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GENE POLYMORPHISMS AND VARIABILITY OF HUMAN APOLIPOPROTEINS

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INTRODUCTION

Recombinant DNA technology has led to the cloning and sequencing of the human apolipoprotein genes and to the development of probes needed to study them. Such studies range from examination of the molecular genetic aspects of their regulated expression to epidemiologic surveys of genetic markers in patients with abnormalities of lipid metabolism.

Because apolipoproteins perform important functions in human lipid metabolism (see below), it was presumed that genetic variation in their levels or structures could influence lipoprotein metabolism and affect an individual's risk of developing atherosclerosis. An intensive effort has begun (see 10, 16, 42, 56 for recent reviews) to characterize such variations. Researchers hope by this means to elucidate the pathogenesis of heart disease and to identify genetic markers that can be used to detect individuals susceptible to coronary artery disease who would benefit from preventive measures.

Here we review the role of apolipoproteins in human lipid metabolism, summarize methods for the examination of apolipoprotein gene variability, and discuss variation in individual apolipoprotein genes. With few exceptions, we do not discuss protein variations not characterized at the genetic level; the interested reader should consult recent reviews (20, 34, 61, 103) for this information.

Cholesterol and triglycerides are transported in the circulation in lipoproteins, macromolecular complexes containing apolipoproteins and lipids. Details of the pathways of lipoprotein transport and metabolism have been reviewed extensively elsewhere (35). Below, we briefly discuss highlights of these pathways.

Transport of Exogenous (Dietary) Lipids

Intestinal and pancreatic lipases hydrolyze the lipids of a fat-containing meal. Free fatty acids and cholesterol are absorbed and reesterified in the intestinal epithelium to form triglycerides and cholesteryl esters, respectively. These lipids are then packaged together with phospholipids, free cholesterol, and at least two apolipoproteins (apoA-I and apoB-48) to form chylomicrons, which are secreted into the intestinal lymph and pass through the thoracic duct into the peripheral circulation. In the circulation, chylomicrons acquire additional apolipoproteins, mainly apoE and several forms of apoC. Triglycerides, which constitute most of the chylomicron mass, are immediately hydrolyzed by lipoprotein lipase at the capillary endothelium, apoC-II serving as a cofactor for this hydrolysis. The free fatty acid products of this hydrolysis are

transferred primarily to adipose tissue for storage as triglycerides or to muscle tissue for beta-oxidation. The lipoprotein particles, now smaller and denser because they have lost most of their triglyceride content, are called chylomicron remnants. They have retained virtually all of their cholesteryl ester content and have transferred some of their apolipoproteins (apoC and apoA-I) primarily to high-density lipoprotein (HDL); they have become enriched with apoB-48 and apoE. These remnants are bound and internalized in part via hepatic membrane receptors that recognize the apoE on the particles. By this mechanism, dietary cholesterol is delivered to the liver, where it plays a role in the regulation of hepatic cholesterol metabolism. Under normal circumstances, chylomicrons and their remnants are short lived in the circulation. Usually no lipoproteins of dietary origin remain in the plasma of normal individuals following a 12-hr fast.

Transport of Endogenous Lipids

The liver secretes a class of lipoproteins called very-low-density lipoproteins (VLDL). These contain free and esterified cholesterol, triglycerides, phospholipids, and a characteristic set of apolipoproteins, notably apoB-100, apoC, and apoE. Like chylomicrons, VLDL particles exchange apolipoproteins with other circulating particles and deliver triglycerides to adipose tissue, muscle, and other tissues via lipoprotein lipase. In the process, they become smaller and denser and are termed VLDL remnants or intermediate-density lipoproteins (IDL). Some of these remnant particles are taken up via hepatic cell membrane receptors, while some proportion undergo conversion to low-density lipoproteins (LDL). The latter process involves removal of the remaining triglycerides and all apolipoproteins except apoB-100; it results in a particle made up almost entirely of cholesteryl esters and apoB-100. A specific LDL receptor (also known as the apoB,E receptor) that recognizes, binds, and internalizes LDL is present on most cell membranes. By this mechanism, LDL particles can deliver cholesterol to most tissues. LDL particles can circulate in the plasma for several days.

HDL and Reverse Cholesterol Transport

In contrast to chylomicrons and VLDL, which are secreted into the circulation as mature particles, HDL are secreted from the liver and small intestine as nascent discoidal particles composed primarily of phospholipids and proteins (apoE, apoA-I, and apoA-II). The particles accept cholesterol from tissues; this cholesterol is esterified via the lecithin–cholesterol acyltransferase (LCAT) reaction. Part of the cholesteryl ester is incorporated in the core of

HDL, making it a spherical particle, while part of it is transferred to chylomicron remnants, IDL, and LDL via the cholesteryl ester transfer protein. Since LDL and remnants of chylomicron and VLDL metabolism can be taken up by the liver, tissue-derived cholesterol can return to the liver (reverse cholesterol transport). The liver can then excrete cholesterol in bile.

