Concentrations and compositions of plasma lipoprotein subfractions of *Lpb^5-Lpu^1* homozygous and heterozygous swine with hypercholesterolemia

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Abstract Pigs with two mutant epitopes, Lpb5 of apolipoprotein B (apoB) and Lpu1 of a yet undefined apolipoprotein, specified by a haplotype *Lpb^5-Lpu^1* and fed a cholesterol-free low fat diet show hypercholesterolemia. The purpose of this study was to establish whether a direct relationship exists between the swine lipoprotein concentration/composition and the genotype for the *Lpb^5-Lpu^1* haplotype; i.e., homozygote versus heterozygote. Lipoproteins of fasted plasma from hypercholesterolemic swine, homozygous (HmHC) and heterozygous (HtHC) for *Lpb^5-Lpu^1*, and from normolipidemic (NL) pigs of other *Lpb-Lpu* haplotypes were separated into five layers by density gradient ultracentrifugation. Layer 1 contained particles of $d < 1.019$ g/ml and layer 5 particles of $d > 1.073$ g/ml. Layers 2, 3, and 4 represented subfractions of low density lipoproteins (LDL). The plasma total cholesterol (TC) of the HmHC group (300 ± 84 mg/dl) was different ($P < 0.05$) from the HtHC group (200 ± 80 mg/dl) and in both HmHC and HtHC, TC was significantly higher ($P < 0.0005$ and $P < 0.005$, respectively) than that of the NL group (69 ± 14 mg/dl). The elevation in plasma TC was due to the increased TC in layers 2 and 3: a 13- and 7-fold increase in HmHC and a 7- and 4-fold increase in HtHC in layers 2 and 3, respectively. Parallel increases in unesterified cholesterol were observed in these two layers. Marked increases in apoB were also observed in layers 2 and 3 of HmHC and intermediate increases in apoB in the same two layers of HtHC. In addition, layer 2 of HmHC was cholesteryl ester (CE)-rich (46.4 ± 3.5%), protein-poor (18.9 ± 2.1%), and triglyceride-poor (2.4 ± 2.7%) in comparison to NL (CE 33.3 ± 9%, protein 23.9 ± 4.6% and triglyceride 14.4 ± 11.3%). Layer 2 of HtHC showed intermediate values between HmHC and NL. Layer 3 was triglyceride-poor subfraction for both HmHC and HtHC groups. The concentrations and compositions of layers 1, 4, and 5 were similar among the three groups. These data establish that a direct relationship exists between the swine *Lpb^5-Lpu^1* genotype and the concentrations and compositions of two LDL subpopulations. — **Lee, D. M., T. Mok, J. Hasler-Rapacz, and J. Rapacz.** Concentrations and compositions of plasma lipoprotein subfractions of *Lpb^5-Lpu^1* homozygous and heterozygous swine with hypercholesterolemia. *J. Lipid Res.* 1990. **31:** 839-847.

Supplementary key words gentics • apolipoprotein epitope • apoB • lipoprotein distribution • immunochemical • subpopulations

Hyperlipidemia is recognized as a major risk factor in the development of coronary artery disease. Family studies in humans and animals have revealed strong hereditary influences on quantitative variations of cholesterol and its main carrier, low density lipoproteins (LDL) (1-7). It is conceivable that mutations in the structural gene for apolipoprotein B (apoB), the major protein moiety of LDL, may produce polymorphic epitopes associated with defective catabolism of lipoproteins.

Sixteen of 22 immunologically defined apolipoprotein epitopes (allotypes, markers) in swine were found to be associated with apolipoprotein B-100 (apoB-100) (8) and cosegregated with restriction fragment length polymorphisms (RFLPs) of the swine *apoB* gene (9). Family studies revealed eight distinct multi-epitope-apoB haplotypes, specified by eight *apoB* alleles (*Lpb^1-Lpb^8*) which gave rise to 36 *apoB* genotypes (10, 11). A survey of different breeds and studies of five-generation families revealed that plasma with elevated LDL and cholesterol in pigs fed a low fat diet has been derived invariably from homozygous *Lpb$^{5/5}$* pigs, exhibiting normally functioning LDL receptors (1, 8). The hyperlipidemic phenotype was established to be heritable and modified by other genes, including likely two mutations, *Lpr^1* and *Lpu^1*, of yet undefined apolipoproteins. The *Lpr^1* gene segregates independently of *apoB*

Abbreviations: LDL, low density lipoproteins; HDL, high density lipoproteins; apoB, apolipoprotein B; Lpb, epitope of apoB; *Lpb*, *apoB* gene locus or allele; apoU, apolipoprotein U; Lpu, epitope of apoU; *Lpu*, *apoU* gene locus or allele; TG, triglycerides; UC, unesterified cholesterol; CE, cholesteryl esters; TC, total cholesterol; HmHC, homozygous hypercholesterolemic; HtHC heterozygous hypercholesterolemic; NL, normolipidemic; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; GLC, gas-liquid chromatography.

