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Genetic Studies of Human Apolipoproteins

XIII. Quantitative Polymorphism of Apolipoprotein C-III in the Mayans of the Yucatán Peninsula

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Abstract. Apolipoprotein C-III (APO C-III) is a structural component of very-low-density and high-density lipoprotein particles and is an inhibitor of lipoprotein lipase. In a study of genetic variation of apolipoproteins in the Mayan population of the Yucatán peninsula, we observed a quantitative polymorphism in APO C-III levels. This polymorphism is expressed as variation in immunoblot staining intensity following isoelectric focusing and as variation in plasma levels of APO C-III determined by radial immunodiffusion. This variation is consistent with the presence in Mayans of an allele associated with low levels of plasma APO C-III which we have designated *APO C-III*D*. Analysis of the distribution of APO C-III levels yields a gene frequency estimate for the deficiency allele of 0.59. There is a significant positive correlation between total plasma APO C-III levels and total plasma cholesterol and triglyceride levels, the lowest levels of cholesterol and triglycerides being seen in individuals homozygous for the deficiency allele. This observation is consistent with the proposed role of APO C-III in lipoprotein metabolism. Family data to determine whether this deficiency allele is due to mutation at the APO C-III structural locus were not available. However, molecular analysis using cloned probes from the APO A-I/C-III/A-IV gene cluster revealed no gross DNA rearrangement or deletion of sequences in this region in homozygous deficient individuals.

Introduction

Apolipoprotein C-III (APO C-III) is the most abundant of the C group apolipoproteins of human plasma. It is a monomeric protein of 79 amino acids containing a carbohydrate side chain composed of galactosamine, galactose and sialic acid O-linked to a threonine residue at position 74 [Vaith et al., 1978]. Variation in the number of terminal sialic acid residues on the carbohydrate side chain gives rise to a biochemical polymorphism characterized by the presence of 3 major APO C-III isoforms in normal plasma. These contain 2 (APO C-III-2), 1 (APO C-III-1) or 0 (APO

esidues [Brewer et al.,
While this intraindi-
ot under genetic con-
987] have reported a
tation which prevents
leads to elevated lev-
in heterozygotes. Ele-
O C-III-2, the highly
ve been reported in
hyperlipoproteinemia
1a; Holdsworth et al.,

thesized primarily by
due propeptide which
20-residue signal pep-
O C-III [Levy-Wilson
et al., 1984; Karatha-
PO C-III accounts for
n very-low-density li-
particles and 2% of
y lipoprotein (HDL)
contribution of APO
minor, because of the
nt of HDL, 60% of
s associated with HDL
n VLDL [Curry et al.,

ctions have been attri-
APO C-III is an inhib-
ipase activity [Brown
] and inhibits the he-
lyceride-rich lipopro-
ants [Shelburne et al.,
., 1980; Windler and

f APO C-III are lim-
eficiency of APO A-I
5.5-kilobase inversion
III/A-IV gene cluster
ome 11q has been de-
asis et al. [1984]. How-
ion of the resulting

APO C-III deficiency to the prematur
coronary artery disease associated wit
this complex rearrangement could not b
separated from the effects of profoun
APO A-I deficiency in the same individu
als. Several DNA polymorphisms hav
been reported in this gene cluster and use
as markers for association studies, bu
these studies have yielded little insight int
the role of APO C-III in normal lipopro
tein metabolism [Breslow, 1988].

In a study of the occurrence and fre
quency of genetically determined varia
tion in the apolipoprotein genes in the
Mayan population of the Yucatán penin
sula, we observed interindividual varia
tion in the intensity of staining of APO
C-III isoprotein bands after isoelectric
focusing (IEF), which suggested the pres
ence of a deficiency allele. We present evi
dence for a quantitative polymorphism at
the APO C-III locus among Mayans.

