Apolipoprotein B signal peptide insertion/deletion polymorphism is associated with Ag epitopes and involved in the determination of serum triglyceride levels

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Abstract We have investigated the insertion/deletion polymorphism in the signal peptide region of the apoB gene in 106 Finnish individuals from North Karelia. The relative frequency of the insertion allele in this sample was 0.73. Strong linkage disequilibrium was detected between this apoB insertion/deletion polymorphism and the Ag(c/g) epitope pair of apoB, while weak linkage disequilibrium was detected between the polymorphism and the four other reported Ag epitope pairs [(a1/d), (x/y), (h/i) and (t/z)], as well as the apoB PvuII and the XbaI RFLPs. Using one-way analysis of variance there was a statistically significant association ($P < 0.05$) between the apoB insertion/deletion polymorphism and serum triglyceride levels in this sample. Individuals homozygous for the insertion allele had higher triglyceride levels than individuals homozygous for the deletion allele, while individuals heterozygous for the polymorphism had intermediate levels. These differences were reduced when individuals were consuming a low fat diet but were statistically significant when the individuals returned to their normal diet. It is possible that insertion or deletion of three hydrophobic amino acids (leu-ala-leu) from the signal peptide of apoB may have a direct effect on plasma triglyceride levels by altering the intracellular processing of apoB or apoB-containing lipoproteins in the liver or intestine. —**Xu, C-f., M. J. Tikkanen, J. K. Huttunen, P. Pietinen, R. Bütler, S. Humphries, and P. Talmud.** Apolipoprotein B signal peptide insertion/deletion polymorphism is associated with Ag epitopes and involved in the determination of serum triglyceride levels. *J. Lipid Res.* 1990. **31:** 1255–1261.

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Apolipoprotein (apo) B is the principal structural apoprotein of chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein, and low density lipoprotein (LDL). It is important in the assembly and secretion of both chylomicrons from the small intestine and VLDL from the liver (1), and also functions as a ligand for the LDL-receptor, thus mediating the cellular uptake of cholesterol (2). Due to the central role played by apoB in the transport of lipids, genetic variations in the apoB gene could help explain the between-individual difference in lipid levels and susceptibility to coronary heart disease (CHD) reported in population studies.

The first evidence for structural variants of apoB and LDL came from serological studies of multiple blood-transfused patients (3, 4). Five closely linked loci, with two co-dominant alleles each, have been identified: Ag (a1/d), Ag (c/g), Ag (x/y), Ag (h/i), and Ag (t/z) (5, 6). The successful cloning and sequencing of the apoB gene (7–10) have made it possible to study apoB variation at the DNA level. A number of RFLPs of the apoB gene have since been reported (11, 12). As a consequence, four of the five Ag loci have been localized to four specific polymorphic sites in the apoB gene identified with restriction enzymes: Ag (c/g), with restriction enzymes BSP1286I (13) and ApaLI (14), at amino acid (aa) residue 71; Ag (a1/d), with AluI (15), at aa 591; Ag (h/i), with MspI (16), at aa 3611 and Ag (t/z), with EcoRI (17, 18), at aa 4154.

Based on population and case control studies, associations between specific apoB epitopes and lipid levels have been reported (19–21). In addition, several groups have shown that genotypes of the apoB XbaI RFLP, which does not itself alter an amino acid in the protein, are associated with significant differences in serum cholesterol

Abbreviations: *ins/del*, insertion/deletion; apo, apolipoprotein; chol, cholesterol; Tg, triglyceride; LDL, low density lipoprotein; HDL, high density lipoprotein; RFLP, restriction fragment length polymorphism; PCR, polymerase chain reaction; SD, standard deviation.

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levels (22–24). Furthermore, this polymorphism shows linkage disequilibrium with the Ag epitopes (17, 18, 25). Boerwinkle and Chan (26) recently reported a rapid method for detecting a 9 base pair (bp) insertion/deletion polymorphism in the signal peptide region of the apoB gene, encoding amino acid -16 to -14 (leu-ala-leu). This polymorphism was first noted by comparison of the apoB gene sequence reported by Cladaras et al. (8) and others (7, 9). In this study, we have examined the association between this polymorphism and the Ag epitopes and apoB RFLPs in 106 Finnish individuals. These individuals had all participated in the North Karelia dietary intervention studies (27–29), which demonstrated that a switch to a diet low in saturated fat and cholesterol produced a dramatic lowering in plasma LDL-, HDL-cholesterol, apoB, and apoA-I levels. We were thus able to study the association between this polymorphism and plasma lipid, lipoprotein, and apolipoprotein levels in individuals on high fat and low fat diets.