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and *Lpu* loci (12), while the Lpu^1 gene forms with the Lpb^5 allele a very rare Lpb^5-Lpu^1 haplotype (10, 11).

Previous studies on metabolic and physicochemical properties of LDL particles were carried out on hypercholesterolemic (mixture of homozygotes and heterozygotes) and normal swine phenotypes (13). It was concluded that the elevation of LDL in the hypercholesterolemic group was restricted to a buoyant LDL subspecies and that hypercholesterolemia resulted from a defect in LDL catabolism (13). In the present study we have expanded the earlier studies by separating homozygotes and heterozygotes in order to investigate the possible direct relationship between the Lpb^5-Lpu^1 genotypes and the concentration and composition of swine lipoproteins.

MATERIALS AND METHODS

Animals

The pigs (eight males and eight females ranging in age from 5 to 31 months) used in this study were derived from the Immunogenetic Project Herd, University of Wisconsin, Madison, which carries components of at least six breeds, have been lipoprotein phenotyped by 22 apolipoprotein epitope specific antibodies, and genotyped for at least eight generations. The pigs were selected and divided into three groups on the basis of their lipoprotein genotype at two very closely linked loci: *Lpb* and *Lpu* as shown in **Table 1**: group 1, normolipidemic (NL) pigs carrying in their genotype haplotypes other than Lpb^5-Lpu^1; group 2, hypercholesterolemic pigs being homozygous (HmHc) $Lpb^{5/5}$-$Lpu^{1/1}$, and group 3, hypercholesterolemic pigs being heterozygous (HtHC) carrying one Lpb^5-Lpu^1 haplotype in their *Lpb-Lpu* genotype.

Pigs were fed ad libitum a diet containing 0% cholesterol and 5% fat (University of Wisconsin Gestation Diet).

Plasma collection

Blood from animals fasted overnight was drawn from the jugular vein into a vacuum bottle containing anticoagulant (15 ml/100 ml blood) consisting of 0.8% citric acid, 2.2% sodium citrate, 2.45% dextrose, anhydrous and 0.1% EDTA (14). In addition, a small volume of blood was collected without anticoagulant for preparation of serum. The blood was kept on ice and centrifuged immediately at 2000 *g* for 30 min at 4°C. The plasma was transferred into a plasma bag to which a preservative mixture was added to final concentrations of penicillin-G 500 units/ml, streptomycin sulfate 50 μg/ml, Na_2EDTA 0.1%, glutathione, reduced form 0.05%, and ε-amino caproic acid 0.13% (15). The plasma (180–200 ml) and serum aliquots were shipped to Oklahoma City overnight on wet ice and isolation of lipoproteins was begun within 24 h of blood drawing.

Subfractionation of lipoproteins

Subfractionation of lipoproteins was carried out by a single spin density gradient ultracentrifugation according to the method established for human plasma (16). This method separated lipoproteins into five layers: layer 1 ($d < 1.019$ g/ml) was characteristic of triglyceride-rich lipoproteins; layer 2 (d 1.021–1.028 g/ml), layer 3 (d 1.032–1.043 g/ml), and layer 4 (d 1.054–1.073 g/ml) were

TABLE 1. Immunogenetically defined swine homozygous (Hm) and heterozygous (Ht) for the Lpb^5-Lpu^1 haplotype with hypercholesterolemia (HC) and normolipidemic (NL) swine with Lpb^{non5}-Lpu^2 haplotypes

No.	Sex	Age	Genotype Expressed in *Lpb-Lpu* Haplotypes
		months	
NL swine			
1	F	7.0	Lpb^2-Lpu^2/Lpb^4-Lpu^2
2	F	5.0	Lpb^1-Lpu^2/Lpb^3-Lpu^2
3	M	10.0	Lpb^2-Lpu^2/Lpb^4-Lpu^2
4	M	7.0	Lpb^1-Lpu^2/Lpb^3-Lpu^2
5	M	6.5	Lpb^2-Lpu^2/Lpb^6-Lpu^2
HmHC swine			
6	F	6.0	Lpb^5-Lpu^1/Lpb^5-Lpu^1
7	F	9.0	Lpb^5-Lpu^1/Lpb^5-Lpu^1
8	M	22.0	Lpb^5-Lpu^1/Lpb^5-Lpu^1
9	F	5.7	Lpb^5-Lpu^1/Lpb^5-Lpu^1
10	M	5.7	Lpb^5-Lpu^1/Lpb^5-Lpu^1
11	M	6.5	Lpb^5-Lpu^1/Lpb^5-Lpu^1
HtHC swine			
12	M	6.3	Lpb^5-Lpu^1/Lpb^8-Lpu^2
13	F	31.0	Lpb^5-Lpu^1/Lpb^5-Lpu^2
14	M	7.5	Lpb^5-Lpu^1/Lpb^3-Lpu^2
15	F	9.0	Lpb^5-Lpu^1/Lpb^5-Lpu^2
16	F	6.5	Lpb^5-Lpu^1/Lpb^3-Lpu^2

