

ISOLATION AND PARTIAL CHARACTERIZATION OF APOLIPOPROTEIN D: A NEW PROTEIN MOIETY OF THE HUMAN PLASMA LIPOPROTEIN SYSTEM

W.J. McCONATHY and P. ALAUPOVIC

*Lipoprotein Laboratory, Oklahoma Medical Research Foundation,
and Department of Biochemistry and Molecular Biology,
University of Oklahoma School of Medicine,
Oklahoma City, Oklahoma 73104, USA*

Received 10 September 1973

1. Introduction

Immunochemical studies of the major density classes established clearly the antigenic heterogeneity of the human plasma lipoprotein system [1–3]. Recent chemical investigations have confirmed these earlier findings and demonstrated the presence of six antigenically distinct polypeptides designated as A-I, A-II, C-I, C-II, C-III and LP-B* [4–6]. An additional antigenic component detected predominantly in HDL [7–10] has been designated 'thin-line' polypeptide due to its characteristic appearance on double diffusion analyses of HDL with anti-HDL or anti-whole serum. Under such experimental conditions, the 'thin-line' polypeptide appears as a characteristic thin precipitin line nearer the antigen well, while the major precipitin band due to LP-A is closer to the antibody well [9, 11]. The occurrence of 'thin-line' polypeptide has also been demonstrated in LDL [11] and

VHDL [12]. On 7% polyacrylamide gel electrophoresis, the 'thin-line' polypeptide migrates into the region between the A-II and C-II polypeptides [10]. A preliminary estimate indicates that this polypeptide accounts for 1–2% of the total protein content of normal HDL [10].

This report describes the isolation and partial characterization of this antigenic determinant previously referred to as 'thin-line' polypeptide. On the basis of these studies we suggest that 'thin-line' polypeptide be designated as apolipoprotein D and its corresponding lipoprotein as lipoprotein family D (LP-D).

2. Materials and methods

2.1. Isolation of human plasma HDL₃

Plasma samples from both fasting and non-fasting healthy young men and women were obtained by plasmapheresis. Due to relatively low concentrations of 'thin-line' polypeptide, at least one liter of pooled plasma was used as starting material for each isolation. Plasma density was adjusted to 1.27 g/ml by adding solid KBr, and the solution was centrifuged in the Ti-60 rotor of the Spinco Model L 2-65B ultracentrifuge at 150 000 g for 22 hr at 5°C. The density (d) of the top layer collected by tube-slicing was adjusted to 1.12 g/ml, and the solution was centrifuged under the same conditions. After discarding lipoprotein floating at d 1.12 g/ml, the infranate was adjusted to d 1.27 g/ml and washed twice by centrifugation at 150 000 g for 44 hr. After washing, the top

* *Abbreviations:* LDL, low density lipoproteins, lipoproteins of d 1.006–1.073 g/ml; HDL, high density lipoproteins, lipoproteins of d 1.073–1.210 g/ml; VHDL, very high density lipoproteins, lipoproteins of d > 1.21 g/ml; α -LP, α -lipoproteins, lipoproteins with an electrophoretic mobility of α -globulins; LP-A, lipoprotein family A, lipoproteins characterized by the presence of apolipoprotein A (ApoA); A-I and A-II, constitutive polypeptides of LP-A; LP-B, lipoprotein family B, lipoproteins characterized by the presence of apolipoprotein B (ApoB); LP-C, lipoprotein family C, lipoproteins characterized by the presence of apolipoprotein C (ApoC); and C-I, C-II, and C-III are constitutive polypeptides of LP-C. The ABC-nomenclature used in this paper has been described previously [19].

layer (d 1.12–1.27 g/ml, HDL₃) was dialyzed exhaustively against distilled water and lyophilized.

2.2. Isolation of 'thin-line' polypeptide

Delipidization of lyophilized HDL₃ was carried out by five successive extractions with chloroform–methanol (2:1, v/v) followed by two washes with diethyl ether. Delipidized HDL₃ (apoHDL₃) was dissolved in 10 ml of 8 M urea in 0.001 M K₂HPO₄, pH 8.0. The apoHDL₃ solution was diluted to 2 M with respect to urea by addition of 0.001 M K₂HPO₄ (pH 8.0) and applied to a hydroxylapatite–cellulose column. The column (30 × 2.2 cm) was packed to a height of 15 cm with a mixture of two volumes of settled hydroxylapatite (Bio-Rad Lab., Richmond, Calif.) and one volume of settled microcrystalline cellulose (Baker, Phillipsburg, N.J.). Prior to sample application, the column was washed with 1 M K₂HPO₄, pH 8.0, and equilibrated with 0.001 M K₂HPO₄, pH 8.0. After application of the sample, the column was eluted with 40–50 ml of 0.001 M K₂HPO₄ buffer, pH 8.0. The eluted fraction was rechromatographed under identical conditions. After the second chromatography, the eluted fraction was examined by basic polyacrylamide gel electrophoresis (PAGE). If more than one band was detected, rechromatography under identical conditions was repeated until only one band could be detected on PAGE. To remove urea, the purified fraction was lyophilized and then chromatographed on a Sephadex G-100 column (90 × 2.5 cm) which had been equilibrated with 2 M acetic acid. Fractions of 5.0 ml were collected at a flow rate of 25 ml/hr, and the protein content of the eluant was monitored by absorbance at 280 nm. Fractions corresponding to the principal protein peak were combined and lyophilized.

