Does the EcoRI Polymorphism in the Human Apolipoprotein B Gene Affect the Binding of Low Density Lipoprotein to the Low Density Lipoprotein Receptor?

J.J. Gallagher and N.B. Myant

In human populations, there is an association between coronary artery disease and a polymorphism in the apolipoprotein B (apo B) gene detected with the enzyme EcoRI. This polymorphism gives rise to two apo B alleles, one (E+) encoding glutamic acid and the other (E−) encoding lysine at position 4,154 in apo B-100, the protein of low density lipoprotein (LDL). We have tested the hypothesis that this amino acid substitution indirectly influences proneness to coronary artery disease by affecting the binding of LDL to LDL receptors. The receptor-binding affinities of LDLs from eight pairs of subjects with genotypes E+/+ and E−/− who were matched for other apo B genotypes were determined in vitro. There was no significant difference between the binding affinities of LDLs from the two groups of subjects. Our results strongly suggest that the amino acid at position 4,154 does not influence the function of the receptor-binding domain in apo B-100 and that the association between coronary artery disease and the EcoRI polymorphism is not mediated by an effect of the polymorphism on serum LDL concentration. In view of our findings, it would be of interest to examine the effect of the amino acid substitution on the binding of LDL to arterial proteoglycans and on the oxidizability of LDL by cells in culture. (Arteriosclerosis and Thrombosis 1992;12:256-260)

Polymorphism in the human apolipoprotein B (apo B) gene detected with the restriction enzyme EcoRI gives rise to two codominant apo B alleles, one with the EcoRI recognition site (E+) and the other without it (E−).1 The E+ allele encodes glutamic acid (Glu) at residue 4,154 in apo B-100 (the protein component of low density lipoprotein [LDL]), while the E− allele encodes lysine (Lys) at this position. The EcoRI polymorphism in the apo B gene, which corresponds to the t/z polymorphism of the Ag system,2 appears to be universally present in humans, with the E− allele occurring at a frequency of between 0.10 and 0.15 in most human populations (see Reference 3). We4 and others5,6 have shown that the frequency of the E− allele is significantly higher in men with coronary artery disease (CAD) than in healthy men from the same population.

The major pathway for removal of LDL from the plasma is mediated by high-affinity binding of the apo B-100 in LDL particles to LDL receptors on the surfaces of cells. The change from Glu to Lys at position 4,154 causes a charge alteration of two units, which might be expected to affect the binding properties of the protein. Hence, it is reasonable to suppose that LDL particles containing apo B-100 encoded in the E− allele (E− apo B-100) are catabolized more slowly and therefore reach a higher serum concentration than do particles with apo B-100 encoded in the E+ allele (E+ apo B-100). This could contribute to the association, in human populations, between CAD and the E− allele, as coronary atherosclerosis is related positively to serum cholesterol concentration.7 Although a statistically significant association between the E− allele and serum cholesterol level was not observed in the previously cited studies, in one study6 the mean serum cholesterol level in patients and controls was highest in E−/− subjects, lowest in those with E+/+, and intermediate in those with E+/−. This is consistent with the possibility that the EcoRI polymorphism is one among several factors contributing to population variability in serum cholesterol level.

In the work described in this report, we have tested the hypothesis that the receptor-binding affinity of LDL from E−/− homozygotes is lower than that of LDL from E+/+ homozygotes.
Methods

