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Evidence for linkage of the apolipoprotein A-II locus to plasma apolipoprotein A-II and free fatty acid levels in mice and humans

(atherosclerosis/sib pair/animal models/complex genetic traits)

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ABSTRACT **Although it has been hypothesized that the synteny between mouse and human genes provides an approach to the localization of genes that determine quantitative traits in humans, this has yet to be demonstrated. We tested this approach with two quantitative traits, plasma apolipoprotein A-II (apoAII) and free fatty acid (FFA) levels. ApoAII is the second most abundant protein of high density lipoprotein particles, but its function remains largely unknown. We now show that, in a backcross between strains *Mus spretus* and C57BL/6J, apoAII levels correlate with plasma FFA concentrations on both chow ($P < 0.0001$) and high-fat ($P < 0.0003$) diets and that apoAII levels are linked to the apoAII gene ($P < 0.0002$). To test whether variations of the apoAII gene influence plasma lipid metabolism in humans, we studied 306 individuals in 25 families enriched for coronary artery disease. The segregation of the apoAII gene was followed by using an informative simple sequence repeat in the second intron of the gene and two nearby genetic markers. Robust sib-pair linkage analysis was performed on members of these families using the SAGE linkage programs. The results suggest linkage between the human apoAII gene and a gene controlling plasma apoAII levels ($P = 0.03$). Plasma apoAII levels were also significantly correlated with plasma FFA levels ($P = 0.007$). Moreover, the apoAII gene exhibited linkage with a gene controlling FFA levels ($P = 0.003$). Evidence for nonrandom segregation was seen with markers as far as 6–12 centimorgans from the apoAII structural locus. These data provide evidence, in two species, that the apoAII gene is linked to a gene that controls plasma apoAII levels and that apoAII influences, by an unknown mechanism, plasma FFA levels. The results illustrate the utility of animal studies for analysis of complex traits.**

Although genetic linkage has been a powerful tool to identify genetic loci for Mendelian disorders in humans, it has been less successful for complex non-Mendelian disorders such as atherosclerosis. Because of their complexities, linkage study of non-Mendelian qualitative traits (i.e., diseases such as atherosclerosis) is inherently less statistically powerful. A suggested approach has been to initially localize quantitative trait loci in rodent models and then extend those results to humans, utilizing the synteny between mouse and human genes. We now apply this approach to two traits involved in lipid metabolism.

Apolipoprotein A-II (apoAII) is a major constituent of human high density lipoprotein (HDL), composing ≈20% of the protein on these particles. At least six, but not all, previous studies have reported significant correlation of plasma apoAII levels with occurrence of myocardial infarction (1–9). In each of the six studies, myocardial infarct patients had significantly lower apoAII levels than their matched controls, and in one study apoAII levels were the phenotype that was most discriminative of myocardial infarct survivors from their controls (3). Population studies of the apoAII gene that used a relatively uninformative *Msp* I restriction fragment length polymorphism have suggested possible associations with apoAII levels (8, 10–12) and triglyceride (TG) levels (13). Genetic studies in mice, involving naturally occurring variations and transgenic animals, have demonstrated important effects of the apoAII gene on lipoprotein metabolism and the development of preatherosclerotic fatty streak lesions (14, 15). Therefore, we have examined in detail the possible effects of apoAII gene variations on traits relevant to coronary artery disease (CAD). For studies with humans, we have used family rather than population studies to avoid assumptions relating to linkage disequilibrium. Our results are consistent in both species and demonstrate that the apoAII gene influences plasma apoAII levels, with possible consequences for HDL metabolism, including reverse cholesterol transport. Moreover, apoAII levels were significantly correlated with levels of free fatty acids (FFAs), also relevant to processes related to CAD (16). Thus, these results may explain, in part, the previously observed associations between plasma apoAII levels and myocardial infarction. These data also provide a clear example of the utility of genetic studies with animal models in identifying loci for complex human genetic traits.

