Association of DNA Polymorphism at the Apolipoprotein B Gene Locus with Coronary Heart Disease and Serum Very Low Density Lipoprotein Levels

Bernhard Paulweber, Walter Friedl, Franz Krempler, Steve E. Humphries, and Friedrich Sandhofer

The role of genetic variation at the 3' end of the apolipoprotein B gene locus in the development of coronary heart disease and the regulation of the serum levels of various lipoproteins was studied by using two common restriction fragment length polymorphisms detected with the enzymes Xba I and EcoR I. A group of 106 male patients with coronary heart disease and 118 matched controls of Austrian origin were investigated. The frequency of the R2 allele of the EcoR I polymorphism at cDNA position 12 669 defined by the absence of the polymorphic EcoR I cutting site was significantly higher among patients than among controls. The controls with the R2 allele had significantly higher levels of total triglycerides, very low density lipoprotein (VLDL) triglycerides, and VLDL cholesterol than did the controls without this allele. Among the patients, the R2 allele was associated with higher serum VLDL apolipoprotein B levels. The chemical composition of VLDL in individuals with different genotypes for the EcoR I polymorphism did not differ significantly. For the Xba I polymorphism at cDNA position 7673, no correlation with coronary risk could be demonstrated. Patients and controls homozygous for the X2 allele characterized by the presence of the polymorphic Xba I cutting site showed a higher total and low density lipoprotein cholesterol level than did subjects with the genotype X1X1 or X1X2. This difference, however, was not statistically significant. These findings indicate that the R2 allele of the EcoR I polymorphism is associated with the occurrence of coronary heart disease and that variation at the 3' end of the apo B gene is involved in the regulation of VLDL metabolism.

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It is well established that elevated serum levels of low density lipoprotein (LDL) cholesterol and apolipoprotein (apo) B1,2 are correlated with increased risk for coronary heart disease. However, the role of triglyceride-rich lipoproteins in the development of atherosclerosis is still unclear. Apo B-100 is the main apoprotein constituent of plasma LDL and very low density lipoprotein (VLDL) particles.3 The cloning and sequencing of the apo B-100 gene by several groups4-5 allows a new approach to the study of the role of the genetic variation of apo B-100. A number of restriction fragment length polymorphisms (RFLPs) of the apo B gene have been reported.6-9 These RFLPs provide deoxyribonucleic acid (DNA) markers for association studies in populations and for linkage analysis in families.

A DNA variation at cDNA position 7673 in exon 26 gives rise to a polymorphic restriction site for Xba I7 but does not result in a change in the amino acid sequence. This polymorphism has been associated with the Ag(x/y) and the Ag(c/g) immunoechemical polymorphism10,11 and with elevated serum levels of apo B,12 cholesterol, and triglycerides.12,13,14 In patients with type III hyperlipidemia, the allele frequency for the Xba I RFLP differed significantly from the frequency in normolipidemic individuals and in those with other types of hyperlipidemias.15 A DNA base change at cDNA position 12 669 creates a polymorphic EcoR I cutting site.7 This DNA change causes a lysine-to-glutamic acid substitution in the mature apoprotein. There is evidence that this point mutation also causes the Ag(t/z) immunoechemical polymorphism.11,16

Table 1 summarizes the results obtained by various authors using apo B RFLPs. They have reported significantly higher frequencies of the rare alleles of the Xba I and EcoR I polymorphisms in 84 survivors of myocardial infarction than in healthy controls.17 Serum lipoprotein levels of subjects with different genotypes for these RFLPs did not differ significantly in this study. In a sample...
from the London population, the X1 and the R2 alleles were found significantly more frequently among patients with coronary artery disease and with peripheral arterial disease than among healthy controls.16 In this study, there was also no significant association between these RFLPs and serum lipid levels. In patients with type IV and V hyperlipemia, the R2 allele was observed significantly more frequently among patients than among healthy controls.18 In this study, the presence of the cutting site throughout the alleles defined by these apo B RFLPs is associated with the occurrence of coronary heart disease or with serum levels of various lipid and lipoprotein parameters in a homogeneous sample from the Austrian population.14,19,20

The aim of this study was to determine whether one of the alleles defined by these apo B RFLPs is associated with the occurrence of coronary heart disease or with serum levels of various lipid and lipoprotein parameters in a homogeneous sample from the Austrian population.