Subjects

To increase our chances of detecting a small effect of the EcoRI polymorphism on receptor binding of LDL, the E+/+ and E−/− subjects were matched pairwise for sex, as far as possible for age, and for polymorphisms in the apo B gene known to be associated with CAD or plasma lipid concentration (those detected with the enzymes Pvu II, Xba I, and Msp I) (see Table 1 legend for genotypes). To ensure that any differences observed between the behavior of LDLs from the two groups of subjects were not attributable to differences in lipid composition of the LDL, the subjects were matched for serum lipid concentrations. In addition, the apo B (arginine^350-glutamine) mutation present in familial defective apolipoprotein B-100 (FDB) was excluded in all subjects. Despite these restraints, we were able to assemble eight pairs of subjects. The 16 individuals who met the aforementioned criteria were identified by screening a total of 521 people, including 174 patients from the Hammersmith Hospital Cardiac Clinic, 264 patients from the Hammersmith Hospital Lipid Clinic, 36 unaffected relatives and spouses of index patients with FDB, and 47 healthy volunteers from other sources. None of the 16 subjects selected had been diagnosed as having familial hypercholesterolemia or any other disorder known to influence LDL metabolism, and none was taking drugs known to affect plasma lipoprotein concentrations. Five had a history of myocardial infarction, two of whom were E+/+ and three of whom were E−/− (see Table 1). The mean ages, serum LDL cholesterol concentrations, and serum triglyceride concentrations did not differ significantly between the E+/+ and E−/− groups of subjects.

Analysis of DNA and Plasma Lipids

DNA was prepared from whole blood by the method of Kunkel et al. Genotypes were determined as previously described. Serum lipid and lipoprotein concentrations were determined in blood samples taken after an overnight fast, as described elsewhere. 

Low Density Lipoprotein Binding

LDL (d=1.019–1.063 g/ml), prepared by sequential ultracentrifugation, was labeled with 125I as described. The specific radioactivity of the stock solution used for each experiment was adjusted to =100 cpm/ng by addition of unlabeled LDL. The binding of LDL to LDL receptors on normal human skin fibroblasts at 4°C was determined by two methods. Direct binding was assayed by the method of Goldstein et al. Dissociation constants (Kd) were calculated from the binding curves by Scatchard analysis. The ability of the test sample of LDL to compete with a standard sample of radioactive LDL for binding to LDL receptors on fibroblasts was determined by a competitive-binding assay.

Experimental Design

The same normal cell line was used for testing all LDL samples. However, because of the time taken to

![Figure 1. Low density lipoprotein (LDL) binding and competition curves from experiment 1. Upper panel: High-affinity binding of 125I-labeled E−/− (○, □) and E+/+ (●, △) LDL by skin fibroblasts at 4°C as a function of LDL concentration (µg protein/ml) in the incubation medium. Lower panel: Amounts of a standard sample of E+/+ 125I-LDL bound to skin fibroblasts at 4°C in the presence of competing unlabeled E−/− (●, ●) or E+/+ (○, □) LDL at increasing concentrations in the medium. For the competition curves, the concentration of the standard 125I-LDL was 3 µg LDL protein/ml of medium in each well. For other details, see “Methods.” All values are means of duplicate estimations. LDL samples were obtained from subjects 9 (●), 10 (●), 11 (□), and 12 (○) (See Table 1).]
Analysis of Results

A two-way analysis of variance was used to test for significance of the differences between mean reciprocal $K_d$ values and mean LDL concentrations giving 50% competition estimated for the two genotypes (E+/+ and E−/−). Differences were tested for significance by determining the $F$ ratios. In the competitive-binding studies, the amounts of radioactive LDL bound to the cells (nanograms per milligram of cell protein) as a function of the concentration of the test sample of LDL (micrograms of protein per milliliter) were fitted to the equation

$$y = Ae^{-kx} + C$$

(\text{where } y \text{ is the amount of cell-bound radioactive LDL and } x \text{ is the LDL concentration in the medium})

by the method of nonlinear least squares, and the values of $A$, $k$, and $C$ were determined by use of a EUREKA program.14 The value of $x$ for $y=50\%$ (the concentration of the test sample required to displace 50% of the bound radioactive LDL) was calculated from the equation

$$x = \frac{1}{k} \left( \ln \frac{50-C}{A} \right)$$

Results

Figure 1 shows the direct-binding and competition curves obtained from the two pairs of E+/+ and E−/− LDL samples used for the same experiment. $K_d$ values were derived from the direct-binding curves by Scatchard analysis. Tables 1 and 2 show the results of