Materials and Methods

Sample Collection

The lowland Maya Indians inhabit the Yucatán
peninsula and other parts of Central America. The
population examined in this report was collected
from a series of local villages, all but one practising
slash-and-burn (rotated-field) agriculture in the Yu
catán. Samples were collected in 1985 and 1987 as
part of a study of chronic disease risk factors in
Amerindians. Ten to twenty milliliters of EDTA-
anticoagulated whole blood were drawn into vacu
tainers, maintained on wet ice and returned to the
laboratory within 3–5 days.

IEF/Immunoblotting

Typing of APO A-I, A-II, A-IV and C-II by nar
row-range, thin-layer IEF followed by immunoblot
ting was performed after the method of Kamboh et
al. [1987]; APO C-III typing followed the same
method with the following modifications. The pH
gradient was established with 2% Pharmalyte pH
4.2–4.9 (Pharmacia, Uppsala, Sweden), gels were
prefocused for 30 min at 1,000 V, 10 W, 250 mA, and
then focused at 2,000 V, 30 W, 250 mA, for 3 h. Gels
were blotted for 2 h under a 1-kg weight and sequen
tially probed with monospecific, polyclonal, goat
antihuman APO C-III (Daiichi, Tokyo, Japan)
followed by rabbit antigoat IgG conjugated with
bacterial alkaline phosphatase for 2 h each. APO
C-III isoprotein bands were visualized by histochem
ical staining of alkaline phosphatase as described
elsewhere [Kamboh et al., 1987].

APO C-III Quantitation

Plasma APO C-III was quantitated by single ra
dial immunodiffusion (SRID) [Mancini et al., 1965]
using commercial plates and control standards from
Daiichi. Means of triplicate determinations were
computed from APO C-III standards included on
each plate, after incubation at room temperature for
48 and 72 h. Total plasma cholesterol and trigly
ceride levels were determined manually using enzy
matic methods [Allen et al., 1974; Bucolo and David,
1973].

DNA Analysis

High-molecular-weight genomic DNA was iso
lated from frozen buffy coats and Southern blot anal
ysis of the APO A-I C-III A-IV gene cluster carried
out as described by Cole et al. [1989] using the DNA
probes pA12.2 and pA13.2 described elsewhere [Kess
ling et al., 1985, 1988].

Statistical Analysis

Resolution of the overall distribution of APO C-
III levels into component gaussian distributions was
done by the method of Bhattacharya [1967]. In this
method, the number of component distributions,
means and variances of the components and the mix
ture proportions are estimated by approximating the
density within a frequency class by a cubic and the
logarithm of class frequency by a quadratic.

To test the null hypothesis of equality of mean
cholesterol levels among APO C-III genotypic
classes we have used analysis of covariance. Since
there were dissimilarities in the distributions of con
comitant variables in the various genotypic classes,
we adjusted the individual cholesterol values with re
spect to these concomitant variables and then tested
the equality of adjusted mean cholesterol values.

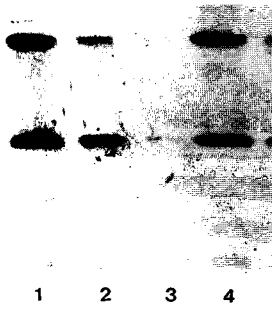

Fig. 1. APO C-III IEF
served in the Mayan pop
from individuals homozyg
are shown in lanes 1 and 4.
presumed to be homozygo
are shown in lanes 3 and
sumed heterozygotes are sh

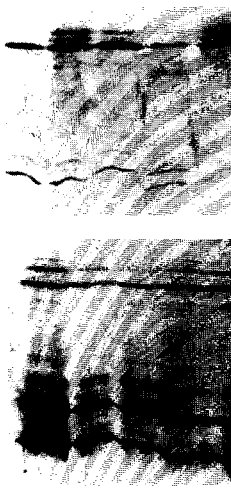

Fig. 2. IEF/immunob
A-II, A-IV and C-II fro
APO C-III phenotypes. S
in figure 1.