MATERIALS AND METHODS

Subjects

Fifty five men and 51 women aged 30–50 years with no history of hyperlipidemia, from a rural community in North Karelia, Finland, participated in this study. Complete details of the selection criteria for these individuals have been presented elsewhere (27–29). Briefly, selected individuals were healthy spouse-pairs recruited from a country-wide screen. This population has been very stable for the last several hundred years, because of the language used and rural nature of the area, with practically no influence of Swedish-speaking individuals. Details of the methods used to determine lipid, lipoprotein, and apoprotein levels on the basal high fat diet, low fat intervention diet, and switch-back period are given elsewhere (27–30). Alcohol consumption was monitored by dietary inventory and did not change during the period of the study. The Ag phenotyping, RFLP genotyping, lipid measurement, and DNA extraction have been previously described (16, 30).

Polymerase chain reaction (PCR)

Oligonucleotides were synthesized on a Pharmacia Gene Assembler (Pharmacia, Sweden). Purification was performed using an NAP-10 column (Pharmacia). The primer pair was designed to amplify the first exon on the apoB gene using the oligonucleotide 5'-CAGCTGGCGATGGACCCGCCGA-3' as the 5' primer and 5'-ACCGGCCCTGGCGCCCGCCAGCA-3' as the 3' primer (26). The amplification reaction was carried out in a final volume of 50 μl containing 200 ng of genomic DNA, 35 pmol of each primer, and 0.75 unit of Taq polymerase (Perkin Elmer-Cetus) in the reaction buffer recommended by the manufacturer, using a Cambio Intelligent Heating block (Cambio). Fifty μl of liquid paraffin was added to the top to prevent evaporation during the process. The denaturation step of the PCR was performed at 95°C for 5 min and annealing and extension at 65°C for 1.5 min for the first round. Fifty subsequent cycles were as follows, 95°C for 1 min and 65°C for 1.5 min.

Gel electrophoresis

Fifteen μl of the PCR product was loaded onto a 5% NuSieve agarose (FMC, Catalog No. 10699) and 2% of electrophoresis grade agarose (BRL, Catalog No. 5510UB) gel. The polymorphic allele difference could be directly typed under UV after 5 h electrophoresis in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, pH 7.7) at 50 volts.

Statistical analysis

Chi-square analysis was used to test the null hypothesis that there is no association between the apoB signal peptide *ins/del* polymorphism and each pair of the Ag epitopes or the apoB PvuII and XbaI RFLPs. The strength of the associations was estimated using the correlation coefficient delta (31). Confidence limits (95% of delta) were calculated using the z transformation (32). Multiple linear regression was used to adjust serum lipid, lipoprotein, and apolipoprotein levels for age, gender, and recorded body mass index (BMI) on baseline, intervention, and switch-back (mean ± SD 25.7 ± 3.1, 25.4 ± 3.0, 25.3 ± 3.0) at the end of each dietary period, respectively. Triglyceride levels were skewed to higher values, and statistical analysis was also carried out on $\log_{10}$ transformed data, which resulted in a distribution of values in the sample not significantly different from normal. Distribution of all other lipid traits was not significantly different from normal in this sample. A one-way analysis of variance (ANOVA) was performed on the adjusted lipid levels to test the null hypothesis that phenotypic variation is not associated with the apoB genetic *ins/del* polymorphism. The significance of the differences observed was estimated using the F test, to compare mean square values between and within different genotype groups. The percentage of sample variance in lipid traits associated with the *ins/del* polymorphism ($R^2 \times 100$) was estimated by linear regression. The change in lipid traits "delta" between the baseline and low fat diet was estimated for each individual after adjustment of each trait for gender, age, and recorded BMI. Delta = adjusted base-line value – adjusted intervention value. The association between delta and genotype was assessed by ANOVA. Statistical analysis was carried out using the MINITAB (State College, PA) computer package. We considered statistical significance to be at the 0.05 level.

