Apolipoprotein B Gene Mutations in Austrian Subjects With Heart Disease and Their Kindred

Walter Friedl, Erwin H. Ludwig, Maureen E. Balestra, Kay S. Arnold, Bernhard Paulweber, Friedrich Sandhofer, Brian J. McCarthy, and Thomas L. Innerarity

In a group of 110 subjects with severe coronary artery disease, two were heterozygous for the apolipoprotein (apo) B arginine$_{3,500}$ → glutamine mutation that characterizes familial defective apo B-100. Both affected subjects were moderately hypercholesterolemic, and their low density lipoproteins (LDLs) were deficient in binding to the LDL receptor. Pedigree analysis of the two probands' families established a correlation between the apo B mutation, defective LDL, and a particular apo B haplotype that was characterized by 10 apo B gene markers. In addition to having one allele carrying the arginine$_{3,500}$ → glutamine mutation, one family member may harbor a second mutant apo B allele that causes its gene product to be present in plasma at a lower than normal level, despite the fact that the affinity of the protein for the LDL receptor appears to be normal. The metabolic basis for the underrepresentation of this second allotype remains to be elucidated. (Arteriosclerosis and Thrombosis 1991;11:371-378)

Apolipoprotein (apo) B-100, a very large protein of 4,536 amino acids, plays a central role in lipoprotein metabolism. Apo B-100 is essential for the assembly and secretion of the triglyceride-rich very low density lipoproteins (VLDLs) by the liver. The VLDLs are converted to intermediate density lipoproteins and finally to low density lipoproteins (LDLs) by lipoprotein lipase. The LDLs, which transport about two thirds of the plasma cholesterol, are catabolized primarily by the hepatic LDL receptor pathway. Apo B-100, the exclusive apolipoprotein of LDL, is the protein ligand that binds to the LDL receptor. Several lines of evidence indicate that the plasma levels of LDL are mainly controlled by LDL receptor–mediated catabolism. For example, the overexpression of hepatic LDL receptors introduced into transgenic mice markedly reduces the level of circulating LDL, and the lack of LDL receptors in the homozygous state of familial hypercholesterolemia leads to LDL levels that are six to eight times greater than normal.

Recently, the role of apo B-100 in maintaining cholesterol homeostasis has been exemplified by studies of mutations of the apo B gene. The two genetic abnormalities attributed to mutations in the apo B gene are familial hypobetalipoproteinemia, which is associated with low plasma LDL cholesterol levels, and familial defective apo B-100 (FDB), which is characterized by high plasma cholesterol levels.

Familial hypobetalipoproteinemia is caused by mutations in the apo B gene that prematurely terminate translation and result in the production of truncated proteins. As a consequence, affected subjects who are heterozygous for this gene have only 25–50% of the normal levels of VLDL and LDL, and individuals with the homozygous genotype have few, if any, apo B-100–containing lipoproteins in their plasma. Investigators have recently obtained evidence of other types of mutations that result in plasma deficient in LDL but that do not cause premature termination of translation.

FDB is associated with a single-nucleotide substitution in the apo B gene: CGG is changed to CAG in codon 3,500, causing a glutamine-for-arginine substitution in the protein at this site. As a consequence of this mutation, the apo B-100 in the LDL binds weakly, if at all, to the LDL receptor and accumulates in the plasma. Because there is one copy of apo B-100 per LDL particle, individuals heterozygous for this disorder possess two populations of LDL, one with normal...
 apo B-100 and one with defective-binding apo B-100. The LDL from FDB heterozygotes has about 32% of normal receptor-binding activity, and essentially all of that activity is attributable to the normal LDL.\textsuperscript{4,5} Clinically, these individuals are characterized by moderate to severe hypercholesterolemia.\textsuperscript{4,5}

In this study, we report the results of screening 318 Austrians for FDB. One hundred ten of these subjects were male patients with severe coronary heart disease, and 208 were men without evidence of ischemic heart disease. In the coronary heart disease population, we identified two cases of FDB. In studying the kindreds of the two probands, we also identified an individual who may have a second mutation at the apo B locus. One apo B allele has the FDB mutation in codon 3,500 that causes hypercholesterolemia, and the second allele produces an LDL-associated apo B that is significantly underrepresented in the plasma. Because one mutation of apo B causes hypercholesterolemia and a putative second mutation in the other apo B allele causes hypocholesterolemia, the net result is normal cholesterol levels in this individual. Furthermore, by using haplotype analysis of the apo B gene, we may be able to identify the allele responsible for this second mutation.

