Effects of Three Genetic Loci in a Pedigree With Multiple Lipoprotein Phenotypes

Mitsuru Emi, Robert M. Hegele, Paul N. Hopkins, Lily L. Wu, Rosemarie Plaetke, Roger R. Williams, and Jean-Marc Lalouel

In the course of familial investigations of coronary artery disease, we identified an extended kinship in which several members were affected with type IIa hyperlipoproteinemia (HLPIIa), type III dyslipoproteinemia (DLP III), or hypobetalipoproteinemia (HBLP). To study the genetic defects responsible for plasma lipoprotein abnormalities in this pedigree and to investigate the phenotypic effect of different genotypic combinations, we used molecular markers for apolipoprotein (apo) B, apo E, and the low density lipoprotein (LDL) receptor to characterize segregation at each locus. Linkage analysis showed that elevated LDL cholesterol levels and the HBLP phenotype were due to defects at the LDL receptor and the apo B loci, respectively. One pedigree member, who inherited both an LDL receptor allele linked with elevated LDL cholesterol levels and an apo B allele linked with HBLP, had a normal lipoprotein phenotype. Seven patients who simultaneously inherited the defective LDL receptor allele and one or two apo E2 alleles manifested DLP III. The E2 alleles in this pedigree were shown by DNA sequence analysis to be the common E2 158 (arginine→cysteine) allele. These findings suggested a possible interaction between the abnormal LDL receptor and apo E2 alleles, resulting in the expression of DLP III in the presence of a single copy of apo E2. (Arteriosclerosis and Thrombosis 1991;11:1349-1355)

Plasma lipid and lipoprotein levels reflect in part the influence of multiple genetic loci. Defects at some of these loci account for specific types of dyslipoproteinemia occurring with regularity among family members. For example, heterozygous familial hypercholesterolemia (FH) is caused by the inheritance of a single copy of a defective low density lipoprotein (LDL) receptor gene. It is recognized clinically by striking elevations of LDL cholesterol (about twofold above normal, or usually >99th percentile) affecting both children and adults. Affected families display bimodally distributed LDL cholesterol levels consistent with an autosomal dominant trait. Typically, there is a striking family history of early coronary disease. Tendinous xanthomas are highly indicative of FH but are an insensitive marker for the affected individual. In general, only LDL cholesterol levels are elevated, and the finding of elevated triglycerides or other lipid abnormalities may bring into question the diagnosis of FH and may suggest other inherited primary lipid disorders such as familial combined hyperlipidemia.

The genetic mechanisms responsible for most other types of familial dyslipoproteinemia appear to be more complex. There are three common apolipoprotein (apo) E isoforms designated 2, 3, and 4. Hence, six combinations are possible, corresponding to the six commonly observed apo E phenotypes (apo E2-2, 3-2, 3-3, 4-2, 4-3, and 4-4), with apo E3-3 being the most common. Type III hyperlipoproteinemia, characterized by the accumulation in plasma of chylomicron and very low density lipoprotein (VLDL) remnants or \( \beta \)-VLDL, xanthoma striata palmaris, tuberous xanthomas, and a high incidence of premature vascular disease, has been associated with homozygosity for apo E2. More than 95% of individuals with type III hyperlipoproteinemia are found to have the apo E2-2 phenotype (compared with a prevalence of 1% for those with apo E2-2 in the general population). Apo E2 is defective in binding to the apo B,E (LDL) receptor, an apparently necessary step for processing VLDL remnants to LDL. Presumably, binding is also deficient to the apo E or chylomicron remnant receptor, although this apo E receptor has not been well characterized.
However, while homozygosity for apo E2 appears necessary for expression of type III hyperlipoproteinemia in most cases, it is not by itself sufficient because only 1–4% of individuals with the apo E2-2 phenotype actually develop type III hyperlipoproteinemia. A second factor, either genetic or environmental, also seems to be necessary for expression. Untreated total plasma cholesterol and triglyceride levels are about 450 and 700 mg/dl, respectively, and treatment can alter levels markedly. Indeed, after treatment such as weight loss or effective medication, persons with type III hyperlipoproteinemia may have normal or near-normal total plasma cholesterol and triglycerides, but β-VLDL may still be detected by gel electrophoresis of plasma. This condition has been called dysbetalipoproteinemia by some investigators (although this term has been used interchangeably with type III hyperlipoproteinemia by others). β-VLDL may also be detected in many apparently normal persons with apo E2-2.

