Apolipoprotein E ε4 Allele as a Genetic Risk Factor for Left Ventricular Failure in Homozygous β-Thalassemia

By Effrosini Economou-Petersen, Athanassios Aessopos, Athina Kladi, Panagiota Flevr, Fotis Karabatsos, Christina Fragodimitri, Peter Nicolaidis, Helen Vrettou, Dimitris Vassilopoulos, Markissia Karagiorga-Lagana, Dimitrios Th. Kremastinos, and Michael B. Petersen

In homozygous β-thalassemia, the organ damage is mainly attributed to excessive iron deposition through the formation of oxygen free radicals. Despite appropriate transfusion and chelation therapy and low ferritin levels, patients still develop organ failure, heart failure being the main cause of death. This study was designed to determine whether the decreased antioxidant activity of the apolipoprotein E (APOE) ε4 allele could represent a genetic risk factor for the development of left ventricular failure (LVF) in β-thalassemia homoygotes. A total of 251 Greek β-thalassemia homoygotes were studied. Patients were divided in three groups: group A (n = 151) with no cardiac impairment, group C (n = 47) with LVF, and 53 patients with LV dilatation and normal LV systolic function constituted the group B. DNA was obtained from all patients, and the polymerase chain reaction was used to analyze the polymorphism at the APOE locus. The APOE allele frequencies were compared with those of a Greek control sample of 216 healthy blood donors. Patients with no cardiac impairment had an APOE ε4 allele frequency (7.9%) not different from population controls (6.5%, P > 0.05), while patients with LVF had a significantly higher frequency of APOE ε4 (12.8%) than the controls (P < 0.05, odds ratio = 2.11, 95% confidence interval 1.03 to 4.32). The APOE ε4 allele may represent an important genetic risk factor for the development of organ damage in homozygous β-thalassemia.

THE HEMOGLOBINOPATHIES are the most common monogenic disorders in the world population, and they were the first diseases to be analyzed by recombinant DNA technology. 1-3 More is known about their molecular pathology than any other genetic disease, and it has been possible to trace almost all of the diverse pathophysiologic features back to primary molecular defects in single genes. 4 In monogenic disorders, most of the phenotypic variability is expected to be due to allelic heterogeneity, although siblings with the same genotype can show big phenotypic differences. The molecular basis of such difference in clinical expression is not yet fully understood.

Homozygous β-thalassemia is characterized by severe hemolytic anemia associated with chronic tissue damage, disease- or treatment-related organ failure and premature death.

Heart failure remains the main cause of death and is traditionally attributed to iron overload because of regular transfusions, increased iron intestinal absorption, and ineffective erythropoiesis during the life span of the patients. 5 6 During the last two decades, a striking improvement in life quality and life expectancy has been observed, mainly due to proper transfusion and effective chelation therapy to prevent secondary hemosiderosis. 7

Regular chelation therapy with deferoxamine mesylate, a naturally occurring trihydroxamic acid produced by Streptomyces pilosus, increases urinary and fecal iron excretion, resulting in amelioration of cardiac dysfunction 8 9 and increased survival. 7 Although many patients benefit from this therapy, others continue to have organ dysfunction and die, sometimes despite intensive treatment with deferoxamine. 10 It can thus be hypothesized that either the deferoxamine therapy is not effective in those patients, or they have reduced defense mechanisms against the iron overload. The present study was designed to test the hypothesis whether a postulated genetically determined decreased antioxidant (and iron binding) activity in some patients with homozygous β-thalassemia could represent an independent risk factor for the development of heart failure.

Apolipoprotein E (apoE) is a plasma protein with known functions in cholesterol transport and metabolism and Alzheimer's disease. 11 12 The APOE gene is located on chromosome 19, and a polymorphism exists at the APOE locus with the three most common alleles designated e2, e3, and e4, corresponding to three isoforms of the apoE protein. 13 It was recently shown that apoE at physiologic levels has isomorph-specific effects in protecting a rat neuronal cell line from oxidative cell death, and that these effects correlated with apoE's antioxidant activity in vitro assays (ranked E2 > E3 > E4). 14 The demonstrated metal binding ability (including iron) of apoE was postulated to be one mechanism accounting for its antioxidant properties. 14 We hypothesized that a decreased antioxidant activity of apoE4 in β-thalassemia homoygotes might be a genetic risk factor for the development of left ventricular failure (LVF). We tested our hypothesis by comparing the frequency of APOE allele e4 in β-thalassemia major patients divided into three groups according to their cardiac status with the e4 frequency in a Greek control sample.