Methods

Subjects

DNA was prepared from white blood cells of 318 Austrian subjects from Salzburg.\textsuperscript{12} One hundred ten subjects were patients suffering from heart disease, and 117 were matched subjects who did not have heart disease (both groups are described in Reference 13). The remaining 91 healthy subjects were randomly selected from a plasmapheresis center in Salzburg.

DNA Analysis

Subjects in two kindreds found to have FDB were haplotyped using 10 DNA markers (Figure 1). These markers consisted of a hypervariable repeat (HVR) marker 3,250 bp 5' to the transcriptional start, a signal-peptide (SP) polymorphism consisting of a 9-bp insertion/deletion within exon 1,\textsuperscript{14} ApaLI, HincII, PvuII, AluI, XbaI, MspI, and EcoRI restriction fragment length polymorphisms (RFLPs), and a 3' HVR.\textsuperscript{15} All RFLP markers were assayed by polymerase chain reaction (PCR) amplification and treatment with the cognate restriction enzyme, followed by acrylamide or agarose gel electrophoresis. For the 5' and 3' HVRs, PCR amplification was followed by denaturing acrylamide gel electrophoresis, whereas the SP PCR was run on an acrylamide gel. Primers and PCR conditions for the SP, ApaLI, XbaI, MspI, the arginine \textsubscript{500} \rightarrow glutamine mutation, and 3' HVR are described elsewhere.\textsuperscript{10,14-16} For the 5' HVR, the two primers were 5' ATTCCTTTCCTAGATGGTGCACTGCTTGC 3' and 5' AACTCTTAAACATTGATAGTGGAACTGTGC 3'. For the HincII RFLP, the primers were 5' GTGAGGGTAGTTTTCAGCATGCTTGC 3' and 5' GCCACAGCAGCCAGGTGCCCTCTGGG 3'. For the PvuII RFLP, the primers were 5' GCCTCCTGGTGGACACTGCGCCAAGGCAA GCT GAGGGTAAGITTTTCAGCATGCTTGC 3'. For the AluI RFLP, the two primers were 5' GAATTGCTGATATTTACTTG 3' and 5' GTAGGCTGATGTITTTTCAGCATGCTTGC 3'. The amplification reactions described here contained 100–500 ng of genomic DNA, 200 ng of each primer, buffer, and deoxynucleotide triphosphate according to Saiki et al.\textsuperscript{17} Conditions for the 30 cycles of these amplifications were as follows: 94°C, 1-minute denaturation; 55°C, 1-minute annealing; and 72°C, 1-minute extension. Haplotypes were deduced unequivocally in all family members, assuming Mendelian inheritance and no recombination.

Lipoprotein Isolation, Cell Cultures, and Low Density Lipoprotein Binding Studies

Plasma samples from probands F.F. and I.R. and from their families were obtained after overnight fasting. LDLs were isolated by standard methods.\textsuperscript{18,19} For the binding competition experiments, normal LDLs were radiolabeled by the iodine monochloride procedure.\textsuperscript{18} Normal human fibroblasts were cultivated, and LDL competition experiments were performed as described.\textsuperscript{18,19} The amount of unlabeled sample LDL

FIGURE 1. Diagram showing position of DNA markers within and near the apolipoprotein (apo) B gene and deduced haplotypes. Upper portion: The apo B gene is indicated by the thick horizontal bar and 5' and 3' regions by the thin horizontal line; relative positions of restriction fragment length polymorphisms are shown by the angled lines underneath the bar. Two hypervariable regions (HVRs) flanking the gene are indicated as hatched boxes. Location of the markers is given as cDNA position, intron number, or number of base pairs (bp) 5' or 3' of the cDNA. Lower portion: Haplotypes, defined by numbers of 5' and 3' hypervariable elements and the presence (+) or absence (−) of DNA markers.