MATERIALS AND METHODS

Ascertainment and Inclusion Criteria for CAD Families. The study sample consisted of 25 multiplex multigenerational Caucasian pedigrees ascertained through a proband with documented (surgically or angiographically) CAD identified at Cedars-Sinai Medical Center. Family inclusion criteria required that at least one other blood relative be affected with CAD. A total of 306 individuals from these 25 families agreed to participate in this study (6–33 members per family). The investigation was approved by the institutional Human Subjects Protection Committee.

Plasma Biochemical Analysis. Overnight fasting blood from participating family members was drawn into EDTA-containing tubes. Plasma concentrations of cholesterol and TG were measured in duplicate using enzymes and reagents

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Abbreviations: apoAI and apoAII, apolipoprotein A-I and A-II, respectively; FFA, free fatty acid; HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglyceride; CAD, coronary artery disease; BSB, (C57BL/6J × *Mus spretus*) × C57BL/6J backcross mice; IBD, identical by descent; cM, centimorgan(s); BMI, body mass index.

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as described (17). Surveillance samples measured quarterly using these protocols met the criteria of standardization required by the Centers for Disease Control and Prevention. Plasma FFA levels were determined enzymatically (Wako Chemical, Austin, TX) (18). Plasma apoAII concentrations were determined in triplicate using a turbidimetric assay with standards and antisera obtained from Boehringer Mannheim.

Genetic Markers. Polymerase chain reaction amplification of the human apoAII $(CA)_n$ repeat was performed using previously described primer pairs (19). The reaction mixture, containing 50 ng of template DNA and all four dNTPs (each at 0.2 μM), was cycled 25 times using a program of 92°C (1 min), 55°C (30 sec), and 72°C (1 min). The amplified products were resolved by electrophoresis on 5% polyacrylamide gels. Allele sizes and frequencies for the apoAII $(CA)_n$ repeat (in parentheses) from 432 scored chromosomes were as follows: 123 bp (0.007), 129 bp (0.002), 131 bp (0.176), 133 bp (0.037), 137 bp (0.345), 141 bp (0.005), 143 bp (0.317), 145 bp (0.106), and 147 bp (0.005). Two loci near the apoAII gene were chosen to increase informativeness. Probes for loci *D1S74* (cYNA13) (20) and *D1S75* (OS-6) (21) were from the American Type Culture Collection (catalog numbers 59364 and 59440, respectively). These markers were typed by Southern blot hybridization, after digestion with *Taq* I, as previously described using high-stringency washes at 65°C (22).

Statistical Analysis. The methodology of robust sib-pair test of linkage was used to test the hypothesis that there is linkage between a quantitative trait and a polymorphic marker (23, 24). The underlying basis for this approach is to compare the quantitative variation in a trait between siblings as a function of the alleles they share identical by descent (IBD). The actual sib-pair linkage analyses and ordering of marker loci are performed by utilizing the SIBPAL subroutine program of SAGE, Version 2.1 (25). In addition, by using the apoAII gene and two nearby flanking markers to construct haplotypes, exact assignment is possible in the majority of sib pairs, allowing both additional calculations for the linkage regression line and determination of the correlations between sibs as a function of genetic marker IBD status. Additional analyses were performed on sib pairs sharing exactly two, one, or zero alleles IBD and on sib pairs sharing just two or zero alleles IBD. The results from these latter analyses were very similar to the analyses presented, and therefore, the additional analyses are not presented here. The marker locus correlations in the proportion of genes the sib pairs shared IBD were used to estimate the recombination between different marker loci and, therefore, to order the marker loci (26).