Methods

Study Subjects

Patients

Consecutive patients were taken from the Department of Cardiology of the Landeskrankenanstalten Salzburg and the Rehabilitation Centre for Heart Disease at Gossenmain near Salzburg. This group consisted of 106 unrelated male patients under the age of 55 with proven severe coronary heart disease; 95 of them had suffered at least one myocardial infarction as verified by an electrocardiogram (ECG) and a typical pattern of specific serum enzymes. Eleven patients had a history of angina pectoris and a more than 70% narrowing of at least one coronary artery as documented by angiography. Only four patients had serum cholesterol levels above 280 mg/dl, and five had serum triglyceride levels above 250 mg/dl. Six patients were taking a lipid-lowering drug (bezafibrate). As assessed on the basis of questionnaire responses, all individuals studied were of Austrian descent.

Controls

This group contained 118 unrelated men (volunteers from the Austrian army and various sports clubs) who were matched with the patients by age and ethnic background. None of them showed signs of coronary heart disease in medical history or resting or exercise ECG. There was no preselection by serum lipid levels in this group. Two of these subjects had serum cholesterol levels above 280 mg/dl, and five had serum triglyceride levels above 250 mg/dl. None was taking a lipid-lowering drug. Seven persons in this group had relatives who had clinical manifestations of coronary heart disease and were younger than 55 years.

In both groups, those individuals with disorders causing secondary hyperlipemia (such as diabetes mellitus,
hypothyroidism, chronic renal and liver diseases, and chronic alcohol abuse) were excluded. None was kept on a special diet, and all subjects gave their informed consent to the study.

**DNA Preparation and Southern Analysis**

Blood samples were collected in ethylenediaminetetraacetic acid (EDTA) tubes and were kept at -70°C until used. Total genomic DNA was prepared from whole blood by a Triton X-100 lysis method. Each restriction enzyme digestion was carried out by using 5 μg of total genomic DNA and 2 to 5 units of enzyme/μg DNA under conditions recommended by the supplier (Amersham International, Buckinghamshire, England). DNA fragments were separated by size on a 1% (Xba I) or 0.8% (EcoR I) agarose gel and were transferred to nylon hybridization membranes (Amersham International) after procedures described elsewhere.

**DNA Probes for Hybridization**

Probes were isolated from plasmids, prepared by electrophoresis on low melting point (LMP) agarose, and subsequently labeled with 32P-deoxyctydine triphosphate at 800 Ci/MM (Amersham International) by using a random oligonucleotide primer method. A 3.5 kilobase (kb) EcoR I genomic fragment of the apo B gene was used to detect the Xba I polymorphism, and a 0.5 kb cDNA fragment was used to detect the EcoR I RFLP.

**Lipid and Lipoprotein Analysis**

All blood samples were drawn after a fasting period of 12 to 14 hours not less than 6 weeks after a myocardial infarction. Total serum cholesterol, serum triglyceride, and high density lipoprotein (HDL) cholesterol levels were determined enzymatically with commercial kits from Boehringer GmbH, Mannheim FRG. VLDL and LDL cholesterol levels were determined after ultracentrifugation following standard procedures. Serum levels of apo A-I, apo A-II, apo B, and lipoprotein (a) [Lp(a)] were quantified by radial immunodiffusion with a commercially available kit (Immuno-Austria, Vienna, Austria).

**Statistical Analysis**

The significance of differences in allele frequencies (obtained by gene counting) between patients with coronary heart disease and healthy controls was tested by a 2×2 contingency table and a χ² test of independence. Each of the lipoprotein parameters was adjusted by polynomial regression to correct for variations due to age, weight, and height. The body mass index was calculated with the formula: body mass index = weight[kg]/[height[m]]². The null hypothesis, that phenotypic variance is not determined by genetic variation at the apo B locus, was tested with a multivariate analysis of variance (MANOVA) of the lipoprotein data with nesting by genotypes or by patient/control status, was also tested by a multivariate analysis of variance (MANOVA) of the lipoprotein data with nesting by genotypes or by patient/control status (SPSS-X, Release 3.1). The degree of linkage disequilibrium between the two RFLPs was estimated by using the correlation coefficient A. Since no chromosome of the haplotype X2R2 was observed unambiguously, the haplotypes of individuals doubly heterozygous for the two RFLPs were all assumed to be X1R2 and X2R1. A result was considered to be statistically significant at ρ<0.05.