MATERIALS AND METHODS

Subjects. The subjects were 251 β-thalassemia major patients of Greek origin (117 men and 134 women), followed systematically by three major thalassemia treatment units in the Athens area belonging to the public health system. Transfusion therapy had started in all patients before the age of 5 years. Each patient was receiving blood transfusions every 15 to 25 days to maintain a hemoglobin level above 9 g/dL during all of the years of follow-up. All patients were receiving chelation therapy with deferoxamine, 500 mg orally every 15 to 25 days to maintain a ferritin level below 3000 μg/L. Regular chelation therapy with deferoxamine mesylate, a naturally occurring trihydroxamic acid produced by Streptomyces pilosus, increases urinary and fecal iron excretion, resulting in amelioration of cardiac dysfunction and increased survival. 7

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therapy with subcutaneous deferoxamine. The therapy had been initiated in each patient after the serum ferritin level had reached 2,000 ng/mL. To evaluate the transfusion therapy and the hemosiderosis level, we obtained the mean pretransfusion hemoglobin level and mean serum ferritin level in each patient over the last 2 and 5 years of follow-up, respectively. Serum ferritin was measured two times per year in each patient by an enzyme-linked immunosorbent assay (ELISA) method (Abbott, Chicago, IL), and values were rounded off to the nearest 10.

Clinical and laboratory cardiac evaluation included medical history, clinical examination, electrocardiographic (ECG), as well as echocardiographic studies. The echocardiographic examination was performed as follows: two-dimensional and M-mode echocardiograms were obtained using instruments with a 3-MHz transducer. A two-dimensional study was first performed to identify the overall cardiac anatomy and motion. Long-axis and parasternal short-axis views at the midventricular level were used to derive the following M-mode measurements: left ventricular end-systolic and diastolic dimensions, left atrial and right ventricular cavity dimensions, thickness of interventricular septum and posterior left ventricular wall according to the recommendations of the American Society of Echocardiography.15 Four- and two-chamber apical views were used to estimate ventricular systolic and diastolic volumes, which were calculated using the discs method.16 Left ventricular ejection fraction (LVEF) was calculated as [(end-diastolic volume minus end-systolic volume) divided by end-diastolic volume] multiplied by 100.

The patients were divided into three groups according to the severity of heart disease based on clinical evaluation and echocardiographic findings. Group A patients had no symptoms or signs of heart failure, and their echocardiographic study was within normal limits. Group C patients had symptoms and signs of LVF and concomitant echocardiographic findings. These patients exhibited dyspnea on exertion (New York Heart Association [NYHA] functional class I-IV) and fulfilled at least two major Framingham criteria for heart failure diagnosis. Finally, patients in group B were asymptomatic, but exhibited LV dilatation (LV end-diastolic diameter index [LVEDDI] higher than 30 mm/m²) without left ventricular systolic dysfunction (LV fractional shortening [FS] higher than 28%), as assessed echocardiographically. Patients in this group did not receive treatment.

Another type of heart failure in β-thalassemia major is associated with right ventricular dilatation. Patients of this kind were excluded from the study, as right ventricular failure in β-thalassemia major is due to different pathophysiological reasons.17,18

APOE genotyping. Genomic DNA was extracted from EDTA blood samples by a salting out procedure.19 Genotyping of polymorphic APOE alleles was done after polymerase chain reaction amplification of genomic DNA, digestion with HhaI restriction enzyme, and agarose gel electrophoresis as described previously.20,21 The control sample for which data on APOE genotype were available consisted of a random sample of 216 voluntary, healthy Greek blood donors.22 The blood donors were 146 men and 70 women, and the mean age was 35.6 years (range, 19 to 64 years).22

Statistical analysis. We used analysis of variance (ANOVA) for multiple groups to assess differences regarding echocardiographic measurements between the three patient groups and the χ² test to compare the e4 allele frequency in the different patient groups with that of the population controls.

RESULTS

From the 251 patients studied, 151 belonged to group A, 53 to group B, and 47 to group C. Three cases were excluded from the study, as they presented a clinical picture of congestive heart failure and echocardiographic findings of profound right ventricular dilatation, good LV function, and pulmonary hypertension at the Doppler study.

Hematologic and hemosiderosis parameters of the patients are shown in Table 1. No statistically significant differences were found between the hemoglobin and ferritin levels of the patient groups.

