Apolipoprotein B Gene Haplotypes

Yuanhong Ma, John A.A. Ladias, Rene Büttler, Verne N. Schumaker, Stylianos E. Antonarakis, Aldons J. Lusis, Camilla Heinzman, and Peter O. Kwiterovich

Berg et al. (Clin Genet 1986;30:515-520) have reported that an Xba I DNA polymorphic site in exon 26 of the apolipoprotein (apo) B gene is associated both with the Ag(x/y) immunoochemical polymorphism and with elevated serum lipoprotein levels. Ma et al. (Arteriosclerosis 1987;7:301-305) have reported that the same Xba I polymorphism is associated with a different immunoochemical polymorphism, Ag(c/g). To extend and clarify these observations, we have determined the Ag and Xba I polymorphism for 106 individuals. We find that the Xba I restriction fragment length polymorphism is in linkage disequilibrium with both Ag(x/y) and Ag(c/g) loci; thus, all 31 Xba I(X1/X1) genotypes observed in this study are also Ag(y/y). All but one of 22 Xba I(X2/X2) genotypes are also Ag(g/g). For individuals homozygous at either two or three of these loci, it was possible to determine the haplotypes for 128 apo B alleles. Of the eight possible apo B haplotypes, only four were represented in this unambiguous subpopulation, although other minor haplotypes were present in the total population from which it was derived. The identification of major apo B haplotypes in human populations may simplify the search for significant correlations between certain apo B alleles and lipid levels and atherosclerosis.

We have performed a more extensive study on the relationship between the Xba I RFLP and the two Ag sites, Ag(c/g) and Ag(x/y) using 106 individuals. In this communication, we report that the Xba I RFLP of apo B is associated with both the Ag(c/g) and the Ag(x/y) sites. All of the Xba I(X1/X1) phenotypes examined in this study are also Ag(y/y). All but one of the Xba I(X2) alleles appear to be Ag(g). We also show that these data are explained by the existence of four major alleles associated with human apo B, although other minor alleles must exist.

Lending additional interest are studies providing evidence both for and against an association between human apo B polymorphisms and serum lipid and apo B levels and myocardial infarction. In the studies reported here, however, no statistically significant differences in lipid levels were found to be associated with any of the apo B alleles.

Methods

Study Population

The Johns Hopkins University Coronary Artery Disease (JHU-CAD) Study is a consecutive case study of men ages 50 years or less and of women ages 60 years or less who were undergoing elective nonemergency coronary arteriography at The Johns Hopkins Hospital. The spouses of the index cases were also studied. In all, 47 male index cases, 29 female index cases, 6 male spouses, and 24 female spouses were included in our sample. The details of the study will be published elsewhere, but the pertinent information is briefly summarized here. After informed consent, each index case had a medical and family history and brief physical examination. After an overnight fast of at least 12 hours,
blood was collected into tubes containing solid EDTA (1 mg/ml blood). Plasma was separated from the buffy coat and erythrocytes by centrifugation at 10°C. Plasma was removed for the determination of plasma lipids, lipoproteins, andapolipoproteins as described by Kwi-terovich et al.13 The leukocytes were saved at 4°C and DNA was isolated as described by Kunkel et al.14

**Genomic DNA Preparation and Southern Analysis**

The DNA preparation and Southern analysis were performed in a manner similar to that previously described,4 using AB6 and AB14 as probes.15 Briefly, human DNA form 106 individuals was prepared from white blood cells in peripheral blood. After cleavage with Xba I restriction enzyme, 5 to 10 μg of DNA from each individual was electrophoresed through 1% agarose gels, and was transferred onto nylon nitrocellulose filters. Apo B cDNA fragments were radiolabeled with alpha-32P-dCTP to specific activities ranging from 8 × 10⁶ to 2 × 10⁷ cpm/μg. Hybridizations and autoradiographs were then performed as described by Ma et al.4

