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A Splice-Junction Mutation Responsible for Familial Apolipoprotein A-II Deficiency

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Summary

The first case of familial apolipoprotein A-II (apo A-II) deficiency was recently reported from Hiroshima, Japan, and designated apo A-II$_{Hiroshima}$. The proband had no immunologically detectable apo A-II in her plasma. DNA sequence analysis showed that the proband was homozygous for a G→A transition at position 1 of intron 3 of the apo A-II gene. A sister of the proband, who had an intermediate level of plasma apo AII, was shown to be heterozygous for this base substitution. This splice-junction alteration is most likely responsible for apo A-II deficiency, since it would be expected to completely block splicing of intron 3 from the primary transcript and therefore prevent formation of functional mRNA. This deficiency seems to have little influence either on lipid and lipoprotein profiles or on the occurrence of coronary artery disease.

Introduction

Apolipoproteins A-I (apo A-I) and A-II (apo A-II) are the major proteins (90%) of human plasma high-density lipoprotein (HDL) particles (Herbert et al. 1983). Plasma HDL, as well as apo A-I and A-II levels, were shown to be inversely correlated with the development of premature coronary heart disease (Miller and Miller 1975; Frager et al. 1979; Maciejko et al. 1983). Apo A-II, which on average contributes approximately 22% of the protein in mass of human HDL, is synthesized primarily by the liver and, after being processed (cleavage of pre- and pro-peptides and sialylation), is incorporated into HDL as a homodimer (Gordon et al. 1983; Lackner et al. 1985). Normal plasma apo A-II levels fall in the range of 25–35 mg/dl. HDL is composed of two populations of particles which differ in apo A content. Some contain both apo A-I and A-II, while others have only apo A-I (Cheung and Albers 1984; Cheung 1986). In addition, HDL particles contain apolipoproteins A-IV, C-I, C-II, C-III, D, and E, lecithin:cholesterol acyltransferase (LCAT), and cholesteryl ester transfer protein (CETP). The latter two proteins have been postulated to participate in the transfer of cholesterol from tissues to plasma (Albers 1985; Reichl and Miller 1986). It has been recently shown that only the apo A-I–containing HDL particles are active in promoting cholesterol efflux from cultured adipose cells (Barbaras et al. 1987). In vitro studies have shown that apo A-II can displace apo A-I from HDL particles (Logocki and Scanu 1980), activate hepatic lipase (Jahn et al. 1983), and inhibit LCAT (Fielding et al. 1972). The physiological role of apo A-II, however, remains to be established, and no anomalies in either the synthesis or amino acid sequence of this protein have been described.

Recently, two of us (K.T. and G.K.) described the only known kindred with familial apo A-II deficiency (Takata et al. 1988). We now present evidence that the deficiency in this kindred is most likely due to a single-base substitution at the donor (5′) splice junction of the third intron of the apo A-II gene, which had been assigned earlier to the q21-q23 region of chromosome 1 (Middleton-Price et al. 1988) and recently had been sequenced in its entirety (Knott et al. 1985; Tsao et al. 1985).

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Material and Methods

Kindred with APO A-II Deficiency

The Hiroshima kindred in which plasma apo A-II deficiency was observed is shown in figure 1. A brief description of the lipid and lipoprotein profiles of individuals in this family has appeared elsewhere (Takata et al. 1988). Plasma apo A-I (indicated in fig. 1) and A-II levels were determined by immunoassays (Cheung and Albers 1977). Apolipoprotein B was determined according to the method of Albers et al. (1975). Plasma lipid determinations were according to procedures described by Warnick (1986).

Preparation and Analysis of Genomic DNA

Plasma and leukocytes were obtained from the proband (II-3) and her sister II-7 for lipid analysis and DNA preparation. DNA was prepared by phenol extraction and ethanol precipitation on an Applied Biosystems (Foster City, CA) model 340A nucleic acid extractor according to the manufacturer's instructions. Restriction-enzyme analysis was performed according to a method described elsewhere (Maniatis et al. 1982; Deeb et al. 1986).

PCR Amplification and Nucleotide Sequencing

Oligodeoxynucleotide primers for amplification and sequencing were synthesized on an Applied Biosystems model 380B. The sequence and position of these primers are given in table 1.