Results

In a general survey of gene and gene product variation at apolipoprotein loci in a Mayan population from the Yucatán peninsula, Mexico, we observed a clear quantitative variation in the products of the APO C-III locus which suggested the presence of an allele associated with decreased levels of APO C-III. Figure 1 shows the IEF/immunoblot phenotypes observed in the plasma of individuals of 3 postulated APO C-III genotypes. The usual IEF/immunoblot phenotype consists of 3 prominent APO C-III isoprotein bands focusing in the pH range 4.2–4.9. These correspond to the asialo form of the mature protein, APO C-III-0, and the sialylated forms containing 1, APO C-III-1, or 2, APO C-III-2, terminal sialic acid residues. A second pattern consisting of the 3 normal isoforms, but at greatly reduced staining intensity, and a third pattern of intermediate intensity were observed. Occasional splitting of the APO C-III-0 band was seen in these samples. Haase et al. [1988] have suggested that this is due to in vivo proteolysis of the mature protein.

In order to rule out the possibility that the observed APO C-III patterns resulted from sample degradation, plasma samples of all 3 phenotypes were analyzed for variation in the products of the APO A-I, A-II, A-IV and C-II loci (fig. 2) as well as at the APO H and APO E loci (data not shown). Among individuals of the various APO C-III phenotypes, variation at the other apolipoprotein loci is minimal and does not correlate with APO C-III phenotype. This suggests that the quantitative APO C-III variation is not due to sample degradation or to gross dyslipoproteinemia. The latter point was verified by d termination of quantitative levels of ch lesterol. Variation in sample loading w controlled by using multiple immunoblo from single gels as described by Kambc et al. [1987].

In order to test whether the variatic detected by the IEF/immunoblot proc dure represents true quantitative variation in plasma APO C-III, we determined total plasma APO C-III levels by SRID for the entire sample. Figure 3 depicts the frequency distribution of APO C-III levels among Mayans. The observed distribution deviates significantly from the normal distribution, and inspection of the distribution suggests some underlying heterogeneity. Resolution of the distribution into its underlying gaussian components by the procedure of Bhattacharya [1967] gave evidence of 4 overlapping normal distributions. The component distributions have estimated mean (±SE) APO C-III concentrations of 3.0 (± 0.23), 7.6 (± 0.17), 12.4 (± 0.25) and 17.4 (± 0.51) mg/dl. The estimated mixture proportions were, respectively, 0.1308, 0.5773, 0.2277 and 0.0639. From the estimated SE of the mean values, it is clear that the overlaps among the 4 component distributions are not large; that is, the distributions are fairly well separated. The mean of the upper 2 distributions, 12.4 and 17.4 mg/dl, are similar to those reported by Gross et al. [1983] in a European population. For an individual, the quantitative level suggested by IEF/immunoblot staining intensity is reflected in its total plasma APO C-III level.

We propose that the IEF/immunoblot data and APO C-III quantitative data are explained by the presence of a mutant allele at the APO C-III locus in the Mayan

