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Expressed Hypervariable Polymorphism of Apolipoprotein (a)

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Summary

Elevated plasma lipoprotein (a) (LP(a)) levels are an independent predictor of the development of premature atherosclerosis in humans. The LP(a) particle consists of two disulfide-linked proteins, apolipoprotein (APO) B and APO(a). The APO(a) is a highly glycosylated protein which carries the LP(a) antigen. Genetic polymorphism in the APO(a) molecule has been reported, and, depending on the sensitivity of the method used, 6–11 alleles at the APO(a) structural locus have been documented in the literature. In this investigation, we have used a high-resolution SDS-agarose electrophoresis method followed by immunoblotting to screen APO(a) polymorphism in 54 families with 130 offspring. This method identified a total of 23 different APO(a) isoforms, and their genetic basis was confirmed in families. In addition to the detectable products of 23 APO(a) alleles, the family data predict the existence of a "null" allele. Of the total 270 individuals tested, 209 (77.4%) revealed double-banded phenotypes and 61 (22.6%) revealed single-banded phenotypes. In the unrelated sample of 140 individuals, however, 114 (81.4%) and 26 (18.6%) had double- and single-banded phenotypes, respectively. When the segregation pattern of single-banded phenotypes in the unrelated sample was followed in families, only nine (6.4%) were found to be true homozygotes, and the remaining 17 (12.2%) were classified as heterozygotes for the null allele. Of the 276 possible phenotypes predicted for 23 alleles in a large population, we observed 115 (42%) phenotypes in our restricted sample. On the basis of our results from the family data, we hypothesize the existence of at least 24 alleles, including a null allele, at the APO(a) structural locus. The calculated frequencies of the *APO(a)*N* (null) and *APO(a)*1–APO(a)*23* alleles in the unrelated sample are as follows: N = 6.1%; 1 = 0.7%; 2 = 4.3%; 3 = 1.4%; 4 = 6.1%; 5 = 8.9%; 6 = 4.3%; 7 = 2.5%; 8 = 11.8%; 9 = 3.9%; 10 = 9.3%; 11 = 4.6%; 12 = 2.9%; 13 = 5.7%; 14 = 2.1%; 15 = 8.2%; 16 = 3.2%; 17 = 5.4%; 18 = 4.6%; 19 = 1.4%; 20 = 0.4%; 21 = 0.4%; 22 = 1.4%; and 23 = 0.4%. The average heterozygosity at the APO(a) structural locus was 94%, which is the highest value for any protein polymorphism reported to date.

Introduction

The lipoprotein (a) (LP(a)) was first discovered in human plasma by Berg (1963) as a genetic variant of low-density lipoprotein (LDL). LP(a) is an LDL-like particle which contains, in addition to APOB, a disulfide-linked molecule of a protein known as apolipoprotein (a) (APO(a)) which carries the LP(a) antigen (Fless et al. 1986). APO(a) shares remarkable structural homology to the fibrinolytic protein zymogen, plasminogen (PLG) (Eaton et al. 1987; McLean et al. 1987), including the protease domain and the lysine-binding domains, known as kringles. While PLG has a single copy of kringles 1–5, APO(a) has both one copy of kringle 5, with 95% identity to the PLG kringle 5, and 15–37 copies of kringle 4, with 75%–85% identity to the PLG kringle 4. Although the APO(a) proteolytic domain shares 94% identity with the PLG protease domain, the cleavage site for tissue PLG activator is changed from arginine to serine in APO(a), and current evidence is that APO(a) lacks proteolytic activity. In addition to their structural homology, APO(a) and PLG are genetically linked on chromo-

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some 6 (Drayna et al. 1988; Weitkamp et al. 1988; Lindhal et al. 1989).

Several studies indicate a strong association between elevated levels of LP(a) and premature development of atherosclerosis (reviewed in Brown and Goldstein 1987; Morrisett et al. 1987; Utermann 1989; Loscalzo 1990) involving both coronary arteries (Armstrong et al. 1986; Dahlen et al. 1987; Rhoads et al. 1986; Murai et al. 1986; Durrington et al. 1988; Hoefler et al. 1988; Rosengren et al. 1990; Sandkamp et al. 1990) and cerebral arteries (Murai et al. 1986; Jürgens and Költringer 1987). Elevated LP(a) levels are found two to five times more frequently in patients with premature heart disease than in controls. LP(a) levels above 20 mg/dl, which are present in about 20% of people, pose a major risk factor for developing coronary atherosclerosis (Sandkamp et al. 1909). It is not yet clear whether the increased heart disease risk associated with LP(a) is due to a role in lipid metabolism as a structural component of the LP(a) particle or is due to its involvement in the fibrinolytic process because of the structural similarity of APO(a) to PLG. Recent experimental data suggest that at physiologic concentrations LP(a) may regulate fibrinolytic activity but that at elevated concentration it may inhibit fibrinolysis by competing with PLG for cellular binding sites on the endothelial surface (Gonzalez-Gronow et al. 1989; Hajjar et al. 1989; Miles et al. 1989). LP(a) also inhibits the binding of PLG to plasmin-modified immobilized fibrinogen or fibrin (Harpel et al. 1989) and thereby may increase the risk of thrombosis.