APOLIPOPROTEIN GENES

Chromosomal Localization

The genes for apoA-I, apoC-II, and apoA-IV are located in a cluster on the long arm of chromosome 11 that spans approximately 22 kilobases (kb) (46). The apoC-III gene is approximately 2.6 kb downstream from the apoA-I gene but is in the opposite transcriptional orientation. The apoA-IV gene is about 7.5 kb downstream from the apoC-III gene and is in the same transcriptional orientation as the apoA-I gene.

The genes for apoE, apoC-I, and apoC-II are on chromosome 19 (57). The apoE and apoC-I genes are within 4 kb of each other and are located on the long arm of this chromosome. In addition, there is an apoC-I pseudogene, which apparently is not transcribed, located 7.5 kb downstream from the first apoC-I gene (84). The apoC-II gene, although tightly linked in pedigree analyses to the apoE gene, is physically closer to the apoC-I gene. Linkage and in situ hybridization data suggest that the gene for apoC-II is within 2 centimorgans of the apoE/apoC-I complex in the middle of the long arm of chromosome 19 (22).

The genes for apoB and apoA-II are on the short arm of chromosome 2 and the long arm of chromosome 1, respectively (41, 50). The gene for apoD is on the long arm of chromosome 3 (24).

Some characteristics of the apolipoproteins and their genes are summarized in Table 1. ApoB-48, synthesized in human intestine, apparently results from co- or posttranscriptional modification of apoB-100 mRNA so that codon 2153 (CAA) becomes a translational stop codon (UAA) (67a).

Evolutionary Considerations

The chromosomal arrangement of the apolipoprotein genes noted above and the repetitive features found among them (see below) have suggested a model for the evolution of these genes. This model groups together the genes for apoA-I, A-II, A-IV, C-I, C-II, C-III, and E into a multigene family having a common ancestral gene (for recent reviews see 55, 84). ApoB is not thought to be part of this family, although regions of amino acid homology among apoB, apoE, and apoA-IV of up to 39% have been reported (55). In spite of that, the exon-intron organization of the apoB gene (9) is so different from

TABLE 1 Human apolipoproteins[a]

Apolipoprotein	Mol. wt. (kd)	A.A.[b]	Gene length (kbp)	Exons/introns
A-I	28	243	1.9	4/3
A-II	17	77	1.4	4/3
A-IV	46	376	2.6	3/2
B-100	550	4536	44	29/28
B-48	275	2152	–[c]	–[c]
C-I	7	57	4.4	4/3
C-II	9	79	3.4	4/3
C-III	9	79	3.2	4/3
D	33	169	12	5/4
E	38	299	3.6	4/3

[a] Based on Breslow (16) and Drayna et al (24).
[b] Amino acids of mature protein.
[c] See text.

that of the other apolipoprotein genes that it is unlikely to belong to this multigene family.

The strongest evidence for the common ancestor gene hypothesis for the non-apoB genes comes from the finding that the general architecture (intron-exon organization) of these genes is similar and from protein and DNA sequence data demonstrating common structural elements. With the exception of the apoA-IV gene, the genomic structure of a family member has four exons and three introns; the apoA-IV gene is missing the first intron, and its first exon represents the fusion of the analogous first and second exons of the other family members.

Homologies among the apolipoproteins were appreciated first when amino acid sequence data for them became available (64; see below for more details). As more extensive amino acid and nucleotide sequence data were collected, it became clear that homologous repeated elements were also present in apoA-II, A-IV, C-I, C-II, C-III, and E genes.

Conclusions drawn from both amino acid and nucleotide sequence comparisons are not always unequivocal. As recently reviewed (11), the computer analysis of much sequence data is dependent upon many arbitrary assumptions, including the algorithm used for the analysis. For example, comparing the DNA sequences of two genes is not equivalent to comparing the amino acid sequences and may lead to different conclusions. Typically, DNA sequence comparisons are less sensitive than are protein sequence comparisons, especially when rapidly evolving proteins are involved, since critical features of the protein (structural features, active site mechanisms, etc) can be conserved even if there are many changes in the nucleotide sequence (11). In fact,

this appears to be the case for the apolipoprotein genes. An 11-mer amino acid repeat is found in a variety of copy numbers usually in the third and fourth exons of the members of the apoprotein gene family. However, there is less sequence homology among the repeats than there is conservation of the properties of the amino acids. For example, if a hydrophobic amino acid occupies a particular position in the repeat in one copy, usually a hydrophobic amino acid (but not necessarily the same one) is found at that position in another copy.