subfractions of LDL with layer 3 as the major fraction of human plasma LDL, while layer 5 (d > 1.073 g/ml) contained high density lipoproteins (HDL) and all plasma proteins (16). HDL was separated from the plasma proteins by flotation of layer 5 at d 1.21 g/ml. For lipid distribution studies, analyses of isolated fractions were carried out without washing in order to achieve 100% recovery of lipoproteins. The total recovery of lipids was verified with cholesteryl ester (CE) and unesterified cholesterol (UC) values (16). For chemical composition analyses, fractions 2–5 were washed once at the upper density limit to remove the contaminating albumin. Since swine lipoproteins are colorless, no banding pattern was visible. A sample of human plasma was centrifuged simultaneously. All tubes were marked for the separated five layers according to the human lipoprotein banding pattern. The five layers were analyzed for chemical composition and apoB content was determined for six pigs. From the results, it was established that, similar to human plasma, layers 1 through 4 were apoB-containing lipoproteins while layer 5 represented HDL with a small amount of apoB-containing lipoproteins. Thus, the same pattern was used for all swine lipoprotein separation.

Chemical analyses

For determination of lipoprotein concentrations, separated fractions in three ultracentrifugal tubes were analyzed for each pig. Triplicate aliquots were taken from all five layers from each tube for lipid analyses. Triglyceride (TG), UC, and CE were determined by GLC according to the method described by Kuksis et al. (17). Phospholipid was determined according to a micromethod of Gerlach and Deuticke (18). Neutral lipids were determined for samples from both serum and plasma. The average ratio between the plasma and serum values represented the dilution factor in plasma due to anticoagulant and preservatives. All plasma lipid values were then corrected to their undiluted volume, i.e., to serum values. Protein was assayed by the method of Lowry et al. (19) using human serum albumin as standard. Sodium dodecyl sulfate was included in the reaction mixture to clear the turbidity caused by lipids (20).

Quantitation of apoB

Antibodies against apoB were produced in a sheep (#3292) inoculated with pig apoB-100. The antigen was isolated by ultracentrifugation at d 1.038–1.050 g/ml from plasma of a $Lpb^{5/5}$ swine and purified by SDS-polyacrylamide gel electrophoresis (PAGE). A gel slice containing apoB-100 was mixed with Freund's incomplete adjuvant and the animal was injected at 2-week intervals. The immune serum used in this study was collected 7 days after the fourth inoculation and was tested for the apoB-100 specificity by immunoblot using the donor LDL. The immune serum (0.18%) was used to quantitate apoB by single radial immunodiffusion (21) in 0.7% agarose (Bio-Rad), as described for swine apoB quantitation earlier (1, 22). Serum samples of all 16 experimental swine and the subfractionated plasma layers of six pigs, two per each genotype group, were tested in duplicate together with an apoB standard. The tests were allowed to equilibrate for 5 days at room temperature before photographs were taken. The surface area of the precipitation zone (halo area) on the photograph was marked with a pen. The photographs were enlarged 10 times, the diameter was measured, and the surface area was estimated in cm^2 for each of the samples. The apoB content was then interpolated from a calibration curve (surface area vs protein content) established for the apoB standard. Bovine immunoglobulin-G (IgG) was used as the protein standard for the Bradford assay (23); values were adjusted to the Lowry estimates. The Bradford value/Lowry value was 0.71. The protein concentration of an affinity-purified (24) LDL fraction (d 1.03–1.05 g/ml) from a $Lpb^{3/3}$ pig was established and was used as the apoB standard. An additional single radial immunodiffusion test was used for samples with antigen concentrations below 7 mg/dl. The volume of these samples was increased in the second test three- to sixfold to obtain larger precipitation areas (>22 cm^2) to minimize the estimation error (<6%) between the duplicate samples for the apoB concentration. To establish acceptable deviation from chance variation, quality control tests were performed. The test included three plasma samples, each representing phenotypes of one studied genotype, fractionated into five lipoprotein subfractions. Each fraction was tested four times in four different volumes ($4 \times 4 = 16$) on the same plate (intra-assay), and in triplicate plates (inter-assay). Coefficient of variation was 0.41–1.46% for intra-assay and 4.1–10.3% for inter-assay. From this 7% was adapted as the control limit of chance variation for the experimental samples.

Statistical method

Student's *t*-test was used for evaluating the significance of difference.

Polyacrylamide gel electrophoresis in SDS

Lipoprotein subfractions were analyzed by 3.3% PAGE in SDS according to the method of Fairbanks, Steck, and Wallach (25) as modified previously (26).