Quantitative LP(a) levels in human plasma vary from 1 mg/dl to 150 mg/dl, and these levels are under genetic control (Sing et al. 1974; Berg 1983; Hasstedt et al. 1983; Morton et al. 1985; Hasstedt and Williams 1986). Recently, Utermann et al. (1987, 1988*a*, 1988*b*; Kraft et al. 1988) have suggested that LP(a) quantitative variation is directly determined by a multiallelic polymorphism at the APO(a) structural locus. However, the postulated relationship is not a simple one, because the reported contribution of the APO(a) size polymorphism accounts for only about 42% of the genetic variation in LP(a) levels (Boerwinkle et al. 1989). In their original study Utermann et al. (1987, 1988*b*) identified six distinguishable APO(a) isoforms and a "null" allele, and they detected no APO(a) isoform in about 50% of individuals tested. In a subsequent study Utermann et al. (1989) reported the presence of at least one APO(a) isoform in about 94% of the population tested. In a recent study Gaubatz et al. (1990) have resolved 11 different APO(a) isoforms, and only about 1% of individuals failed to show at least one APO(a) isoform.

In the present report, we describe a high-resolution SDS–agarose electrophoresis method which can detect at least 23 different APO(a) allelic isoforms. The genetic basis of these allelic isoforms is confirmed in families, and the incidence of the null allele is estimated using family data.

Subjects and Methods

Family data

A subset of 54 families with 130 children was drawn from the Rochester Family Heart Study (Moll et al. 1989) for APO(a) typing. For the purpose of APO(a) allele frequency estimation, the random sample of 140 was established by pooling genotypes from parents (N = 108), those grandparents whose phenotypes were different from those of their offspring (N = 28), and laboratory personnel (N = 4).

Electrophoresis and Immunoblotting

Samples were prepared by mixing 10–15 μl of plasma with 30 μl of reducing buffer (1:2:10 ratio of β-mercaptoethanol/0.5% bromophenol blue in 5% glycerol/5% SDS) and heating the mixture for 5 min at 100°C. The sample temperature was equilibrated by leaving the sample at room temperature for about 15 min, after which it was either immediately applied to the gel or frozen overnight and used the next morning. Electrophoresis was performed on 1.5% agarose submarine gel by using a Hoefer submarine-gel unit and an LKB power supply. Agarose gels (15 cm × 25 cm) were cast using a solution of 150 ml of 1.5% ultrapure agarose (BRL) dissolved in 90 mM Tris, 90 mM boric acid, 2 mM EDTA, and 0.1% SDS. Fifteen microliters of reduced samples were applied in 1.5-mm-wide and 2-mm-deep wells made 3 cm from the cathode on the gel. Electrophoresis was carried out in tank buffer (45 mM Tris, 45 mM boric acid, 2 mM EDTA, and 0.1% SDS) for 7–8 h at constant 25 W at 4°C. Alternatively, gels were run for 15–16 h at constant 10 W. Each gel contained a control cocktail of six APO(a) isoforms prepared by mixing plasma samples from three individuals with different APO(a) types, to control for gel-to-gel variation in migration and to aid in differentiating closely migrating isoforms.

After electrophoresis, proteins were transferred to a nitrocellulose membrane (pore size 0.45 μm) by electroblotting using a Hoefer Transphor Cell at 90 V for

3 h in 10 mM Tris, 40 mM glycine, and 5% methanol. After protein transfer, the nitrocellulose membrane was incubated for 60 min in a solution of 5% powdered skim milk dissolved in deionized water, to block the remaining protein-binding sites. The filter was then immersed overnight in monospecific, polyclonal rabbit anti-human APO(a) antiserum (Behringwerke) at 1:500 dilution. The filter was washed extensively in Tris-buffered saline (pH 7.5) and incubated with a second antibody, goat anti-rabbit IgG, conjugated with alkaline phosphatase enzyme (Pel Freez) at 1:2,500 dilution for 3 h. After extensive washings, the APO(a) banding pattern was visualized by histochemical staining for alkaline phosphatase.

Results

APO(a) Isoforms and Their Nomenclature

Routine APO(a) typings on plasma samples were performed using the high-resolution SDS-agarose-gel electrophoresis method, followed by immunoblotting using commercially available APO(a) antiserum. The specificity of the observed banding pattern was confirmed both using a monospecific polyclonal goat anti-human APO(a) antiserum provided by Dr. Joel Morrisett (Baylor College of Medicine, Houston) and using a mouse monoclonal anti-human Lp(a) provided by Dr. David Usher (University of Delaware, Newark). In each individual's plasma sample, either one or two bands were observed. A total of 23 different APO(a) isoforms, which focused in different regions on the SDS-agarose gel, were immunolocalized. Since, compared with previously reported methods, our system detects a large number of APO(a) isoforms, we have developed our own nomenclature for their designation. A numerical numbering system, i.e., 1–23, was assigned to each different isoform, with 1 being the highest-apparent-molecular-weight species migrating near the cathode and with 23 being the lowest-apparent-molecular-weight species migrating near the anode end of the gel (figs. 1–3). A control, containing

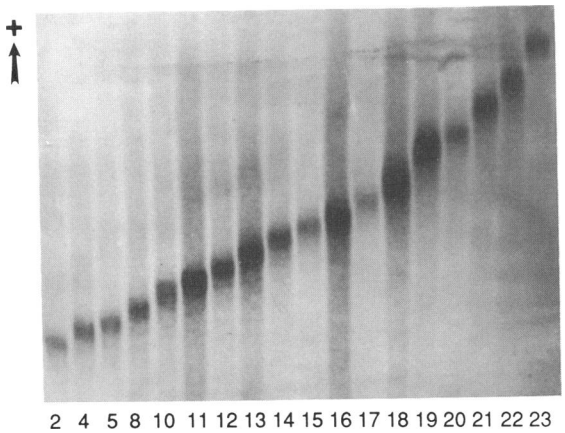

Figure 1 Eighteen APO(a) single-banded APO(a) isoforms resolved in 1.5% SDS–agarose-gel electrophoresis followed by immunoblotting. The APO(a) phenotype of each isoform is given beneath each sample track.