Computer analyses have led to a suggested consensus structure of the mRNAs for the apolipoprotein family members (shown in Figure 1) that emphasizes the repetitive and organizational features described above. A specific example will illustrate certain aspects of this model.

Examination of the apoA-I amino acid sequence (64) revealed the existence of six homologous 22-amino-acid-long sequences in the carboxy-terminal position of the protein. The 22-mer repeat was, in turn, found to consist of two related 11-mers. Nucleic acid sequence analysis showed that in codons 99–230 of the mRNA there were six 66-bp tandemly repeated DNA regions that corresponded to the amino acid repeats. The data suggest that intragenic recombination resulting in duplications of the repeats expanded the apo-I gene after the gene diverged from an evolutionary precursor (28). Since the repeating units are found in the other family members, the current view of the evolutionary relationships among these genes is that there was a common ancestor with divergence resulting mainly from a series of duplication events. Two evolutionary trees for apolipoprotein genes have recently been proposed (55, 84). They agree about there being a common ancestor gene and about the interrelatedness of the genes for apoA-I, II, IV, C-I, II, III, and E. However, there are disagreements (based upon technical arguments, discussed elsewhere) about the specific relationships among the family members (11, 55, 84).

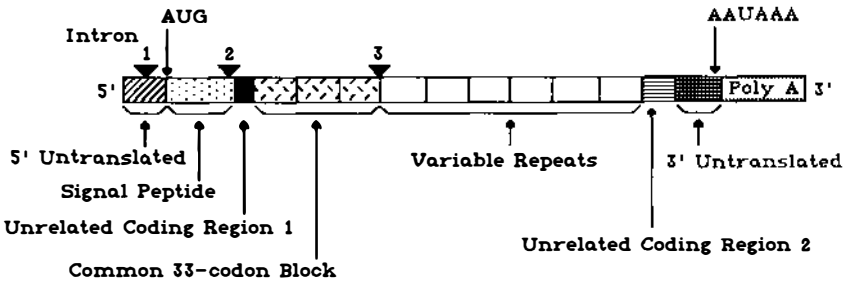

Figure 1 Consensus structure of mRNA for a member of the apolipoprotein multigene family (as suggested in Ref. 55).

APOLIPOPROTEIN GENE VARIATION

Methods to Study Variability of Apolipoprotein Genes

In human populations, the approaches to the study of apolipoprotein genetic variations include:

1. *Biochemical genetics*. A physical property of the protein itself (e.g. the isoelectric point) that varies among individuals is characterized; its mode of inheritance is determined, and functional correlations related to it are sought. Examples of this approach are the investigations of apoE (90, 105; also, see below).

2. *DNA sequencing*. The sequence of the gene from a subject with a disease condition of interest is compared to the sequence from a normal individual. Mutations are sought that may be involved in the pathogenesis of the disease. For example, it has been shown that a variety of mutations, many arising from nonhomologous recombination events, are associated with particular subtypes of familial hypercholesterolemia (53). DNA sequencing is relatively labor intensive, but the new method of polymerase chain reaction, in which a target region of DNA from an individual can be amplified and directly sequenced without having to be cloned first (73), has allowed for rapid determinations of sequence data.

3. *Genetic linkage*. Two related but distinct techniques are used to show an association between a genetic marker and a condition of interest. These are segregation (or linkage) analysis in families and linkage disequilibrium in populations. The basic principle in each is to identify a genetic determinant or marker close enough to the locus carrying the mutation of interest that the two are not separated during meiosis, when recombination events normally occur (92).

The genetic markers most commonly used in both types of studies are restriction fragment length polymorphisms (RFLPs). These are differences in the length of DNA fragments (as analyzed by Southern blotting) after digestion with DNA sequence–specific restriction endonucleases. As originally proposed in 1980 (12), a variety of polymorphisms arising from insertions, deletions, substitutions, etc can be detected as long as a change in the normal position of the recognition sequence of a restriction enzyme is involved.

Linkage of RFLPs with phenotypes can then establish a genetic marker for the phenotype. The polymorphism will not itself necessarily be the mutation underlying the phenotype, however. For example, although it has been established that in familial Type 3 hyperlipidemia, where a structural mutation in the apoE gene results in an amino acid substitution, there is a tightly linked RFLP for this disease that maps well away from this mutation, in the

promoter region of the apoC-I gene (48, 80). Although some RFLPs are indeed coincident with underlying mutations (e.g. in sickle cell anemia), they usually represent a particular chromosomal background upon which the disease-causing mutation occurred and can provide information about chromosomal localization.