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required to displace 50% of labeled normal LDL was measured and compared with a normal value.

**Solid-Phase Radioimmunoassays**

The ability of plasma samples and LDL to bind to monoclonal antibodies was determined by solid-phase competition radioimmunoassay (RIA) according to previously described methods.20

The basis for the assay was to compare the ability of various test LDLs to compete with standard LDL for binding to the monoclonal antibody MB19. LDLs in phosphate-buffered saline (PBS, 0.5 μg/well) from an MB19/MB19 homozygote were bound to Removeawell plates (Dynatech Labs, Inc., Alexandria, Va.) by incubation for 3 hours at room temperature. The excess LDLs were removed from the wells by washing four times with washing buffer (PBS containing 0.3 mM EDTA, 0.05% Tween-20, 0.04% aprotinin, and 0.1% RIA-grade bovine serum albumin [BSA]). Exposed protein-binding sites on the plastic surface were blocked by incubation for 45 minutes at room temperature with blocking buffer (PBS containing BSA). The blocking buffer was removed by washing four times with washing buffer. The assay was performed by adding 25-μl aliquots of the test LDL diluted in dilution buffer (washing buffer containing BSA) followed by 25 μl MB19 (ascites diluted 1:10,000 in dilution buffer). The plates were incubated overnight at 4°C and then washed five times with washing buffer. The amount of MB19 bound was determined by incubation for 4–5 hours with a second radioactive antibody (~400,000 cpm 125I-labeled sheep anti-mouse immunoglobulin G/well).

The amount of apo B in plasma samples from the kindred was determined by a solid-phase RIA using the monoclonal antibody MB3 and a normal LDL as a standard. The MB47/MB3 ratios for the probands and their families were determined by RIA.21 This measurement is an indicator of the presence of the apo B codon 3,500 mutation.

**Results**

Our screening of 318 Austrian subjects (110 patients with severe coronary heart disease, 117 matched controls, and 91 individuals randomly selected from a plasmapheresis center) for a specific point mutation that changes arginine to glutamine at position 3,500 of apo B showed that two subjects from the heart disease group were heterozygous for this mutation. The mutation did not occur in the control or the randomly selected group.

**Haplotype Analysis**

The families of the two probands (F.F. and I.R.) were studied to determine their apo B haplotypes and to identify the family members carrying the codon 3,500 mutation. The inheritance of the haplotypes in the F. family and the R. family is illustrated in Figure 2. The pedigree analysis was very informative even though the R. family is very small. In the F. family, the specific codon 3,500 mutation could be identified through four generations (Figure 2A; Table 1). Using the DNA markers in conjunction with the occurrence of the codon 3,500 mutation, we observed 15 different haplotypes. The haplotypes were designated A-O, and they are listed in the lower part of Figure 1. In both families, all haplotypes could be deduced unequivocally on the assumption that no recombination event occurred. In whomever the codon 3,500 mutation occurred in either the F. or the R. family, it occurred exclusively on haplotype A. Subject J.H., who is discussed below, inherited haplotype A from his father and haplotype E from his mother.