RESULTS

Concentrations and distributions of lipids in lipoprotein subfractions of swine

Table 2 shows the TG concentrations in lipoprotein subfractions from HmHC, HtHC, and NL swine. Similar

TABLE 2. Triglyceride concentrations in lipoprotein subfractions of plasma from immunogenetically defined swine homozygous and heterozygous for the Lpb^5-Lpu^1 haplotype with hypercholesterolemia and normolipidemic swine with Lpb^{non5}-Lpu^2 haplotypes

Lipoprotein Subfractions	Normals (5)[a]	Homozygotes (6)	Heterozygotes (5)
		mg/dl of plasma	
Layer 1 (d<1.019 g/ml)	12.38 ± 4.25[b]	19.99 ± 12.30	22.33 ± 5.84[c]
Layer 2 (1.021-1.028 g/ml)	3.00 ± 0.84	5.97 ± 3.36	4.13 ± 1.89
Layer 3 (1.032-1.043 g/ml)	0.98 ± 0.70	1.94 ± 1.56	1.58 ± 1.19
Layer 4 (1.054-1.073 g/ml)	1.62 ± 1.09	1.75 ± 2.08	0.97 ± 0.56
Layer 5 (d>1.073 g/ml)	0.36 ± 0.43	0.19 ± 0.15	0.32 ± 0.28
Whole plasma	18.34 ± 4.53	29.84 ± 16.9	29.33 ± 6.79[c]

[a]Number in parentheses represents number of animals.
[b]Mean ± SD.
[c]Significance of difference at $P < 0.01$, compared to the corresponding normolipidemic group.

to human plasma, the majority of the plasma TG was present in layer 1 for all swine. Although TG in whole plasma and layer 1 of HmHC and HtHC showed higher than normal levels, only those in HtHC represented a significant difference ($P < 0.01$) from normals. The TG concentrations or percent distributions decreased with increasing density for both HmHC and HtHC groups. The NL group had a similar trend except that layer 4 showed higher TG than layer 3 inasmuch as layer 4 represented the major lipoprotein fraction for NL group (see Table 3).

Table 3 shows the total cholesterol (TC, expressed as the cholesterol moiety) and the UC concentrations of lipoprotein subfractions in HmHC, HtHC, and NL groups. The plasma TC in the HmHC group (300.05 ± 84.40 mg/dl) was 50% higher than that in the HtHC group (200.12 ± 80.82 mg/dl) ($P < 0.05$) and both were significantly higher ($P < 0.0005$ and $P < 0.005$, respectively) than that in the NL group (68.70 ± 13.64 mg/dl). The elevation in plasma TC was due to the increased concentrations in layers 2 and 3 of HC groups. More than a 13-fold increase in layer 2 and a 7-fold increase in layer 3 were observed in the HmHC when compared to the NL group. The HtHC group showed the intermediate values between HmHC and NL with a 7-fold increase in layer 2 and a 4-fold increase in layer 3. Although an increase in layer 4 was observed for both HmHC and HtHC, the values were not statistically significant. Total cholesterol in layers 1 and 5 was not affected by hypercholesterolemia.

As shown in Table 3, the plasma UC in HmHC (75.97 ± 20.40 mg/dl) and in HtHC (51.72 ± 25.76

TABLE 3. Total cholesterol and unesterified cholesterol concentrations in lipoprotein subfractions of plasma from immunogenetically defined swine homozygous and heterozygous for the Lpb^5-Lpu^1 haplotype with hypercholesterolemia and normolipidemic swine with Lpb^{non5}-Lpu^2 haplotypes

	Total Cholesterol			Unesterified Cholesterol		
Lipoproteins Subfractions	Normals (5)[a]	Homozygotes (6)	Heterozygotes (5)	Normals (5)	Homozygotes (6)	Heterozygotes (5)
		mg/dl of plasma			*mg/dl of plasma*	
Layer 1 (d<1.019 g/ml)	2.25 ± 0.74[b]	3.60 ± 1.79	3.19 ± 1.63	0.90 ± 0.27	1.89 ± 0.90	1.73 ± 1.14
Layer 2 (1.021-1.028 g/ml)	7.66 ± 5.67	101.88 ± 40.7[c]	52.75 ± 45.57[g]	1.71 ± 1.05	27.26 ± 10.90[c]	13.73 ± 9.72[f]
Layer 3 (1.032-1.043 g/ml)	19.93 ± 10.95	138.49 ± 40.4[c]	91.57 ± 48.30[e]	4.53 ± 2.31	35.62 ± 11.30[c]	24.48 ± 16.89[f]
Layer 4 (1.054-1.073 g/ml)	23.72 ± 8.12	41.08 ± 17.8	32.12 ± 13.35	4.91 ± 2.33	8.60 ± 3.74	7.79 ± 4.65
Layer 5 (d>1.073 g/ml)	15.13 ± 3.46	15.00 ± 9.41	20.49 ± 7.37	2.96 ± 0.76	2.61 ± 1.64	3.99 ± 1.58
Whole plasma	68.70 ± 13.64	300.05 ± 84.4[c]	200.12 ± 80.82[d]	14.99 ± 3.50	75.97 ± 20.4[c]	51.72 ± 25.76[e]

