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DNA Polymorphisms in and around the Apo-A1-CIII Genes and Genetic Hyperlipidemias

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SUMMARY

We have studied the frequency of DNA polymorphisms in and around the apolipoprotein A-1 (Apo-A1) and apolipoprotein CIII (Apo-CIII) gene loci in 53 persons of Caucasian descent with genetic hyperlipidemias. Three restriction-fragment-length polymorphisms (RFLPs) have previously been located 5′ and 3′ to the Apo-A1 gene and in the Apo-CIII gene and were detected after digestion with *Xmn*I, *Pst*I, and *Sst*I, respectively, and hybridization with a 2.2-kb fragment of the Apo-A1 gene. These RFLPs are in linkage equilibrium. The rare variant sites for *Xmn*I (X2) and *Sst*I (S2) were more frequent in familial combined hyperlipidemia (FCH) than in controls and persons with other genetic hyperlipidemias. When considered as a haplotype, this difference was significant ($P < .03$). The findings in this study suggest that the previously reported association between S2 and hypertriglyceridemia may be accounted for, in part, by inclusion of numerous patients with FCH. Our data provide further evidence that these RFLPs around and within the Apo-A1/Apo-CIII genes do not participate in unmasking clinical expression in persons with familial dysbetalipoproteinemia.

INTRODUCTION

It has recently been proposed that a polymorphism around the human apolipoprotein A1 (Apo-A1) and apolipoprotein CIII gene (Apo-CIII) loci may be

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related to hypertriglyceridemia (Rees et al. 1983, 1985). This restriction-fragment-length polymorphism (RFLP) is due to a variable *Sst*I site that has been mapped in or close to the 3′ noncoding region of the Apo-CIII gene. Using enzymes *Xmn*I and *Pst*I, Kessling et al. (1985) have detected two further RFLPs around the A1 locus: the variable *Xmn*I site is ~2.5 kb 5′ to the Apo-A1 gene while the variable *Pst*I site is at the 3′ side of the Apo-A1 gene. They found a statistically significant increase in the rarer allele after digestion with *Sst*I and *Xmn*I and hybridization with an Apo-A1 probe in nondiabetic persons with type IV and type V hyperlipidemia in terms of the Frederickson classification (Brunzell et al. 1983).

Hypertriglyceridemia is the most common hyperlipidemia and occurs in ~10% of the general population (Assmann 1982). In most instances it is secondary to diabetes mellitus (Brunzell et al. 1978), obesity, alcoholism, and drugs such as thiazide diuretics (Helgeland et al. 1978) and, occasionally, β-adrenergic blockers (Helgeland et al. 1978).

Investigation of families with persistent hypertriglyceridemia has revealed that ~30% of persons are suffering from a common genetic disorder called familial combined hyperlipidemia (FCH) (Brunzell et al. 1976). This disease, which appears to be inherited as an autosomal dominant trait, is present in ~1% of the general population but affects ~12% of men with coronary atherosclerosis below the age of 60 (Goldstein et al. 1973). It is characterized by the presence of multiple lipoprotein phenotypes in the same family. This variability of lipid values is reflected in varying electrophoretic patterns, since patients with FCH and their affected relatives may be characterized as type IV, type II, and, less commonly, type V phenotypes in terms of the Frederickson classification (Brunzell et al. 1983). Several lipoprotein phenotypes may be seen in the same individual on different occasions. In contrast to such other hypertriglyceridemia-causing disorders as familial hypertriglyceridemia, very-low-density-lipoprotein (VLDL) apolipoprotein-B synthetic rates are elevated in patients with FCH and this elevation appears to be an important marker for this disease.

Familial dysbetalipoproteinemia (FD) is defined by an absolute deficiency in one of the three phenotypes of apolipoprotein-E (Apo-E), namely, Apo-E3 (Utermann et al. 1980). The E3 apolipoprotein is crucial for the clearance of VLDL and chylomicron remnants. Despite the high frequency (1 in 100) of E3 deficiency, the phenotypic expression of the disease as characterized by hypertriglyceridemia and hypercholesterolemia is uncommon (1 in 5,000) (Utermann et al. 1977). The factors important for the expression of the disease in persons with the genetic deficiency of E3 are not well understood.

