Apolipoprotein B and a Second Major Gene Locus Contribute to Phenotypic Variation of Spontaneous Hypercholesterolemia in Pigs


Abstract The Lpb\(^b\) apolipoprotein B (apoB) allele occurs in pigs with spontaneous hypercholesterolemia. Low-density lipoprotein (LDL) from these pigs binds to the LDL receptor with a lower affinity and is cleared from the circulation more slowly than control pig LDL. However, the severity of hypercholesterolemia in pigs with the mutant apoB allele is highly variable. This study aimed to determine the metabolic basis for the phenotypic heterogeneity among Lpb5 pigs. Lpb5 pigs were divided into two groups: those with plasma cholesterol greater than 180 mg/dL (Lpb5.1) and those with plasma cholesterol less than 180 mg/dL (Lpb5.2). LDL from both Lpb5.1 and Lpb5.2 pigs was catabolized in vivo and in vitro at a similarly reduced rate. The difference in plasma cholesterol between the two phenotypic groups was in part due to a higher buoyant LDL production rate in Lpb5.1 pigs than in Lpb5.2 pigs. The in vivo LDL receptor status was evaluated by measuring the catabolism of LDL chemically modified to abrogate LDL receptor binding. Approximately 50% of LDL clearance in normal and Lpb5.2 pigs was via the LDL receptor; in Lpb5.1 pigs, 100% of LDL clearance was LDL receptor independent. Quantitative pedigree analysis of the segregation of the plasma cholesterol phenotype suggested that two major gene loci (the apoB locus and a second apparently unlinked locus) contribute to the determination of plasma cholesterol levels in this pig population. (Arterioscler Thromb. 1994;14:409-419.)

Key Words • LDL • mutation • cholesterol • genetics • hyperlipidemia • animal models • LDL receptor

Population genetic studies have demonstrated that heredity contributes significantly to hyperlipidemia. In some instances, particular hyperlipidemic syndromes have been shown to be caused by single-gene mutations. However, the frequency of the known single-gene mutations is still far below the frequency of hyperlipidemia. The population studies therefore predict that multiple gene loci are involved and that in some instances there may be interactions between gene loci to produce a clinical syndrome.

Apolipoprotein B (apoB) may be one such gene locus. ApoB is the primary protein component of low-density lipoprotein (LDL), which carries the bulk of cholesterol in human plasma, and is a ligand for the LDL receptor; thus, apoB functions in the assembly of the LDL particle and mediates its clearance from the blood. Given the central role of apoB in LDL metabolism, apoB mutations alone or in combination with other defects probably contribute significantly to hypercholesterolemia in the human population. One recently discovered apoB point mutation (Arg\(_{590}\)—>Gln) results in LDL particles with reduced LDL receptor binding activity and in some patients is associated with a reduced rate of LDL clearance from the plasma.\(^1\) This mutation is relatively common in the human population, suggesting that clinically relevant mutations in apoB may be abundant.

Hypercholesterolemia is also highly heritable in pigs. Rapacz et al\(^2\) described a strain of pigs bearing an immunogenetically defined lipoprotein-associated marker, designated Lpb5,\(^3\) and having marked hypercholesterolemia and premature atherosclerosis. The antiserum used to identify the Lpb5 pigs was shown to react only with apoB-100,\(^2\) and the apoB gene from Lpb5 pigs has a unique 283-bp insertion at intron 28.\(^4\) In addition, Lpb5 pigs have LDL with defective binding to the LDL receptor\(^5\) and delayed plasma clearance.\(^6\) These observations strongly suggest an association between apoB polymorphisms and hypercholesterolemia in pigs. However, pigs with the Lpb\(^b\) allele of apoB are phenotypically heterogeneous; the more severe phenotype (designated Lpb5.1) involves twofold to fourfold increases in plasma cholesterol, whereas a second phenotypic group (designated Lpb5.2) has only a moderate plasma cholesterol elevation.\(^7\)

In this study, we further characterize pigs with the Lpb5.1 and Lpb5.2 phenotypes to clarify the metabolic contributions to variation in plasma cholesterol in pigs. In addition, quantitative pedigree analysis was applied to suggest a genetic model consistent with the segregation pattern of the plasma cholesterol phenotype.

Methods

Description of the Pig Population

The study population consisted of pigs representing the Lpb5.1, Lpb5.2, and normocholesterolemic phenotypes. The founders of this population carrying the Lpb\(^b\) allele of apoB were donated by Dr Jan Rapacz from the University of Wisconsin Immunogenetic Herd. Non-Lpb5 founder pigs were...
obtained from the University of Wisconsin Arlington Swine Farm. The pigs were housed at the University of Wisconsin-Madison and maintained on a 0% cholesterol, 5% fat diet (University of Wisconsin Gestation Diet).

The pedigree analyzed in this study included 22 sires, 27 dams, and 320 offspring. Blood samples were obtained from all offspring at 3 to 4 months of age (6 weeks after weaning) for determination of plasma cholesterol levels and isolation of genomic DNA for apoB genotype determination as described below. In all, plasma cholesterol determinations from a total of 337 pigs (91 Lpb5.1 pigs, 160 apoB Lpb5.2 pigs, and 86 non-Lpb5 p pigs) were included in the pedigree-phenotype dataset.