Although superficially similar, population and family studies have different underlying assumptions. Linkage disequilibrium in a population occurs when, despite many generations of random mating, the locations of a marker (itself a locus) and another locus are not randomized, implying they are reasonably close to each other. Sufficient random mating among the population to achieve complete equilibration of many unrelated loci has not occurred, however, which can lead to erroneous conclusions. For example, if a particular ethnic group predominated in either the control or disease population sample, a marker may be associated with ethnicity rather than with the disease status of the group.

Linkage analysis, on the other hand, depends upon the cosegregation of the marker with the phenotype through generations of a family. Associations between the marker and the phenotype do not depend upon linkage disequilibrium, since markers millions of base pairs apart will cosegregate in a family [because the probability of recombination is only 0.01 per million base pairs per meiosis (56)]. In addition to linkage information, the mode of inheritance and a more accurate functional characterization of mutated alleles can be obtained from family studies.

Population studies are sometimes easier to conduct, but because of their limitations (discussed below), some geneticists recommend that any associations disclosed by a population study be followed up by linkage analysis using informative families (for example, see 56). Situations exist in which a haplotype that includes information about a number of RFLPs is a more useful genetic marker than any single RFLP, since the chromosomal background upon which a particular mutation occurred is more completely characterized. Haplotype analyses can be done in both family and population studies.

When defective gene products are beyond speculation (e.g. in the case of cystic fibrosis), an alternative to the approach of using specific gene probes ("candidate genes") is to test random probes from a variety of locations within the human genome to see if any are potential markers for the phenotype under investigation. This process has been dubbed "reverse genetics."

ApoA-I/C-III/A-IV Gene Cluster

For several reasons, RFLPs for the apoA-I gene were among the earliest to be reported. First, apoA-I was among the first human apolipoprotein genes to be cloned and sequenced. In addition, epidemiologic studies showed the risk of

developing coronary artery disease to be inversely correlated with plasma HDL cholesterol and apoA-I levels (15), a finding that sparked considerable interest in the genetics of apoA-I.

Before DNA probes were available, apoA-I genetic variation was studied mainly by the detection of protein variants using isoelectric focusing. Approximately one dozen variants have been described resulting from structural mutations; but these variants are rare, altogether found in less than 0.1% of the general population (69). Perhaps the best known is apoA-I_{Milano}, in which the arginine at amino acid residue 173 is replaced by a cysteine. Although A-I_{Milano} is associated with low HDL levels (31), not all of the protein variants are, and the functional consequences of most are not well known. Two variants, apoA-$I_{Marburg}$ ($lys_{107} \rightarrow 0$) and apoA-$I_{Giessen}$ ($pro_{143} \rightarrow$ arg), have been reported deficient in the ability to activate LCAT (70).

The availability of DNA probes quickly resulted in numerous population studies using RFLPs of the apoA-I/C-III/A-IV gene cluster to identify genetic markers in linkage disequilibrium with such clinical phenotypes as low HDL levels and coronary artery disease. We use one of the first, that of Schaefer's group (67), to illustrate the typical strategy involved. Using the restriction enzyme PstI to define two alleles, the frequencies of these alleles in three populations were determined. In a group of normal controls, the minor allele frequency was only 2–3%. However, in the index cases of 12 kindreds with familial hypoalphalipoproteinemia, an autosomal dominant disorder in which HDL cholesterol levels are below the 10th percentile for sex and age, the minor allele frequency was 42%. In addition, the minor allele frequency was 17% in patients who developed coronary artery disease before the age of 60. The authors concluded that the frequency of the minor allele was significantly elevated in the two study groups, indicating that it may be a marker for some phenotypes involving low HDL cholesterol levels and/or increased risk of coronary artery disease.

The PstI polymorphism had previously been mapped to the 3' end of the apoA-I gene—i.e. the polymorphism of the DNA sequence detected was located outside of the coding region. As in practically all of the RFLP studies to be described, it is unclear whether the polymorphism itself or a linked abnormality (i.e. a mutation elsewhere) is responsible for the observed phenotypes. Since the genes for apoA-I, C-III, and A-IV are clustered, a polymorphism of one of these genes will also be linked to the others. Many investigators therefore would interpret a finding of a polymorphism using an apoA-I gene probe as a polymorphism not simply of the apoA-I gene itself but rather of the apoA-I/C-III/A-IV cluster.

There are other reported associations of RFLPs of the apoA-I/C-III/A-IV gene cluster with a variety of phenotypes. For example, RFLPs identified using SacI have been associated with increased risk of myocardial infarction

(27), low HDL levels (2), and high triglyceride levels (71). RFLPs identified using MspI have been associated with increased risk of myocardial infarction in England but with resistance to coronary artery disease in Germany (27, 32).