RESULTS

The alleles of the *ins/del* polymorphism in the signal peptide region of the apoB gene detected by PCR and NuSieve gel electrophoresis are presented in **Fig. 1.** We designated the longer allele of 93 bp the *ins* allele and the shorter allele of 84 bp the *del* allele, with frequencies of 0.73 and 0.27, respectively. The distribution of genotypes observed is that expected for a sample in Hardy-Weinberg equilibrium. The frequency of the *ins* allele in this Finnish population is significantly higher than that reported previously (26) (gene counting $X^2 = 6.6$, 1df, $P < 0.01$).

The 106 individuals were each typed for the five Ag polymorphisms and the number of individuals exhibiting each antigen type and the *ins/del* polymorphism combination is presented in **Table 1.** By means of X^2 analysis and calculation of correlation coefficient, delta, we found a highly significant association between the Ag (c/g) epitope pair and the *ins/del* polymorphism ($P < 0.0001$), with the (g) antigen being associated with the *ins* allele, and the (c) antigen with the *del* allele. There was a statistically significant association between the *ins/del* polymorphism and the Ag (a1/d) ($P < 0.0001$), and the (x/y) ($P < 0.005$) epitope pairs and the apoB XbaI RFLP ($P < 0.0001$). We detected weak linkage disequilibrium between this polymorphism and the Ag (h/i) and Ag (t/z) epitope pairs as well as the apoB PvuII RFLP in intron 4 (33) (Table 1). In the absence of family studies it is not possible unambiguously to determine haplotypes at this locus. However, inspection of the data shows that while the *ins* allele occurs on many haplotypes, the *del* allele occurs unambiguously on at least three haplotypes defined by the other seven polymorphic sites (data from Table 1 and not shown).

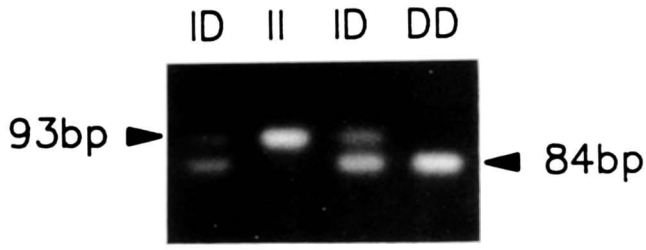

Fig. 1. The 5' insertion/deletion polymorphism of the apoB gene. "I" represents the *ins* allele and "D" the *del* allele.

All individuals had taken part in the North Karelia dietary intervention study in 1985 (27-29). One-way analysis of variance was first used to compare mean serum lipid, lipoprotein, and apolipoprotein levels with different

TABLE 1. Genotype association among Ag epitopes, apoB RFLPs, and the apoB 5' insertion/deletion polymorphism

	Position (aa)	II[a]	ID[a]	DD[a]	Total	χ^2	Delta (Confidence Limit)
cc		0	0	5	5		−0.98
cg	71	0	45	0	45	204	(−0.97)-(−0.98)
gg		54	2	0	56	$P < 0.0001$	
a1a1		26	6	0	32		+0.50
a1d	591	21	32	0	53	36.4	(+0.39)-(+0.59)
dd		7	9	5	21	$P < 0.0001$	
V1V1		35	40	5	80		−0.24
V1V2	IVS 4	16	7	0	23	8.3	(−0.11)-(−0.36)
V2V2		3	0	0	3	$P > 0.05$	
xx		9	0	0	9		+0.34
xy	?	18	25	0	43	15.8	(+0.21)-(+0.45)
yy		27	22	5	54	$P < 0.005$	
X1X1		29	12	0	41		+0.45
X1X2	2488	22	27	0	49	38.4	(+0.34)-(+0.55)
X2X2		3	8	5	16	$P < 0.0001$	
hh		0	0	0	0		−0.22
hi	3611	3	7	2	12	6.5	(−0.09)-(−0.34)
ii		51	40	3	94	$P > 0.1$	
tt		33	37	5	75		−0.25
tz	4154	19	10	0	29	6.9	(−0.12)-(−0.37)
zz		2	0	0	2	$P > 0.1$	
Total		54	47	5	106		

Based on the five *del/del*-cc individuals and the two *del/ins*-gg individuals, the *del* allele occurs unambiguously only on the following haplotypes: *1*) *del*-c-d-V1-y-X2-h-t (2 chromosomes); *2*) *del*-c-d-V1-y-X2-i-t (8 chromosomes); *3*) *del*-g-a1-V1-y-X2-i-t (2 chromosomes).