**Determination of Ag Phenotypes**

The Ag(c/g) and Ag(x/y) phenotypes were determined using the passive hemagglutination inhibition method.16 A representative assay for Ag(x) will be briefly described: Ag(x+) LDL were chemically coupled to red blood cells, which were then agglutinated with anti-Ag(x+) antiserum in microtiter plates. If an unknown serum was added, agglutination was inhibited if the unknown LDL were Ag(x+). The original assay was modified chiefly by the replacement of diazotized benzidine with chromium chloride for fixation of the LDL to the red cells and by the use of microtiter plates.17

**Determination of Unambiguous Haplotypes**

For 64 of the 106 individuals, it was possible to determine the haplotypes for each of their two apo B genes. Thus, for individuals who are homozygous at all three sites or heterozygous at only one site, their haplotypes can be constructed directly from knowledge of their Ag and RFLP phenotypes. For example, the homozygous Ag(c/c), Ag(y/y), Xba I(X1/X1), determines the haplotype for each of the two apo B genes as Ag(c), Ag(y), Xba I(X1). (Here the ordering of the three sites is totally arbitrary, and does not reflect the actual order in the gene which is unknown at this time.) For individuals heterozygous at a single site, such as Ag(c/c), Ag(y/y), Xba I(X1/X2), the haplotypes for the two apo B alleles will be Ag(c), Ag(y), Xba I(X1) and Ag(c), Ag(y), Xba I(X2). We were not able to determine unique haplotypes for persons who were heterozygous at more than one site.

**Results and Discussion**

**Significant Linkage Disequilibrium Found between All Three Sites**

Either a 5 kb or an 8.6 kb fragment, caused by the presence or absence, respectively, of an Xba I restriction

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ag(x/x)</th>
<th>Ag(x/y)</th>
<th>Ag(y/y)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>X1X1</td>
<td>0</td>
<td>0</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>X1X2</td>
<td>0</td>
<td>20</td>
<td>33</td>
<td>53</td>
</tr>
<tr>
<td>X2X2</td>
<td>4</td>
<td>16</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>36</td>
<td>66</td>
<td>106</td>
</tr>
</tbody>
</table>

p<0.005.

**Table 3. Association between Ag(x/y) and Ag(c/g) Polymorphism of Human Apoprotein B**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Ag(c/c)</th>
<th>Ag(c/g)</th>
<th>Ag(g/g)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ag(x/x)</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Ag(x/y)</td>
<td>2</td>
<td>8</td>
<td>26</td>
<td>36</td>
</tr>
<tr>
<td>Ag(y/y)</td>
<td>8</td>
<td>43</td>
<td>15</td>
<td>66</td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
<td>51</td>
<td>45</td>
<td>106</td>
</tr>
</tbody>
</table>

p<0.05.
Table 4. Ag-Restriction Fragment Length Polymorphisms Haplotyping for Ag (c/g), Ag (x/y), and Xba I Polymorphic Sites

<table>
<thead>
<tr>
<th>Possible haplotypes (2^[a] = 8)</th>
<th>Number of alleles of that haplotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
</tr>
<tr>
<td>(1) - c - x - x - X1 - -</td>
<td>0</td>
</tr>
<tr>
<td>(2) - g - x - x - X1 - -</td>
<td>0</td>
</tr>
<tr>
<td>(3) - c - y - y - X1 - -</td>
<td>38</td>
</tr>
<tr>
<td>(4) - g - y - y - X1 - -</td>
<td>35</td>
</tr>
<tr>
<td>(5) - c - x - x - X2 - -</td>
<td>0</td>
</tr>
<tr>
<td>(6) - g - x - x - X2 - -</td>
<td>23</td>
</tr>
<tr>
<td>(7) - c - y - y - X2 - -</td>
<td>0</td>
</tr>
<tr>
<td>(8) - g - y - y - X2 - -</td>
<td>32</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
</tr>
</tbody>
</table>

*p<0.005.

Ag(g) alleles were also Xba I(X2). Interestingly, as seen in the first column, about 90% of the Ag(c) alleles appear to be Xba I(X1), while 70% of the Xba I(X1) alleles appear to be Ag(c), as seen in the first row of Table 2.