A number of factors limit the conclusions derived from population-based RFLP studies, such as the difficulty of determining the nature of the underlying genetic abnormality, interactions among the phenotypes, and, especially, the fact that heart disease is multifactorial in origin. Both environmental (e.g. diet, exercise, stress, etc) and genetic influences act on a variety of lipoproteins, apolipoproteins, enzymes, and tissue-specific events involved in lipoprotein metabolism, all highly regulated and potentially related to causation. Other host factors, such as vascular wall biology and platelet function, may also be etiologic variables.

Thus, by considering the group of apolipoprotein genes as the "candidate" gene pool, only a small—albeit important—fraction of the genetic component (which itself is a subset of risk factors) is examined. Furthermore, technical factors such as relatively small sample sizes and artifacts from the ethnic composition of study and control groups have also contributed to the limited nature of the conclusions to date (86).

In addition, there are situations in which genetic variations affecting apoA-I are not linked to its structural gene, so that apoA-I/C-III/A-IV RFLPs would not be expected to be informative. For example, in humans, Tangier disease is an autosomal recessive condition characterized by extremely low apoA-I and HDL levels. This disorder is most likely caused by the increased catabolism of normal apoA-I (52), and not by a mutation in the structural gene for apoA-I.

Alternative strategies can overcome some of the problems that limit conclusions from population-based genetic studies. In general, the study of well-defined phenotypes would be expected to reduce genetic heterogeneity of the underlying causes and lead to more specific results. The use of haplotype analysis, which considers combinations of RFLPs, may bring out relationships not obtained from single RFLP studies—e.g. the association between the PstI+/SacI− haplotype and low HDL cholesterol levels in Italian kindreds (78).

ApoA-II Gene

A RFLP using the enzyme MspI has been reported. A polymorphic site in the DNA sequence exists 3' to the gene; Southern blotting analysis defines two alleles (3.0 kb and 3.7 kb, major and minor, respectively) (77). The minor allele frequency in Caucasians has been reported to be from 11–19%. In one study, men who were homozygous for the minor allele had higher serum apoA-II concentrations than men homozygous for the major allele (35.4 vs

29.4 mg/dl) (77). This finding was not confirmed, however, in a later study by the same authors (47). It has also been reported that the major allele occurred more frequently in hypertriglyceridemic patients (or the minor allele occurred less frequently—9% in patients vs 19% in controls). However, the relevance of these findings to coronary artery disease is not clear since a later study of this RFLP among patients with atherosclerosis indicated no association (75). Structural variants of apoA-II have been found in mice but not (so far) in humans.

ApoB Gene

Although apoB was the last major apolipoprotein to be cloned (owing to technical problems related to the size and nature of the protein), studies of this gene using cDNA probes were quickly begun because of its central importance to lipoprotein metabolism.

EARLY IMMUNOLOGIC STUDIES Some information about genetic variation of apoB had been obtained previously by immunologic techniques. The sera from multiply transfused patients were examined for allo-antibodies to LDL. Careful characterization of these antibodies revealed ten distinct factors of LDL that formed a heritable antigenic group termed the Ag system (1). These antigenic variants were organized into five pairs of alleles designated Ag-(c)/(g), (x)/(y), (t)/(z), (a_1)/(d), and (h)/(i). Close genetic linkage between the Ag system locus and the apoB gene was subsequently shown, implying that the Ag system reflects protein polymorphisms of apoB (8).

The antigenic determinant Ag-(y) is associated with elevations of plasma cholesterol and triglyceride (5, 7). The determinant Ag-(c) has been found to be associated with the reactivity of a monoclonal antibody designated MB-19, which distinguishes three phenotypes of apoB that have strong, weak, and intermediate binding to the antibody (76, 97). No differences in LDL composition or density among these phenotypes have been reported (100). Since there are 32 different possible haplotypes of the Ag system, with at least 14 already reported (17), there is a significant number of markers that can be used for comprehensive genetic studies to discern relationships among the Ag system, lipid metabolism, and the development of coronary artery disease.

APOB RFLP ANALYSIS Numerous RFLPs of the apoB gene have been reported. The enzymes XbaI, EcoRI, and MspI define polymorphisms that are strongly linked to the Ag-(x) and Ag-(y) alleles in family studies (8). Digestion of the apoB gene with XbaI detects two bands on Southern blotting—one 8.6 kb, the other 5.0 kb (the corresponding alleles are designated X1 and X2, respectively, and are present with equal frequency in the general population)

(8). In a population study, a strong association was found between Ag-(x) and the 8.6 kb band. The XbaI polymorphism cannot be the site of the variation in the apoB protein that is represented by Ag-(x), however, since the change in the DNA does not result in an amino acid substitution. It has been suggested that the XbaI polymorphism is linked to amino acid changes elsewhere in the apoB molecule, since patients who are X2/X2 (homozygous for the smaller allele) had the lowest LDL fractional catabolic rate when compared to subjects having genotypes X2/X1 and X1/X1 (39).