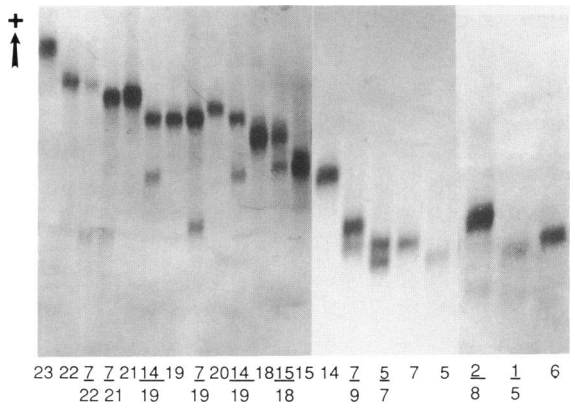

Figure 2 APO(a) phenotypes resolved in 1.5% SDS–agarose-gel electrophoresis followed by immunoblotting. The APO(a) phenotypes are indicated beneath each sample track.

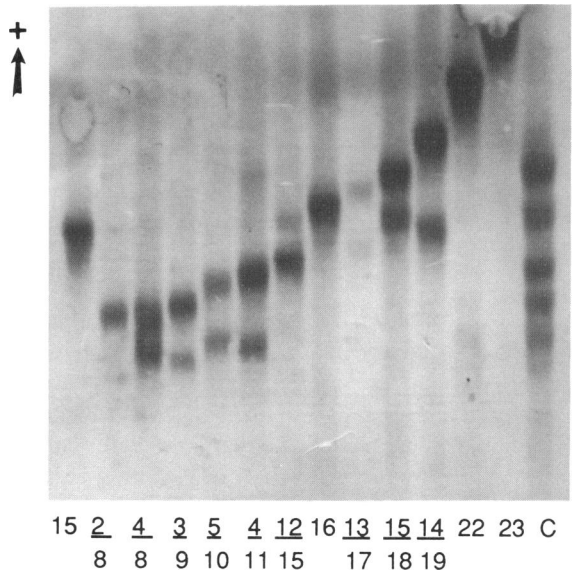

Figure 3 Various APO(a) single- and double-banded APO(a) phenotypes resolved in 1.5% SDS–agarose-gel electrophoresis followed by immunoblotting. The APO(a) phenotypes are given beneath each sample track. The control mixture (lane C) contains six APO(a) isoforms with designations, from cathode to anode, 2, 5, 8, 11, 15, and 18.

six known distinct isoforms, was run in the middle of each gel to aid in the detection of new alleles and to control for gel-to-gel variation in the migration of APO(a) species. This step was necessary to ensure reliable classification of closely migrating bands. We propose that the synthesis of these 23 different APO(a) isoforms is encoded by multiple alleles at a single structural locus, and these alleles are numbered *APO(a)*1–APO(a)*23*. Single-banded phenotypes are considered either homozygous for the given allele or heterozygous for the presence of a null allele (discussed below), and double-banded phenotypes are heterozygotes due to codominant expression of two different alleles.

In addition to the major isoforms, some faint, low-molecular-weight bands were occasionally observed, probably because of partial proteolysis of APO(a) in stored samples. The presence of these faint bands did not interfere in the reliable classification of APO(a) phenotypes.

Inheritance of APO(a) Polymorphism

The genetic basis of various APO(a) isoforms was confirmed in 54 families with a total of 130 children (table 1). Each parental cross was unique because the same parental combination was not observed in more than one cross. A total of 72 distinct single- and double-banded APO(a) phenotypes were observed in children; 25 of these 72 were in addition to the distinct 86 phenotypes observed in the unrelated sample. The segregation pattern in 41 families confirmed the autosomal codominant inheritance of APO(a) isoform phenotypes. In 13 families (families 13, 16, 26, 34, 35, 36, 38, 40, 42, 46, 48, 49, and 51 in table 1) 14 of 34 children showed isoforms traceable to their parents. The remaining 20 children, however, exhibited a single band rather than the expected two. This situation happened only when (*a*) at least one parent had a single band phenotype and (*b*) the missing band in the offspring was traceable to the single-banded parent. We conclude, therefore, that in such cases the single-banded parents were heterozygous for the null allele whose allele product was undetectable on electrophoresis gels and that they transmitted this null allele to some of their children. This does not rule out the formal possibility that no true null exists but that our method lacks the sensitivity to detect the product of a "functional" null.

The segregation of an apparent null allele in two pedigrees (13 and 36 in table 1) is shown in figure 4. In the first pedigree, one parent has the double-banded phenotype designated 14-19, and the other parent expresses a single-banded phenotype designated 14. Both of their daughters have a single intense band at position 14 and thus appear to be homozygous for the *APO(a)*14* allele inherited from each of their parents. Similarly, their sons have at position 19 a single band, which they have inherited from their mother, but lack a paternal band in the expected position 14. It means that the father is not homozygous for the *APO(a)*14* allele but is a heterozygous for the null allele, and, therefore, his phenotype is designated as N-14. On the basis of this hypothesis, both male offspring have inherited the null allele from their father, and so their phenotypes are designated as N-19. Although their two daughters appear to be homozygous for the *APO(a)*14* allele, it is possible that they carry the null allele and are N-14 heterozygotes.

In the second pedigree, the mother has a single band at position 15, and the father has double bands at positions 10 and 17. Their one son has inherited from his mother the solitary band at position 15 and one of his father's two bands at position 10, and he is, therefore, heterozygous 10-15. His two brothers, however, have inherited their father's second band at position 17 but not their mother's solitary band at position 15. It is, therefore, logical to believe that the mother is heterozygous for the null allele and has phenotype N-15 and that she has transmitted the null allele to two (of three) sons, who have phenotype N-17.