We have tried to avoid false-positive results by the following analytic measures. (*i*) The linkage study of human plasma apoAII levels to the apoAII locus was a test of a specific prior hypothesis based on the mouse work, not a standard random multiple loci linkage analysis. Therefore, the appropriate level of statistical significance was set at a P value of 0.05. (*ii*) Linkage of FFA levels with the apoAII locus provided a second subsequent prior hypothesis that was tested with flanking markers. Again this allowed a standard level of significance. (*iii*) We have excluded from our analyses those sibs with extreme trait values, i.e., greater than the mean ± 3 SD. (*iv*) We excluded those sib pairs with very large squared trait differences, i.e., greater than the mean ± 3 SD. (*v*) We used the more conservative unweighted least-squares option during sib-pair analysis. (*vi*) Finally, to be maximally conservative, we performed all analyses on both the raw and logarithmic-transformed data, with and without regression adjustments of age, sex, and body mass index (BMI, kg/m^2). A true positive result will usually be robust to different transformations.

Analysis of the linkage of the mouse apoAII gene to genes underlying quantitative traits and the intraclass correlations of traits with each other were determined by standard parametric and nonparametric statistics using the STATVIEW and SUPERANOVA programs (Abacus Concepts, Berkeley, CA) on the Macintosh.

Mouse Studies. C57BL/6J mice were obtained from The Jackson Laboratories and cousin-bred *Mus spretus* (Spain) were obtained from Michael Potter (National Institutes of Health, Bethesda, MD). A backcross of (C57BL/6J × *Mus spretus*) × C57BL/6J was performed. We have designated these backcross progeny as BSB mice. Mice were fed standard commercial rodent chow containing 12% of calories as fat from weaning. At 3–4 months of age, the mice were maintained on a moderate-fat high-cholesterol diet (Teklad 90221, Teklad, Madison, WI) containing 30% of calories as fat for 5 weeks before sacrifice (15).

Mice were fasted for ≈15 hr before transport to the laboratory and collection of blood through the retroorbital sinus. Blood samples were collected in plasma separator tubes containing EDTA, placed on ice, and centrifuged to prepare plasma. Plasma TG and FFA concentrations were determined by enzymatic procedures employing colorimetric endpoints (15). Plasma apoAII levels were determined by immunoblot analysis as described (14), except that detection was by a chemiluminescent procedure. Quantitation of apoAII levels was done using a United States Biochemical SciScan model 5000 densitometer. Samples were quantitated in quadruplicate, compared to a C57BL/6J pooled plasma standard, and expressed as mg of apoAII protein per dl. The protocol was shown to be linear with amount of apoAII added over the ranges of plasma used. Genomic DNA was isolated as described (22). The mouse apoAII gene was genotyped using an *Eco*RI restriction fragment length variation (27).

RESULTS

Relationship of the apoAII Gene, Plasma apoAII Levels, and FFA in Mice. One hundred ninety-eight BSB backcross mice were examined for linkage between the apoAII gene and plasma apoAII levels on both chow and high-fat diets. Levels of the apoAII protein were significantly correlated with the apoAII genotype. Analysis of variance of the apoAII genotype and plasma apoAII levels yielded a P value of 0.0001 on a chow diet (n = 198), genotype "BB" (homozygous for C57BL/6J alleles) vs. "SB" (heterozygous for *Mus spretus* and C57BL/6J alleles) and a P value of 0.0002 on a high-fat diet (n = 96). On a chow diet, mice with the BB genotype had apoAII levels of 8.7 ± 0.5 mg/dl (n = 100; mean ± SEM) and mice with the SB genotype had apoAII levels of 6.2 ± 0.38 mg/dl (n = 98). On the high-fat diet, mice with the BB genotype had apoAII levels of 5.8 ± 0.38 mg/dl (n = 45), and mice with the SB genotype had apoAII levels of 4.0 ± 0.27 mg/dl (n = 51). The values for apoAII levels were adjusted for the effects of age. These results are consistent with the previous results from studies with mice in indicating the genetic variations of the apoAII gene modulate plasma apoAII levels (15, 28).