The nucleotide change associated with the Xba I restriction site is a silent T → C alteration affecting only the third nucleotide of a threonine codon at amino acid 2488.19 Since no amino acid change is involved, this nucleotide change cannot cause either the Ag(c/g) or the Ag(x/y) immunochemical polymorphisms. Moreover, if the Xba I restriction site were to coincide with either Ag determinant, the corresponding data presented in Tables 1 or 2 would lie entirely on a principal diagonal; off-diagonal terms are sufficient to exclude identity.

The relationship between the Ag(x/y) phenotype and the Ag(c/g) phenotype was analyzed for 146 individuals and the results are shown in Table 3. The hypothesis of linkage equilibrium is rejected at the p>0.05 level. Inspection of these data shows that most Ag(c) are also Ag(y), although the converse is not true. Also, most Ag(x) are also Ag(g); again, the converse is not true.

That linkage disequilibrium exists between the Ag(x/y) and Ag(c/g) loci is not a new observation; for example, it is clearly apparent from the haplotype analysis presented by Böttler et al.1 A quantitative assessment of linkage disequilibrium is given by the delta value,19,20 with a value of 0 representing linkage equilibrium and a value of 1 representing complete linkage disequilibrium; according to this measure, the Xba I locus shows the same degree of linkage disequilibrium with Ag(x) and Ag(c), with delta values of 0.56 and 0.59, respectively. Between Ag(x/y) and Ag(c/g), the delta value is smaller at 0.31. The 95% confidence limits for these three delta values are 0.48/0.64, 0.52/0.67, and 0.25/0.37, respectively.

Haplotype Analysis

To better define the overall relationship between the three polymorphic sites, Ag(x/y), Ag(c/g), and the Xba I RFLP, unambiguously determined allelic haplotypes were identified for 64 individuals (128 alleles) from this population of 106 persons, and the results are shown in Table 4. The allelic haplotypes for the remaining 42 persons were ambiguous because of their heterozygosities at more than one polymorphic site. All of the eight possible allelic haplotypes, which can be generated through random DNA recombination between the three sites are listed in Table 4. Assuming allele frequencies for Ag(c) of 0.3, Ag(g) of 0.7, Ag(x) of 0.25, Ag(y) of 0.75, Xba I(X1) of 0.54, and Xba I(X2) of 0.46, the expected number of alleles possessing each haplotype was computed and listed in parallel with the number observed. Of the eight possible haplotypes, only four were unambiguously found in this subpopulation, although other minor haplotypes must be present in the total population: the probability that this uneven distribution was due to chance was *p<0.005.

Moreover, for these four major alleles, all Xba I(X1) were also Ag(y), all Xba I(X2) were Ag(g), all Ag(c) were Ag(y), and all Ag(x) were Ag(g). It can be seen that the presence of four major haplotypes in the subpopulation studied explains in a conceptually simple way the linkage disequilibria apparent in Tables 1, 2, and 3.

When the double or triple heterozygous individuals are deleted in forming the subpopulation, the estimates of haplotype frequencies become biased. This results in an overestimation of the frequencies of the common haplotypes and an underestimation of the frequencies of minor haplotypes. These minor haplotypes are apparent in three cases where an Xba I(X2) must be an Ag(c) (Table 2) and in two cases where an Ag(c) must be an Ag(x) (Table 3).

Cholesterol, Triglyceride, and Apoprotein B Levels

Because of the considerable interest in a possible association between cholesterol, triglyceride, and apo B levels with the possession of certain apo B alleles, an attempt was made to demonstrate significant correlations within this population. Although some small differences in mean levels were observed in agreement with the reports of Law et al.,2 Berg et al.,3 Tikkanen et al.,4 and Robinson,5 there were no statistically significant differences in either cholesterol or total plasma apo B between any of the haplotypes, or with any of the individual markers.

References

4. Ma Y, Schumaker VN, Böttler R, Sparkes RS. Two DNA restriction fragment length polymorphisms associated with Ag(lz) and Ag(c/g) antigenic sites on human apolipoprotein B. Arteriosclerosis 1987;7:301-305

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Y H Ma, J A Ladias, R Bütler, V N Schumaker, S E Antonarakis, A J Lusis, C Heinzman and P O Kwiterovich

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