Of the 41 pedigrees which demonstrated simple autosomal codominant inheritance patterns of APO(a) isoforms, selected examples, illustrated in figures 5 and 6, show the inheritance of some of the alleles observed during this investigation. In all of these families, four different alleles are present in the two parents, and in most cases inheritance of APO(a) alleles is demonstrated in three generations. Of the 24 alleles observed, the autosomal codominant inheritance pattern of 22 alleles was confirmed in families. Because of lack of informative family data, we did not observe the segregation of the *APO(a)*20* and *APO(a)*21* alleles. The APO(a) 21 isoform was detected in a single grandparent, while the APO(a) 20 isoform was observed in the plasma sample of a laboratory control.

Intensity of APO(a) Isoforms

Generally, APO(a) isoforms migrating toward the anode (i.e., those isoforms having low molecular weight) stained more intensely than did cathodally migrating (i.e., high-molecular-weight) bands. However, we observed several exceptions to this rule.

Table 1

Inheritance of APO(a) Types in 54 Families with 130 Children

Family	Parental Mating	No. of Children	Phenotype(s) of Children (no. of children)
1	15-18 × 8-15	3	8-15 (1), 8-18 (1), and 15-15 (1)
2	15-18 × 12-18	2	18-18
3	3-9 × 8-9	2	3-9 and 8-9
4	8-18 × 15-16	2	8-16
5	1-15 × 11-17	2	1-11 and 11-15
6	8-17 × 5-13	3	5-17 (2) and 8-13 (1)
7	2-13 × 13 or N-13	2	2-13 and 13/N-13
8	4-10 × 5-11	4	4-5 (1), 10-11 (1), 4-11 (1), and 5-10 (1)
9	3-19 × 3-17	1	17-19
10	3-5 × 6-19	1	3-19
11	N-4 × 4-22	3	4-22 (1) and 4/N-4 (2)
12	4-12 × 9-10	3	4-9 (1) and 10-12 (2)
13	N-14 × 14-19	4	N-19 (2) and 14-14 (2)
14	5-15 × 5 or N-5	2	5/or N-5
15	8-13 × 8-13	3	13-13
16	N-8 × 10 or N-10	2	N-10 and 8-10
17	2-15 × 10 or N-10	4	10-15 (2) and 2-10 (2)
18	11-18 × 4 or N-4	4	4-11 (3) and 4-18 (1)
19	5-8 × 5-15	3	8-15 (2) and 5-5 (1)
20	6-14 × 15-17	2	6-17 and 14-17
21	2-8 × 4-9	1	4-8
22	4-12 × 12-18	2	4-12 and 12-12
23	10-15 × 12-17	4	10-12 (1), 15-17 (1), and 12-15 (2)
24	2-8 × 2-7	1	2-2
25	5 or N-5 × 8-9	2	5-8 and 5-9
26	N-17 × 6-10	3	6-17 (2) and N-6 (1)
27	4-8 × 7-9	3	4-9 (1), 7-8 (1), and 8-9 (1)
28	8-11 × 2-11	3	11-11 (1), 8-11 (1), and 2-8 (1)
29	4-8 × 8-18	2	4-8
30	5-6 × 7-17	5	5-17 (1) and 5-7 (4)
31	5-22 × 7-9	2	5-7 and 7-22
32	6-15 × 15-23	2	15-15 and 15-23
33	1-4 × 5-8	2	1-5 and 1-8
34	N-8 × N-13	2	N-8 and N-13
35	N-9 × 17 or N-17	2	N-17
36	N-15 × 10-17	3	10-15 (1) and N-17 (2)
37	6-17 × 5-6	2	6-6
38	N-16 × 11-14	2	N-11 and 11-16
39	2-2 × 10 or N-10	1	2-10
40	N-10 × 7-9	2	N-9 and 9-10
41	4-16 × 5 or N-5	3	4-5 (2) and 5-16 (1)
42	N-11 × 6-10	3	N-10 (1) and 7-11 (2)
43	5-6 × 6-22	2	6-6 and 6-22
44	7-13 × 10-16	3	7-10 (2) and 7-16 (1)
45	8-11 × 11-15	2	8-11
46	5-10 × N-22	1	N-5
47	5-18 × 15-18	3	5-18
48	N-16 × 5-13	3	N-5 (1) and 5-16 (2)
49	N-10 × N-13	3	N-10 (2) and N-13 (1)
50	5-10 × 13-17	2	5-17 and 10-13
51	N-10 × 12-15	4	10-12, 10-15, N-12, and N-15
52	11-13 × 13-18	1	11-18
53	8-17 × 13-15	1	15-17
54	2-10 × 8-11	1	2-11

NOTE.—A total of 72 different APO(a) types were observed in children; 25 of these are present only in children.

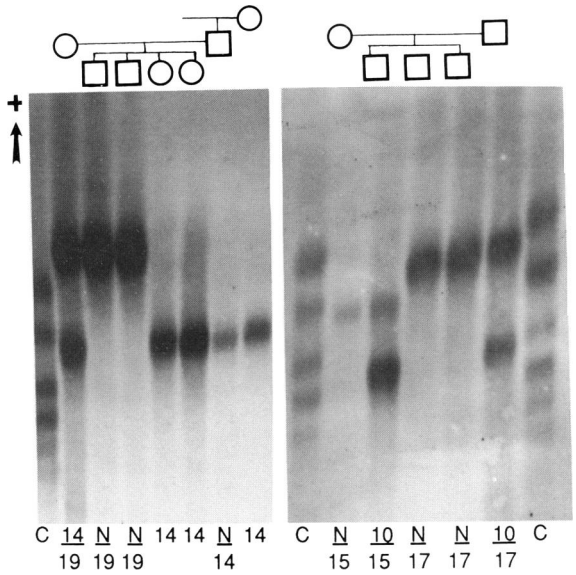

Figure 4 Inheritance of the APO(a) null allele in two families. The APO(a) phenotypes are indicated beneath each sample track. The control mixture (lane C) contains six APO(a) isoforms with designations, from cathode to anode, 2, 5, 8, 11, 15, and 18.