Plasma apoAII protein levels were significantly correlated with plasma FFA levels in the BSB backcross mice. FFA and apoAII measured from mice that were on a high-fat diet showed a statistically significant correlation, whether tested by regression ($P < 0.0003$; $r = 0.34$) or tested by the nonparametric Spearman rank correlation coefficient ($P < 0.001$) (n = 108). This correlation was still significant after adjusting for the effects of age and body weight ($P < 0.0003$). When plasma FFA and apoAII levels were measured in chow-fed mice, both the regression ($P < 0.0001$; $r = 0.39$) and Spearman rank correlation coefficients ($P < 0.0001$) were statistically significant (n = 187). This correlation remained significant after adjusting for the effects of age and body weight ($P < 0.0019$). Thus, the correlation of apoAII levels

with FFA occurred regardless of diet. The apoAII gene, however, did not show statistically significant evidence of linkage, with the current sample size, to FFA levels in BSB backcross mice.

ApoAII Transgenic Mice. We have produced transgenic mice containing the mouse apoAII gene and expressing about 3-fold elevated plasma apoAII as compared to nontransgenic littermates (29). Because of the above linkage studies showing a correlation between FFA and apoAII levels, we examined these mice for differences in FFA levels. As predicted, the transgenic mice exhibited higher plasma FFA levels than nontransgenic littermates (1910 ± 80 and 1530 ± 71 μmol/liter, respectively; $P < 0.007$).

Linkage of the apoAII Gene, Plasma apoAII Levels, and FFA Levels in Humans. The clinical characteristics and baseline values for total and HDL cholesterol, TG, and FFA from the CAD subjects are summarized in Table 1. Sib-pair linkage analysis of these families showed that the apoAII, *D1S74*, and *D1S75* loci cover 12 centimorgans (cM) of chromosome 1. *D1S74* is ≈5.7 cM from the apoAII gene, and *D1S75* is ≈5.6 cM from *D1S74* and 12 cM from the apoAII gene (data not shown). By using the robust sib-pair methodology (25), analysis of 78 sib pairs by SAGE programs suggested linkage between the apoAII gene and a gene controlling plasma apoAII levels ($P = 0.03$; Table 2). No significant evidence for linkage was detected between a locus determining apoAII levels and the *D1S74* and *D1S75* loci. Analysis of the data using only those sib pairs unequivocally classified as sharing two, one, and zero apoAII alleles IBD also confirmed that a gene controlling plasma apoAII levels was linked to the apoAII locus ($P = 0.017$). The regression of apoAII alleles shared IBD with squared trait differences for plasma apoAII is shown in Fig. 1*A*. Furthermore, the correlation of apoAII levels between two members of a sib pair was 0.74 when the sibs shared two alleles IBD and was −0.05 when the sibs shared zero alleles IBD.

We also detected significant evidence of linkage between a gene controlling FFA levels and three independently scored loci within or around the apoAII gene. Linkage of logarithmic-transformed FFA levels was detected with the apoAII ($P = 0.003$), *D1S74* ($P = 0.033$), and *D1S75* ($P = 0.005$) loci (Table 3). Similar results were obtained with the square root-transformed data (data not shown). Again, reanalysis of the data using only those sib pairs unequivocally classified as sharing two, one, and zero apoAII alleles IBD also supported the hypothesis that a gene controlling plasma FFA levels was linked to the apoAII locus ($P = 0.003$). The regression of apoAII alleles shared IBD with squared trait differences for plasma FFA is shown in Fig. 1*B*. The correlation of FFA levels between sibs was 0.33 when the sibs shared two alleles IBD and was −0.26 when the sibs shared zero alleles IBD.