[a]Number in parentheses represents number of animals.
[b]Mean ± SD.
Statistical evaluation between hypercholesterolemic groups and the corresponding normolipidemic group for the significance of difference at: [c], $P < 0.0005$; [d], $P < 0.005$; [e], $P < 0.01$; [f], $P < 0.025$; [g], $P < 0.05$

mg/dl) was 5- and 3-fold higher, respectively, than that of the NL group (14.99 ± 3.50 mg/dl). These increases were significantly different ($P < 0.0005$ and $P < 0.01$, respectively). Similar to TC, the increased plasma UC was mainly due to the increasing UC in layers 2 and 3 of HC groups. There was a 16-fold increase in layer 2 and an 8-fold increase in layer 3 of HmHC group compared to NL, with $P < 0.0005$ for both layers. An intermediate 6-fold increase in layer 2 and a 5-fold increase in layer 3 of HtHC was observed ($P < 0.001$ and $P < 0.05$, respectively). Thus, the increase in TC in layers 2 and 3 of HC groups was due to the increase of both UC and CE of these two fractions.

The percent distributions of UC among all lipoprotein fractions resembled those of TC distribution, for each of the three swine groups (figures not shown). Layer 4 represents the major TC fraction in normal swine. In fact, the combined layers 3, 4, and 5 in NL group contained 86% of plasma TC and 82% of plasma UC. These TC and UC major peaks shifted in HtHC to layer 3 with skew to layer 2 and with more skew to layer 2 in the HmHC group (figures not shown).

Intermediate values were found between the NL and the HmHC group for all lipoprotein fractions of HtHC group in UC distribution, TC distribution, UC concentration, and TC concentration (except layer 5), regardless of whether the difference was significant or not. Such an intermediate relationship was not observed in the values of TG concentration or TG distrubution, confirming that the *Lpb*^{5}-*Lpu*^{1} genotype is indeed associated with esterified and unesterified cholesterol and not with triglycerides.

Concentrations of apoB in plasma and in lipoprotein subfractions of swine

The mean concentrations of apoB in plasma in NL, HmHC, and HtHC groups from all pigs studied were 43.9 ± 7.2 mg/dl, 166.0 ± 43.9 mg/dl, 113.3 ± 42.6 mg/dl, respectively. The differences in apoB between groups corresponded very closely to the differences observed for TC and UC. The estimated correlation between plasma TC and apoB for all 16 pigs studied was high ($r = + 0.96$). **Fig. 1** illustrates an example of apoB distributions in lipoprotein subfractions of individual pigs each representing a close apoB concentration phenotype to the mean plasma apoB of the three genotype groups. The mean apoB values for the distribution in the five lipoprotein subfractions were obtained from testing the layers of six pigs, two per each genotype group. The estimated apoB values for the 1–5 layers were 1.25, 3.25, 15.77, 22.11, and 4.93 mg/dl for the NL group; 1.83, 32.29, 131.62, 32.39, and 8.38 mg/dl for the HmHC group; and 1.76, 20.56, 70.56, 29.51, and 6.69 mg/dl for the HtHC group. These results demonstrated that apoB contents were markedly increased in layers 2 and 3 of hypercholesterolemic groups. HmHC group had 10- and 8-fold increase in layers 2 and 3, respectively, over the NL group, while HtHC group had approximately 6- and 4-fold increase, respectively, in the corresponding layers. These intermediate apoB values in layers 2 and 3 of HtHC group between HmHC and NL groups resemble the characteristics observed in TC and UC.

Compositions of lipoprotein subfractions

To assess whether the increase in UC and CE concentrations in HC swine was due to the increase in lipoproteins of normal composition or altered composition, the chemical compositions were determined for all subfractions isolated from the three groups of swine plasma and shown in **Table 4**. Close resemblance was observed in the chemical compositions of layer 1, the TG-rich lipoproteins, among all three fasting groups.

Comparing the chemical compositions of three LDL subfractions (layers 2, 3, and 4) from the three groups of swine (table 4) it was found that, in general, the relative protein content increased with increasing density. The TG decreased with increasing density. The phospholipids and the UC did not change much with the density. These compositional trends were similar to human LDL subfractions (16, 27, 28). Layer 4, the major fraction of normal swine LDL, showed similar composition in HmHC, HtHC, and NL groups, except that the phospholipids