An intermediate classification seems appropriate for patients with the chemical abnormalities of classical type III hyperlipoproteinemia but who lack tuberous or palmar xanthomas. This condition will be designated herein as type III dyslipoproteinemia (DLPIII). Definite DLPIII may be defined by the criterion developed by Fredrickson et al10 as a VLDL cholesterol to plasma triglyceride ratio greater than or equal to 0.30 (with plasma triglycerides between 150 and 1,000 mg/dl). In probable DLPIII the VLDL cholesterol to plasma triglyceride ratio is greater than or equal to 0.25. This criterion was developed as a means to chemically define type III hyperlipoproteinemia. By use of a definite criterion for DLPIII, the presence of significantly elevated levels of β-VLDL determined by electrophoresis was detected with fair sensitivity and good specificity. A lower level of total plasma triglycerides was delineated because the specificity of the VLDL cholesterol to plasma triglyceride ratio was poor when plasma triglycerides were below 150 mg/dl and not because plasma triglycerides above this cutoff were considered elevated. The upper limit was chosen because sensitivity of the ratio was lost when chylomicrons were present. Elevations of total plasma cholesterol and triglycerides may be mild to severe in DLPIII but may not be as striking as in patients with classical type III hyperlipoproteinemia. Another potential difference between type III hyperlipoproteinemia and DLPIII is the plasma LDL cholesterol concentration. In type III hyperlipoproteinemia, plasma LDL cholesterol is generally low. In DLPIII, LDL cholesterol may be low, normal, or elevated, depending on the case.

In the course of family studies of coronary artery disease, we ascertained a pedigree in Utah and Idaho, designated K625, through five sisters who experienced early myocardial infarction and hyperlipoproteinemia. Extended study of the pedigree identified six individuals with type III hyperlipoproteinemia (HLPIIia; LDL cholesterol >95th percentile, with normal plasma triglycerides) and seven individuals with definite or probable DLPIII. A branch of the pedigree included four subjects with hypobetalipoproteinemia (HBLP), an autosomal dominant disorder characterized by low levels of LDL cholesterol in heterozygotes. This branch was extended to ascertain a large pedigree designated K635 that contained 16 individuals affected with HBLP. In K635 we had demonstrated cosegregation of HBLP with DNA markers at the apo B locus.

Pedigrees K625 and K635 with several individuals affected with HLPIIia, DLPIII, or HBLP provided an opportunity to use molecular markers to examine the combined phenotypic effects of several genes involved in lipoprotein metabolism. Our analyses led to three conclusions: 1) HLPIIia in the pedigree resulted from a defect at the LDL receptor gene locus; 2) DLPIII appeared to result from the simultaneous inheritance of at least one apo E2 allele and a defective LDL receptor allele; and 3) a normal lipid profile was observed in one individual who had inherited both a defective LDL receptor allele responsible for HLPIIia and a defective apo B allele responsible for HBLP.

Methods

Pedigree K625 was ascertained through five sisters (individuals 1, 10, 28, 50, and 72 in Figure 1) who had early myocardial infarction and hyperlipoproteinemia. K625 and K635 overlapped in a branch formed by the mating between individuals 94 and 95.

Blood samples collected after 12–16 hours of fasting were prepared according to guidelines described in the Lipid Research Clinics Program Manual of Laboratory Operations. Lipid and lipoprotein concentrations were measured by a microscale procedure developed in our laboratory. Plasma cholesterol and triglyceride concentrations were assayed enzymatically with Baker reagents on an Enzymatic Autoanalyzer (Baker Instrument Corp., Allentown, Pa.). Concentrations of HDL cholesterol were determined by the MgCl2–dextran precipitation method. Plasma lipoproteins (in 200 μl EDTA plasma) were centrifuged in a Beckman TL-100 tabletop ultracentrifuge for 4 hours at 60,000 rpm at room temperature and thereafter separated into top (VLDL) and bottom (LDL plus high density lipoprotein [HDL]) fractions by tube slicing. LDL cholesterol was determined by subtracting the HDL cholesterol concentration from LDL plus HDL cholesterol. Although VLDL cholesterol can be measured either directly or indirectly, we followed an accepted convention of measuring it indirectly by subtracting LDL cholesterol and HDL cholesterol from total cholesterol concentrations in plasma. Apo E phenotyping was performed by isoelectric focusing on polyacrylamide gels after delipidation of the plasma VLDL fraction as described by Warnick et al.