Previously, Lpb5 pigs were immunologically identified by the presence of an LDL-borne allotype, designated Lpb5.2. These animals are now identified using the polymerase chain reaction to amplify the target genomic DNA and detect a 283-bp fragment (data not shown). Unreacted glucose and reducing agent were removed by gel filtration on a Sephadex G25 column, followed by dialysis against PBS/EDTA (1 mM) cocktail as described previously. Very-low-density lipoprotein (VLDL, d < 1.006 g/mL), intermediate-density lipoprotein (IDL, d = 1.006 to 1.019 g/mL), and LDL (d = 1.019 to 1.063 g/mL) were isolated and purified as described previously. For some experiments, LDL was subfractionated and the fractions were pooled into two density ranges: d = 1.019 to 1.038 g/mL (buoyant LDL) and d = 1.038 to 1.063 g/mL (dense LDL). The density cutoff at 1.038 g/mL was used for all animal LDL separations and represents the most common density at which there was a trough in the mutant LDL density profile. The protein concentration of each subspecies was determined by the modified Lowry method of Markwell et al. Cholesterol concentrations in whole plasma and lipoprotein fractions were determined by enzymatic assay (Sigma Diagnostic Kit No. 351, Sigma Chemical Co).

Lipoprotein Iodination

LDL and LDL subspecies were iodinated with 125I (Du Pont) or 131I (ICN) as described previously. Each preparation was assayed for proper relative electrophoretic mobility and lack of bacterial contamination. LDL specific activities were 50 to 450 cpm/μg protein. Greater than 95% of the radioactivity in the LDL preparations was precipitable with trichloroacetic acid (TCA; final concentration, 10%), and less than 2% of the radioactivity was chloroform soluble. ApoE was not detected in any of the LDL preparations by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. All iodinated LDL preparations to be used for in vivo studies were injected into animals 2 days after iodination. An aliquot of each iodinated lipoprotein preparation was again subjected to density gradient ultracentrifugation. All iodinated LDL preparations used for each experiment). The animals were fasted 12 hours before the start of each experiment, fed 6 hours after the initial injection, and fed twice daily thereafter. During the 96-hour experimental period, 15 6-mL samples were collected from each animal via vena cava puncture into Vacutainer tubes containing EDTA (final concentration, 1.5 mg/mL). Plasma samples were precipitated in TCA (10% wt/vol, 4°C), and pellet radioactivities were determined in a model 5001 gamma counter (Packard).

The plasma disappearance curves for each unfractonated LDL preparation were analyzed by a two-pool model described by Matthews, and kinetic parameters were estimated using the nonlinear least-squares curve-fitting program KINETIC (as modified by G.A. McPherson, Elsevier-BIOSOFT) as described previously. This program uses a multieponential algorithm to calculate the slopes and intercepts of each exponential component. The average of the area of the biexponential fit was used to estimate the fractional clearance rate, and the F test was used to determine significant differences between each pair of curves generated from each animal.

Transport Rates Between LDL Subspecies

Iodinated autologous LDL subspecies (eg, 125I-buoyant LDL and 131I-dense LDL) were simultaneously injected into nonanesthetized Lpb5.2 pigs, and plasma samples were collected as described above. Plasma samples were dialyzed overnight at 4°C against an NaBr solution (d = 1.04 g/mL) to determine the purity of the injected doses.

**Fig. 1. Bar graph shows plasma and lipoprotein cholesterol concentrations in Lpb5 and control pigs. Very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), low-density lipoprotein (LDL), buoyant LDL (d = 1.019 to 1.038 g/mL), dense LDL (d = 1.038 to 1.063 g/mL), and high-density lipoprotein (HDL) were separated by density gradient ultracentrifugation. Values are means from control (pig Nos. 8, 9, 10, 11), Lpb5.2 (pig Nos. 15, 16, 17, 18), and Lpb5.1 (pig Nos. 29, 30, 31, 32) pigs.**
TABLE 1. Genotypes and Phenotypes of Pigs Used in Metabolic Studies

<table>
<thead>
<tr>
<th>Pig</th>
<th>Lpb</th>
<th>Phenotype</th>
<th>Plasma Cholesterol, mg/dL</th>
<th>LDL Protein, mg/dL</th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>Non-5</td>
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<td>104</td>
<td>12.4</td>
</tr>
<tr>
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<td>Control</td>
<td>83</td>
<td>8.4</td>
</tr>
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<tr>
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<td>Non-5</td>
<td>Control</td>
<td>73</td>
<td>9.7</td>
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<td>Non-5</td>
<td>Control</td>
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<td>6.9</td>
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<td>8.7</td>
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<tr>
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<td>Lpb5.2</td>
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<tr>
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<td>5/5</td>
<td>Lpb5.2</td>
<td>116</td>
<td>18.7</td>
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<td>Lpb5.2</td>
<td>93</td>
<td>14.9</td>
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<tr>
<td>18</td>
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<td>Lpb5.2</td>
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<td>18.5</td>
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<tr>
<td>22</td>
<td>5/5</td>
<td>Lpb5.2</td>
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<td>Lpb5.1</td>
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<td>71.0</td>
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<tr>
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<td>5/5</td>
<td>Lpb5.1</td>
<td>380</td>
<td>82.3</td>
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</table>

LDL indicates low-density lipoprotein. For buoyant LDL, \(d=1.038 \text{ g/mL}\); for dense LDL, \(d=1.063 \text{ g/mL}\).