Two genomic DNA fragments of the apo A-II gene were PCR amplified (Saiki et al. 1985) using oligonucleotide pairs 1+4 and 2+6 (table 1) as primers. The fragments encompassed the entire apo A-II gene, including 280 bp of the 5′ upstream regulatory sequences. The double-stranded amplified DNA fragments were purified by electrophoresis on a 1% low-melting-temperature agarose gel (SeaPlaque; FMC Bioproducts, Rockland, ME), phosphorylated at the 5′ ends by T4 polynucleotide kinase (Maniatis et al. 1982) and cloned into the *Sma*I site of pGEM3 (Promega Corp., Madison, WI). Sequencing was performed by the dideoxy chain termination method (Sanger et al. 1977) on double-stranded circular DNA templates by using T7, SP6, and oligonucleotides 3 and 5 (table 1) as primers. The Sequenase DNA sequencing kit (U.S. Biochemical Corp., Cleveland) was used.

Direct sequencing of the PCR-amplified DNA fragment containing the A→G mutation was also performed. The procedure used was essentially that described by Gibbs et al. (1989), with the following modifications: The double-stranded DNA PCR product was purified by electrophoresis on a 1.0% low-melting-temperature agarose (SeaPlaque; FMC Bioproducts, Rockland, ME); the unincorporated nucleotides and primers were separated from the single-stranded DNA product by a Centricon 30 (Amicon Corp., Danvers, MA), and the sequencing reactions were incubated at 37°C for 10 min.

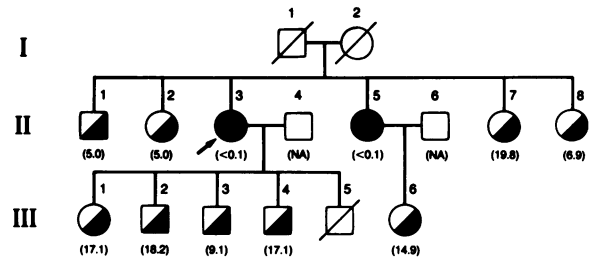

Figure 1 Pedigree of the family displaying apo A-II deficiency. Filled symbols denote homozygotes for apo A-II deficiency; half-filled symbols denote obligate and presumptive heterozygotes for apo A-II deficiency. The proband is indicated with an arrow. Numbers in parentheses under symbols represent plasma apo A-II levels in milligrams per deciliter. (NA) = not analyzed.

Table 1

Sequency of Oligonucleotides Used as Primers

Number	Sequence	Position[a]
1	5′ ATCCTGCTTCCTGTTGCAT 3′	632–650
2	5′ GAGCAGCATCCAAAGAG 3′	970–986
3	5′ AGATTCACTGCTGTGGAC 3′	1415–1432
4	5′ CACGGTCTGGAAGTACTG 3′	1554–1537
5	5′ TCAGACTTCTGTGGGACC 3′	1698–1681
6	5′ ACATACCAGGCTCAGAGCT 3′	2260–2242

[a] According to the numbering system in Knott et al. 1985.

Results

Familial Apo A-II Deficiency

The kindred shown in figure 1 represents the first known case of familial apo A-II deficiency (Takata et al. 1988). Two individuals—the proband (62 years of age) and her sister II-5 (59 years of age)—were found to have undetectable levels (<0.1 mg/dl) of apo A-II in their plasma, as determined by radioimmunoassay. Obligate heterozygotes (III-1, III-2, III-3, III-4, and III-6) and, in addition, four presumptive heterozygotes (II-1, II-2, II-7, and II-8) had levels (indicated under the symbols in fig. 1) that were one-sixth to two-thirds of the mean value for the normal range (25–35 mg/dl). Fast-

ing plasma lipid and lipoprotein levels of the proband and of her apo A-II–deficient sister II-5 are given in table 2. Except for the low level of apo A-I, values for the proband are quite normal. Her sister II-5 has total cholesterol (TC), triglycerides (TG), apo A-I, and apo B-100 levels that are higher than those for the proband yet close to the normal values. These differences are most likely due to environmental factors, as both subjects are homozygotes for the A-II mutant allele. Apo A-II deficiency therefore has little influence on lipid and lipoprotein levels. Furthermore, coronary angiography performed on the proband showed no significant abnormalities (Takata et al. 1988). No evidence of consanguinity was found in this kindred.