2.3. Immunochemical methods

Double-diffusion and immunoelectrophoresis in 1% agarose gels were carried out according to previously described procedures [9]. Immunoprecipitin lines were stained with Amido black 10B and Oil red O. The preparation and characterization of monospecific antisera to human A-I, A-II, C-I, C-II, and C-III polypeptides and LP-B have already been described [9]. Rabbit antisera to human α -LP and albumin were purchased from Behringwerke A.G., Marburg an der Lahn, GFR. The commercial antiserum to α -LP gave two precipitin lines with HDL₃ when tested by double dif-

fusion analysis. One line corresponded to the 'thin-line' polypeptide and the other to LP-A [6,9,11].

The immunization of a rabbit with 'thin-line' polypeptide was carried out with an isolated preparation further purified by preparative 7% polyacrylamide gel electrophoresis on a Buchler Fractophorator (Buchler Instr., Fort Lee, N.J.). Preparative PAGE was carried out on a larger scale according to the analytical procedure described by Davis [13]. Eight molar urea was used in preparing the separating gel (4 ml) and 4 M urea in preparing the stacking gel (2 ml). Approximately 8–10 mg of sample, dissolved in the stacking gel solution, was applied to the polyacrylamide gel. Tris–glycine, pH 8.3, was used as the electrode and elution buffer. Fractions were collected at 2.5 min intervals utilizing a constant current of 10 mA. The elution pattern was monitored by absorbance at 280 nm and by analytical PAGE. Fractions displaying a single band on PAGE were combined and lyophilized. A rabbit was injected intraperitoneally with 2.5 mg of this purified 'thin-line' polypeptide homogenized in 1 ml of Freund's complete adjuvant. After one week, the animal was injected with an identical antigenic preparation. Antiserum was drawn by cardiac puncture three weeks following the initial injection.

2.4. Analytical methods

Basic 7% polyacrylamide gel electrophoresis has been described elsewhere [9]. Amino acid analyses were performed on a Beckman Model 120C amino acid analyzer by an accelerated automatic procedure on spherical resins [11]. Half-cystine was determined by the performic acid oxidation procedure. The values for cysteic acid were corrected for the 94% recovery found by Moore [14]. Tryptophan was determined by the *p*-toluenesulfonic acid hydrolysis procedure [15]. Glucosamine content was estimated from the amino acid analyses [11].

3. Results

Two successive fractionations on a hydroxylapatite–cellulose column were usually sufficient to yield a 'thin-line' polypeptide preparation which was characterized by a single band on 7% PAGE (fig. 1). However, in some cases a minor component was present near the junction of the separating and stacking gels.

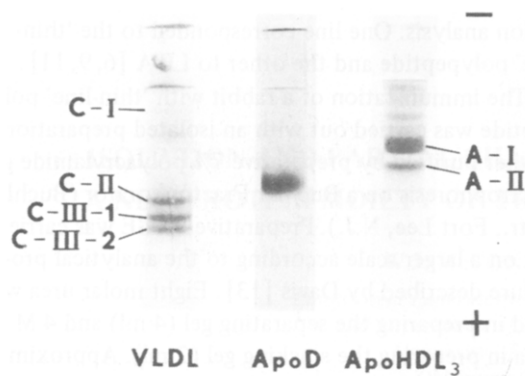


Fig. 1. Basic polyacrylamide gel electrophoresis patterns of VLDL, ApoD ('thin-line' polypeptide) and ApoHDL₃.

To remove this minor component and urea, gel filtration on Sephadex G-100 was performed and 'thin-line' polypeptide was eluted as a major symmetrical peak (fig. 2).

After lyophilization, this major fraction reacted only with antiserum to 'thin-line' polypeptide (fig. 3). On double diffusion analyses, it gave a negative reaction with antibodies to LP-A, LP-B, A-I, A-II, C-I, C-II, C-III, and albumin (fig. 3). On basic 7% PAGE, the 'thin-line' polypeptide exhibited a single band with mobility between the A-II and C-II polypeptide bands (fig. 1).