Rarer genetic causes of hypertriglyceridemia include familial hypertriglyceridemia (Glueck et al. 1973) and autosomal recessive disorders such as lipoprotein lipase deficiency (Brunzell et al. 1980) and apolipoprotein-CII deficiency (Cox et al. 1978).

In previous reports documenting the relationship between Apo-A1 polymorphisms and hypertriglyceridemia, patients have been subdivided into groups for comparison primarily on the basis of their lipoprotein-electrophoretic profiles (Kessling et al. 1985; Rees et al. 1985). No clear distinction has been

made as to whether subjects were suffering from a primary genetic disorder or whether the hyperlipidemia was secondary to environmental factors.

In an effort to explore whether the previously reported finding reflects an association with a particular genetic hyperlipidemia, we chose to classify a group of hypertriglyceridemia patients on the basis of their primary genetic disorder, irrespective of their lipoprotein-electrophoretic pattern at the time of testing. In this way an association between polymorphisms around the Apo-A1/Apo-CIII loci and the genes for FCH, FD, and familial hypercholesterolemia (FH) was investigated.

Variations in the distribution of the *Sst*I polymorphism in the Apo-CIII gene have been shown in nonhyperlipidemic individuals of different ethnic groups (Rees et al. 1985, 1986). The variant *Sst*I site is present in 65% of Chinese and in 30% of Africans and Indian Asians, whereas no normotriglyceridemic Caucasians demonstrated this polymorphism. To remove this bias, we studied only persons of Caucasian ancestry.

METHODS

Patients

Unrelated hyperlipidemic patients were selected from the Lipid Clinic at Shaughnessy Hospital. Patients with secondary hyperlipidemias, such as diabetics, were excluded from this group. Renal, hepatic, and thyroid function were normal in these subjects, and they were not taking any drugs known to cause hyperlipidemia. All of the participants were off alcohol for 72 h prior to testing. These subjects had undergone detailed dietary assessments and were following diets in which intake of saturated fat was <30% of total kilocalories and cholesterol intake was <300 mg/day. Twenty-four persons (11 females and 13 males) were diagnosed as FCH, based on the presence of hypertriglyceridemia and/or hypercholesterolemia in their first-degree relatives and apolipoprotein-B above the level determined as the normal upper limit in our laboratory (table 1). The diagnosis of FD was made in 14 patients (seven females and seven males) on the basis of characteristic clinical features (planar or tuberous xanthomata), the presence of a floating β-migrating lipoprotein band on ultracentrifugation and electrophoresis, and the absense of Apo-E3 after phenotyping for Apo-E. Fifteen patients (10 females and five males) with FH were diagnosed on the basis of hypercholesterolemia, characteristic clinical features (tendon xanthomata), and the presence of a parent and/or sib with hypercholesterolemia.

Thirty-eight control subjects (18 males and 20 females) were healthy, normal Caucasian volunteers who had serum cholesterol and triglyceride levels between the 25th and 75th percentiles for their age. The mean age of the patients was 36 ± 4 years, and that of the controls was 33 ± 3 years.

MATERIAL AND METHODS

DNA Analysis

DNA was prepared from frozen whole blood by means of a modification of the procedure of D. Hoar (personal communication). Aliquots (5 μg) of DNA

TABLE 1

MEAN ± SD TRIGLYCERIDE, CHOLESTEROL, HDL, AND APO-B IN CONTROLS AND PERSONS WITH GENETIC HYPERLIPIDEMIAS

Serum Component	Controls (38)	Familial Hypercholesteremia (15)	Familial Combined Hyperlipidemia (24)	Familial Dysbetalipoproteinemia (14)
Triglyceride	110 ± 10	150 ± 65	301 ± 184***	676 ± 460***
Cholesterol	185 ± 89	345 ± 77*	301 ± 51*	416 ± 138**
HDL	50 ± 6	45 ± 14	43 ± 13	33 ± 12**
Apo-B	102 ± 7	162 ± 55	179 ± 52**	87 ± 33

NOTE.—Underlined numbers represent values significantly different from numbers not underlined. Numbers in parentheses are number of subjects.
* $P < .05$.
** $P < .001$.
*** $P < .0001$.