Southern Blot Analysis

Genomic DNA from the proband was first subjected to Southern blot analysis after digestion to completion with each of the restriction enzymes *Hin*dIII, *Msp*I, *Eco*RI, and *Pvu*II. The bands that hybridized to a full-length cDNA probe (previously prepared in our laboratory) were identical to those generated from genomic DNA isolated from normal, unrelated individuals (data not shown). An RFLP exists at an *Msp*I site 528 bp downstream from the 3′ end of the apo A-II gene (Tsao et al. 1985). The proband was found to be homozygous for the common 3.3-kb allele (presence of the restriction site).

PCR Amplification and Sequencing

Oligonucleotides 1 and 4 (table 1) were used as primers to amplify the 5′ region of the apo A-II gene (nucleotides 632–1554) of the sequence reported by Knott et al. (1985) from both a normal individual and the apo A-II–deficient proband. This segment includes 280 bp of 5′-upstream sequences; exons 1, 2, and part of 3; and introns 1 and 2. Another pair of primers (2 and 6, table 1) was used to amplify the middle and 3′ regions of the apo A-II gene which includes exons 2–4 and introns 1–3. The PCR products were cloned into the plasmid vector pGEM3 and sequenced as described in Material and Methods, by using oligonucleotide primers 3, 5 (table 1), and those corresponding to the T7 and SP6 promoters. A G→A transition at position 1617 and representing the first base of intron 3 was found in the sequence of the A-II gene from the proband (fig. 2). All eight independent clones sequenced from two separate amplification reactions had this single base substitution at the exon 3/intron 3 splice junction. Direct sequencing of the PCR-amplified DNA confirmed the homozygosity of the proband for this base substitution. Examination of the DNA of the proband's sister II-7, who has intermediate levels of plasma apo AII, showed that she is heterozygous for this mutation (data not shown). The normal 5′ (donor) splice site of intron 3 has the sequence GGCCAA:gtaa, where upper- and lowercase letters represent exon and intron sequences, respectively (Knott et al. 1985; Tsao et al. 1985). The dinucleotide gt is highly conserved and forms part of the recognition signal for the splicing machinery. This dinucleotide has been changed to at in the apo A-II–deficient individual and therefore could likely lead to loss of splicing at the exon 3/intron 3 junction. Since apo A-II is synthesized in the small intestine and liver, it was not possible to obtain, from the apo A-II–deficient patients, biopsies of these tissues for RNA analysis as a means of confirming the loss of splicing.

Table 2

Plasma Lipid and Lipoprotein Levels in A-II-deficient Individuals

Subjects	Concentration (mg/dl)				
	TC	HDL-Cholesterol	TG	A-I	B-100
Proband	155	47	102	99	94
Sister II-5	235	43	191	121	133
Normolipidemic (mean ± SD; n = 30)	211 ± 40	48 ± 17	116 ± 45	163 ± 31	89 ± 18

[a] Fasting plasma values are indicated for the proband and for her sister II-5, who were found to be homozygous for the A-II mutation and have undetectable levels of A-II in their plasma. Values for normolipidemic individuals are those reported recently by Punnonen et al. (1987) for postmenopausal Japanese women.