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Results

Phenotypes of Lpb5.1 and Lpb5.2 Pigs

In this study, total plasma cholesterol levels were elevated by 200% to 300% in the Lpb5.1 pigs relative to

Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin G, and 100 µg/mL streptomycin (PS, GIBCO) as described previously. Cells were grown to subconfluence in Corning six-well plates and washed with PBS, and the medium was replaced with 2 mL DMEM containing 5% lipoprotein-deficient serum plus PS 48 hours before experiments. For competition experiments, fibroblasts were incubated in the presence of 125I-labeled LDL (2 µg/mL) and increasing concentrations of unlabeled LDL in DMEM, 25 mmol/L HEPES, and BSA (5 mg/mL), pH 7.4, for 4 hours at 4°C on a rotary shaker. Cell surface-bound LDL was released with dextran sulfate and quantified according to the method of Goldstein et al18 as described previously. Data were analyzed using the EBDPA program (Elsevier-BIOSOFT) for determination of the concentration at which 50% of radiolabeled LDL binding was inhibited (=IC50). For degradation experiments, cells were incubated at 37°C with 125I-labeled control LDL. Protoxidative degradation was quantified as described except that silver nitrate was used to precipitate free iodide. The cells were washed and dissolved in 2N NaOH, and cell-associated LDL was measured by gamma counting.

Quantitative Pedigree Analysis of Plasma Cholesterol

Complex segregation analysis was performed on plasma cholesterol levels using a modification of the Pedigree Analysis Package20 as previously described. Four models were evaluated (Table 5). Before analysis, data were corrected for age. There was an age effect in males only, with a decrease in total cholesterol of 2 mg/dL per month. Segregation analysis results therefore were standardized to age 6 months. Cholesterol levels are 37 mg/dL higher in nursing animals. In all segregation analysis models, cholesterol levels were allowed to be influenced by sex, genotype at the apoB locus, multiple genes with small effects (polygenes), and random environmental factors. The simplest model (last line of Table 5) assumed that all variation in cholesterol levels was attributable to these factors alone. The remaining three models all included an effect of a major gene in addition to the apoB locus but differed in their assumptions about the linkage of the major gene to the apoB locus. In one, the loci were constrained to be unlinked (recombination fraction θ=0.5); in the second, the loci were assumed to be very tightly linked or identical (θ=0); and in the third, θ was estimated.

For each model, we estimated the parameter values that maximized the likelihood of observing the pedigree data. Parameters estimated included allele frequencies at the major locus and the apoB locus, parameters describing the means and standard deviations of the cholesterol distributions corresponding to each genotype, the polygenic heritability h2 (the proportion of the variance around each genotypic mean that is attributable to polygenes), genetic disequilibrium (the degree of association between specific alleles at the apoB locus and the second major locus), and a sex effect. Of the four models evaluated, the most general is model 1, the two-locus model with θ estimated. Other models were compared with this one by using likelihood ratio statistics to compute λi (where i denotes degrees of freedom), obtained as minus twice the difference between the log likelihoods of the models. This difference is approximately distributed as χ² with degrees of freedom equal to the difference in the numbers of parameters estimated in the two models. The best model is the one requiring the fewest estimated parameters and exhibiting a likelihood that is not significantly smaller than that for model 1.

Results

Phenotypes of Lpb5.1 and Lpb5.2 Pigs

In this study, total plasma cholesterol levels were elevated by 200% to 300% in the Lpb5.1 pigs relative to

Competition and Degradation Experiments

Normal human fibroblasts (TJ-6F) were kindly provided by Dr Lynn Allen-Hoffman (University of Wisconsin–Madison). Pig skin fibroblasts were obtained from a control pig by punch biopsy from the hind thigh. The cells were maintained on Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 10% fetal bovine serum (HyClone), 100 U/mL penicillin G, and 100 µg/mL streptomycin (PS, GIBCO) as described previously. Cells were grown to subconfluence in Corning six-well plates and washed with PBS, and the medium was replaced with 2 mL DMEM containing 5% lipoprotein-deficient serum (LDS) for 48 hours before experiments. Medium was changed to DMEM+10% LDS containing penicillin G, and 100 µg/mL streptomycin (PS, GIBCO) as described previously. Cells were prepared similarly and incubations carried out at 4°C in DMEM+25 mmol/L HEPES+5 mg/mL bovine serum albumin containing 2.0 µg/mL control 125I-LDL plus varying concentrations of unlabeled competing LDLs. Bound LDL was released by dextran sulfate and counted; results are expressed as percent of maximum bound.

Fig. 2. Graph shows representative distributions of low-density lipoprotein (LDL) protein after density gradient ultracentrifugation of control (No. 8), Lpb5.2 (No. 17), and Lpb5.1 (No. 30) pig plasma.