[a]I, insertion allele; D, deletion allele.

TABLE 2. Mean (± SD) lipid, lipoprotein, and apolipoprotien levels with different apoB 5' insertion/deletion genotypes in the sample from North Karelia

Fraction		II[a] (n = 47)	ID[a] (n = 44)	DD[a] (n = 5)	F
Chol	(mmol/l)	6.3 ± 1.1	6.1 ± 1.2	6.5 ± 1.3	0.50
Tg	(mmol/l)	1.3 ± 0.7	1.0 ± 0.4	0.8 ± 0.4	3.3[b]
LDL-chol	(mmol/l)	4.6 ± 1.1	4.5 ± 1.1	4.7 ± 1.0	0.25
HDL-chol	(mmol/l)	1.4 ± 0.3	1.4 ± 0.3	1.6 ± 0.4	1.0
ApoB	(mg/dl)	120 ± 29	113 ± 26	119 ± 22	0.72
ApoA-I	(mg/dl)	158 ± 20	155 ± 25	174 ± 24	1.7
ApoA-II	(mg/dl)	44 ± 5	42 ± 6	45 ± 4	1.4

[a]I, insertion allele; D, deletion allele.
[b]$P < 0.05$.

genotype of the *ins/del* polymorphism on the baseline diet (**Table 2**). No significant association was found between the *ins/del* polymorphism and apoB or LDL-cholesterol levels, nor with apoA-I, apoA-II, HDL-, or total-cholesterol levels. However, as shown in Table 2, individuals homozygous for the *ins* allele have higher triglyceride levels than individuals homozygous for the *del* allele, with individuals heterozygous for both alleles having intermediate levels, compatible with a co-dominant effect of this polymorphism. These differences are statistically significant ($P < 0.05$). When the data were analyzed in men and women separately, a similar effect on triglyceride levels associated with *ins/del* genotype was seen in both groups (not shown). The distribution of the triglyceride levels in this sample is skewed to higher values; however, after $\log_{10}$ transformation, the difference associated with genotype remained statistically significant ($F = 3.3$, $P < 0.05$). By linear regression the apoB *ins/del* polymorphism explained 6.7% of the phenotypic variance in serum triglyceride levels in this sample.

We then investigated the associations between the *ins/del* polymorphism and serum lipid levels in the same individuals who had consumed a low fat diet for 6 weeks and after they returned to their original high fat diet. On the low fat diet the mean serum triglyceride levels of the whole sample fell by 2.7% (1.13 ± 0.58 to 1.10 ± 0.45 mmol/l) and returned to the original levels (1.12 ± 0.56 mmol/l) on switch-back. As shown in **Table 3,** the effect of the polymorphism on the serum triglyceride levels diminished during the dietary intervention period and reappeared on the switch-back diet. No other significant associations between the polymorphism and dietary response in any plasma lipid, lipoprotein, and apolipoprotein levels were observed.

For each individual we also estimated the change in each lipid trait, "delta," between the base-line and low fat diets. Only for plasma triglycerides was there evidence of a significant difference associated with *ins/del* genotype. Individuals with the genotype *ins/ins* had a mean reduction in plasma triglycerides of 0.11 (± 0.46) mmol/l while individuals with one or more *del* allele showed a mean increase in plasma triglycerides of 0.07 (± 0.53) mmol/l. These differences were statistically significant ($F = 4.99$, $P = 0.03$).

DISCUSSION

We have examined the association between the *ins/del* polymorphism in the signal peptide of apoB and polymorphisms in apoB detected as the Ag epitopes. The Ag (c/g) epitope pair is created by a T-C transition at nucleotide 421 on the cDNA (the 4th exon of the apoB gene), resulting in an isoleucine-threonine substitution (13). Compared with other Ag antigen pairs, this amino acid change is the closest to the NH_2- terminal end of the protein, in

TABLE 3. Mean (± SD) triglyceride levels of individuals with different *ins/del* genotypes in base-line, intervention, and switch-back diets

Diet	II[a] (n = 47)	ID[a] (n = 44)	DD[a] (n = 5)	F	R^2
		mmol/l			
Base-line	1.28 ± 0.68	1.00 ± 0.42	0.85 ± 0.40	3.3	6.7[b]
Intervention	1.15 ± 0.49	1.07 ± 0.42	0.90 ± 0.21	0.8	1.6
Switch-back	1.27 ± 0.71	1.00 ± 0.33	0.81 ± 0.19	3.7	7.2[b]

[a]I, insertion allele; D, deletion allele.
[b]$P < 0.05$.

the T4 peptide, and thus the closest to the *ins/del* polymorphism. It is, therefore, not surprising that these two polymorphisms show very strong linkage disequilibrium.