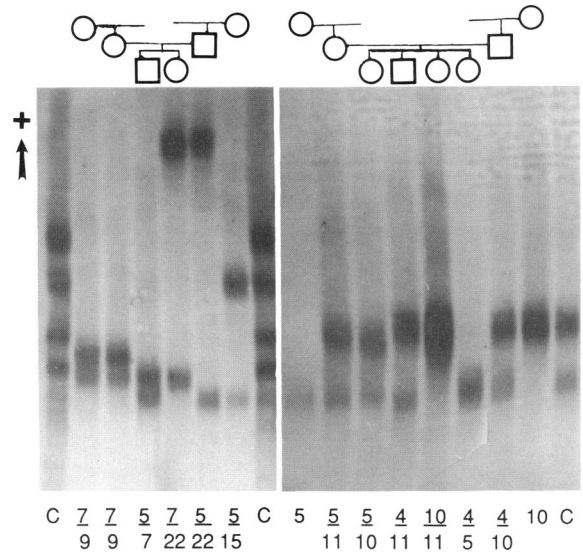

Figure 5 Segregation of *APO(a)*5, APO(a)*7, APO(a)*9, APO(a)*15,* and *APO(a)*22* alleles in family on left, and inheritance of *APO(a)*4, APO(a)*5, APO(a)*10,* and *APO(a)*11* alleles in family on right. The control (right lane C) in the family on the right contains two APO(a) isoforms, 4 and 10, while the control mixture (left lane C) in the family on the left contains six APO(a) isoforms with designations, from cathode to anode, 2, 5, 8, 11, 15, and 18. The APO(a) phenotypes are indicated beneath each sample track.

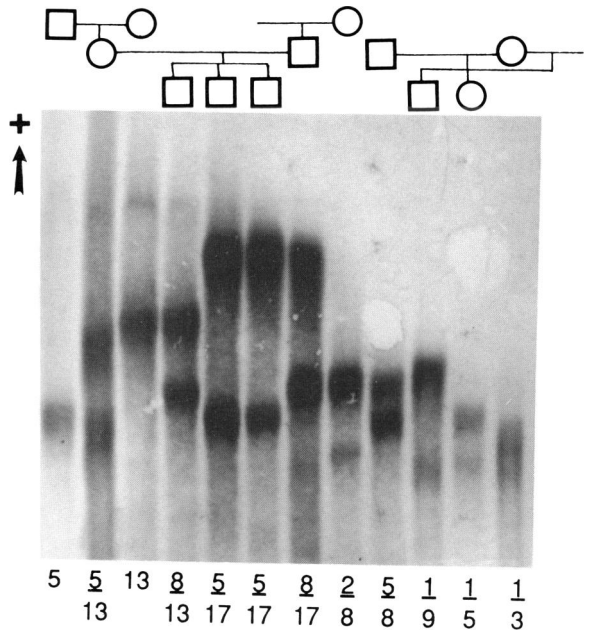

Figure 6 Two families illustrating segregation patterns of *APO(a)*1, APO(a)*2, APO(a)*3, APO(a)*5, APO(a)*8, APO(a)-*13,* and *APO(a)*17* alleles. The APO(a) phenotypes are given beneath each sample track.

APO(a) isoforms 15, 17, and 20 in figure 1 show less intensity compared with the next-migrating high-molecular-weight isoforms. Similarly, compared with the higher-molecular-weight band at position 12, a double-banded phenotype designated 12-15 in figure 3 has less immunoreactivity at position 15. Within families, however, the intensity of various APO(a) isoforms remained constant. In figure 7, this point is illustrated in one family (family on left) which shows the inheritance of a less-intense isoform at position 16 over three generations. Figure 7 also demonstrates a family (family on right) in which intensity of the same isoform was different in two different individuals and in which this intensity difference was transmitted to the next generation. The isoform in question is at position 9, which is present in both parents in generation II. The father (phenotype 8-9) has an intense band at position 9 and has transmitted this intensity to one of his sons having the 3-9 phenotype. The mother (phenotype 3-9) has a very weak maternal band at position 9 and has transmitted this weak-intensity band to her second son, who has the 8-9 phenotype. It is important to note here that the son having the 8-9 phenotype appears to have a single band at position

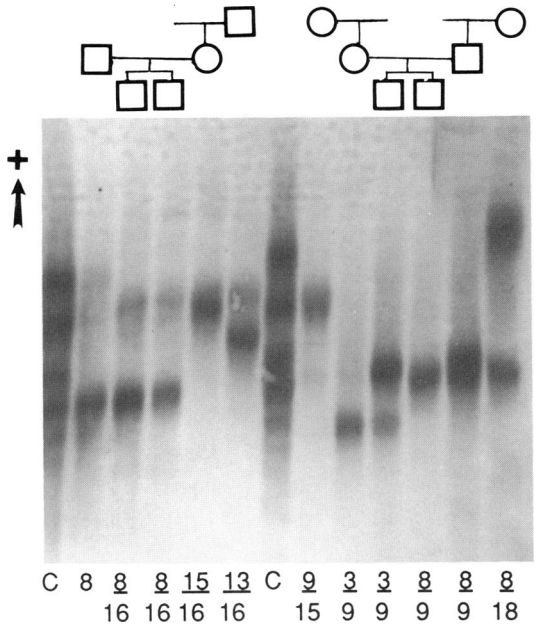

Figure 7 Genetic transmission of *APO(a)*3, APO(a)*8, APO(a)*9,* and *APO(a)*16* alleles from parents to offspring in two families. Note that the low-molecular-weight band at position 16 is less intense than the high-molecular-weight bands at positions 8, 13, and 15 in the family on the left and that this low intensity at position 15 is inherited through three generations. In the family on right, the APO(a) band at position 9 has different intensities in two individuals, and these differences are inherited. APO(a) phenotypes for each family member are given beneath each sample track. The control mixture (lanes C) contains six APO(a) phenotypes with designations, from cathode to anode, 2, 5, 8, 11, 15, and 18.