Fig. 3. Top, Line graph shows degradation of control, Lpb5.1, and Lpb5.2 low-density lipoprotein (LDL). Pig fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM)+10% lipoprotein-deficient serum (LDS) for 48 hours before experiments. Medium was changed to DMEM+10% LDS containing 2.0 µg/mL of each 125I-LDL, and cells were incubated at 37°C for indicated times. Precipitation and quantitation were performed as described in "Methods." Bottom, Line graph shows competition for binding of control, Lpb5.1, and Lpb5.2 LDL to human fibroblasts. Cells were prepared similarly and incubations carried out at 4°C in DMEM+25 mmol/L HEPES+5 mg/mL bovine serum albumin containing 2.0 µg/mL control 125I-LDL plus varying concentrations of unlabeled competing LDLs. Bound LDL was released by dextran sulfate and counted; results are expressed as percent of maximum bound.
non-Lpb5 pigs, whereas the Lpb5.2 pigs displayed a mean 30% increase in total plasma cholesterol (Fig 1 and Table 1). VLDL was elevated twofold in both Lpb5.2 and Lpb5.1 pigs, but this made only a minor contribution to total plasma cholesterol (Fig 1). In both the Lpb5.1 and Lpb5.2 pigs, the primary contributor to the plasma cholesterol increase was LDL. However, the distribution of LDL cholesterol and protein within the LDL density range differed between the phenotypic groups. In normal pigs, most LDL was in the dense LDL density range, whereas the Lpb5.1 pigs manifested the increase in LDL predominantly in the lower-density buoyant LDL subpopulation (Table 1 and Fig 2). The buoyant LDL concentration in the Lpb5.2 pigs was as much as twofold higher than in the control pigs but only 25% to 50% of the level found in Lpb5.1 pigs (Table 1).

Relative Affinities for the LDL Receptor

LDL from the hypercholesterolemic Lpb5.1 pigs has a reduced affinity for the LDL receptor. Because the Lpb5.2 pigs apparently have the same apoB allele as Lpb5.1 pigs (see "Discussion"), we investigated the possibility that Lpb5.2 LDL also has a reduced affinity for the LDL receptor. As previously reported, Lpb5.1 LDL was degraded more slowly than control LDL in cultured fibroblasts. The Lpb5.2 LDL degradation rate was similar to that of Lpb5.1 LDL (Fig 3, top). In vitro competition binding experiments demonstrated that Lpb5.1 and Lpb5.2 LDLs were equally poor competitors for the LDL receptor on fibroblasts when compared with control pig LDL; IC50 values for Lpb5.1 and Lpb5.2 LDL were not significantly different from each other (Lpb5.1, 2.39±0.15 μg/mL; Lpb5.2, 2.83±1.01 μg/mL), whereas control pig LDL was a significantly better competitor than either Lpb5.1 or Lpb5.2 LDL (IC50=1.25±0.70 μg/mL; P<.016) (values are the average of at least four determinations per pig type; representative competition curves are shown in Fig 3, bottom). Thus, both Lpb5.2 and Lpb5.1 pigs have LDL with a similarly reduced affinity for the LDL receptor in vitro.

Plasma Clearance of Lpb5.2 and Lpb5.1 LDLs

Previous experiments showed that LDL from Lpb5.1 pigs was cleared from the circulation more slowly than LDL from non-Lpb5 pigs. To ascertain whether the LDL particles from Lpb5.2 pigs also had a decreased plasma clearance rate, the in vivo catabolism of control, Lpb5.2, and Lpb5.1 LDL particles was compared. When Lpb5.1 and Lpb5.2 LDLs were labeled with 131I and 125I and simultaneously injected into control pigs, their plasma clearance rates were essentially identical (Fig 4, right). When simultaneously injected into a control pig, Lpb5.2 LDL was cleared from the plasma approxi-

### Table 2. Plasma Clearance of Lpb5.2 Low-Density Lipoprotein in Control Pigs

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Donor</th>
<th>Lpb5.2 LDL</th>
<th>Control LDL</th>
<th>F Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>0.032</td>
<td>0.047</td>
<td>P&lt;.001</td>
</tr>
<tr>
<td>2</td>
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<td>P&lt;.001</td>
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</tr>
<tr>
<td>4</td>
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<td>P&lt;.001</td>
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<tr>
<td>5</td>
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<td>P&lt;.001</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>0.023</td>
<td>0.039</td>
<td>P&lt;.05</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein. Each control pig (recipient) was injected simultaneously with autologous control LDL (d=1.019 to 1.063 g/mL) and LDL from an Lpb5.2 pig (donor). Fisher's F test was used to determine significant differences between plasma disappearance curves for Lpb5.2 and control LDL within each animal. Mean fractional clearance rate for Lpb5.2 LDL was significantly different (P<.05) from the mean control LDL fractional clearance rate by paired Student's t test.
TABLE 3. Plasma Clearance of Lpb5.2 Low-Density Lipoprotein in Lpb5.2 and Lpb5.1 Pigs

<table>
<thead>
<tr>
<th>Recipient Pig</th>
<th>Genotype</th>
<th>Plasma Cholesterol, mg/dL</th>
<th>Donor Pig and Radiolabel</th>
<th>19, 125I</th>
<th>20, 131I</th>
<th>26, 125I</th>
<th>27, 131I</th>
<th>F Test</th>
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<tbody>
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<td>0.030</td>
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<td>0.024</td>
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<tr>
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<td>5/5 (Lpb5.2)</td>
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Iodinated Lpb5.1 and Lpb5.2 low-density lipoproteins (LDLs, d=1.019 to 1.063 g/mL) were injected simultaneously into each pig. Fisher's F test was used to determine significant differences between plasma disappearance curves within each animal. Mean fractional clearance rates for autologous Lpb5.2 and Lpb5.1 LDLs were significantly different (P<.05) as determined by Student's t test.