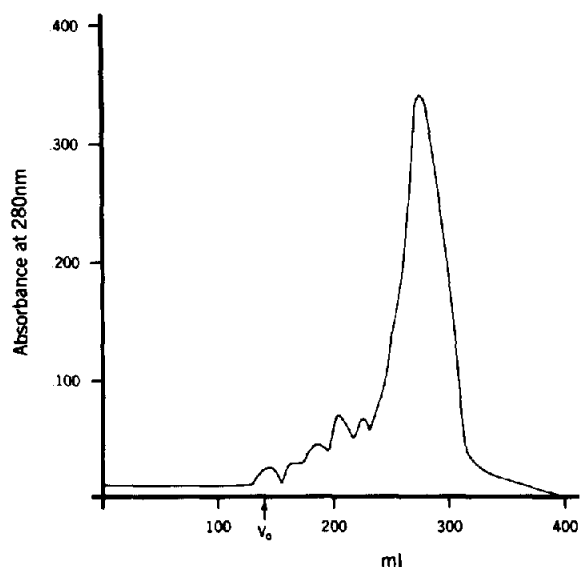


Fig. 2. Elution pattern of ApoD on Sephadex G-100. Elution carried out with 2 M acetic acid. Arrow indicates void volume.

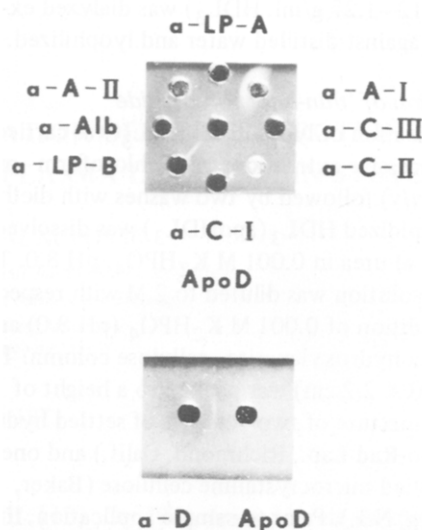


Fig. 3. Double diffusion analysis in 1% agarose gel of principle fraction eluted from the Sephadex G-100. Center well contains ApoD. a = anti.

Table 1
Amino acid composition of apolipoprotein D.

Amino acids	ApoD ^a	A-I ^c	A-II ^d
	moles/100 000 g		
Aspartic	83.5	64.0	33.0
Threonine	44.8	35.8	61.0
Serine	35.3	46.0	50.7
Glutamic	99.4	130.4	155.2
Proline	66.9	34.0	40.1
Glycine	33.8	41.0	30.6
Alanine	50.7	64.9	50.9
Valine	50.2	45.8	60.1
Half-cystine	9.7 ^b	0	10.0
Methionine	6.3	7.7	8.9
Isoleucine	37.2	1.8	9.8
Leucine	56.2	135.0	90.2
Tyrosine	25.9	21.0	40.7
Phenylalanine	29.1	21.3	40.7
Lysine	58.8	65.2	94.3
Histidine	10.7	18.9	0
Arginine	17.6	56.0	0
Tryptophan	3.2	24.0	0
Glucosamine	29.2		

^a Average of three 24 hr hydrolyses.

^b Determined by performic acid oxidation.

^c Data from Edelstein et al. [16].

^d Data from Scanu et al. [17].

Amino acid analysis of 'thin-line' polypeptide indicated the presence of all the common amino acids, including half-cystine and tryptophan, and glucosamine (table 1). The amino acid composition differed from those of the well characterized polypeptides of the human plasma lipoprotein system [4, 5].

Antiserum to the 'thin-line' polypeptide reacted with HDL₃ and the purified polypeptide (fig. 4). Fusion of these two precipitin lines indicated clearly the identity of the isolated and delipidized 'thin-line' polypeptide and its corresponding intact form as it exists in HDL₃. Furthermore, the precipitin line of purified 'thin-line' polypeptide fused with one of the two precipitin lines of HDL₃ when tested with antibodies to α -LP (fig. 4). As mentioned earlier, the second precipitin line of HDL₃, closer to the antibody well, is that of LP-A. The antiserum to the 'thin-line' polypeptide gave no reaction with either A-I, A-II, C-I, C-II, C-III polypeptides, LP-B or albumin.

The precipitin line of 'thin-line' polypeptide in HDL₃ stained for both lipid and protein.

4. Discussion

We have concluded on the basis of immunological properties, electrophoretic mobility in 7% polyacrylamide gel and characteristic amino acid and glucosamine composition that 'thin-line' polypeptide is a newly isolated, distinct polypeptide of the human plasma lipoprotein system. It does not react with monospecific antibodies to A-I, A-II, C-I, C-II and C-III polypeptides, LP-B or albumin (fig. 3). Immunization of a rabbit with this polypeptide induced the production of an antiserum which reacted positively only with its corresponding antigen. The mobility of 'thin-line' polypeptide on 7% PAGE was intermediate between those of A-II and C-II polypeptide bands (fig. 1) and did not correspond to any of the previously characterized lipoprotein polypeptides including the so-called arginine-rich polypeptide [18].