Significant evidence of linkage was not detected to any of these three loci for the untransformed, logarithmic-transformed, or square root-transformed data ($0.22 < P < 0.92$) of other quantitative traits, including plasma total cholesterol, HDL cholesterol, or TG. Consistent with the observation that the apoAII gene is linked to genes underlying both plasma apoAII and FFA levels, the levels of plasma apoAII were significantly correlated with the levels of plasma FFA ($P = 0.007$; $r = 0.17$). This correlation was also significant after both FFA and apoAII had been adjusted for linear effects of sex, age, and BMI ($P = 0.02$; $r = 0.15$).

Table 1. Phenotype values of the members of the CAD families ascertained through a CAD proband (151 males and 155 females)

Trait	*n*	Data
Age, years	306	52 ± 18
BMI, kg/m^2	306	26 ± 4
Total cholesterol, mg/dl	306	201 ± 42
HDL cholesterol, mg/dl	306	55 ± 17
TG, mg/dl	306	103 ± 70
FFA, μmol/liter	269	596 ± 247
apoAII, mg/dl	281	35 ± 6

Data are the mean ± SD.

Table 2. *P* values of sib-pair linkage analyses for apoAII levels with the apoAII locus

Number of sib pairs	Without adjustment, *P* value: log apoAII	Without adjustment, *P* value: Raw apoAII	With adjustment for age, sex, and BMI, *P* value: log apoAII	With adjustment for age, sex, and BMI, *P* value: Raw apoAII
78*	0.030	0.033	0.010	0.021
ApoAII linkage on those sib pairs sharing exactly two and zero apoAII alleles				
23	0.013	0.015	ND	0.022

ND, not determined.
*Using SIBPAL program of SAGE (see text).

DISCUSSION

Our results indicate that genetic variations at the apoAII gene locus may influence plasma apoAII levels and FFA levels in humans and that variations in apoAII protein modulate FFA levels in both mice and humans. As further support for the specificity of the linkage of quantitative FFA and apoAII levels for the apoAII locus, we found no such evidence for linkage with other quantitative lipid-related traits. Although the biological significance of these results is strengthened by their occurrence in two species, the linkage results in humans require independent confirmation. The role of apoAII in plasma lipoprotein metabolism has been unclear. Studies in mice have shown that altered expression of apoAII influ-

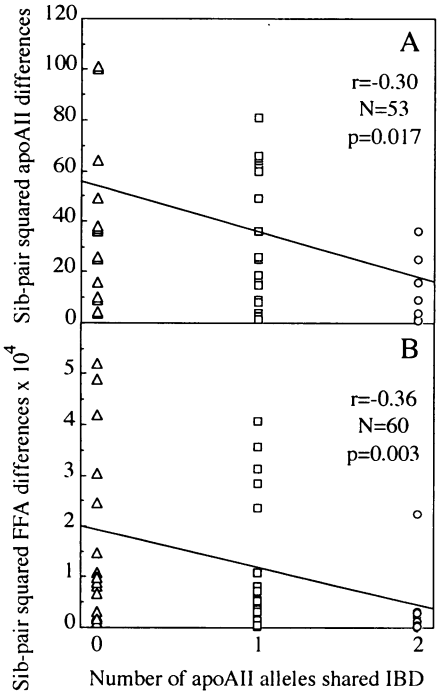

FIG. 1. Significant regression of squared trait differences for unadjusted plasma apoAII and FFA levels on apoAII alleles that the sib pairs shared IBD. This figure includes only those individuals whose apoAII haplotypes could be definitely assigned to IBD sharing of exactly two (○), one (□), or zero (△) alleles of the apoAII locus. Similar statistical significance was observed with the SIBPAL program on all sib pairs (see text). (*A*) Plasma levels of apoAII. (*B*) Plasma levels of FFA.