In this sample the *del* allele occurs unambiguously on three haplotypes defined by the other seven polymorphic sites. Two of these are related to each other by a single base change in the gene that creates the h/i epitope pair. The other haplotype contains two additional changes at amino acid 71 (c/g) and 591 (a1/d). These observations could be explained by recombination in the apoB gene leading to loss of linkage disequilibrium, or multiple events creating the deletion allele. It is not possible to distinguish between these possibilities with the present data.

It has been reported by several groups that polymorphisms at the apoB gene locus are associated with between-individual differences in plasma lipid levels (21–24). In addition, a rare amino acid substitution (Arg-Gln) at residue 3500 alters receptor binding ability in patients with familial defective apolipoprotein B-100 (34) causing hypercholesterolemia, which in some patients is associated with premature atherosclerosis (35). In this study we have observed that a recently reported polymorphism of apoB, created by the insertion or deletion of three amino acids in the signal peptide region of the protein (8, 26), is significantly associated with serum triglyceride levels. The insertion allele, with 27 amino acids as the signal peptide of apoB, is associated with higher serum triglyceride levels, while the deletion allele, with only 24 amino acids, is associated with lower levels. Significant differences in mean triglyceride levels were also observed when the subjects were grouped according to their Ag(c/g) immunophenotypes. The (g) allele was associated with higher triglyceride levels while the (c) allele was associated with the lower levels ($P < 0.05$) (data not shown). The fact that the same association was observed in a duplicate estimate of lipid traits (switch-back) strengthens the conclusions in this group of individuals, though the association needs to be confirmed in a genetically independent sample.

The function of signal peptides in the biosynthesis and secretion of proteins is to direct proteins across membranes during the initial stage of export from their site of synthesis to their final destination (36–38). After insertion of the signal peptide and the growing protein chain into the membrane of the endoplasmic reticulum, the signal peptide is cleaved off (36–38). The three amino acids leu-ala-leu, included in the apoB insertion allele and excluded in the deletion allele, may alter the hydrophobicity of the signal peptide of the alleles. It could, therefore, alter the rate of translocation of newly synthesized apoB from the cytoplasm into the endoplasmic reticulum. This might result in a different rate of secretion of apoB from either the epithelial cells of the small intestine (B-48) as chylomicrons, or from the hepatocytes in the liver (B-100) as VLDL. In this sample of individuals on a high fat diet, we observed differences in plasma triglycerides in the absence of any significant differences in plasma apoB levels. Since there is one apoB protein in each lipoprotein particle (1, 7–9) this suggests that the triglyceride content of the apoB-containing particles in individuals with different *ins/del* genotypes is altered. If this association is confirmed in larger samples, it suggests that this polymorphism may therefore have a direct effect on intracellular handling of apoB protein and the apoB-containing lipoproteins. Alternatively, the polymorphism may be functionally silent and may be in linkage disequilibrium with a functionally important sequence change in the coding or the promoter regions of the gene.

The effect associated with the insertion/deletion polymorphism in this population is modulated by diet. On the basal high fat, low P/S diet, the effect of the polymorphism on triglyceride levels was significant, explaining 6.7% of the phenotypic variance. When individuals changed to a low fat, high P/S diet, this effect disappeared, but when they returned to the original diet the effect reappeared, explaining 7.2% of the phenotypic variance in this sample. Individuals homozygous for the *ins* allele have a significantly greater reduction in plasma triglycerides on the low fat diet than those individuals with one or more *del* allele. We speculate that the postulated difference in intracellular handling of apoB associated with the *ins/del* polymorphism disappears on the low fat diet because of overall reduced synthesis and secretion of lipoproteins. Cell biology experiments and in vivo turnover studies on individuals with different *ins/del* genotypes would clarify this hypothesis and help elucidate the functional difference resulting from the sequence variation in the region of the apoB signal peptide.

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