In another population study using the XbaI RFLP, the larger band was associated with lower cholesterol and apoB levels, while the smaller band was associated with elevations of triglycerides and cholesterol (5). Since the large band is genetically linked to Ag-(x), these studies are consistent with previous reports that individuals who were Ag-(x-) [i.e. Ag-(y)] had higher fasting levels of triglycerides and cholesterol than those with Ag-(x) (7). Thus, the XbaI large band, linked to Ag-(x), would be expected to be associated with lower lipid levels.

Another apoB RFLP is due to the variation in the repeat number of an adenine:thymine (AT)-rich sequence in the 3' end of the gene (36). This RFLP can be detected by a number of enzymes including MspI and BamHI. With MspI, the major allele is the one with fewer repeated sequences. Using the XbaI, MspI, and EcoRI RFLPs (EcoRI defines a major allele of 11 kb and a minor allele of 13 kb) to study a population of patients who had suffered myocardial infarctions, it was found that the frequencies of the large XbaI, minor EcoRI, and the minor MspI alleles were all significantly higher in the patient population than in controls, although there were no significant associations between any allele and levels of LDL or apoB. The authors concluded that genetic variation at the apoB locus may be a risk factor for myocardial infarction independent of variations in plasma levels of LDL cholesterol or apoB (36).

Related to this conclusion is the report of an epitope of apoB recognized by a monoclonal antibody designated as BIP-45 whose strength of binding to the antibody is linked to the XbaI alleles. However, although the risk of ischemic heart disease was associated with the epitope, there was no association between the epitope and lipid levels. The authors suggest that the genetic variations of the apoB locus represented by the XbaI and BIP-45 alleles were involved independently in the determination of serum lipid levels and the development of heart disease (26; see 66 for a related study of patients with peripheral vascular disease).

Other RFLPs of the apoB gene (see 56 for review) are being actively investigated in clinical studies; in the future, other associations among RFLPs and disease states are likely to be identified. However, the study of apoB

polymorphisms at the protein level has continued to provide valuable insights into genetic variation. For example, a kindred with deficient binding of apoB to normal LDL receptors has been characterized (45). This condition is inherited in an autosomal codominant manner, and the only abnormality so far identified is a substitution at the 3500 position of apoB of glutamine for arginine (82). The defective apoB has enhanced binding to the monoclonal antibody designated MB-47 whose epitope is between amino acid residues 3350 and 3506 (94). Since apoB is such a large protein, it is likely that more apoB variants with dysfunctions will be described. Indeed, a variety of such proteins have been reported (74, 101). It should be noted that not all variants will necessarily result in lipid metabolism abnormalities (for example, see 98).

OTHER GENETIC STUDIES There are other clinical conditions in which genetic variants of apoB may be involved. Anderson's disease is a disorder identified in infants with fat malabsorption who have apoB-48 deficiency and the inability to produce chylomicrons. However, apoB-48 can be identified within small intestinal epithelial cells by immunocytochemical techniques, so that the defect appears to be in chylomicron assembly or secretion (13). In contrast to Anderson's disease is the report of patients with a selective apoB-100 deficiency and a lipoprotein profile termed normotriglyceridemic abetalipoproteinemia (38, 60). These patients can make apoB-48 and can mount an appropriate chylomicron response to fat feeding, but hepatically derived lipoproteins containing B-100 are absent.

The most severe form of abetalipoproteinemia is an autosomal recessive condition characterized by severe fat malabsorption, the lack of all apoB-containing lipoproteins in plasma, and neurologic abnormalities (37). However, there are apparently no gross rearrangements of the apoB gene in probands (51) and no linkage of the phenotype to the apoB gene in family studies (40). In addition, some patients have normal length apoB protein and mRNA species in liver (25, 51). Taken together, these findings indicate that the defect in this form of abetalipoproteinemia is at the posttranslation level.

A superficially similar condition is hypobetalipoproteinemia. Homozygotes have fat malabsorption and a severe deficiency of apoB-containing lipoproteins; heterozygotes have approximately half-normal plasma levels of apoB, indicating a codominant mode of inheritance (37). This disorder is quite heterogeneous, but most of the phenotypes are compatible with defects in the structure or regulation of the apoB gene (54, 72, 99).

Finally, there are conditions with increased levels of apoB. One is hyperapobetalipoproteinemia, in which there is an elevation of LDL-apoB with near-normal levels of LDL cholesterol (81, 85). LDL particles in the plasma

of these patients are smaller and denser than normal. They were originally identified in a large percentage of heart attack victims (81). It was subsequently determined that in individuals with normal LDL-apoB levels, an increase in the proportion of dense vs light LDL was associated with increased risk of myocardial infarction (16). Despite the obvious similarities of the dense LDL subclass phenotype to the hyperapobetalipoproteinemia phenotype, the exact relationship between them is not clear.