LDL from four Lpb5.2 littermates, two Lpb5.2 and two Lpb5.1 pigs, was isolated and differentially iodinated with either 125I or 123I. Each of the four animals was simultaneously injected with autologous LDL and LDL from a littermate of the opposite phenotype. In the Lpb5.2 pigs, Lpb5.1 and Lpb5.2 LDLs were cleared at the same rate. Similarly, in the Lpb5.1 pigs, the clearance rates of the two types of LDL were not significantly different (Table 3).

This defective behavior of Lpb5.1 and Lpb5.2 LDL in vivo paralleled the abnormal behavior of these particles in their ability to compete for binding to the LDL receptor in cultured fibroblasts. Thus, particle differences between Lpb5.1 and Lpb5.2 LDL cannot account for the difference in plasma LDL levels between the two phenotypic groups.

**In Vivo LDL Receptor-Mediated Clearance**

Although Lpb5.1 and Lpb5.2 LDL particles behaved identically in vivo and in vitro, Lpb5.1 pigs cleared LDL particles (regardless of their source) more slowly than did Lpb5.2 pigs (Table 3). Moreover, whereas normal and Lpb5.2 pigs cleared Lpb5.1 and Lpb5.2 LDL more slowly than normal LDL, Lpb5.1 pigs cleared LDL from all three types of pigs at the same rate (Table 3 and Reference 8), suggesting that they lacked the ability to recognize the structural defect in Lpb5 apoB.
It therefore was likely that the Lpb5.1 pigs were not expressing normal levels of the LDL receptor in vivo despite the fact that fibroblasts from these pigs appear to express normal LDL receptor activity. To test the possibility that the amount of receptor-mediated LDL clearance differed among the three phenotypic groups, we measured LDL receptor and nonreceptor in vivo clearance differed among the three phenotypic groups, modified LDL, like native LDL, was cleared most rapidly in control pigs and differed in each phenotypic group. Modified LDL, like native LDL, was cleared most rapidly in control pigs and differed in each phenotypic group. LDL indicates low-density lipoprotein. Autologous buoyant (d=1.019 to 1.038 g/mL) and dense (d=1.038 to 1.063 g/mL) LDLs were injected simultaneously into Lpb5.2 pigs. Fractional rate constants \( k_{\text{b}} \) and \( k_{\text{d}} \) were calculated by dividing LDL flux by corresponding LDL concentration (eg, \( F_{\text{b0}}/\text{buoyant LDL}=k_{\text{b}} \)). These rates represent direct clearance from the pool and not total plasma clearance. \( F_{\text{b0}} \) and \( F_{\text{d0}} \) represent direct input into buoyant and dense LDL, respectively. \( F_{\text{b1}} \) and \( F_{\text{d1}} \) represent flux from buoyant to dense LDL and flux from dense to buoyant LDL, respectively. \( F_{\text{b0}} \) and \( F_{\text{d0}} \) represent total catabolic rate of buoyant and dense LDL, respectively. \( k_{\text{b}} \) and \( k_{\text{d}} \) represent fractional rate constants for the clearance of buoyant and dense LDL, respectively.

\*Values for Lpb5.1 and control pigs are means of n=5 reported in Checovich et al. 10

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Plasma Cholesterol, mg/dL</th>
<th>LDL Protein, mg/dL</th>
<th>Flux, (μg/kg)/h</th>
<th>Fractional Rate Constants, Pools/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buoyant</td>
<td>Dense</td>
<td>Production</td>
</tr>
<tr>
<td>25</td>
<td>110</td>
<td>20.0</td>
<td>37.1</td>
<td>64</td>
</tr>
<tr>
<td>21</td>
<td>130</td>
<td>26.2</td>
<td>29.3</td>
<td>129</td>
</tr>
<tr>
<td>22</td>
<td>117</td>
<td>18.9</td>
<td>34.1</td>
<td>163</td>
</tr>
<tr>
<td>23</td>
<td>112</td>
<td>16.6</td>
<td>21.8</td>
<td>123</td>
</tr>
<tr>
<td>24</td>
<td>135</td>
<td>28.2</td>
<td>30.0</td>
<td>117</td>
</tr>
<tr>
<td>Mean</td>
<td>121</td>
<td>21.9</td>
<td>30.4</td>
<td>119</td>
</tr>
<tr>
<td>SD</td>
<td>10</td>
<td>4.4</td>
<td>5.3</td>
<td>32</td>
</tr>
</tbody>
</table>

LDL fluxes in Lpb5.1 pigs

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Plasma Cholesterol, mg/dL</th>
<th>LDL Protein, mg/dL</th>
<th>Flux, (μg/kg)/h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Buoyant</td>
<td>Dense</td>
</tr>
<tr>
<td>Mean</td>
<td>218</td>
<td>63.2</td>
<td>20.5</td>
</tr>
<tr>
<td>SD</td>
<td>25</td>
<td>15.9</td>
<td>5.6</td>
</tr>
</tbody>
</table>

LDL fluxes in control pigs

<table>
<thead>
<tr>
<th>Pig No.</th>
<th>Plasma Cholesterol, mg/dL</th>
<th>LDL Protein, mg/dL</th>
<th>Flux, (μg/kg)/h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>89</td>
<td>8.6</td>
<td>22.0</td>
</tr>
<tr>
<td>SD</td>
<td>15</td>
<td>2.8</td>
<td>6.2</td>
</tr>
</tbody>
</table>