**Results**

Table 2 summarizes the clinical and lipoprotein parameters of the patient and control groups. It confirms the well-known correlation between several lipoprotein parameters and the risk for coronary heart disease. A diagram of the restriction map of the 3' end of the apo B gene and the restriction fragment patterns of the Xba I and the EcoR I RFLPs are shown in Figure 1. For Xba I, we designate the 3.5 kb fragment caused by the presence of the cutting site as the X2 allele, and for EcoR I, the 12.5 kb fragment caused by the absence of the cutting site as the R2 allele (Figure 1B).

Table 3 shows the allele frequencies of these polymorphisms in patients and controls. The observed genotype frequencies did not differ significantly from those expected for a sample from a population in Hardy-Weinberg equilibrium. The haplotype, X2R2, was not observed unambiguously in any of the individuals, indicating a degree of linkage disequilibrium between these two RFLPs (Δ=0.46). There was no difference in the allele frequencies of the Xba I polymorphism between patients and controls. The frequency of the rare allele (R2) of the EcoR I polymorphism was significantly higher among patients than among controls; 42% of the patients carried the R2 allele compared to only 27% of the controls. From the results shown in Table 2, it can be predicted that the proportion of patients with high Lp(a) levels will be greater than in the control group. This raises the possibility of an association between apo B RFLP genotypes and Lp(a) levels. To investigate this, the genotype and allele frequencies of the EcoR I polymorphism and Lp(a) levels was tested with a one-way analysis of variance (ANOVA) of the lipoprotein data by using the Minitab statistical computing system (State College, Philadelphia, PA). Levels of serum total, LDL, and VLDL triglycerides were skewed to the right. Therefore, statistical calculations were carried out after logarithmic transformation of the data. For these calculations, the six patients taking bezafibrate were omitted. The F statistic was used to test the significance of differences among the lipid levels of subgroups with different genotypes. When significant results were obtained, Scheffe's tests were performed to calculate the statistical significance of differences in mean lipoprotein levels between the subgroups with different genotypes (homozygotes for allele 1 vs. homozygotes for allele 2, heterozygotes vs. homozygotes for allele 1 or 2). The significance of differences among the lipid levels of patients and controls was tested by using Student’s t test. To correct for the many statistical tests performed, Bonferroni corrections were carried out for all the p values. The null hypothesis, that phenotypic variance is not determined by genotypes or by patient/control status, was also tested by a multivariate analysis of variance (MANOVA) of the lipoprotein data with nesting by genotypes or by patient/control status (SPSS-X, Release 3.1). The degree of linkage disequilibrium between the two RFLPs was estimated by using the correlation coefficient A. Since no chromosome of the haplotype X2R2 was observed unambiguously, the haplotypes of individuals doubly heterozygous for the two RFLPs were all assumed to be X1R2 and X2R1. A result was considered to be statistically significant at ρ<0.05.
Table 2. Clinical, Lipid, and Lipoprotein Parameters of Patients with Coronary Heart Disease and Matched Controls

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Patients (n=106)</th>
<th>Controls (n=118)</th>
<th>Statistical evaluation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age (years)</td>
<td>47.3±5.7</td>
<td>49.7±5.4</td>
<td>ns</td>
</tr>
<tr>
<td>Smokers (%)</td>
<td>45.3</td>
<td>25.4</td>
<td>ns</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>27.2±7.2</td>
<td>25.2±2.7</td>
<td>ns</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>220.3±35.6</td>
<td>206.4±37.7</td>
<td>ns</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>40.9±8.1</td>
<td>49.9±16.1</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>HDL II cholesterol</td>
<td>12.3±3.8</td>
<td>17.4±10.6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>HDL III cholesterol</td>
<td>28.8±5.6</td>
<td>32.8±9.9</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>155.9±35.9</td>
<td>137.4±35.6</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>VLDL cholesterol</td>
<td>25.2±16.0</td>
<td>19.2±18.1</td>
<td>ns</td>
</tr>
<tr>
<td>Total triglycerides</td>
<td>146.8±74.0</td>
<td>124.9±73.4</td>
<td>ns</td>
</tr>
<tr>
<td>LDL triglycerides</td>
<td>45.5±15.6</td>
<td>42.3±22.9</td>
<td>ns</td>
</tr>
<tr>
<td>VLDL triglycerides</td>
<td>91.5±66.2</td>
<td>69.9±64.3</td>
<td>ns</td>
</tr>
<tr>
<td>Apo B</td>
<td>107.8±22.0</td>
<td>98.1±24.4</td>
<td>ns</td>
</tr>
<tr>
<td>LDL apo B</td>
<td>96.0±19.1</td>
<td>80.2±18.7</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>VLDL apo B</td>
<td>11.8±10.4</td>
<td>17.9±15.4</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>122.0±23.3</td>
<td>147.4±34.9</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>46.3±8.6</td>
<td>51.3±12.9</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td>24.9±28.9</td>
<td>14.6±17.6</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are means±SD and are given in mg/dl unless otherwise indicated.
*After Bonferroni correction.
ns=nonsignificant, MANOVA=multivariate analysis of variance, HDL=high density lipoprotein, LDL=low density lipoprotein, VLDL=very low density lipoprotein, apo=apolipoprotein.