Double diffusion analyses (fig. 4) demonstrated that the isolated polypeptide was identical with the thin immunoprecipitin lines of HDL or HDL₃. The affinity of the thin immunoprecipitin lines of HDL or HDL₃ for both lipid and protein stains indicated its lipoprotein character. The appearance of separate precipitin lines of intact 'thin-line' lipoprotein and LP-A

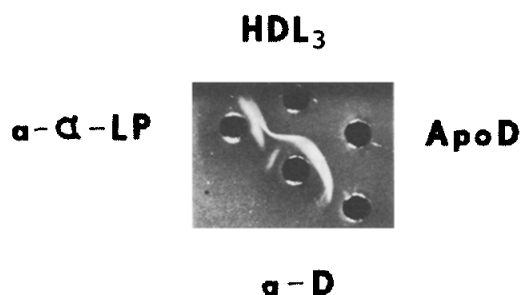


Fig. 4. Double diffusion analyses in 1% agarose gel of antiserum to ApoD (a-D) and reactions of identity of antigens and antibodies to ApoD. a = anti.

(fig. 4) and the already demonstrated non-identity of LP-A and 'thin-line' polypeptide [11] represent evidence that 'thin-line' polypeptide is not a constituent of LP-A molecules.

These results suggest that the 'thin-line' polypeptide is the apolipoprotein of a distinct lipoprotein family present mainly, although not exclusively, in HDL₃. According to the ABC-nomenclature [19], this protein moiety is designated as apolipoprotein D (ApoD) and its corresponding lipoprotein(s) as lipoprotein family D (LP-D).

Acknowledgements

We thank Mrs. Carolyn Linhardt and Mr. H.J. Cho for their skillful technical assistance and Mrs. M. Farmer and Mr. R. Burns for their help in the preparation of the manuscript.

This study was supported in part by Grants HL-6221 and HL-7005 from the U.S. Public Health Service, and by the resources of the Oklahoma Medical Research Foundation.

References

- [1] Aladjem, F., Lieberman, M. and Gofman, J.W. (1957) *J. Exptl. Med.* 105, 49.
- [2] Scanu, A. (1966) *J. Lipid Res.* 7, 295.
- [3] Alaupovic, P. (1968) *Prog. Biochem. Pharmacol.* 4, 91.
- [4] Shore, V. and Shore, B. (1968) *Biochemistry* 7, 3396.
- [5] Brown, W.V., Levy, R.I. and Fredrickson, D.S. (1969) *J. Biol. Chem.* 244, 5687.
- [6] Alaupovic, P., Kostner, G., Lee, D.M., McConathy, W.J. and Magnani, H.N. (1972) *Exposés Ann. Biochim. Med.* 31, 145.

- [7] Ayrault-Jarrier, M., Levy, G. and Polonovski, J. (1963) *Bull. Soc. Chim. Biol.* 45, 703.
- [8] Kook, A.I., Eckhaus, A.S. and Rubinstein, D. (1970) *Can. J. Biochem.* 48, 712.
- [9] Alaupovic, P., Lee, D.M. and McConathy, W.J. (1972) *Biochim. Biophys. Acta* 260, 689.
- [10] Kostner, G. and Alaupovic, P. (1972) in: *Proceedings of the XIX Annual Colloquium on the Protides of Biological Fluids* (Peeters, H., ed.), p. 59, Pergamon Press, Oxford and New York.
- [11] Lee, D.M. and Alaupovic, P. (1970) *Biochemistry* 9, 2244.
- [12] Alaupovic, P., Sanbar, S.S., Furman, R.H., Sullivan, M.L. and Walraven, S.L. (1966) *Biochemistry* 5, 4044.
- [13] Davis, B.J. (1964) *Ann. N.Y. Acad. Sci.* 121, 404.
- [14] Moore, S. (1963) *J. Biol. Chem.* 238, 235.
- [15] Liu, T.-Y. and Chang, Y.H. (1971) *J. Biol. Chem.* 246, 2842.
- [16] Edelstein, C., Lim, C.T. and Scanu, A.M. (1972) *J. Biol. Chem.* 247, 5842.
- [17] Scanu, A.M., Lim, C.T. and Edelstein, C. (1972) *J. Biol. Chem.* 247, 5850.
- [18] Havel, R.J. and Kane, J.P. (1973) *Proc. Natl. Acad. Sci. U.S.* 70, 2015.
- [19] Alaupovic, P. (1972) in: *Proceedings of the XIX Annual Colloquium on the Protides of Biological Fluids* (Peeters, H., ed.), p. 9, Pergamon Press, Oxford and New York.