Pig No. of LDL subpopulations

Lpb5.1 pigs overproduce buoyant LDL. We investigated whether buoyant LDL overproduction also contributed to the moderate hypercholesterolemia associated with the Lpb5.2 pig. Compared with values previously obtained in control and Lpb5.1 pigs, the Lpb5.2 pigs produced significantly more buoyant LDL than did control pigs and significantly less buoyant LDL than did Lpb5.1 pigs (Table 4). In the Lpb5.2 pigs, direct input into dense LDL (\( F_{\text{d0}} \)) was greater than direct input into buoyant LDL (\( F_{\text{b0}} \)). Because Lpb5.1 pigs produced less dense LDL than either control or Lpb5.2 pigs, the total LDL production (input into buoyant and dense LDL, \( F_{\text{b0}}+F_{\text{d0}} \)) was actually similar for Lpb5.1 and Lpb5.2 pigs.

Quantitative Pedigree Analysis of Plasma Cholesterol Phenotype

The existence of severely hypercholesterolemic Lpb5.1 pigs and moderately hypercholesterolemic Lpb5.2 pigs that apparently share the same apoB allele is inconsistent with a genetic model whereby hypercholesterolemia in these pigs is solely due to a single mutant allele at the apoB locus. We therefore subjected our pedigree-phenotype dataset, consisting of plasma cholesterol levels and known genotypes at the apoB locus, to quantitative pedigree analysis.

Four models were evaluated for their ability to describe the inheritance pattern of plasma cholesterol phenotypes. As shown in Table 5, the model that does not include a second major gene in addition to apoB (model 4) can be rejected when compared with the most general model considered (model 1), with \( P<.000001 \). We can also reject a model that has a major locus tightly linked to the apoB locus (model 2, \( \theta=0, P<.001 \)). The
model that includes a major locus unlinked to the apoB locus (model 3) cannot be rejected ($P = .0645$) and is the best-fitting (most parsimonious) model for our pedigree-phenotype dataset. The parameters of this model include (Table 6) allele frequencies at the apoB locus and the major locus. The major locus appears to be unlinked to apoB, although power to estimate $\theta$ is low; the estimated recombination fraction in model 1 is 0.21, but with a log of the odds score of only 0.74. The major locus has a nearly recessive allele determining high cholesterol levels, with an effect that is approximately equal to the effect of the apoB locus. The frequency of this allele is 0.37, and the frequency of the $Lpb^s$ apoB allele is 0.42. These are maximum likelihood estimates determined from founders (individuals whose parents are not included in the pedigree). The two loci together account for 64% of the variance in total cholesterol, and polygenes account for an additional 24% (data not shown).

We also tested the hypothesis of mendelian transmission at the major locus by evaluating a model in which the parameter values were set equal to those of the two-locus mendelian model, but the transmission probabilities associated with the three major locus genotypes were estimated rather than being restricted to their mendelian values (results not shown). The two-locus mendelian model could not be rejected compared with this more general model, and the estimated transmission probabilities were not significantly different from mendelian values. We further tested an environmental heterogeneity model, with parameter values equal to those of the two-locus mendelian model but with genotype of offspring independent of parental genotype (results not shown). This model was rejected compared with the more general model in which transmission probabilities were estimated. These tests provide further evidence that a major locus, in addition to the apoB locus, influences total cholesterol in our pig population.

**Discussion**

We previously characterized spontaneously hypercholesterolemic pigs carrying a defined allele for apoB ($Lpb^s$). The animals had been selected on the basis of apoB allotypes, and those allotypes correlated with specific markers in apoB genomic DNA. The hypercholesterolemia phenotype always segregated with the apoB genomic markers; i.e., all hypercholesterolemic pigs carried the $Lpb^s$ apoB allele and all normocholesterolemic pigs carried non-$Lpb^s$ alleles. This led us to hypothesize that a structural defect encoded by the $Lpb^s$ allele could be responsible for the hypercholesterolemia phenotype.

<table>
<thead>
<tr>
<th>Model</th>
<th>Gene Loci</th>
<th>Recombination</th>
<th>Parameters</th>
<th>$\chi^2$</th>
<th>$P^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Two loci</td>
<td>apoB</td>
<td>$\theta$ estimated</td>
<td>12</td>
<td>2926.54</td>
<td>...</td>
</tr>
<tr>
<td>(2) Two loci, tightly linked</td>
<td>apoB</td>
<td>$\theta = 0$</td>
<td>11</td>
<td>2937.42</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>(3) Two loci, unlinked</td>
<td>apoB</td>
<td>$\theta = 0.5$</td>
<td>11</td>
<td>2929.96</td>
<td>.0645</td>
</tr>
<tr>
<td>(4) One locus</td>
<td>apoB</td>
<td></td>
<td>7</td>
<td>2992.85</td>
<td>&lt;.000001</td>
</tr>
</tbody>
</table>

apoB indicates apolipoprotein B; MG, major gene; PG, polygenic effects; and $\theta$, recombination fraction.

*For comparison with the two-locus model with $\theta$ estimated (model 1).