8 because the immunoreactivity present in isoform 9 is low.

Population Distribution of APO(a) Polymorphism

Among the 140 unrelated individuals tested, 86 distinct single- and double-banded APO(a) phenotypes were identified. Inclusion of an "operational null" (N) allele detected in family studies raised the total number of phenotypes to 91 (table 2). Before the analysis of family data, 26 individuals (19%) were classified as homozygotes and 114 (81%) as heterozygotes. After family studies, 17 of the 26 single-banded individuals were reclassified as heterozygotes carrying the N allele (table 2, first row), and only nine individuals (6%) of the total unrelated sample were found to be true homozygotes (table 2, first diagonal row).

On the basis of evidence that a null allele exists in addition to the detectable products of 23 alleles, we calculated allele frequencies of all 24 alleles in the unrelated sample (fig. 8). Except for the four alleles, *APO(a)*1, APO(a)*20, APO(a)*21,* and *APO(a)*23,* all were observed at a polymorphic frequency. However, there are only four alleles—*APO(a)*15, APO(a)*5, APO(a)*10,* and *APO(a)*8*—which have appreciable frequencies of 8%–12%. The frequency of the N allele, which is about 6%, is close to these values. There are eight other alleles whose frequencies are 4%–6%, and the remaining seven alleles have allele frequencies of 1%–3%. It is likely that the present estimate of APO(a) allele frequencies will change as the number of unrelated individuals tested increases.

Hardy-Weinberg Equilibrium Test

The conventional test of Hardy-Weinberg equilibrium is not appropriate in this case because we observed only 91 of the expected 300 phenotype classes, and only a small number of individuals were observed in each phenotype class. However, we performed the following available alternative χ^2 analyses. Expected values in all 300 phenotype classes were estimated using allele frequency data as listed in the legend to figure 8. Although we observed only 91 phenotype classes, and despite small in each class, we first performed the standard χ^2 analysis. On the basis of this test ($\chi^2 = 248.67$, df = 299, $P > .60$), the observed and expected values were in excellent agreement. In the second test, we followed the conventional procedure—i.e., if there are classes with expected values <1 and if df >1, then the smaller classes can be combined. In this analysis, 24 groupings of observed and expected values were obtained by adding numbers in columns and rows separately, and the equilibrium hypothesis was accepted in both cases ($\chi^2 = 5.95$, df = 23, $P > .99$; $\chi^2 = 7.20$, df = 23, $P > .99$). In the third test, we combined all the homozygote and heterozygote classes separately and, on the basis of χ^2 analysis, these values also do not differ significantly ($P > .80$) from equilibrium expectation.

Discussion

In the present investigation, we describe the application of high-resolution SDS–agarose-gel electrophoresis followed by immunoblotting to detect the expressed hypervariable polymorphism at the APO(a) glycoprotein locus. The method is extremely sensitive, as it detects at least one APO(a) isoform in all individuals tested and has resolved 23 discrete APO(a) allelic isoforms. The SDS-agarose method for routine

Table 2

APO(a) Phenotypes in 140 Unrelated Individuals

		Band 2																					
Band 1	N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
N		...	...	...	1	1	...	...	2	1	3	1	...	2	1	1	2	1	...	...	...	...	1	...
1		...	...	...	1	...	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...
2			1	...	...	...	...	1	3	...	2	1	...	1	1	1	...	...	...	...	...	...	...	...
3				...	...	1	...	...	...	1	...	...	...	...	...	...	...	1	...	1	...	...	...	...
4					1	...	...	...	4	1	2	1	2	...	...	...	2	...	...	...	...	...	1	...
5						2	3	...	2	...	4	1	...	2	...	5	...	...	1	...	...	...	1	...
6							...	...	1	...	2	...	...	...	1	1	...	1	...	1	1	...	1	...
7								...	1	3	...	...	...	1	...	...	...	1	...	...	...	...	...	...
8									...	2	...	3	1	3	...	2	...	3	3	2	...	1	...	...
9										...	1	...	1	...	...	...	...	1	...	...	...	...	...	...
10											3	...	...	...	1	1	3	...	1	...	...	...	...	...
11												...	...	1	1	2	...	1	1	...	...	...	...	...
12													...	...	...	1	...	1	2	...	...	...	...	...
13														1	...	1	1	1	1	...	...	...	...	...
14															...	...	...	...	1	...	...	...	...	...
15																...	1	1	4	...	...	...	...	1
16																	...	...	...	...	...	...	...	...
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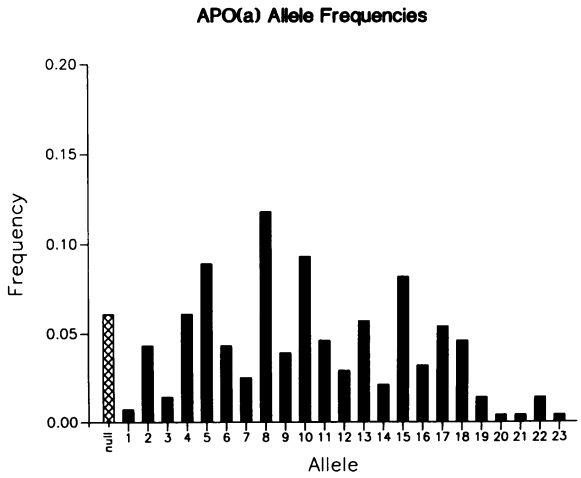