Table 3. Genotype Distribution and Relative Allele Frequencies of RFLPs Detected with Enzymes, Xba I and EcoR I, in Patients and Controls

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Genotype distribution*</th>
<th>Relative allele frequencies†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X1X1</td>
<td>X1X2</td>
</tr>
<tr>
<td>Xba I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>30 (0.28)</td>
<td>56 (0.53)</td>
</tr>
<tr>
<td>Controls</td>
<td>29 (0.25)</td>
<td>65 (0.55)</td>
</tr>
<tr>
<td></td>
<td>X1= 8.5 kb</td>
<td>X2= 3.5 kb</td>
</tr>
<tr>
<td>EcoR I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients</td>
<td>R1R1 62 (0.58)</td>
<td>37 (0.35)</td>
</tr>
<tr>
<td>Controls</td>
<td>R1R2 86 (0.73)</td>
<td>30 (0.25)</td>
</tr>
<tr>
<td>Patients with Lp(a)&gt;30 mg/dl</td>
<td>R2R2 16 (0.53)</td>
<td>11 (0.37)</td>
</tr>
<tr>
<td>Controls with Lp(a)&gt;30 mg/dl</td>
<td>R1= 10.5 kb</td>
<td>R2= 12.45 kb</td>
</tr>
</tbody>
</table>

*The number of individuals (relative genotype frequencies in parentheses).
†The significance of differences was calculated from allele frequencies obtained by gene counting.

In the patient group, homozygotes for the rare allele (R2) of the EcoR I polymorphism had significantly higher serum levels of VLDL apo B than did heterozygotes or homozygotes for the frequent allele (R1). The same trend, although not significant, was observed in the control sample. Significant associations between the R2 allele and higher serum levels of total triglycerides, VLDL triglycerides, and VLDL cholesterol were found in the control group. A trend for these associations was also observed among the patients but did not reach statistical significance (Table 4).

A trend for an association between the Xba I polymorphism and differences in serum total and LDL cholesterol levels was found for patients and controls. Patients and
controls with the genotype X2X2, had higher total and LDL cholesterol levels than did those with the genotype X1X2 or X1X1 (Table 5). When subjects with Lp(a) levels above 30 mg/dl (30 patients and 24 controls) were omitted, the same trend was observed. Total cholesterol in patients was: X1X1 (n=16), 218.5±40.8 mg/dl; X1X2 (n=40), 214.1±32.8 mg/dl; and X2X2 (n=14), 233.9±41.6 mg/dl. The total cholesterol in controls was: X1X1 (n=21), 205.2±43.5 mg/dl; X1X2 (n=54), 200.2±36.9 mg/dl; and X2X2 (n=19), 213.3±39.8 mg/dl. However, these trends were not statistically significant.

None of the other lipoprotein parameters listed in Table 2 was significantly associated with any RFLP genotype. To examine if there was any difference in the composition of VLDL particles of subjects with different genotypes, the ratios of VLDL cholesterol/VLDL triglycerides, VLDL apo B/VLDL triglycerides, and VLDL apo B/VLDL cholesterol were calculated for all subjects. No statistically significant associations between genotypes and these ratios were observed (data not shown).