The cardiologic characteristics of the three patient groups are depicted in Table 2. Twenty-eight patients of group C were in severe cardiac failure of NYHA class III-IV and 19 of class I-II. All patients in groups B and C had LV dilatation and LVEDDI higher than 30 mm/m². Groups A and B had good LV function with a significant difference in fractional shortening and ejection fraction with the group C (Table 2). All 47 patients of group C were receiving treatment with angiotensin-converting enzyme (ACE) inhibitors, diuretics ≥ digitalis. The 53 patients of group B and the 151 patients of group A were not in cardiac failure and had echocardiographically normal LV function.

Table 3 gives the APOE e4 allele frequencies in the three patient groups, as well as in controls. The APOE e4 allele frequency was significantly higher than in controls (χ² = 5.08, P < .05). The e4 frequency in patients with heart failure (12.8%) was significantly higher than in controls (χ² = 4.34, P < .05, odds ratio = 2.11, 95% confidence interval 1.03 to 4.32), and so was the e4 frequency in group B patients (12.3%, χ² = 4.04, P < .05). Taking patient groups B and C together, the e4 frequency of 12.5% was significantly higher than in controls (χ² = 6.45, P < .02). All β-thalassemia homozygotes irrespective of cardiac diagnosis had an e4 frequency (9.8%) not different from the population of healthy blood donors (χ² = 3.30, P > .05). Four patients were homozygous for the e4 allele, the three belonged to group C and one to group B.

DISCUSSION

The severity of iron toxicity in β-thalassemia major seems to be related to the magnitude of the body iron burden.23,24 The exact mechanism of iron overload toxicity has been uncertain for many years. Via the iron-driven Fenton and Haber-Weiss reactions, the nontransferrin plasma iron, in its bivalent or

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<th>Table 1. Hematologic Characteristics of Patients With β-Thalassemia Major Stratified by Cardiac Status</th>
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*The mean pretransfusion hemoglobin level and mean serum ferritin level was obtained in each patient as the mean of the values over the last 2 and 5 years of follow-up, respectively.
can result in oxidative stress and human disease. In the heart, oxygen free radicals and antioxidant defense mechanisms involve membrane lipids and proteins. Imbalance between production of oxygen free radicals and antioxidant defense mechanisms may also contribute to the pathogenesis of infectious myocarditis in a certain number of patients with homozygous β-thalassemia. As shown in animal models, oxygen free radicals may also contribute to the pathogenesis of infectious myocarditis. Apart from iron overload, it has been recently shown by our group that myocarditis appears to be involved in the pathogenesis of LVF in a certain number of patients homozygous for the β-thalassemia. It represents the first demonstration of a genetic factor unlinked to the β globin gene cluster contributing to the clinical manifestations of the disease. The e4 allele could represent a predictive marker for development of LVF in patients with β-thalassemia major and LV dilatation (our patient group B), suggesting a closer follow-up of such patients. As the APOE e4 frequency was found in only 12.8% of patients with homozygous β-thalassemia and LVF, it is obvious that other genetic and environmental factors, as for instance, the number of transfusions, iron overload, chelation therapy, and viral myocardial inflammation, play a role in the development of organ damage. The fact that patients in groups B and C, as shown in Table 1, did not have either higher ferritin levels or were transfused more inadequately than those without cardiac impairment, supports the role of APOE e4 allele as a genetic risk factor for the development of LVF. Furthermore, some patients of group A with e4 allele could still develop LVF. Additional evidence for the relationship between the e4 allele and heart disease comes from an analysis of patients homozygous for the e4 allele, of whom three had LVF and one LV dilatation, but numbers in this category are small. The increase in the e4 allele frequency among patients with cardiac disease comes with a reduction in e3 rather than e2 allele frequency, but the e2 allele frequency is already very low in the Greek population, and something similar might apply to β-thalassemia.

As the APOE e4 allele was found in patients of advanced age with no cardiac impairment, the presence of the e4 allele does not guarantee the development of LVF in β-thalassemia. In addition, as more than 85% of patients with LVF do not carry the e4 allele, this is neither a necessary prerequisite for the development of LVF. This is equivalent to the association of APOE e4 with Alzheimer’s disease, where the association has been confirmed in almost 100 studies around the world. A consensus statement on APOE genotyping in Alzheimer’s disease concluded that APOE genotyping can be used as an adjunct to other diagnostic tests, but that prospective investigations of dementia as a function of APOE genotype are needed, and something similar might apply to β-thalassemia.