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Subject</th>
<th>Age (yr)</th>
<th>LDL-C (mmol/l)</th>
<th>Triglyceride (mmol/l)</th>
<th>EcoRI genotype</th>
<th>$1/K_d$</th>
<th>Concentration at 50% competition (µg/ml)</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>63</td>
<td>4.2</td>
<td>3.62</td>
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<td>2</td>
<td>3</td>
<td>37</td>
<td>3.1</td>
<td>0.57</td>
<td>−/−</td>
<td>0.32</td>
<td>5.0</td>
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<tr>
<td>3</td>
<td>4</td>
<td>48</td>
<td>4.2</td>
<td>2.75</td>
<td>+/+</td>
<td>0.63</td>
<td>6.9</td>
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<tr>
<td>4</td>
<td>5</td>
<td>37</td>
<td>3.1</td>
<td>0.52</td>
<td>+/+</td>
<td>0.36</td>
<td>6.4</td>
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<tr>
<td>5</td>
<td>6</td>
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<td>10.1</td>
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<td>0.97</td>
<td>7.4</td>
</tr>
<tr>
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<td>4.1</td>
<td>1.00</td>
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<td>1.67</td>
<td>7.6</td>
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<tr>
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<td>60</td>
<td>3.7</td>
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<td>8.7</td>
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<tr>
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<td>3.9</td>
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<td>0.71</td>
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<td>0.98</td>
<td>2.9</td>
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<tr>
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<td>60</td>
<td>3.4</td>
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<td>0.83</td>
<td>3.0</td>
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<tr>
<td>15</td>
<td>16</td>
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<td>4.8</td>
<td>2.13</td>
<td>+/+</td>
<td>0.81</td>
<td>3.9</td>
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<td>16</td>
<td>47.6±11.4</td>
<td>3.5±0.4</td>
<td>1.87±1.3</td>
<td>0.70±0.23</td>
<td>5.05±2.5</td>
<td></td>
<td></td>
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<tr>
<td>Mean E+/+ ±SD</td>
<td>51.5±9.8</td>
<td>4.1±0.6</td>
<td>1.65±0.8</td>
<td>0.90±0.40</td>
<td>5.76±1.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Collect blood samples from subjects from different parts of England, all 16 samples of LDL could not be tested in the fresh state on cells grown to optimal density on the same day. In each of four separate experiments, direct and competitive-binding assays were performed on samples from two pairs of matched donors on the same day. In each experiment, cells stored in liquid nitrogen were thawed, transferred to tissue-culture flasks in 15 ml of growth medium containing 10% fetal calf serum, and incubated at 37°C. When the cells had grown to confluence, the medium was removed and the cells were trypsinized and suspended in growth medium containing 10% fetal calf serum, and incubated at 37°C. When the cells had grown to confluence, the medium was removed and the cells were trypsinized and suspended in growth medium containing 10% fetal calf serum. One milliliter of the cell suspension containing 1×10⁶ cells was added to each 35-mm well, and the wells were incubated at 37°C for 5 days. On day 5, the medium was changed to one in which the calf serum was replaced by 5% lipoprotein-deficient human serum, and the incubation was continued for 48 hours. All assays were performed on day 7. The blood samples used for a single experiment were taken over a period of not more than 3 days, and the interval between experiments was in no case longer than 3 weeks.

High-affinity binding of radiolabeled low-density lipoprotein (LDL) to skin fibroblasts at 4°C in vitro was measured in duplicate at concentrations of 0–20 µg LDL protein/ml medium. Values for $K_d$ were calculated by Scatchard analysis of the binding curves. For statistical analysis, $K_d$ values were normalized by taking their reciprocals. For competitive-binding assays at 4°C, the unlabeled test sample of LDL was added to the incubation wells in duplicate at increasing concentrations (0–20 µg LDL protein/ml medium) together with a fixed amount of a standard sample of radiolabeled E+/+ LDL (3 µg LDL protein/ml medium). The concentration of unlabeled LDL required to reduce the amount of cell-bound radiolabeled LDL to 50% of the baseline value was calculated as described in "Methods." Subjects 2 and 4 were female. All others were male. All subjects had genotypes $Pvu$ II+/+, $Xba$ I+/+, or $Msp$ I+/+. LDL-C, serum LDL cholesterol concentration; −/−, restriction site absent; +/+, restriction site present; *, clinical history of myocardial infarction.
EcoRI Polymorphism and LDL Binding