Genomic DNA was prepared from lymphocytes as previously described. The LDL receptor cDNA clone pHII was used to detect Neo I and Msp I diallelic restriction fragment length polymorphisms.
FIGURE 1. Pedigrees K625 and K635. Members are identified by numbers below each symbol. Descendants of members 01 and 02 form K625, and descendants of members 201 and 202 form K635; the two pedigrees overlap in a branch formed by mating between members 94 and 95. Phenotypes and genotypes are shown respectively above and below a centerline in each symbol. Definitions of phenotypes are as follows: LDL-c >95%, low density lipoprotein cholesterol (LDL-c) greater than the age- and sex-specific 95th percentile; LDLPIII, very low density lipoprotein cholesterol to plasma triglyceride ratio >0.25 when plasma triglyceride concentrations were >150 mg/dl; HBLP, hypobetalipoproteinemia defined by joint segregation analysis of total cholesterol, and LDL cholesterol, and plasma apolipoprotein (apo) B concentrations. Definitions of genotypes are as follows: LDL receptor defect, carrier of defective LDL receptor allele assigned by risk calculation, with use of LDL cholesterol level and presence of the F haplotype; Apo E2, presence of at least one E2 isoform (E2-2, E3-2, or E4-2); Apo B defect, carrier of apo B haplotype C cosegregating with HBLP. Circles denote females and squares denote males.
one in 50,000. By contrast, several recombinants were observed between LDL cholesterol concentrations and the apo B alleles. This implies that a molecular defect in the LDL receptor locus present on haplotype F is most likely responsible for the high LDL cholesterol concentrations in this pedigree. Because haplotype F was not unique in this pedigree, carrier status was assigned by risk calculations with the use of LDL cholesterol concentration and genotypes at the LDL receptor locus for any subject with a probability in excess of 0.95, as determined by complex segregation analysis.

Possible Interaction Between Low Density Lipoprotein Receptor and Apolipoprotein E Loci

In seven of the 22 individuals with haplotype F and elevated LDL cholesterol, probable or definite DLPIII was diagnosed on the basis of a ratio of VLDL cholesterol to plasma triglycerides in excess of 0.25 and total triglycerides above 150 mg/dl. In fresh plasma from all seven of these DLPIII individuals, we observed β-migration of isolated VLDL on agarose gel electrophoresis, thus confirming the presence of β-VLDL. As shown in Table 1, all seven FH individuals with DLPIII carried at least one apo E2 allele; two were E2-2 homozygotes. By contrast, all six HLPIIa subjects were apo E3-3. This association of DLPIII with apo E2 was highly significant by Fisher’s exact test (p<0.0001). The association remained significant when the two apo E2-2 homozygotes were excluded (p=0.002), suggesting that a single apo E2 allele may lead to the accumulation of cholesterol-rich VLDL in the DLPIII subjects who carry the LDL receptor defect.

Sequence of Apolipoprotein E Receptor-Binding Domain

The apo E2 allele detected by gel electrophoresis usually corresponds to an arginine to cysteine substitution at position 158 of the mature apo E lipoprotein. However, rare apo E2 and E3 isoforms with structural mutations affecting receptor binding have been described. Consequently, we employed DNA amplification, M13 cloning, and sequencing of genomic DNA to examine the DNA sequence encoding the receptor-binding domain of apo E in five DLPIII subjects with the apo E2-3 phenotype. Typical E3 and E2 alleles were observed in all cases, confirming the apo E electrophoretic phenotyping. Likewise, dot-blot analysis of apo E genotypes confirmed homozygosity for E2 (cysteine158) in two DLPIII subjects and homozygosity for E3 (arginine158) for all six HLPIIa subjects included in the analysis (see Figure 2). Thus, the possibility that the observed association of DLPIII with apo E2 could have been due to unusual or rare apo E isoforms was excluded.