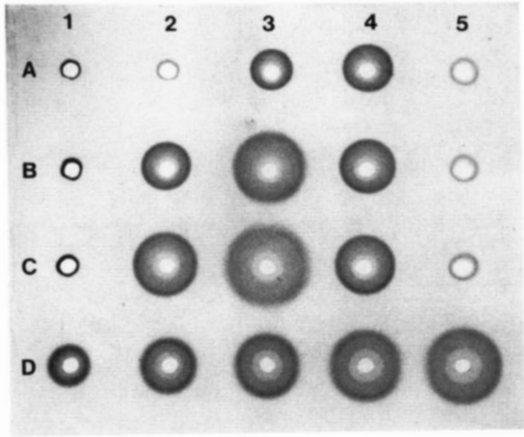

Fig. 1. Quantitation of apoB in lipoprotein subfractions of swine plasma by single radial immunodiffusion. Plasma was subfractionated individually into five layers by density gradient ultracentrifugation (see Methods). Aliquots of layers 1–5, each corresponding to 2 μl of plasma, were applied to individual wells marked 1–5, respectively, in rows A, B, and C. A: Layers from a normolipidemic *Lpb*^{2}-*Lpu*^{2}/*Lpb*^{4}-*Lpu*^{2} pig with plasma apoB 44.77 mg/dl (TC 69.5 mg/dl) with the distribution per layer: 1.25,2.11, 14.51, 21.27 and 5.63 mg/dl for layers 1–5, respectively; B: layers from a heterozygous *Lpb*^{5}-*Lpu*^{1}/*Lpb*^{8}-*Lpu*^{2} pig with plasma apoB 116.07 mg/dl (TC 205.0 mg/dl) with the following distribution per layer: 1.41, 20.99, 59.58, 28.03 and 6.06 mg/dl; C: layers from a homozygous *Lpb*^{5}-*Lpu*^{1}/*Lpb*^{5}*Lpu*^{1} pig with plasma apoB 196.64 mg/dl (TC 358.0 mg/dl) with the following distribution: 1.41, 39.58, 112.54, 35.21 and 8.17 mg/dl; D: apoB standards (see Methods). The quantities of the apoB standards applied in wells 1–5 were 15.49, 30.99, 46.48, 61.97, and 77.46 mg/dl, respectively. The apoB concentration values for layers with lower than 7 mg/dl were established by additional tests with larger sample volume (see Methods).

TABLE 4. Chemical compositions of lipoprotein subfractions from plasma of immunogenetically defined swine homozygous and heterozygous for the *LPB$_5$-Lpu^1* haplotype with hypercholesterolemia and normolipidemic swine with *Lpb^{nor5}-Lpu^2* haplotypes

Lipoproteins	Swine (n)	Proteins	Phospholipids	Cholesteryl Esters	Unesterified Cholesterol	Triglycerides
				%		
Layer 1 (d < 1.019 g/ml)	Normal (5)	14.44 ± 2.87[a]	17.32 ± 4.77	6.21 ± 1.96	4.38 ± 0.73	56.64 ± 7.77
	Homozygotes (6)	14.09 ± 2.52	14.87 ± 3.87	7.52 ± 2.54	5.20 ± 1.70	58.38 ± 7.42
	Heterozygotes (5)	14.46 ± 2.06	14.45 ± 3.76	6.44 ± 1.60	4.70 ± 1.53	59.94 ± 6.00
Layer 2 (d 1.021–1.028 g/ml)	Normal (5)	23.90 ± 4.62	19.94 ± 4.98	33.25 ± 9.01	8.46 ± 2.39	14.43 ± 11.29
	Homozygotes (6)	18.92 ± 2.11[c]	21.94 ± 1.68	46.42 ± 3.49[b]	10.10 ± 1.34	2.36 ± 2.74[c]
	Heterozgotes (5)	20.17 ± 2.07	20.96 ± 2.63	44.83 ± 2.84[c]	9.12 ± 0.98	6.85 ± 6.20
Layer 3 (d 1.032–1.043 g/ml)	Normal (5)	25.00 ± 4.70	20.69 ± 3.60	43.97 ± 3.64	8.55 ± 0.35	1.78 ± 0.84
	Homozygotes (6)	25.86 ± 2.59	22.32 ± 2.35	42.14 ± 3.81	9.27 ± 1.27	0.41 ± 0.13[b]
	Heterozygotes (6)	25.36 ± 2.91	21.45 ± 1.30	44.35 ± 1.94	8.36 ± 1.01	0.46 ± 0.24[b]
Layer 4 (d 1.054–1.073 g/ml)	Normal (5)	29.99 ± 1.39	21.42 ± 3.06	39.64 ± 2.64	7.07 ± 1.13	1.19 ± 1.04
	Homozygotes (6)	30.10 ± 4.80	18.41 ± 1.25[d]	42.84 ± 5.15	7.66 ± 1.05	0.79 ± 0.42
	Heterozygotes (5)	30.72 ± 3.33	20.43 ± 2.56	41.17 ± 4.81	6.63 ± 0.61	1.15 ± 0.72
Layer 5 (HDL) (d 1.073–1.21 g/ml)	Normal (5)	40.17 ± 3.69	28.75 ± 9.99	27.22 ± 6.14	3.58 ± 0.98	0.35 ± 0.31
	Homozygotes (6)	42.06 ± 5.24	28.18 ± 8.32	26.97 ± 4.69	3.55 ± 1.13	0.42 ± 0.41
	Heterozygotes (5)	41.53 ± 4.28	24.34 ± 5.78	30.07 ± 4.53	3.77 ± 0.65	0.35 ± 0.23