were digested with each of the following restriction endonucleases: *Sst*I (Bethesda Research Labs, Gaithersburg, MD), *Pst*I (Pharmacia Ltd., Dorval, Québec), and *Xmn*I (New England Biolabs, Beverly, MA). DNA fragments were electrophoresed through a 0.8% agarose gel and transferred to nylon filters by means of Southern blotting (Southern 1975). The filters were prehybridized in 5 × SSPE (pH 7.5), 10 × Denhardt's (0.2% BSA, 0.2% PVP-360, and 0.2% Ficoll), and 0.5 mg sheared denatured salmon sperm DNA/ml, and 0.3% sodium dodecyl sulfate (SDS) and incubated several hours at 65 C. Filters were hybridized overnight at 65 C in 5 × SSPE, 5 × Denhardt's, 0.5 mg salmon sperm DNA/ml, and 0.1% SDS. The genomic probe of the human Apo-A1 gene (pA1-2.2) was a gift from Dr. Steven Humphries (Charing Cross Sunley Research Centre, London). A *Pst*I digest was used to isolate the 2.2-kb fragment containing the Apo-A1 gene. By means of the random oligonucleotide-priming method (Feinberg and Vogelstein 1983, 1984), ~20 ng of this probe was labeled with P^{32} (Amersham, Canada) to 10^8 cpm. A minimum of 1×10^7 cpm/400 cm^2 of filter was used for each hybridization. Filters were washed twice in 0.5 × SSPE, 0.1% SDS for 15 min at 65 C and then once in 0.1 × SSPE, 0.1% SDS for 15 min and exposed to Kodak XAR-5 film with intensifying screens at −70 C for 1–4 days.

Biochemical Analysis

Cholesterol and triglyceride in the ethylenediaminetetraacetate-plasma and lipoprotein fractions of fasting subjects were measured by means of enzymatic methods (Bucculo and David 1973; Allain et al. 1974). High-density-lipoprotein cholesterol (HDL) was measured enzymatically after heparin manganese precipitation of Apo-B-containing lipoproteins, as described by Albers et al. (1978). Plasma Apo-B was measured by means of radial immunodiffusion on commercial plates (Tago, Inc., Burlingham, CA) (Mancini et al. 1958). Isoforms of

Apo-E were determined by means of the one-dimensional gel isoelectric-focusing method of Warnick et al. (1979).

Statistical Analysis

The mean triglyceride, cholesterol, HDL, and Apo-B values in the control and the three patient groups were compared using analysis of variance and Tukey's pairwise comparison procedure ($P < .05$). The analysis of genotypic frequencies in the different groups was performed using χ^2-analysis (with the Yates correction, when appropriate).

RESULTS

The mean ± SD serum triglyceride, cholesterol, HDL, and Apo-B levels for the four different groups are shown in table 1. Apo-B levels in the FCH and FH groups were significantly higher than those in the control and FD groups ($P < .05$). Triglyceride levels were significantly elevated in the FD and FCH groups compared with those in the control group ($P < .05$), whereas HDL levels were significantly reduced in the FD group only. Cholesterol levels were significantly elevated in all three patient groups.

The three polymorphisms detected using *Sst*I, *Xmn*I, and *Pst*I yielded DNA fragments as previously described (Kessling et al. 1985). In the *Sst*I digest, DNA from all persons revealed an invariant fragment of 5.7 kb and additional common fragments of 4.5 kb and, less commonly, of 3.2 kb. After hybridization with the Apo-A1 probe, digestions with *Xmn*I yielded fragments of 8.3 kb as the common allele and of 6.6 kb as the rare allele. After digestion with *Pst*I, the probe detected a common fragment of 2.2 kb and an infrequent fragment of 3.3 kb (figs. 1, 2). For the *Sst*I, *Xmn*I, and *Pst*I digests, the respective common