<table>
<thead>
<tr>
<th>Parameter Estimates</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency of the $Lpb^s$ apoB allele</td>
<td>0.42</td>
</tr>
<tr>
<td>Frequency of the &quot;high&quot; second locus allele</td>
<td>0.37</td>
</tr>
<tr>
<td>Linkage disequilibrium</td>
<td>0.61</td>
</tr>
<tr>
<td>Polygenic heritability ($h^2$)</td>
<td>0.68</td>
</tr>
<tr>
<td>Variation around apoB and second locus genotypes</td>
<td>38.0 mg/dL</td>
</tr>
<tr>
<td>Sex effect (males higher than females)</td>
<td>11.0 mg/dL</td>
</tr>
</tbody>
</table>

**Mean Estimated Plasma Cholesterol, mg/dL**

<table>
<thead>
<tr>
<th>Major Locus Allele</th>
<th>ApoB Allele</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-5/Non-5</td>
</tr>
<tr>
<td>&quot;Low&quot; homozygote</td>
<td>93</td>
</tr>
<tr>
<td>Heterozygote</td>
<td>85</td>
</tr>
<tr>
<td>&quot;High&quot; homozygote</td>
<td>207</td>
</tr>
</tbody>
</table>

apoB indicates apolipoprotein B.
allele was responsible for the hypercholesterolemia of those pigs. This hypothesis was supported by studies that revealed functional defects in LDL particles from pigs carrying the \( \text{Lpb}^{5} \) allele; LDL from the mutant hypercholesterolemic \( \text{Lpb}^{5} \) pigs has a twofold to sixfold lower affinity for the pig LDL receptor\(^2\) and is catabolized more slowly in vivo\(^8\) compared with LDL from non-\( \text{Lpb}^5 \) pigs.

In addition to being biologically defective, LDL particles from the \( \text{Lpb}^5 \) pigs are also structurally unusual.\(^8\) Buoyant cholesterol ester–enriched LDL particles are present at four to eight times the normal concentration in pig plasma and comprise approximately 70% of the total plasma LDL protein in \( \text{Lpb}^5 \) pigs. In vivo turnover studies showed that the accumulation of buoyant LDL was primarily due to a 14-fold increase in the production rate of these particles.\(^10\) We therefore concluded that there were two distinct phenotypes associated with the severe hypercholesterolemia of \( \text{Lpb}^5 \) pigs: slowly catabolized LDL particles and overproduction of buoyant LDL.

However, the \( \text{Lpb}^5 \) pigs displayed phenotypic heterogeneity: some had severe hypercholesterolemia with a prominence of buoyant LDL (\( \text{Lpb}^5.1 \) pigs), and others had very modest hypercholesterolemia with only a small increase in buoyant LDL (\( \text{Lpb}^5.2 \) pigs).\(^9\) Because the same polymorphic markers used to distinguish \( \text{Lpb}^5 \) apoB from the seven other known pig apoB alleles are present in \( \text{Lpb}^5.1 \) and \( \text{Lpb}^5.2 \) apoB, we presume that these two groups of pigs share the same apoB allele, although complete sequence analysis of the \( \text{Lpb}^5.1 \) and \( \text{Lpb}^5.2 \) apoB genes is required before this can be verified. If polymorphism within the \( \text{Lpb}^5 \) apoB gene is responsible for the variation in plasma cholesterol levels among \( \text{Lpb}^5 \) pigs, it is not manifested by altered catabolism of the resulting LDL particles; we have shown that differences in the catabolic properties of the LDL particles could not account for the differences in plasma cholesterol between \( \text{Lpb}^5.1 \) and \( \text{Lpb}^5.2 \) pigs.

One of the more striking observations arising from this study was the apparently complete absence of LDL receptor activity in \( \text{Lpb}^5.1 \) pigs. This may explain why these animals are unable to distinguish \( \text{Lpb}^5 \) LDL from normal pig LDL.\(^8\) In addition, it raises the possibility that buoyant LDL overproduction might be related to the absence of LDL receptor activity. For example, a defect in an enzyme associated with cholesterol metabolism might increase the metabolically active hepatic cholesterol pool. This pool may simultaneously affect LDL receptor expression and influence the cholesterol and/or fatty acid composition of de novo lipoprotein particles. Alternatively, a lack of LDL receptor activity may cause the more extensive production of buoyant LDL by delaying VLDL remnant clearance, resulting in an increased conversion of VLDL to LDL. This would be analogous to the situation in the Watanabe heritable hyperlipidemic rabbit, which displays overproduction of LDL due to slow clearance of LDL.\(^22\) However, Huff and Telford\(^25\) determined that 80% of LDL in miniature pigs is directly synthesized by pathways not involving the VLDL cascade. In \( \text{Lpb}^5.1 \) pigs, we could account for only 20% of buoyant LDL, as have been derived from plasma VLDL, suggesting that “direct” production of buoyant LDL was occurring.\(^8\) As explained by Shames and Havel,\(^24\) one cannot rule out the possibility that there is a rapidly turning over pool of VLDL that is converted to LDL. Because the VLDL pool size in pigs is very small, it is impossible to carry out VLDL turnover studies in pigs in a manner that accurately models the heterogeneity of VLDL.