**Plasma MB47/MB3 Binding Assay**

Previously, we had determined that the apo B monoclonal antibody MB47 bound with enhanced affinity to the LDL of family members from two kindreds with FDB.21 To determine whether the association between enhanced binding of MB47 to LDL from subjects heterozygous for the apo B arginine3,500 → glutamine mutation is absolute, we exam-
the binding of MB47 to the LDL of probands J.R. and F.F. and their relatives. The control apo B monoclonal antibody MB3 was used to normalize differences in LDL concentration because, unlike MB47, MB3 fails to show a difference in binding to FDB LDL compared with the LDL of normal control subjects.21 Thus, the ratio of the binding of LDL to MB47 compared with its binding to MB3 acted as an index of MB47 binding. The LDL from all subjects analyzed in more detail. His LDLs were isolated on five separate occasions and were tested a total of nine times for receptor binding. Their binding activity was compared with 2.9 μg/ml for the unaffected family members and controls. Surprisingly, the LDL from two subjects (J.H. and Fr.F.) was much more defective than LDL from any other FDB subject, either in the two families studied here or when compared with approximately 30 other FDB subjects previously tested. Values of 16% and 22% of normal receptor-binding activity were obtained for J.H. and Fr.F., respectively. By comparison, the average receptor-binding activity of FDB LDL using this assay is 35.6%, and in the present study, this mean value was 35.7%. Although the severely defective binding of the LDL from Fr.F. was confirmed by isolating the LDL on a second occasion, his LDLs were not investigated further. However, the LDLs from J.H. were examined in more detail. His LDLs were isolated on five separate occasions and were tested a total of nine times for receptor binding. Their binding activity was 16.3±3.8% (mean±SD) of normal, with an average of 16.7 μg abnormal LDL/ml required to displace half the labeled LDL ligand (Figure 3; Table 1).

**Affinity of MB19 for Low Density Lipoproteins**

Although LDL isolated from J.H. had 16% of normal receptor binding, his serum cholesterol level was only 205 mg/dl. Consideration was given to the possibility that J.H. carried two different types of mutation in his apo B genes: on one allele, the codon

<table>
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<tr>
<th>Family member/yr</th>
<th>Amino acid residues at position 3,500</th>
<th>MB47/MB3</th>
<th>LDL IC50* (mg/ml)</th>
<th>Percent of control IC50</th>
<th>Plasma concentration (mg/dl)</th>
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<td></td>
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*Amount of unlabeled sample LDL needed to displace 50% of labeled normal LDL.

LDL, low density lipoprotein; TC, total cholesterol; HC, high density lipoprotein cholesterol; TG, triglycerides; AB, apolipoprotein B; Arg, arginine; Glu, glutamine; ND, not determined.
Friedl et al  

**FDB in Austrian Subjects**  

375

FIGURE 3. Curves showing ability of low density lipoprotein (LDL) from J.H., from a familial defective apolipoprotein B (FDB) heterozygote, and from a normal control to compete with normal 125I-labeled LDL for binding to LDL receptors (% of control) on normal human fibroblasts. Dulbecco's modified Eagle's medium containing 10% human lipoprotein-deficient serum, 2 μg 125I-labeled LDL protein/ml, and the indicated concentrations of unlabeled J.H., FDB heterozygote, or normal LDL (μg protein/ml) were added to 22-mm culture wells (0.450 ml/well) containing normal human fibroblasts. After a 2-hour incubation at 4°C, the amount of 125I-labeled LDL bound was measured. The 100% control value was 9.5 ng 125I-labeled LDL.

3,500 mutation (haplotype A), which causes hypercholesterolemia, and on the other allele (haplotype E), a mutation that results in its protein product being underrepresented in the plasma LDL. To explore this possibility, we took advantage of the observation that J.H. was heterozygous for the MB19 polymorphism. The monoclonal antibody MB19 detects a common, functionally neutral apo B polymorphism. Approximately 10% of the population is homozygous for the LDL that binds with high affinity to MB19 (designated MB19\(/_{MB19}\)), approximately 45% is homozygous for the LDL that binds with low affinity to MB19 (MB19\(/_{MB19}\)), and approximately 5% is MB19 heterozygous (MB19\(/_{MB19}\)). The antibody binds to the LDL homozygous for one apo B allotype with an 11-fold higher affinity than to the other. Because of the large difference in affinity of the two MB19 allotypes for MB19, it is possible, by using appropriate standards, to estimate accurately the concentration of the LDL representing each allele in the plasma.

The ability of LDL from an MB19, homozygote, an MB19\(_2\), homozygote, a standard MB19 heterozygote, a typical FDB patient, or subject J.H. to compete with standard MB19, (high-affinity) LDL for binding to the MB19 antibody was determined by solid-phase RIA (Figure 4). As previously reported, three readily distinguishable patterns of displacement were observed for the LDL from the MB19, homozygote, the MB19\(_2\), homozygote, and the standard MB19 heterozygote. The displacement curve of a typical individual heterozygote for FDB indicates that approximately 30% of the total LDL is normal and approximately 70% is the LDL allotype with the arginine-to-glutamine substitution. Clearly, the displacement curve for J.H. indicates an even greater imbalance between the abundance of the MB19, and MB19\(_2\) allotypes.