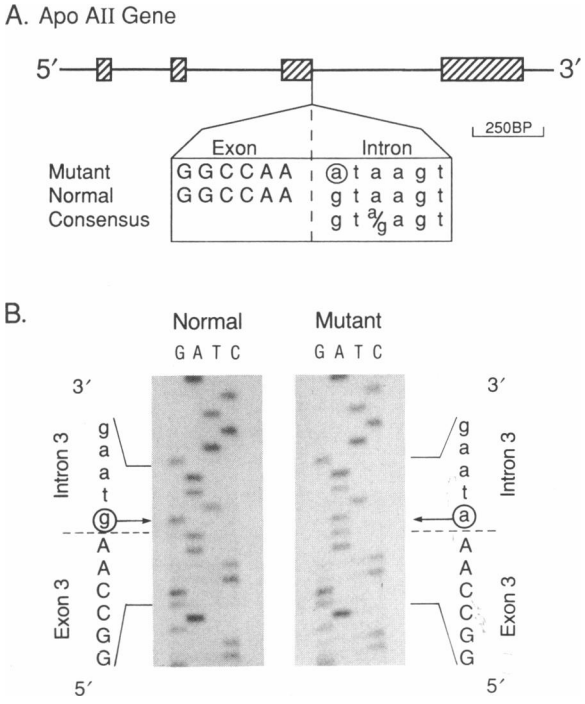

Figure 2 *A*, Schematic representation of the apo AII gene. Exons are represented by hatched bars, which are interrupted by lines representing introns. *B*, Autoradiographs of sequencing gels of DNA of the normal and mutant apo A-II clones at the exon 3/intron 3 splice junction. Genomic clones from the proband and normal individuals were obtained by PCR amplification, cloned into the plasmid vector pGEM3, and sequenced according to the procedures described in Material and Methods. The first base of intron 3 in the normal (g) and proband (a) clones are encircled. The dashed line represents the exon/intron boundary.

Discussion

Genomic DNA of the proband of the only known kindred with familial apo A-II deficiency was analyzed for possible alterations in nucleotide sequence of the apo A-II gene. PCR amplification of the apo A-II alleles of the proband, followed by cloning into a bacterial plasmid and nucleotide sequencing, revealed the presence of a G→A transition at position 1 of intron 3. This single base substitution changed the highly conserved dinucleotide GT→AT, which forms part of the recognition signal at the donor (5′) splice site, the consensus sequence of which is 5′ GT (A/G) AGT (Breathnach and Chambon 1981; Mount 1982). Mutations involving the GT dinucleotide at the 5′ ends of introns have invariably resulted in both loss of splicing and formation of functional mRNA from the primary transcript. For example, mutations (G→A or G→T) have been identified, at position 1 of introns 1 and 2 of the human β-globin gene, that led to a complete loss of normal splicing of these introns and to synthesis of the β-globin chain, giving rise to the β^0-thalassemia phenotype (Orkin 1988). In each of these mutations, other cryptic (not normally used) donor-like splice sites were employed. A potential cryptic donor-like splice site (GTGACT) exists in exon 3 of the apo A-II gene. Analysis of RNA transcripts would reveal whether this site is actually used in the absence of the normal signal. The use of this cryptic site would eliminate 65 nucleotides from the mature mRNA, resulting both in a translational frame shift and in an in-frame termination codon (UGA) at position 30 of the fourth exon. The resulting truncated protein would likely be unstable, as has been observed in the β^0-thalassemia mutants described above. The observation that the proband had no immunologically detectable apo A-II in her plasma is consistent with the above suggestion.

The fact that all eight apo A-II clones isolated from the proband had the same G→A transition would suggest that she is homozygous for this mutation. The proband's sister II-7, who had intermediate levels of plasma apo AII, was shown to be heterozygous for the mutant allele. As no consanguinity is known to exist in this kindred, this mutant allele may not be too uncommon in Hiroshima.

The total absence of apo A-II in the plasma of the proband and her sister II-5 would mean that their HDL particles are all of the apo A-1–containing class. This seems to have little if any consequences insofar as either plasma lipid and lipoprotein profiles or the incidence of coronary heart disease in this family are concerned. It appears, therefore, either that apo A-II plays only a minor role in lipoprotein metabolism or that apo A-I or other apolipoproteins can substitute for it (Cheung et al. 1987). It is of interest to note that certain animals, such as the dog, pig, cow and chicken, have very little or no apo A-II in HDL particles (Chapman 1986), which again suggests a minor role of apo A-II in lipid metabolism. Examination of other families with apo A-II deficiency would, however, be necessary before making any general conclusions, since other genetic or environmental factors may have obviated the need for apo A-II in the kindred described in the present study. Apo A-II may, however, play a significant role in the human blood coagulation system. Recently, Carson (1987) demonstrated that apo A-II inhibits participation of coagulation factor III in the activation of coagulation factor X by factor VIIa.

Acknowledgments

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