Overproduction of VLDL- and LDL-apoB is thought to be present in familial combined hyperlipidemia, an autosomal dominant disorder in which the hyperlipidemias are heterogeneous, even within the same kindred (33). Possible mechanisms for this disorder include alterations in the structure or processing of apoB, in the regulation of its gene, or in pathways such as lipoprotein assembly or catabolism unrelated to apoB per se. Needless to say, a better characterization of disorders associated with apoB abnormalities is being aggressively pursued now that the techniques and probes to deal with this protein and its gene are available.

ApoC-II Gene

As mentioned earlier, apoC-II is an activator of lipoprotein lipase. There is an autosomal recessive human condition in which a marked reduction of plasma apoC-II results in severe hypertriglyceridemia (14). In some probands, a small amount of a mutant apoC-II has been found (29, 59), implying that an unstable or nonfunctional protein is produced in these individuals. Indeed, in one kindred, a mutation in the apoC-II gene leading to production of a protein lacking amino acid residues necessary for lipoprotein lipase activation has been described (18).

Also described are protein variants designated apoC-II-St. Michael (19), apoC-II-Bethesda (83), apoC-II-Padova (62), apoC-II-X, and apoC-II-Y (59). ApoC-II-St. Michael was identified in a family with chylomicronemia and is a nonfunctional protein caused by a frame-shift mutation in the apoC-II gene. ApoC-II-Bethesda was described in a family in which the proband was a compound heterozygote for both the protein variant and a null allele so that his phenotype included elevated triglycerides and apoC-II deficiency. ApoC-II-Padova was described in an Italian family in which the proband had an elevation of plasma triglycerides and a deficiency of apoC-II. Evidence indicates a structural mutation of the protein leading to either its decreased secretion or increased catabolism.

ApoC-II-X and Y were reported in a single kindred. Both forms have altered immunoreactivity to antibodies against apoC-II, but the plasma levels of both variants are present in the near-normal concentrations, implying a structural mutation of apoC-II which results in abnormal function (59). In one

patient with severe deficiency of plasma apoC-II, Southern blotting analysis revealed extensive rearrangements of the 3' end of the apoC-II gene (30). Finally, approximately 12% of African blacks have a structural variant of apoC-II containing a single amino acid substitution, but this mutation has no obvious physiological consequences (65).

RFLP studies have been performed using apoC-II gene probes. The enzymes TaqI, BglI, BanI, and NcoI all define major and minor alleles on Southern blotting analysis (56). Except for a study in some families with Type 1 hyperlipidemia in which the TaqI RFLP was linked to the apoC-II deficiency (91), no significant associations with serum lipid levels have been demonstrated (43, 91). In addition, except for the apoC-II-St. Michael and -Y variants described above, no association has been described between protein variants of apoC-II and atherosclerosis (3). It should be noted that the St. Michael family is consanguineous (19), so that this particular association with vascular disease may be rather genetically distinct.

ApoE Gene

The apoE gene is polymorphic, with three common structural alleles; apoE phenotypes can be identified by isoelectric focusing of delipidated VLDL isolated from plasma (102). The allele products are distinguished on the basis of cysteine and arginine content at two sites (residues 112 and 158) on the 299-amino-acid chain of the mature apoE molecule. E4 has arginine and E2 has cysteine at both sites, while E3 has cysteine at site 112 and arginine at site 158 (68, 95, 104).

The three common alleles, E2, E3, and E4, occur at frequencies of approximately 0.07, 0.78, and 0.15, respectively, in Caucasian populations. However, even among Caucasian populations there are some significant differences in allele frequencies; for example, the frequency of E2 in Finns is 50% lower while the relative frequency of E4 is 50% higher than the overall Caucasian average. In all populations studied, the E3 allele is the most common form, although significant variation is seen in the relative frequencies of these 3 alleles among populations (23).

Several studies have shown that these three common alleles contribute to the variance of lipoprotein levels among normolipidemic individuals. Sing & Davignon have calculated that the apoE gene may account for as much as 14% of the total genetic variability of plasma cholesterol levels (79). The results obtained by Utermann (87) for a German population showed that the mean plasma cholesterol of individuals with the apoE 4/4 phenotype was 197 mg/dl, while that for apoE 2/2 subjects was 140 mg/dl. The other phenotypes were intermediate: 4/3 = 189 mg/dl, 3/3 = 184 mg/dl, and 3/2 = 166 mg/dl. The mean plasma apoE level varied in the opposite direction: 4/4 = 1.9 mg/dl, 3/3

= 2.4 mg/dl, and 2/2 = 5.1 mg/dl. The explanation for this variability appears to be that the E2 form of apoE is markedly defective in binding to the LDL (apoB,E) receptor (44, 93). This results from the amino acid substitution at a region of apoE involved in receptor binding. It is thought that reduced delivery of cholesterol to the liver from apoE-containing HDL, remnants of chylomicrons, and VLDL upregulates LDL receptors, thereby enhancing LDL clearance. Although the E3 and E4 forms of apoE bind to receptors about equally well in vitro, the E4 form appears to be metabolized more rapidly than E3 in vivo. This is thought to downregulate LDL receptors, resulting in higher plasma cholesterol levels (56).

