which both local and systemic activation of complement may contribute to the development of AMD. Decreasing activation of the alternative pathway of complement in the blood and body fluids may protect against development of AMD and provides a target for novel treatment strategies.

**MATERIALS AND METHODS**

**Subjects**

Case and control subjects were consecutively ascertained by the authors under a protocol approved by the Mayo Clinic Institutional Review Board. All patients provided written informed consent. The case and control subjects in this report are derived from a group of Caucasian individuals who met specific sample resource and diagnostic criteria as described in the Supplementary Material.

**Complement protein measurements**

All complement assays were carried out as previously described (28,36,37). Complement components were measured in plasma by enzyme-linked immunoassays (ELISAs) using monoclonal capture antibodies as described in the Supplementary Material.

**Genotyping**

Subjects were genotyped for SNPs selected based on previous reports of association with disease and ability to capture common haplotypes spanning alternative complement pathway genes in Caucasians. A total of 11 SNPs were genotyped using TaqMan SNP genotyping assays (Applied Biosystems, Foster City, CA, USA) as described in the Supplementary Material.

**Statistical analysis**

Logistic regression was utilized to evaluate baseline differences between cases and controls. Nominal P-values of less than 0.05 are reported. Uncorrected plasma levels in cases and controls were summarized using medians owing to the skewness of the distributions and were compared using quantile regression (38). The effects of age, gender, and plasma creatinine levels on plasma complement levels were corrected by taking the residuals after fitting quantile regression. The residuals were standardized for unified interpretation of each plasma level regardless of their scales. Quantile regression was used to assess the impact of each SNP and haplotype on plasma levels of complement proteins. Proteomic and genetic models were created using stepwise logistic regression, and were validated by Bayesian model averaging method (39). Proteins with a significance level of less than or equal to 0.05 in the model are reported. The corresponding receiver-operating characteristic curve was generated and AUC was calculated. Additional information is provided in the Supplementary Material.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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