Allelic variation at the APOE locus has been studied in many populations. Significant differences have been observed between Caucasian, Chinese, Japanese, and black races. Also among Caucasian populations there is a significant variation in the allele frequencies. Average allele frequencies from Caucasians show an e4 allele frequency of 15.0%. The very low e4 frequency in the Greek population is in agreement with the gradient found for the frequency of this allele in Europe, and is supported by a similar low frequency (7.0%) in Greek Cypriots. Our finding of an association between the APOE e4 allele and a high toxicity through the formation of hydroxyl radicals (OH). This leads to peroxidative damage of membrane lipids and proteins. Imbalance between production of oxygen free radicals and antioxidant defense mechanisms can result in oxidative stress and human disease. In the heart, the imbalance between free radicals and antioxidant mechanisms is manifested as impaired function of the mitochondrional inner-membrane respiratory chain resulting in abnormal energy metabolism expressed clinically with fatal cardiomyopathy. Apart from iron overload, it has been recently shown by our group that myocarditis appears to be involved in the pathogenesis of LVF in a certain number of patients homozygous β-thalassemia. As shown in animal models, oxygen free radicals may also contribute to the pathogenesis of infectious myocarditis.

In the present study, we categorized the patients in three groups according to the severity of heart disease. The normal cases of group A and the severely affected cases of group C had clear clinical and echocardiographic characteristics and were easily distinguished. Patients of group B were characterized by LV dilatation and good LV function. In these cases, LV and left atrial (LA) dilatation could possibly represent the first step of LV dysfunction without overt LV decreased contractility, or a compensatory mechanism due to anemia. Some of these patients will eventually develop LVF.

The results of the functional polymorphism at the APOE locus in our study suggest that the e4 allele may be a genetic risk factor for the development of LVF (and other organ damage) in homozygous β-thalassemia. It represents the first demonstration of a genetic factor unlinked to the β globin gene cluster contributing to the clinical manifestations of the disease. The e4 allele could represent a predictive marker for development of LVF in patients with β-thalassemia major and LV dilatation (our patient group B), suggesting a closer follow-up of such patients. As the APOE e4 frequency was found in only 12.8% of patients with homozygous β-thalassemia and LVF, it is obvious that other genetic and environmental factors, as for instance, the number of transfusions, iron overload, chelation therapy, and viral myocardial inflammation, play a role in the development of organ damage. The fact that patients in groups B and C, as shown in Table 1, did not have either higher ferritin levels or were transfused more inadequately than those without cardiac impairment, supports the role of APOE e4 allele as a genetic risk factor for the development of LVF. Furthermore, some patients of group A with e4 allele could still develop LVF. Additional evidence for the relationship between the e4 allele and heart disease comes from an analysis of patients homozygous for the e4 allele, of whom three had LVF and one LV dilatation, but numbers in this category are small. The increase in the e4 allele frequency among patients with cardiac disease comes with a reduction in e3 rather than e2 allele frequency, but the e2 allele frequency is already very low in the Greek population, and other factors may influence the level of this allele.

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and LVF in Greek β-thalassemia homozygotes now needs confirmation from other populations using appropriate population controls.

The well-known isoform-specific influence of apoE on plasma cholesterol level and atherosclerosis cannot explain the association found between APOE allele ε4 and LVF in β-thalassemia homozygotes, as atherosclerosis is not a general feature of the pathophysiology of the disorder. It is recognized now that the severity of a monogenic disorder may be modified by a second locus, depending on the genetic background. Any genes involved in the pathogenic pathway may represent candidate modifier genes. The boundaries between monogenic and polygenic disorders might not always be so clear-cut as previously thought, as more genes and interactions become known from the progress of the Human Genome Project.

The finding of an increased frequency of APOE ε4 allele in β-thalassemia homozygotes with LVF provides additional evidence to the theory of oxygen free radicals as contributing to the organ damage, due to the recently demonstrated antioxidant and iron binding activity of apoE. This suggests that several other genetic loci could be of potential relevance to the oxidative damage of organs in β-thalassemia. Such loci include genes modulating genesis of oxygen free radicals (ie, cytochrome C oxidase), genes for scavenger enzymes (superoxide dismutases, catalase), genes regulating mitochondrial DNA replication, structural genes for membrane lipoproteins, and genes involved in DNA repair mechanisms. It is noteworthy that mutant mice lacking the Mn-superoxide dismutase enzyme suffer neonatal lethality due to dilated cardiomyopathy. Other functional polymorphisms in such genes could be examined for association with organ failure in β-thalassemia. It is also believed that genetic susceptibility for a majority of common diseases will be associated with relatively common alleles of one or several loci.

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