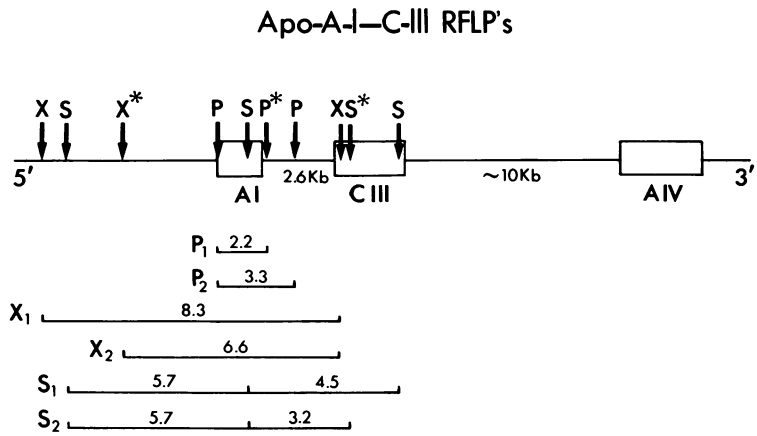

FIG. 1.—Map of the Apo-A1 and Apo-CIII gene loci showing polymorphic sites *Pst*I (P), *Sst*I (S). and *Xmn*I (X) with expected DNA fragment sizes after hybridization with an Apo-A1 probe. The common alleles are designated P1, S1, and X1, and the infrequent alleles P2, S2, and X2 of the polymorphic sites are designated with an asterisk.

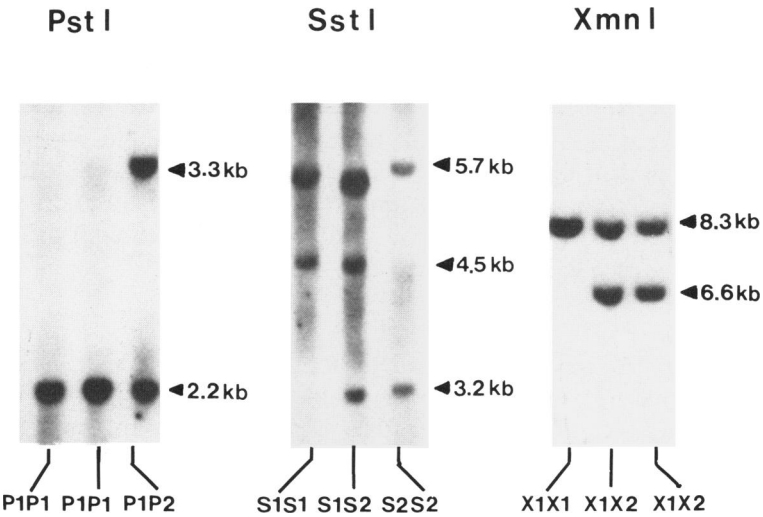

FIG. 2.—RFLPs detected after digestion of human DNA with *Pst*I, *Sst*I, and *Xmn*I and hybridization with the Apo-A1 probe.

alleles have been denoted as S1, X1, and P1, the respective infrequent alleles as S2, X2, and P2 (figs. 1, 2).

The allele frequencies of the three Apo-A1/Apo-CIII RFLPs in unrelated normolipidemic controls are shown in table 2. Analysis of *Pst*I polymorphisms revealed no differences between the control and patient groups (table 2). An increased frequency of the rarer alleles for the *Sst*I and *Xmn*I polymorphism—i.e., alleles S2 and X2, respectively—was seen in patients with FCH. These frequencies did not reach statistical significance ($P = .08$).

The *Xmn*I and *Sst*I polymorphisms are in linkage equilibrium. Four persons of 91 individuals in this study had S2 and X2 alleles. Three of these persons had FCH, and one had FD.

Analysis of the frequency of the S2 and X2 as a haplotype revealed a statistically significant ($P < .03$) increase in persons with FCH compared with either controls or persons with FH.

Analysis of the paired genotypic frequencies for the three Apo-A1/Apo-CIII RFLPs revealed no significant differences between observed and expected values for a population in Hardy-Weinberg equilibrium (data not shown).

DISCUSSION

In the present study, S2 was three times more common in persons with FCH than in controls, and X2 showed a similar trend. These results did not reach statistical significance. If these alleles are combined to form a haplotype (S2 X2), their frequency is significantly higher in persons with FCH than in either controls or persons with FH ($P < .03$).