In 1975, Utermann reported the association of a genetic variant of apoE with familial Type 3 hyperlipidemia (88). Subsequent studies established that more than 90% of Type 3 patients are E2 homozygotes. The remainder are E2 heterozygotes, at least some of whom are compound heterozygotes having rare alleles of apoE that exhibit defective binding (56). Familial Type 3 hyperlipidemia is a rare disorder found in fewer than 1 in 5000 people. Patients have increased plasma cholesterol and triglyceride levels due to the accumulation of β-VLDL, and clinically have xanthomata and atherosclerosis (especially peripheral vascular disease). The fact that fewer than 1% of the population have the apoE 2/2 phenotype, yet 90% of Type 3 patients have the apoE 2/2 phenotype illustrates the strong association between this condition and the E2 allele. However, more than 95% of people with the apoE 2/2 phenotype are normolipidemic; in fact, as noted above, they have lower than average plasma cholesterol levels. It is thought, therefore, that a second factor (genetic or environmental) is present in some subjects with the E2/E2 phenotype that promotes the expression of Type 3 hyperlipidemia. Some examples of contributory factors are familial hypercholesterolemia, familial hypertriglyceridemia, familial combined hyperlipidemia, diabetes, hypothyroidism, and obesity (4, 23). However, this does not explain the recent finding by Klasen et al (48), who reported a RFLP in the apoE gene using the enzyme HpaI, in which the minor allele had a frequency of 0.38 in normolipidemic individuals. ApoE 2/2 homozygotes had a similar frequency of the minor allele; however, among 39 individuals with Type 3 hyperlipidemia, the frequency was 0.97. This suggests that genetic variation is present in or near the apoE gene that is in genetic disequilibrium with the HpaI minor allele and that contributes to the Type 3 phenotype.

The apo-E2 allele is also present in higher frequency in a subset of patients with primary hypertriglyceridemia (Type 4) (58) but not in patients with familial combined hyperlipidemia.

The E4 allele has been reported to be increased in patients with "hypercholesterolemias" defined by plasma cholesterol levels above 260 mg/dl (4) or

280 mg/dl (89). This may represent the extreme of the incremental effect of E4 on LDL, as discussed above.

Finally, a number of rare alleles of apoE have been discovered, some of which are associated with hyperlipidemia or atherosclerosis (16). Two such, E5 and E7, occur in about 5% of Japanese patients with hyperlipoproteinemia and atherosclerosis (96).

Apo(a) Gene

Lp(a) is a lipoprotein consisting of a glycoprotein, apo(a), covalently attached by a disulfide bond to the apoB-100 moiety of an LDL-like particle. The function of Lp(a) remains unclear, but interest in this lipoprotein is high because of reports of an association between high plasma levels of Lp(a) and atherosclerosis (6, 21). The cDNA for apo(a) has recently been cloned (63). Its availability will permit the performance of the genetic studies similar to those undertaken for the other apoproteins. Parallel to this approach to identifying genetic variation of apo(a) is phenotyping of apo(a) by antibodies. Six alleles have been characterized in this way. Their frequencies and the Lp(a) plasma levels associated with homozygosity for the alleles have been determined. For example, in Austria the phenotypes designated B and O have frequencies of 0.013 and 0.6, respectively. The average plasma Lp(a) concentrations for those with the B and O phenotypes differ dramatically (61.7 and 4.4 mg/dl, respectively) (49).

ApoD Gene

ApoD is a minor constituent of HDL whose function is unknown. Recent cloning of its cDNA and gene has provided information about its nucleic and amino acid sequences (24). ApoD does not appear to be related to any of the apolipoproteins already discussed. Its function in lipoprotein metabolism and relationship to other apoproteins are subjects of ongoing investigations.

CONCLUDING REMARKS

Study of apolipoprotein gene variation is still in its early phases. With the tools now in hand to investigate all of the apolipoproteins, and the keen interest in their genetic variants, an explosive growth of knowledge over the next few years can be expected.

Although we have focused here on a few specific research strategies, we emphasize that only the integration of information from a variety of approaches—genetic, physiologic, biochemical, epidemiologic, etc—will lead to an optimal understanding of lipoprotein metabolism and its relationship to cardiovascular disease.

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