Figure 8 Graphic representation of APO(a) allele frequencies. The frequencies of all 24 alleles are as follows: Null = .061; 1 = .007; 2 = .043; 3 = .014; 4 = .061; 5 = .089; 6 = .043; 7 = .025; 8 = .118; 9 = .039; 10 = .093; 11 = .046; 12 = .029; 13 = .057; 14 = .021; 15 = .082; 16 = .032; 17 = .054; 18 = .046; 19 = .014; 20 = .004; 21 = .004; 22 = .014; and 23 = .004.

APO(a) genetic screening was adapted after critical consideration and evaluation of several reported SDS-polyacrylamide methods. In all the reported methods, discontinuous buffer systems have been used to resolve APO(a) isoforms in 3%–12% SDS-polyacrylamide gels. In our hands, even low-percentage (4%) polyacrylamide gels failed to yield consistent, satisfactory separation between various APO(a) isoforms, because of the latters' high molecular weights. However, agarose-gel electrophoresis in 90 mM Tris-borate buffer containing 0.1% SDS provided superior resolution between APO(a) isoforms, most probably because of molecular-sieving effect, because agarose has a higher pore size than does polyacrylamide matrix and thus is suitable for separation of high-molecular-weight proteins such as APO(a). The technical advantages of this method are that (1) it does not require a stacking gel; (2) gel casting is quick and simple; and (3) the same buffer is used for both gel preparation and electrophoretic separation.

The development of the present SDS-agarose

method for routine APO(a) typing was prompted by the fact that the reported methods of SDS-polyacrylamide gels in discontinuous buffer systems using either vertical (Utermann et al. 1988*a*, 1988*b*) or horizonal (Kraft et al. 1988) slab gels were not found to be appropriate for resolving the majority of the APO(a) isoforms. As compared with discontinuous polyacrylamide gels in which APO(a) bands focus in a narrow region close to the bottom half of the resolved gel, in the continuous agarose gel the APO(a) bands migrate in a region which extends from the middle to the upper half of the gel. Compared with the polyacrylamide gel, in the agarose gels there is a four- to sixfold increase in the migration distances between APO(a) isoforms of a given double-banded phenotype. Isoforms that had detectable several different migration positions on agarose gels had indistinguishable and identical positions on polyacrylamide gels (data not shown).

In table 3 the sensitivity and resolving power of the SDS-agarose method for APO(a) typing are compared with those reported discontinuous SDS-PAGE methods. Compared with the reported 6–11 allelic products detected by other methods, the SDS-agarose method differentiated 23 APO(a) isoforms. Six isoforms designated F, B, S1, S2, S3, and S4 were observed in five studies (Kratzin et al. 1987; Utermann et al. 1987, 1988*b*, 1989; Kraft et al. 1988), while 11 isoforms designated 1–11 were reported by Gaubatz et al. (1990) in humans. Similarly, nine APO(a) isoforms have been reported in baboons (Rainwater et al. 1989). In the absence of a direct comparison between the isoforms reported in other human studies and those observed in the present investigation, it is not possible to determine which of the reported six and 11 isoforms correspond to our 23 isoforms. On the basis of electrophoretic mobility of APO B, which is similar both to that of Utermann et al.'s isoform B and to that of Gaubatz et al.'s isoform 3, in our nomenclature either isoform 18 or isoform 19 is similar to isoform B or isoform 3. Utermann et al. and Gaubatz et al. observed, respectively, four and eight isoforms that had molecular weights higher than those of the APO B species, while we have observed at least 17 such high-molecular-weight isoforms. Clearly, the main difference between previously reported methods and ours is the inability of discontinuous SDS-PAGE methods to resolve high-molecular-weight APO(a) isoforms. Thus, several distinct APO(a) isoforms classified on SDS-agarose gel will have identical mobility on SDS-polyacrylamide gel, and the true level of heterogeneity in the APO(a) molecule will be underestimated. Our method has resolved 115 distinct APO(a) phenotypes, compared with the 15 (Utermann et al. 1988*b*) or 43 (Gaubatz et al. 1990) detected by SDS-polyacrylamide gels. The superiority of our method is also evident in the detection of double-banded phenotypes in about 80% of individuals tested: this figure is about double that reported by others.

In our survey of 270 individuals, we have not seen any true example of the null phenotype. This result contrasts sharply with early studies, which reported that almost 50% subjects had undetectable APO(a) bands (Utermann et al. 1987, 1988*a*, 1988*b*). However, improved sample-preparation protocols enabled

Table 3

Frequencies of Single-banded, Double-banded, and Unbanded APO(a) Phenotypes in Unrelated Individuals

Study	Species (*N*)	No. of Alleles Observed	No. of Phenotypes Observed	% of Phenotypes		
				Single Banded	Double Banded	Unbanded
Rainwater et al. 1989	Baboons (165)	9	31	50	45	5
Utermann et al. 1987	Humans (247)	6	12	46	5	49
Kratzin et al. 1982	Humans (73)	6	Not given	52	48	Not given
Utermann et al. 1988*b*	Humans (441)	6	15	50	6	44
Kraft et al. 1988	Humans (194)	5	15	55	8	37
Utermann et al. 1989	Humans (279)	5	15	74	20	6
Gaubatz et al. 1990	Humans (692)	11	43	59	40	1
Present study	Humans (140)	23	86	19[a]	81	0
Present study	Humans (270[b])	23	115	23[c]	77	0

[a] Actual proportion of single-banded phenotypes after confirmation in families was 9/140 = 6%.