Kessling et al. (1985) and Rees et al. (1983, 1985) have studied persons with hypertriglyceridemia and reported increased frequencies of the rare allele for

TABLE 2

GENOTYPE AND ALLELE FREQUENCIES OF THREE APO-A1/APO-CIII RFLPS IN CONTROLS AND HYPERLIPIDEMIC PATIENTS

Group	Genotype Frequency			Allele Frequency		Group vs. Controls (χ^2-Analysis)
	*Pst*I					
	P1P1	P1P2	P2P2	P1	P2	
Controls (38) ...	33	5	0	0.94	0.06	
FCH (24)	23	1	0	0.98	0.02	0.53
FH (15)	14	1	0	0.97	0.03	0.04
FD (14)	12	2	0	0.93	0.07	0.0
	*Xmn*I					
	X1X1	X1X2	X2X2	X1	X2	
Controls (38) ...	31	6	1	0.89	0.11	
FCH (24)	14	10	0	0.79	0.21	2.91
FH (15)	13	2	0	0.93	0.07	0.0
FD (14)	12	2	0	0.93	0.07	0.0
	*Sst*I					
	S1S1	S1S2	S2S2	S1	S2	
Controls (38) ...	33	4	1	0.95	0.05	
FCH (24)	18	5	1	0.85	0.15	0.72
FH (15)	14	1	0	0.97	0.03	0.04
FD (14)	10	4	0	0.88	0.12	0.79

*Xmn*I and *Sst*I, respectively. Given the reported high frequency of FCH in family studies of persons with hypetriglyceridemia (Brunzell et al. 1983), it is likely that persons with FCH were included in their study populations. The increased frequency of the rare alleles for *Xmn*I and *Sst*I in persons with FCH reported in the present investigation could account, at least in part, for the previously reported association between variant alleles for *Xmn*I and *Sst*I and hypertriglyceridemia.

Our investigation differs from previous studies in that the cause for the hyperlipidemia was defined in terms of clinical and biochemical criteria and in some instances by evaluation of lipid values in first-degree relatives. In this way, a more precise diagnosis of a particular genetic hyperlipidemia was possible, which allowed closer examination of the possible causes of this association.

It has previously been shown that Apo-CIII represents a physiological inhibitory modulator of lipoprotein lipase activity, resulting in hypertriglyceridemia (Windler et al. 1980). Genetic variations within Apo-CIII (*Sst* polymorphism) or close to Apo-CIII (*Xmn*I) theoretically could affect Apo-CIII activity, resulting in a higher frequency of hypertriglyceridemia in these individuals.

Recently, Ordovas et al. (1986) reported that 28 of 88 persons with severe coronary heart disease before the age of 60 had absence of the *Pst*I endonuclease site after hybridization with the Apo-A1 probe. The mean ± SD ages of males and females in their study were, respectively, 51 ± 2 and 54 ± 3 years, which suggests that a significant number of these persons had a genetic hyperlipidemia (Hazzard et al. 1973). The frequency of the *Pst* polymorphism in our control population was 5%, which is similar to that reported by Ordovas et al. (4%). However, in contrast to their results, the genetic-hyperlipidemia patients in our investigation did not have an increased frequency of this polymorphism.

The frequency of the *Pst*I polymorphism (P2 frequency = .03) was not increased in the 34 persons in our study population who had a history of angina and/or myocardial infarction. In the study by Ordovas et al. (1986), ~75% of patients with the variant polymorphic site had HDL values below the 10th percentile. This is the most important biochemical criterion for the diagnosis of familial hypoalphalipoproteinemia (Schaefer 1984). Persons with this disorder have been shown to have an increased frequency of the *Pst*I polymorphism (Ordovas et al. 1986). Therefore, it is possible that the prior reported finding of an increased frequency of the variant *Pst*I site in patients with premature coronary artery disease reflects the inclusion of patients with familial hypoalphalipoproteinemia. The failure to confirm the finding of higher frequency of the variant *Pst*I site in this study is, in all likelihood, due to the fact that subjects with familial hypoalphalipoproteinemia were not included in the analysis.

In the present investigation there were no differences between persons with FD and controls in the allele frequencies for the different polymorphisms. Our studies therefore have provided additional evidence that variations in the alleles around Apo-A1 and Apo-CIII are not responsible for the clinical expression of FD in patients who are homozygous for Apo-E2 (Vella et al. 1985).

Further studies on larger numbers of well-defined patient groups are needed to confirm these initial observations. The gene locus or loci for FCH are not known. Linkage analysis in appropriate families will help to determine the relationship between the gene for FCH and the Apo-A1/Apo-CIII gene cluster. The availability of cloned DNA probes will make it possible to look for other associations between genetic disorders of hyperlipidemia and RFLPs. In this way greater understanding of the genes important in lipoprotein metabolism will be obtained.

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