Table 3. *P* values of sib-pair linkage analyses for FFA with each of three loci, at the apoAII locus and within 12 cM of the apoAII locus

Genetic locus	Without adjustment			With adjustment for age, sex, and BMI		
	Number of sib pairs	*P* value		Number of sib pairs	*P* value	
		log FFA	Raw FFA		log FFA	Raw FFA
apoAII	86*	0.003	0.070	85	0.025	0.049
D1S74	97*	0.033	0.022	96	0.045	0.068
D1S75	75*	0.005	0.094	74	0.21	0.34
	FFA linkage on those sib pairs sharing exactly two and zero apoAII alleles					
apoAII	25	0.027	0.015	25	ND	0.046

ND, not determined.
*Using SIBPAL program of SAGE (see text).

enced HDL levels (15) and aortic fatty streak development (30). However, various population studies (10–13), as well as our present results, have failed to reveal significant relationships between apoAII gene polymorphisms and HDL levels in humans. In addition, studies of individuals homozygous for a mutation resulting in apoAII deficiency did not show any striking alterations in lipoprotein levels as compared to the average lipoprotein levels in the Japanese population (31). Our present findings suggest that the apoAII gene may, nevertheless, importantly influence processes related to CAD by affecting plasma levels of apoAII and FFA. This may explain, in part, the previously observed associations between apoAII levels and incidence of myocardial infarction (1–6).

Various studies have shown that the apoAII content of HDL may importantly influence HDL functions (for review, see ref. 32). Thus, separation by immunoaffinity chromatography of HDL particles containing both apolipoprotein A-I (apoAI) and apoAII from HDL particles containing apoAI alone has revealed striking differences in the content of lecithin cholesterol acyltransferase and other proteins (33). Moreover, HDL particles lacking apoAII are more effective in promoting sterol efflux from some cell types than HDL particles containing both apoAI and apoAII (33–35). Our results suggest that the apoAII gene modulates such HDL functional states by controlling the apoAII content of HDL. A previous population study revealed an association between an apoAII gene restriction fragment length polymorphism and the ratio of apoAI to apoAII (36), consistent with our present findings.

The correlation of apoAII levels with FFA levels provides an additional mechanism of potential importance in CAD. (*i*) Plasma FFA levels are known to affect hepatic TG synthesis and very low density lipoprotein (LDL) production (37). *In vitro* studies have also revealed that FFAs decrease the interaction of LDL with the LDL receptor (38) but increase LDL uptake by a pathway independent of the LDL receptor (39). (*ii*) FFAs appear to influence lipid peroxidation and foam-cell formation, both important processes in early atherogenesis (40). Thus FFAs have been shown to protect gastrointestinal membranes from lipid peroxidation, possibly as a result of the ability of FFAs to chelate iron, a potent prooxidant (41). The FFA content of serum also appears to contribute importantly to foam-cell formation *in vitro* (42). (*iii*) Long-chain FFAs have been shown to directly activate calcium channels on ventricular myocardial cells (43), thereby increasing the contractile state of the heart. (*iv*) Finally, since plasma FFA levels are related to insulin resistance (44), it is interesting to speculate that variations of the apoAII gene may contribute to the cluster of abnormalities associated with insulin resistance, designated as syndrome X (45).

The mechanism by which apoAII influences FFA levels is unknown, but hepatic lipase is activated by apoAII *in vitro* (46) and HDL particles containing both apoAI and apoAII interact more effectively with hepatic lipase than HDL particles containing only apoAI (47). There may well be other interactions between apoAII and cellular or plasma proteins that are as yet unknown.

In conclusion, our results indicate that the apoAII locus is linked to a locus that influences plasma apoAII levels and FFA levels in two species, humans and mice. Even though consistent results were generated from humans and mice, the evidence for linkage of the apoAII gene to plasma apoAII levels and to FFA levels needs to be independently confirmed in additional family studies. As discussed above, both traits are likely to be of significance in the genetic susceptibility to CAD. This work also demonstrates that the mouse–human synteny can be utilized to detect loci affecting quantitative traits in humans and that the effect may be detectable as far as map distances of 6–12 cM. Thus, this work illustrates the utility of animal models in the analysis of complex human genetic disorders.

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