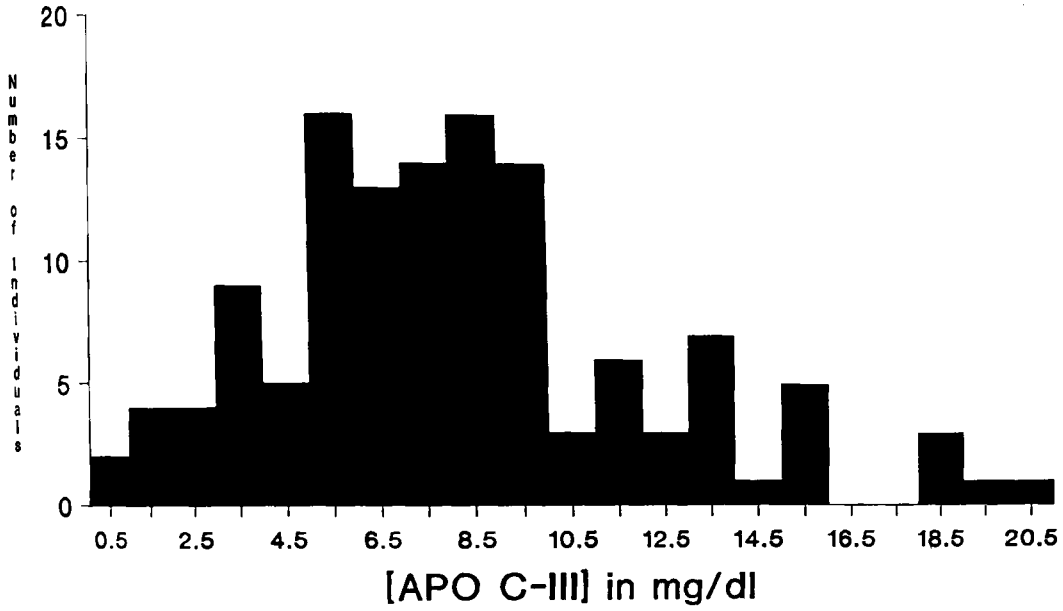

Fig. 3. Distribution of plasma APO C-III levels in the Mayan population of the Yucatán peninsula.

population which is associated with reduced levels of plasma APO C-III. We have designated the postulated allele as *APO C-III*D* to indicate a quantitative deficiency. Individuals homozygous for this allele, APO C-III D-D, have an average plasma APO C-III level of 3.0 mg/dl. Heterozygous, APO C-III N-D, and homozygous normal individuals, APO C-III N-N, have average plasma APO C-III levels of 7.6 and 12.4 mg/dl, respectively. Using the estimated proportion of the population having APO C-III concentrations corresponding to the 3 component APO C-III distributions, we estimated the frequencies of the *APO C-III*N* and *APO C-III*D* alleles as 0.41 and 0.59, respectively. These estimates correspond closely to estimated frequencies based on visual scoring of APO C-III isoprotein intensity patterns from the stained gels. While these estimates are crude, they indicate that the deficiency allele is present at polymorphic frequency in the Mayan population. We have also tried to fit several plausible 3-allele models. We assumed that there are 3 alleles N (normal), D (deficient) and H (high), and estimated allele frequencies and then performed goodness-of-fit X^2 tests by assuming that the phenotypic classes represented various genotypic combinations (for example, that phenotypic class P1 corresponded to individuals of DD and ND genotypes, P2 corresponded to NN, P3 corresponded to HD, and P4 corresponded to HH and NH genotypes). The goodness-of-fit X^2 values were significant at the 5% level for all the various phenotype-genotype combinations that we tried. In other words, 3-allele models

Table 1. Phenotype and proposed genotype classes, mean APO C-III levels and adjusted cholesterol lev in the Mayans of the Yucatan Peninsula

Phenotype classes	n	Proposed genotypes	Mean APO C-III mg/dl	Adjusted mean cholest mg/dl
P1	27	D-D	3.0	144 ± 8
P2	47	N-D	7.6	167 ± 6
P3	12	N-N	12.4	140 ± 11
P4	9	-	17.4	227 ± 13

do not fit the phenotypic distribution that we have observed.

Data on total cholesterol levels were available on the 95 individuals for whom quantitative APO C-III levels were measured. Since it has been reported that APO C-III is an inhibitor of lipoprotein lipase activity [Brown and Baginsky, 1972], it was of obvious interest to investigate the relationship between the APO C-III locus and total cholesterol. For this, we first computed the correlation coefficient between the APO C-III and total cholesterol levels separately for both sexes. The values were 0.56 and 0.45 for males and females, respectively. Both values are significant at the 5% level. In order to determine the impact of the deficiency allele on quantitative levels of plasma cholesterol, we performed an analysis of covariance using sex, age, height, weight, and triceps and subscapular skinfold thicknesses as covariates. Individuals were classified into 1 of 4 classes with respect to APO C-III levels. Each individual was assigned to the class represented by the nearest estimated mean APO C-III level from the prior analysis. The adjusted mean cholesterol level was computed for each phenotype class. None of the regression coefficients associated with the covariates was significantly different from zero at the 5% level. The results are summarized in table 1. The F ratio (= 11.91, d.f. = 3) for testing the equality of adjusted mean cholesterol levels for the 4 APO C-III classes turned out to be significant at $p < 0.001$. For phenotype classes P1, P2 and P4, increasing mean APO C-III levels are associated with increasing average adjusted cholesterol levels. The failure of the P3 class to conform to this pattern may be due to the small sample size in this class (and the resultant large SE of mean total cholesterol level) and/or due to classification errors. It may be noted that even though sex was not a significant covariate, since the sample size for males was small, we repeated the analysis of covariance using females only. The results were concordant.