Combined Phenotypic Effects of Low Density Lipoprotein Receptor and Apolipoprotein B Loci

We previously reported definite evidence of linkage between HBLP and apo B in pedigree K635. The inferred apo B defect was carried by a haplotype designated C. K625 and K635 overlap through a mating involving individuals 94 and 95; individual 94 has the HLPIIa phenotype and was inferred by pedigree analysis to carry the LDL receptor defect. Likewise, individual 95, although deceased, was inferred to have carried the apo B defect responsible for HBLP. Of four living children, two had HLPIIa (subjects 109 and 119), one had HBLP (subject 138),

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### Table 2. Definition of Low Density Lipoprotein Receptor Haplotypes

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Nco I/HHI</th>
<th>Msp I/HHI</th>
<th>Pvu II/P17</th>
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<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
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<tr>
<td>C</td>
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<td>+</td>
</tr>
<tr>
<td>H</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Symbols (+ and −) represent alleles for each enzyme/probe system. Allele sizes: 3.4 kb (+) or 13.0 kb (−) for Nco I system; 0.47 kb (+) or 0.50 kb (−) for Msp I system; 4.9/3.6 kb (+) or 8.5 kb (−) for Pvu II system.

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![Figure 2](http://atvb.ahajournals.org/Downloadedfrom)
and one had normal plasma LDL cholesterol concentrations (subject 137). In our previous analysis of HBLP, subject 137 was inferred to be a carrier of the apo B defect cosegregating with HBLP, with probability greater than 99%. The present analysis allowed us to infer that this subject must also have inherited the LDL receptor defect segregating in this pedigree with probability greater than 99%. We conclude that this subject carries two defects in the heterozygous state: one that usually elevates LDL cholesterol twofold and another that usually lowers LDL cholesterol to less than half-normal concentrations in plasma. In this person the combined expression of two defective genes with opposing effects resulted in a normal lipid profile (total cholesterol, 187 mg/dl; triglycerides, 77 mg/dl; HDL cholesterol, 47 mg/dl; and LDL cholesterol, 125 mg/dl).

Discussion

In this extended pedigree, elevated LDL cholesterol levels were linked with the LDL receptor locus as shown by DNA markers, proving by molecular means that the FH was due to a defective LDL receptor gene. Furthermore, FH patients with at least one E2 allele at the apo E locus frequently displayed DLPIII. Thus, the apo E2 allele had a striking effect on plasma triglyceride and VLDL cholesterol concentrations among individuals in K625 who carried the defective LDL receptor allele; the increase in the means for plasma triglycerides and VLDL cholesterol were, respectively, 100 mg/dl and 55 mg/dl. Individuals with DLPIII usually have low plasma concentrations of LDL cholesterol, but all seven DLPIII individuals in K625 who carried the defective LDL receptor allele; the increase in the means for plasma triglycerides and VLDL cholesterol were, respectively, 100 mg/dl and 55 mg/dl. Individuals with DLPIII usually have low plasma concentrations of LDL cholesterol, but all seven DLPIII individuals in K625 who carried the defective LDL receptor allele; the final level of these patients with FH and DLPIII may be more progressive than in those with simple HLP Ia.

Patients with DLPIII have been known to respond dramatically to dietary and medical therapy.9 We had the opportunity to measure lipoprotein concentrations in the plasma of two patients with FH and DLPIII (subjects 1 and 37) before and after initiation of drug and dietary therapy. With therapy, their phenotype modified to one consistent with simple HLP Ia because of a marked decrease in total triglycerides (41% in both) and VLDL cholesterol (59% and 69%, respectively). Their plasma concentrations of LDL cholesterol, although decreased, still exceeded the 95th percentile for age and sex. Four other individuals (subjects 28, 72, 119, and 12) who carried the same defective LDL receptor allele and one apo E2 allele could be sampled only while they were under medical and dietary treatment. They also exhibited a simple HLP Ia phenotype. The DLPIII phenotypes of the latter four individuals may have been modified by treatment.

Age influences the expression of DLPIII, as manifestations of DLPIII rarely occur before adulthood.9 Two FH patients, individuals 39 and 40, who manifested DLPIII at ages 13 and 9 are rare exceptions. It is noteworthy that these two children as well as the 11-year-old girl with DLPIII described by Hazzard et al.30 were heterozygous for the LDL receptor defect but homozygous for apo E2.

We also observed a family member (subject 137) who had a normal lipid profile but who inherited defective alleles for the LDL receptor and for its ligand, apo B. Defective LDL receptor and apo B genes causing FH and HBLP have opposite effects on plasma LDL cholesterol concentrations. They also have a gene-dosage effect; that is, a homozygote has a more extreme deviation in LDL cholesterol than does a heterozygote. We interpret the phenomenon in subject 137 as the combined effect of two oppositely acting genes. One can assume that these defects result in roughly half the normal amount of gene product in each case. In the patient’s circulation, therefore, a new state of equilibrium can be postulated under the circumstance of half the normal number of ligand molecules (apo B) and half the normal number of LDL receptors; the final level of LDL cholesterol and apo B would thereby be normal.