To determine more precisely the concentration of the LDL that was a product of each allele, we used known mixtures of LDL from MB19, and MB19\(_2\) homozygotes. Based on the 50% displacement of the standard mixtures and of LDL from J.H., we estimate that LDL from J.H. contained about 17% of the MB19 allotype (haplotype E) and about 83% of the MB19\(_2\) allotype (haplotype A) (Figure 5). These results are consistent with the competitive receptor experiments, indicating that LDL from J.H. has only 16% of normal receptor-binding activity.

**Discussion**

There are two major findings in this work. First, of 110 patients with severe coronary heart disease, two

![Figure 4](http://atvb.ahajournals.org/) Curves showing ability of low density lipoprotein (LDL) from five different subjects to compete with a standard MB19, LDL for binding (B/Bmax) to antibody MB19 in a solid-phase radioimmunoassay. LDLs were from an MB19, homozygote (MB19\(_1\), MB19\(_2\); ▲), from subject J.H. (*), from a familial defective apolipoprotein B (FDB) heterozygote who is also an MB19 heterozygote (MB19\(_2\); ■), a standard MB19 heterozygote (MB19\(_2\); MB19\(_1\); MB19\(_2\); MB19\(_1\); MJ; ●), and an MB19, homozygote (MB19\(_1\); MB19\(_2\); ○). Assay conditions are described in "Methods."
inherited FDB. No codon 3,500 mutations in apo B-100 were found in the control or the randomly selected group. Although these numbers are small, they are consistent with other reports of the prevalence of this mutation in groups of subjects with hypercholesterolemia and atherosclerosis.\(^5\) The LDLs of both probands bound poorly to LDL receptors and avidly to the antibody MB47, and both probands were heterozygous for an arginine-to-glutamine substitution at codon 3,500 of the apo B gene. Pedigree analysis of the families of the probands demonstrated a correlation between moderate hypercholesterolemia and the codon 3,500 mutation, the antibody binding, and the receptor binding. The codon 3,500 mutation that produces hypercholesterolemia was confined to one apo B allele (haplotype A). A parallel haplotype analysis using this same approach for 12 other arginine\(^{3,500}\) \(\rightarrow\) glutamine mutant probands indicates that these mutations occur on this same characteristic haplotype.\(^4\) The fact that 15 different haplotypes were distinguishable in the 20 subjects in our study attests to the high resolution provided by this haplotype analysis that uses 10 markers.

The second major finding of this study is the circumstantial evidence that one affected subject (J.H.) may carry two different apo B mutations. As discussed previously, one apo B allele has the codon 3,500 mutation, which causes hypercholesterolemia (haplotype A), and on the other allele there may be another mutation that produces hypocholesterolemia (haplotype E). The following evidence points to a second mutation specific to an apo B allele. The LDLs containing the apo B-100 with the codon 3,500 mutation are essentially devoid of receptor-binding activity. Because of this lack of receptor-mediated clearance, typical FDB heterozygotes are hypercholesterolemic, and the defective LDLs accumulate in their plasma to approximately a 2:1 ratio over normal LDLs. In contrast, the LDL from J.H. displayed about 17% of normal receptor-binding activity, or only about half that of a typical FDB heterozygote. If the severely defective LDLs from J.H. were the result of a mutation in apo B haplotype A that also reduced receptor-mediated clearance of this allele-specific LDL, then J.H. would be expected to be severely hypercholesterolemic. Instead, J.H. has a normal serum cholesterol level, indicating that it is unlikely that the LDLs resulting from the second allotype (haplotype E) also have diminished receptor binding. It would appear, rather, that some other mechanism must explain the larger-than-expected defective binding receptor activity of the LDL.