In order to determine whether the proposed deficiency allele was associated with a major deletion or rearrangement of DNA sequences in the APO A-I/C-III/A-IV gene cluster, high-molecular-weight genomic DNA was isolated from 64 individuals representing all 4 phenotype classes. Genomic digests were prepared with the restriction endonucleases XmnI, PstI, SstI and PvuII. Probe pAI2.2 detects an XmnI polymorphism 5′ to the APO A-I gene, and PstI and SstI polymor-

phisms between the 3 ends of APO A-I and APO C-III genes [Kessling et al., 1985]. Probe pAI3.2 detects a PvuII polymorphism in the first intron of the APO C-III gene and a second polymorphism between the APO C-III and APO A-IV genes. The restriction patterns observed were normal in all phenotype classes, and interindividual differences were restricted to the previously reported restriction fragment length polymorphisms (RFLPS). The frequencies of RFLP alleles in the A-I/C-III/A-IV cluster among Mayans have been reported by Valdez et al. [1988]. No nonrandom association was noted between quantitative phenotype classes and particular RFLP alleles, but the number of individuals for whom DNA typing was available (n = 64) was not sufficient to detect minor departures from equilibrium.

Discussion

In this study, we have presented strong circumstantial evidence for a quantitative polymorphism affecting plasma levels of APO C-III in the Mayans of the Yucatán peninsula, Mexico. We propose that this variation is due to the presence of an APO C-III deficiency allele at the APO C-III structural gene locus. However, formal proof that the observed variation is due to a mutation in the APO C-III structural locus requires detailed family studies or demonstration of a molecular defect. The products of the proposed deficiency allele are indistinguishable from the normal APO C-III as revaled by IEF/immunoblot analysis, and is subject to normal posttranslational glycosylation (fig. 1). A preliminary molecular analysis revealed no molecular alteration in the APO A-I/C-III/A-IV gene cluster.

Whatever the molecular basis for this phenotype, this study represents the first demonstration of APO C-III deficiency with an apparent genetic basis. APO C-III deficiency has previously been reported in association with APO A-I deficiency due to a gross rearrangement of DNA sequences in the APO A-I/C-III/A-IV cluster [Karathanasis et al., 1984]. Because of the profound effect of APO A-I deficiency on lipoprotein levels, it was not possible to separate the effect of the concomitant APO C-III deficiency on quantitative levels of lipoproteins. We have seen that deficiency of APO C-III is associated with reduced levels of plasma cholesterol. This effect may explain why APO C-III deficiency has not been reported since individuals with the deficiency would not be severely dyslipoproteinemic.

In addition to reduced levels of APO C-III due to a postulated deficiency allele, we have observed a small proportion of individuals (6%) with highly elevated levels of APO C-III. This elevation is associated with the highest observed plasma cholesterol levels in this population. Gross et al. [1983] have reported a similar observation in a study of APO C-III levels in the German population. Whether elevated levels of APO C-III and the accompanying elevation in cholesterol levels seen in the Mayans has a genetic basis remains to be determined. It is interesting to note in the context that, similar to our observations, Kashyap et al. [1981b] noted high cholesterol levels in hyperlipoproteinemic patients to be associated with higher APO C-III concentrations. The same authors