[b] Includes 140 unrelated and 130 children.

[c] Actual proportion of single-banded phenotypes in the whole sample after confirmation in families was 29/270 = 11%.

those same authors to detect APO(a) bands in about 94% individuals (Utermann et al. 1989). Recently, Gaubatz et al. (1990) have also reported the detection of APO(a) bands in 99% of individuals tested. Previous immunological findings by Albers et al. (1977) have concluded that there are not true LP(a)-negative individuals, and our immunoblotting results agree with their findings. However, since we have observed two families in which both parents were heterozygous for the null allele, it will not be surprising if we encounter a few examples of the null phenotype in further studies. Gaubatz et al.'s (1990) finding that only about 1% of 692 tested individuals had no APO(a) band appears to be a reasonable estimate of the population frequency of the null phenotype.

Previously, three separate studies have investigated the inheritance of APO(a) isoforms in a total of 18 families with 59 children (Utermann et al. 1987, 1988*a;* Gaubatz et al. 1990), and, with a few exceptions, a pattern of simple Mendelian inheritance was observed. In those families where the autosomal codominant inheritance of APO(a) isoforms was not followed, the existence of the null allele was postulated (Utermann et al. 1987, 1988*a*). In the present investigation, we confirm both the autosomal codominant segregation of APO(a) isoforms and the existence of null allele at the APO(a) locus in a large number of families. Among the 54 families analyzed, the null allele was found to be segregated in 13 families, with 10 children inheriting the null allele from the father and nine inheriting it from the mother. The equal occurrence of the null allele in fathers and mothers—and the equal transmission of it to children—suggest that nonpaternity is not an explanation for these observations. Information derived from children that allows us to determine the presence of the null allele in single-banded parents has enabled us to estimate the frequency of the null allele in the sample of unrelated individuals, a frequency which is about 6%. Previous studies have noted that the population distribution of APO(a) isoforms is not in Hardy-Weinberg equilibrium (Boerwinkle et al. 1989; Gaubatz et al. 1990). However, on the basis of the 24 allele frequencies that we observed, the distribution of observed and expected APO(a) phenotypes were in excellent agreement. Our family and population data support the hypothesis that APO(a) phenotypic variation is due to multiple APO(a) alleles at a single locus and that the observed variation is not due to postsynthetic modifications in the APO(a) molecule. Previously, Utermann et al. (1987) and Gaubatz et al. (1990) have shown that, while neuraminidase treatment affected the molecular sizes, the differences between various APO(a) isoforms were maintained.

The molecular basis of APO(a) protein size variation appears to be due to differences in the number of PLG-like kringle 4 encoding sequences, which are tandemly repeated at least 30 times in the APO(a) gene (McLean et al. 1987). Evidence in favor of this hypothesis has been provided by observations that a direct correlation exists between APO(a) size variations and hepatic RNA transcripts sizes, both in humans (Koschinsky et al. 1990) and in baboons (Hixson et al. 1989). Probably the most direct evidence that APO(a) size variation is due to variation in the kringle 4 encoding sequence is provided by Lackner et al. (1990), who, using pulse-field gel electrophoresis with the cloned kringle 4 used as a probe, have shown a 5–6-kb increment between each APO(a) allele. A careful analysis of mobility differences between the observed 23 APO(a) isoforms differentiated in the present study also provides strong evidence in favor of this hypothesis. When APO(a) isoforms are arranged in the sequence 1–23, as shown in figure 1, they form a nearly linear array. The minimum distance between closely migrating isoforms is about 1 mm. If we consider that a 1-mm mobility difference represents one repeat-unit increment, then we have observed at least 23 kringle 4 repeats in the present investigation. However, there are at least eight regions where the difference between two adjacent APO(a) isoforms is >1 mm, which would indicate that additional APO(a) isoforms should probably fall within these regions. For example, the distance between APO(a) isoforms 9 and 10, 11 and 12, 16 and 17, 17 and 18, and 21 and 22 is about 2 mm each and thus predicts the presence of one additional isoform at each of these five regions. Similarly, there are distances of about 3 mm and about 4 mm, respectively, between isoforms 20 and 21 and between isoforms 18 and 19, distances which predict an additional two and three isoforms, respectively, between the two elements in each of these pairs. The greatest distance, about 5 mm, was observed between isoforms 22 and 23, indicating that four more isoforms may fall within this region. On the basis of these distance calculations, we predict the detection of about 14 additional allele products as the number of individuals tested increases. We have already detected 23 APO(a) isoforms, and with the addition of 14 predicted isoforms the total number of kringle 4 repeat units will reach 37, an estimate similar to those based on cDNA sequencing data (McLean et al. 1987)

and hepatic RNA transcript sizes (Koschinsky et al. 1990).

With the detection of at least 24 alleles, the estimated mean heterozygosity at the APO(a) locus is about 94%, which is the highest value yet reported for any protein-coding locus. This is also the first example of a plasma protein which exhibits extremely high structural polymorphism due to an expressed hypervariable region within the coding sequence of gene. To our knowledge, the only other example of protein polymorphism which has demonstrated hypervariable expressed coding sequences is the human tumor-associated epithelial mucin-type glycoproteins encoded by the PUM locus (Swallow et al. 1987). The PUM polymorphism can be detected in human urine and has been shown to have at least 10 discrete allele products.

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