[a]Mean ± SD; no significant difference was observed between the hypercholesterolemic groups and the normolipidemic group in layers 1 and 5. Significance of difference at: [b], $P < 0.005$; [c], $P < 0.025$; [d], $P < 0.05$ when compared to the corresponding normals.

were slightly lower ($P < 0.05$) in the HmHC group. Significant differences ($P < 0.005$) were observed in percent TG of layer 3 from HmHC and HtHC compared to the NL group. Since the TG was such a minor component in this subfraction, these differences did not affect the relative contents of other components. Thus, the composition of all other components in layer 3 appeared to be similar among the three groups. The most striking differences in chemical compositions were found in layer 2: CE % (46.42 ± 3.49%) was significantly higher in HmHC than in NL (33.25 ± 9.01%) ($P < 0.005$). The increase in percent CE of HmHC was at the expense of protein (18.92 ± 2.11%) and TG (2.36 ± 2.74%). The corresponding percent protein was 23.91 ± 4.62% and TG 14.43 ± 11.29% in NL groups. Thus, the abnormal LDL particles in the HmHC group were characterized with CE-rich, protein-poor, and TG-poor layer 2. The HtHC group also showed higher CE % in layer 2 (44.83 ± 2.48%, $P < 0.025$). The content of all other components in layer 2 of HtHC fell at the intermediate values between HmHC and NL group, though the differences from NL group were not significant. Thus, the abnormal LDL particles in HtHC group were comprised of CE-rich lipoproteins in layer 2.

When all lipoprotein subfractions in NL group were examined, the highest percent CE content was found in layer 3. However, the highest CE content in HmHC group was in layer 2, whereas the highest CE content in HtHC group was found equally in layers 2 and 3.

No significant difference was observed in chemical compositions of HDL among the three groups (Table 4). When HDL was compared to LDL, the protein contents (40–42%) were significantly higher than those in LDL subfractions (19–31%). The phospholipids (24–29%) were higher, and the CE (27–30%) and UC (3.6–3.8%) were lower in HDL than those in LDL subfractions (18–22% phospholipids, 33–46% CE, and 7–10% UC).

Analyses by SDS-PAGE

Fig. 2 demonstrates that apoB-100 is the predominant apolipoprotein present in all three LDL subfractions in NL, HmHC, and HtHC groups of swine. It was verified

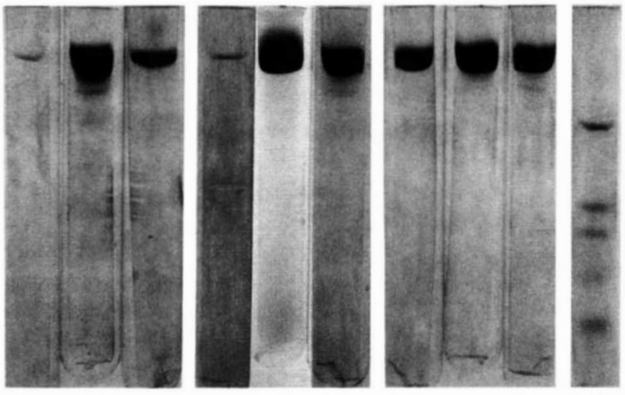

Fig. 2. LDL subfractions from hypercholesterolemic and normolipidemic swine plasma on 3.3% SDS-PAGE. After isolation and washing, the LDL subfractions, not adjusted to the original plasma volume, were applied to 3.3% SDS-PAGE. Eight μl of layer 2 (gels 1–3), 3 μl of layer 3 (gels 4–6), and 5 μl of layer 4 (gels 7–9) were from normolipidemic swine (gels 1, 4, 7), and swine homozygous (gels 2, 5, 8) and heterozygous (gels 3, 6, 9) for the Lpb^5-Lpu^1 haplotype with hypercholesterolemia. Gel S, calibration standards, from top to bottom: myosin, M_r, 200,000; β-galactosidase, M_r, 116,500; phosphorylase B, M_r 92,500; bovine serum albumin, M_r 66,200; and ovalbumin, M_r 45,000.

with gradient slab gel electrophoresis and basic urea-PAGE that apolipoproteins E and C were the minor components in these subfractions (figure not shown) and no difference in apolipoprotein composition was observed between HmHC or HtHC and NL group.

DISCUSSION

Results of this study extend the previous information (1, 8, 13) on association of apolipoprotein polymorphism with hyperlipidemia by demonstrating that under the same diet regimen (with low fat and zero cholesterol intake) and raised in the same living environment, pigs with different *apoB* and *apoU* alleles and genotypes (heterozygous vs homozygous) are characterized with different lipid and apoB levels and different lipoprotein compositions. Among them, pigs with the Lpb^5-Lpu^1 haplotype showed significantly elevated plasma apoB and cholesterol levels. The elevations in these parameters are attributed to the marked increase in concentration of light particles of LDL with abnormal compositions, i.e., CE-rich, protein-poor, and TG-poor particles of apparent density 1.021–1.028 g/ml, and, to a lesser extent, the increase in TG-poor LDL of d 1.032–1.043 g/ml. The cholesterol, apoB, and lipoproteins of pigs heterozygous for Lpb^5-Lpu^1 haplotype are characterized with intermediate values and lipoprotein patterns between homozygotes and normals. These data show that the three cholesterol and apoB phenotypes, correlated with the degree of hypercholesterolemia or hyperapobetalipoproteinemia, are associated with the Lpb^5-Lpu^1 haplotype dose: none in NL, one dose in the heterozygotes, and two doses in the homozygotes.