LDL heterogeneity has been observed in humans.\(^25\) Austin et al\(^22\) proposed that LDL size must be determined by a single major structural locus. Recently Nishina et al\(^32\) reported linkage of the LDL size phenotype to the LDL receptor locus, although recent evidence suggests that the linked locus is not the LDL receptor structural gene.\(^28\) Although variation in LDL receptor activity may certainly occur in this pig population, two observations are incompatible with the LDL receptor being the second major cholesterol-influencing locus. First, cultured fibroblasts from \( \text{Lpb}^5.1 \) pigs appear to express normal LDL receptor activity.\(^2\) Second, the expression of the second major locus is apparently recessive, whereas the known LDL receptor mutations are expressed in a codominant fashion. Taken together, the observations in the mutant pigs and in humans are consistent with multiple factors affecting LDL heterogeneity, either through an effect on LDL receptor expression (in pigs) or perhaps as a consequence of the polymorphism near the LDL receptor structural gene (in humans).

The quantitative pedigree analysis performed on our pig population indicated that the distribution of plasma cholesterol levels was most consistent with the apoB gene and a second major locus, probably unlinked to apoB, contributing to the hypercholesterolemia. These two loci account for a substantial portion (64%) of the variance in plasma cholesterol concentration in this pedigree. Even so, the high polygenic heritability (\( h^2=0.68 \), Table 6) indicates that further genetic effects remain to be identified. The remaining genetic contribution could be either multiple genes with small effects, an additional major gene, or a combination of the two.

A surprising result of the genetic analysis was high gametic disequilibrium, indicating an association between alleles at the two major loci—surprising because the prediction of the same analysis was that the two genes are unlinked. Gametic disequilibrium implies nonrandom transmission of alleles at different loci and not necessarily physical proximity of the two loci. Thus, specific haplotypes occur with higher than random frequency in our pig population. This could result from a bias in the selection of animals for breeding. Indeed, because we had generally bred for \( \text{Lpb}^5 \) pigs with high cholesterol, we most likely selected for pigs carrying the high-cholesterol alleles at each of the two major loci.

Rapacz and Hasler-Rapacz\(^29\) previously determined that three separate immunologically identified genes contribute to hypercholesterolemia in the pig population: \( \text{Lpb}^5 \), \( \text{Lpu}^1 \), and \( \text{Lpr} \). The combination of the \( \text{Lpb}^5 \), \( \text{Lpu}^1 \), and \( \text{Lpr} \) alleles was associated with the most severely hypercholesterolemic pigs, and the original nomenclature of the \( \text{Lpb}^5.1 \) and \( \text{Lpb}^5.2 \) pigs was derived from their haplotypes at the \( \text{Lpb} \) and \( \text{Lpu} \) loci (\( \text{Lpb}^5.1 \) pigs were \( \text{Lpb}^5 \), \( \text{Lpu}^1 \), and \( \text{Lpr} \), while \( \text{Lpb}^5.2 \) pigs were \( \text{Lpb}^5, \text{Lpu}^1 \)). The \( \text{Lpr} \) gene encodes an apolipoprotein unique to pigs termed HPR in rats.\(^29\) In contrast to the initial observation, we were unable to confirm that the genotype at the \( \text{Lpr} \) locus is associated with variation in plasma cholesterol levels.\(^32\) Thus, we do not suspect that...
the second major locus indicated by the pedigree analysis is Lpr. The protein product of the Lpu locus has never been determined, although it is known to be present on pig LDL. A thorough investigation by Lee et al.⁸ of the effect of homozygosity and heterozygosity at the Lpu locus on lipoprotein concentration and composition in Lpb5 pigs revealed that Lpb⁵, Lpu⁴ pigs display the most elevated total plasma cholesterol and buoyant LDL levels. Lpu may be either a separate allele of apoB or tightly linked to the apoB gene, as no recombination between Lpb and Lpu occurred in genetic crosses over 18 generations.⁹ Because Lpu is an LDL-borne allotype, it may represent epitopes unique to Lpb5.1 buoyant LDL, thus accounting for its linkage disequilibrium with apoB and its correlation with severe hypercholesterolemia.

Our finding that a second major gene influences plasma cholesterol levels in pigs is of considerable interest in light of recent findings in humans. Several studies have linked polymorphisms within the apoB gene to variation in plasma triglyceride, cholesterol, or apoB levels¹⁰⁻¹⁴ as well as to the development of atherosclerosis. However, these correlations greatly depend on the population being studied, such that apoB gene polymorphisms may be associated with levels of none, some, or all of the lipoprotein components. Clearly, other genetic factors contribute to hyperlipidemia in humans. Coresh et al.¹⁵ recently found that certain apoB restriction fragment length polymorphisms did not appear to contribute to variation in plasma apoB concentration and that apoB is not the major gene influencing plasma apoB levels in 23 families, as determined by pedigree and sib-pair linkage analysis. Although variation in the apoB gene clearly contributes to apoB and plasma cholesterol levels in pigs, the evidence that another major gene or genes determine cholesterol concentration supports the use of these Lpb5 pigs as a model for human hypercholesterolemia and as a tool for identifying other gene loci involved in the determination of plasma cholesterol levels.

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References


Apolipoprotein B and a second major gene locus contribute to phenotypic variation of spontaneous hypercholesterolemia in pigs.

R J Aiello, D N Nevin, D L Ebert, P J Uelmen, M E Kaiser, J W MacCluer, J Blangero, T D Dyer and A D Attie