**Discussion**

The aim of the present study was to determine whether genetic variation at the 3' end of the apo B gene is involved in the pathogenesis of coronary atherosclerosis and in the regulation of the serum levels of various lipoproteins.

The frequency of the rare allele of the EcoR I polymorphism (R2) caused by the absence of the cutting site for the enzyme was nearly twice as high in the patient group (24.1%) as in the control group (14.4%). This is in agreement with the results obtained by Hegele et al. in a Boston-based study. As has already been pointed out by these authors, it would be rather surprising to find a difference greater than that, since many independent genetic and nongenetic factors are responsible for the development of coronary heart disease. Hegele et al. also reported an increased frequency of the X1 allele of the Xba I polymorphism in the patient group compared to the control group. This result was not confirmed by our study.

In the study by Hegele et al., there was no significant association between serum lipoprotein levels and these DNA polymorphisms. In our study, a strong association was observed between the R2 allele of the EcoR I polymorphism and several VLDL components. In the control group, the R2 allele was correlated with higher levels of total triglycerides, VLDL triglycerides, and VLDL cholesterol. The two individuals with the genotype, R2R2, had lower levels of total and VLDL triglycerides than did individuals with the genotype, R1R2, but that might be explained by the small sample size. The R2 allele of this polymorphism was significantly associated with higher levels of VLDL apo B in the group of patients with coronary heart disease and weakly associated in the control group. Surprisingly, the mean level of VLDL apo B in the patient group was lower than in the control group, and currently we have no explanation for this finding. It appears that an association exists between the R2 allele and increased serum levels of VLDL.

The X2 allele of the Xba I polymorphism has been found to be associated with increased serum levels of cholesterol and triglycerides by others. These results could not be confirmed in our study. Homozygotes for the X2 allele exhibited the highest serum total and LDL cholesterol levels among patients and controls. Heterozygotes for this polymorphism, however, had the lowest total and LDL cholesterol levels in the patient group. The differences were not statistically significant; this finding might be a chance result and needs to be confirmed in a larger study. Thus from our data, a simple co-dominant action of this allele on the regulation of serum cholesterol levels seems to be unlikely. The strong association between the X2 allele and serum levels of apo B reported by Berg was not observed in our study.

In Table 1 we have summarized the similarities and discrepancies in the results obtained by various authors using apo B RFLPs. There are several possible explana-
were of Austrian descent, it is likely that they represented why some of the observations presented here have not at different times on chromosomes with a different background of marker alleles. Since all subjects of our study origins. 17,18
 association of the Xba I and the EcoR I polymorphism.
 been made in samples of populations with mixed ethnic
tions for the discrepancies. First, the clinical criteria applied for the selection of the study groups were different. Second, in many reports the sample sizes are small, and the studies would only have adequate power to detect a difference in allele frequency if the attributable risk associated with the allele were high. Third, in some studies serum lipid levels were measured in nonfasting subjects. Fourth, the fact that there is considerable variation in the frequencies of these RFLPs among different ethnic groups 14-20 is a further argument against the direct comparison of the various studies. An association between a mutation responsible for a certain phenotype and one of these polymorphisms should also be found for the other RFLP. In our study, coronary heart disease and some of the lipoprotein parameters were significantly associated with only one of these RFLPs. This fact could be explained by the low degree of linkage disequilibrium between the two RFLPs. The possibility exists that with a much larger number of individuals, associations with the other RFLP could also be demonstrated. In several other studies, an observed association was also restricted to one of these RFLPs. 11,14,15

Correlation of the R2 allele with an increased risk for coronary heart disease or peripheral arterial disease has now been found in the U.S.,17 the U.K.,18 and the Austrian populations. It is not possible, however, to draw from these statistical data any conclusion on the underlying biochemical mechanisms of this increased risk. The point mutation which gives rise to the EcoR I RFLP also causes a change in the primary structure of the mature apo B protein. There is no direct evidence that this DNA mutation is causally related to the pathogenesis of coronary atherosclerosis, but this explanation cannot be ruled out. However, it is more likely that this RFLP is in linkage disequilibrium with a mutation elsewhere in the apo B
gene or in another nearby gene, which predisposes to coronary heart disease. The most important finding of our study was the association of the R2 allele with coronary heart disease and elevated serum VLDL levels. Further investigation will be needed to determine whether these findings reflect a causal relationship between VLDL levels and increased risk for coronary heart disease.

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