The issue was clarified by demonstrating that the exceptionally defective binding activity of J.H.'s LDL results from the abundance of the binding-defective LDL derived from the allele carrying the codon 3,500 mutation (allootype A). The clarification was possible because J.H. is heterozygous for a common apo B polymorphism that can be detected by the MB19 antibody. By using an assay recently described by Gavish et al\(^8\) and Young et al,\(^20\) we estimated that approximately 83% of the total LDL is derived from allele A and 17% from allele E. Thus, to summarize: the total LDLs from J.H. have approximately 17% of normal receptor-binding activity, the LDLs produced by allele A (which carries the arginine\(^{3,500}\) \(\rightarrow\) glutamine change) have almost no binding activity, and the allotype distribution representing the two alleles (A:E) is 83%:17%. The 83%-17% distribution of LDL from J.H. is significantly different from the \(\sim 65%:35%\) distribution found in the 40 other FDB patients whom we have studied. Moreover, we were able to show that this unequal apo B-100 allotype distribution occurred only in the LDL and not in the VLDL from J.H. Using the MB19 assay, we showed that the VLDL from J.H. contained equal amounts of apo B-100 from allele A and allele E, a finding that establishes that equal amounts of both allotypes were synthesized (M. Balestra et al, unpublished data). The most logical explanation for all these findings and our working hypothesis is that the allele defined by haplotype E is associated with a mutation that causes the gene product to be underrepresented in the plasma because of increased LDL catabolism.

**Figure 5.** Curves showing ability of various low density lipoproteins (LDLs) to compete with a standard MB19, LDL for binding (B/Bmax) to antibody MB19 in a solid-phase radioimmunoassay. Lipoproteins were pure MB19 LDL (MB19, [100%]; ▲), a mixture of 90% MB19 LDL and 10% MB19 (MB19:MB19, [90%:10%]; □), LDL from J.H. (*), a mixture of 80% MB19 and 20% MB19, LDL (MB19:MB19, [80%:20%]; ●), and 100% MB19, LDL (MB19, [100%]). Assay conditions are described in "Methods."
Very recently, Gavish et al. reported that among a group of hypocholesterolemic subjects, a significant number displayed unequal amounts of the two apo B and LDL allotypes in their plasma. Antibody MB19 was used to identify allele-specific differences in the amount of apo B in the plasma in specific individuals and to confirm in family studies that the unequal expression was inherited in an autosomal dominant fashion. DNA markers were used to confirm the linkage of the immunoreactivity phenotype to the apo B gene locus, although the causative mutation was not identified. In the case of our subject J.H., the 83%:17% distribution of the two LDL allotypes clearly conforms to the unequal pattern observed by Gavish et al. However, because of a limited pedigree, we have been unable to demonstrate that the second apo B allele is responsible for this aberrant ratio, which differs both from that of normal subjects and from that of other FDB subjects. The mother of the proband, the only relative available, is an MB19 homozygote.

However, the second apo B allele of J.H. is a very rare one, as deduced from haplotype analysis. For example, alleles containing 35 repeats of the 3' HVR occur in only approximately 1% of individuals in this same Austrian population. Furthermore, haplotype E contains 15 rather than 14 repeats of the 5' HVR, a feature of only approximately 10% of alleles in this population. These two markers alone therefore imply that the second allele carried by J.H. is present in only about 0.1% of the population. While these data do not prove that this haplotype is associated with an apo B mutation that leads to hypocholesterolemia, they do suggest that this is a plausible working hypothesis.

The results of this study suggest that a number of different types of mutations in the apo B gene can affect LDL levels. By determining the LDL receptor-binding activity, the concentration of allele-specific LDL, and the exact apo B haplotype of all the family members of unusual probands, it should be possible to detect the existence of a number of different types of apo B mutations and to assess their effect on plasma cholesterol levels.

Acknowledgments

We thank Tom Rolain and Charles Benedict for the graphics, Kerry Humphrey and Joan Ketchmark for manuscript preparation, Cappy Rall for a critical reading of the manuscript, and Sally Gullatt Seehafer and Al Averbach for editorial assistance.

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**KEY WORDS** • familial defective apolipoprotein B • low density lipoproteins • atherosclerosis • genetic disorders • lipoproteins • low density lipoprotein receptors
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doi: 10.1161/01.ATV.11.2.371

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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