The complete chemical studies on all density fractions indicate that the allelic differences at the *Lpb* and *Lpu* loci have little effect on the quantities and qualtities of the TG-rich lipoproteins (d < 1.019 g/ml), HDL, and the dense subfraction of LDL, characterized by d 1.054–1.073 g/ml.

Our findings on the increasing compositionally abnormal buoyant LDL in hypercholesterolemic swine are in good agreement with the earlier report (13). Checovich et al. (13) grouped the swine based on plasma cholesterol levels. The earlier "mutant" group was composed of Lpb^5-Lpu^1 homozygotes, heterozygotes, and four not completely genotyped hypercholesterolemic pigs (13). In the present studies pigs were selected based solely on the Lpb^5-Lpu^1 genotype. Thus, a direct comparison of lipid and apoB levels between homozygotes and heterozygotes of Lpb^5-Lpu^1 haplotype became possible.

It is interesting to note that the composition of normal human layer 2 falls into the median range between the normolipidemic and the hypercholesterolemic swine layer 2 (16), and that the composition of normal human layer 3 falls into the median range between the normolipidemic and the hypercholesterolemic swine layer 3, with the exception of lower TG in the latter (16).

The accumulation of plasma cholesterol in hypercholesterolemic swine was demonstrated to be due to the slower catabolic rate of abnormal LDL particles, though the LDL receptor activity in hypercholesterolemic swine groups was found to be normal (13). The slower catabolic rate of abnormal LDL particles was not due to abnormal apolipoprotein composition since the latter was not observed between hypercholesterolemic group and normal group. The putative receptor-binding region of apoB carrying Lpb^5 allele was also shown to have the same amino acid sequence as normal pig apoB (9). However, an insertion of approximately 250 bp of extra DNA into intron 28 was found in *Hind*III fragment in Lpb^5, and a *Hinc*II site polymorphism in exon 29 was found present in Lpb^5 and in Lpb^8, but absent in the other alleles (9). These mutations, especially exon mutations, and other single amino acid substitutions found in apoB with Lpb^5 (9) may play a role in altering the conformation of the LDL and result in the slow removal of these abnormal lipoprotein particles.

Segregation data on Lpu, with a lack of recombination among *Lpb-Lpu* haplotypes observed in 18 generations, indicate a close *apoB-apoU* linkage.

While statistical means of cholesterol concentration phenotypes for the three groups correlate well with the *Lpb-Lpu* genotypes, the high standard deviations indicate the existence of other yet undefined genetic variants influencing the observed phenotypic variations. These variations are in agreement with the cosegregation data from the original study (1), indicating polygenic influence on the cholesterol concentration phenotype. A possibility that Lpr^1 allele has some influence on the observed variations is under investigation.

This is the first report establishing that a direct correlation is present in swine between the *Lpb*^{5}-*Lpu*^{1} genotype at the *apoB* and *apoU* loci and the chemical compositions and concentrations of two LDL subpopulations in homozygotes and in heterozygotes. These data suggest that the genotype for *Lpb*^{5}-*Lpu*^{1} haplotype modulates the lipoprotein metabolism reflecting on the concentrations and compositions of LDL subpopulations. In other words, in the absence of dietary and environmental effects, *apoB* and *apoU* genes dictate the concentrations and compositions of LDL subpopulations. Since swine lipoprotein and apoB resemble those of humans, it seems likely that a similar relationship between *apoB* alleles and LDL subpopulations may also exist in humans. In fact, earlier studies by Berg et al. (29) on association between immunologically defined polymorphism of human apoB (Ag system) (30) and plasma lipid levels, showed that persons lacking the Ag(x) epitope (Ag(x –)) had higher levels of cholesterol and triglycerides. Law et al. (31) found that subjects who lacked the DNA *XbaI* restriction site polymorphism (*XbaI* –) had lower cholesterol and triglyceride levels. Berg et al. (32) found that Ag(x –) cosegregates with the presence of the *XbaI* restriction site polymorphism (*XbaI* +), and Ag(x +) with the absence of this restriction site (*XbaI* –).

Most recently, Soria et al. (33) identified that subjects with *apoB* mutation in the codon for amino acid 3500 (CGG→CAG), a CG mutational "hot spot," was associated with defective LDL and hypercholesterolemia.

Thus, genetically defined swine serve as a valuable model for studying apolipoproteins and lipoproteins in relation to atherosclerosis.

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