In summary, by means of genetic markers we have classified members of a large family as carriers of molecular defects at three loci. This genotypic characterization allowed us to explain lipid phenotypes in terms of an apparent interaction between the apo E and LDL receptor loci and the effects of a third locus linked to the apo B gene for HBLP. Our study illustrates the feasibility of an analysis of interactions among multiple loci in genetic investigations of complex metabolic disease. The suggested interaction between the apo E and the LDL receptor loci observed in this pedigree prompted us to further examination of multiple FH pedigrees, described in an accompanying report.31
was performed with the LINKAGE computer pro-

Table I. Lipoprotein Profiles for the 22 Familial Hypercholesterolemia Patients in K625

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<tr>
<th>No.</th>
<th>Age (yr)</th>
<th>Sex</th>
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<th>TG</th>
<th>HDL</th>
<th>LDL</th>
<th>VLDL</th>
<th>r</th>
<th>Apo E</th>
<th>MI (yr)</th>
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<td></td>
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<td></td>
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<tr>
<td>10</td>
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<td>F</td>
<td>479</td>
<td>318</td>
<td>32</td>
<td>320</td>
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<td>3/2</td>
<td>59+</td>
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<tr>
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<td>F</td>
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<td>M</td>
<td>312</td>
<td>273</td>
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<td>140</td>
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FH diagnosis was based on LDL receptor DNA markers and LDL cholesterol levels as described in text. All lipids are expressed in milligrams per deciliter.

FH, familial hypercholesterolemia; LDL, low density lipoprotein cholesterol; TC, total cholesterol; TG, plasma triglycerides; HDL, high density lipoprotein cholesterol; VLDL, very low density lipoprotein cholesterol; r, ratio of VLDL to plasma TG; Apo E, apolipoprotein E phenotype (confirmed by dot-blot analysis); MI, myocardial infarction (age of onset).

(RFLPs) on Southern blots. Other probes used for phenotyping included the LDL receptor genomic clone pRHP17, which detects a Pvu II diallelic RFLP; the apo B cDNA clones pB8 and pB23, which detect Xba I and Msp I diallelic RFLPs, respectively; and the apo B genomic clone pBXB1, which detects an Xba I-Sau I RFLP, reflecting a locus that contains a variable number of tandem repeats.

Enzymatic DNA amplification of a 123-bp region containing the sequence that encodes the apo E receptor–binding domain (amino acid residues 136–160) and dot-blot analysis with allele-specific oligonucleotide probes coding either arginine158 or cysteine158 have been described previously. Genomic DNA from individual 3 was also subjected to amplification of an 831-bp region of the apo E gene that contains the 3' part of intron 3 and the 5' part of exon 4. The amplified fragments of the apo E gene were sequenced after cloning into M13 vectors. Eight clones from each individual were sequenced to obtain sequences of both alleles.

Analysis of relations among lipid measurements and genotypes at the LDL receptor and apo B loci was performed with the LINKAGE computer program. Fisher's exact test was used to compare differences in the prevalence of DLPIII among FH subjects with different apo E phenotypes.

Results

Linkage Between Elevated Low Density Lipoprotein Cholesterol Concentration and the Low Density Lipoprotein Receptor Locus

Pedigrees K625 and K635 (extended pedigrees of a single spouse pair) are shown in Figure 1, with lipoprotein phenotypes and genotypes at the LDL receptor, apo E, and apo B loci. Twenty-two subjects had LDL cholesterol concentrations above the 95th percentile of their age and sex reference values (Table 1). DNA polymorphisms at the LDL receptor locus were characterized by digesting genomic DNA with Nco I, Msp I, or Pvu II. Haplotypes were formed by combining the alleles of the three RFLPs as defined in Table 2. Linkage analysis demonstrated that the high LDL cholesterol phenotype segregated with markers at the LDL receptor locus without recombination, with a maximum log of the odds score of 4.74, corresponding to an odds ratio greater than...
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References


Key Words • linkage analysis • type IIa hyperlipoproteinemia • low density lipoprotein receptor defect • pedigree analysis
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