Table 2. Analysis of Variance of Low Density Lipoprotein Binding to Fibroblasts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Between experiments</th>
<th>Between genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1/K_d), reciprocal of the dissociation constant of low density lipoprotein (LDL) binding to LDL receptors; 50% competition, the concentration of LDL required to reduce the amount of cell-bound radioactive LDL to 50% of the baseline value.</td>
<td>7.2 (p=0.01)</td>
<td>2.57 (p=0.1)</td>
</tr>
<tr>
<td>50% Competition</td>
<td>0.9 (p=0.1)</td>
<td>1.8 (p=0.1)</td>
</tr>
</tbody>
</table>

Discussion

Human apo B-100 is thought to contain a single LDL-receptor-binding domain that includes amino acid sequences extending from residue 3,147 to residue 3,500 (see Scott13). Hence, it might be expected that a charge alteration of two units at position 4,154 would affect the receptor-binding properties of LDL. Our findings provide no evidence for this possibility. However, an association between CAD and the E⁺ allele has been demonstrated only by comparing large numbers of unrelated individuals with and without CAD.4-6 Hence, if the association in the population is caused by a difference in the binding properties of LDL particles with E⁺ and those with E⁻ apo B-100, this difference may be so small that it cannot be demonstrated in a study of eight pairs of subjects, however carefully they are matched. This explanation of our negative findings seems unlikely. Statistical analysis of our observations, using the error variance derived from a two-way analysis of variance, shows that the method used for estimating \(K_d\) would have revealed a minimum difference of 17% between genotypes with 90% confidence. This result, combined with the finding that the concentration required for 50% competition was lower with E⁺ LDL than with E⁻ LDL (although not significantly so), suggests that the presence of a Lys residue at position 4,154 does not alter the function of the LDL-receptor-binding domain of apo B-100. In support of this, an association between plasma cholesterol concentration and the presence of a Lys residue at position 4,154 does not alter the function of the LDL-receptor-binding domain of apo B-100. In support of this, an association between plasma cholesterol concentration and the presence of a Lys residue at position 4,154 does not alter the function of the LDL-receptor-binding domain of apo B-100.

A possible explanation for the association in the population between CAD and the EcoRI polymorphism is that the polymorphism affects the susceptibility of LDL to modification by peroxidation in the vicinity of the arterial wall. Biologically oxidized LDL particles are taken up by macrophages, resulting in the formation of the foam cells that are essential components of atherosclerotic lesions (see Reference 16). The mechanism by which oxidation converts LDL into a potentially atherogenic lipoprotein appears to be the formation of decomposition products of lipoprotein fatty acids, followed by covalent linkage of these products to the e-amino groups of Lys residues in apo B.17 LDL particles in which apo B has been modified in this way are ligands for acetylated-LDL receptors on macrophages. It is conceivable that the substitution of Lys for Glu at a specific position in the apo B molecule favors the formation of oxidized species of LDL in vivo.

It is also possible that E⁺ LDL and E⁻ LDL particles have different affinities for extracellular proteoglycans in the arterial wall. Camejo et al.18 have shown that LDL binds to chondroitin sulfate-rich proteoglycans extracted from the human aorta and that binding is mediated by interaction of basic residues in apo B with acidic groups in the polysaccharide moiety of the proteoglycan. Moreover, Camejo et al.18 in a competitive-binding study with synthetic apo B peptides, have shown that a peptide comprising residues 4,230–4,254 associates strongly with aortic proteoglycan in vitro. This suggests that it would be worthwhile to examine the effect of Lys residue at position 4,154 in apo B on the affinity of LDL for aortic proteoglycans. Finally, it is possible that the EcoRI site is in linkage disequilibrium with another polymorphic site, in or near the apo B gene, that influences proneness to CAD.

Acknowledgments

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References

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**Key Words**: EcoRI polymorphism • apolipoprotein B gene • low density lipoproteins • receptor binding • heart disease
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