Comparative In Vitro Study of the Pro-Apolipoprotein A-I to Apolipoprotein A-I Converting Activity Between Normal and Tangier Plasma

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Abstract. We examined the ability of the plasma of a 52-yr-old male Tangier patient to effect the conversion of radiolabeled pro-apolipoprotein A-I (apo A-I), isolated from hepatoma cell culture media, into mature apo A-I. The conversion was assessed by amino-terminal sequence analysis, isoform patterns with two-dimensional gel electrophoresis, and a rapid assay based on the different solubilities of intact pro-apo A-I and its hexapeptide prosegment in 10% trichloroacetic acid. We found that the converting activity of Tangier plasma was comparable to that exhibited by control normolipidemic plasma and that in both cases pro-apo A-I was correctly processed at the Gln-Asp bond. After ultracentrifugal fractionation of Tangier plasma at $d = 1.21$ g/ml, the pro-apo A-I-to-mature apo A-I converting activity was mainly recovered in the middle fraction of $d = 1.225$ g/ml and was at least 10-fold more effective than the top and bottom fractions. In contrast, in normal plasma the activity was only present in the top and bottom fractions.

It has been previously established that in Tangier plasma the pro-apo A-I/apo A-I ratio is significantly

higher than normal (1 vs. 0.02). Our studies suggest that this abnormal ratio is not the result of a reduced converting enzyme activity and may relate to differences in turnover rates between Tangier and normal plasma apolipoproteins.

Introduction

Tangier disease is a rare autosomal-recessive disorder of lipid metabolism characterized by low or nonexistent plasma high density lipoprotein ($d = 1.063$ – 1.21 g/ml; HDL), low plasma cholesterol, and normal or elevated triglyceride levels. Plasma apolipoprotein A-I (apo A-I)¹ is only ~1% of normal (1). Apparent normal rates of apo A-I synthesis and accelerated catabolism have been reported (2, 3). Work in our laboratories (4) and others (5) has shown that apo A-I is secreted with a hexapeptide amino-terminal extension (pro-apo A-I) and demonstrated that there exists in normal plasma an enzyme that effects the conversion of pro-apo A-I to mature apo A-I (6). Because the ratio of pro-apo A-I to apo A-I is significantly higher in Tangier than in normal plasma (~1 vs. 0.02, reference 7), we wanted to establish whether the abnormal ratio could be the consequence of a defective enzyme activity. For this purpose, we have studied the plasma pro-apo A-I processing enzyme of a newly discovered Tangier subject and compared the results with those obtained in normal plasma.

Methods

Description of Tangier case

A 52-yr-old man (O.P.) was referred to the Ospedale Maggiore, University of Milan, because of a syringomyelia-like syndrome. His

1. Abbreviations used in this paper: Apo A-I, apolipoprotein A-I; PMSF, phenylmethylsulfonylfluoride.

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father died at age 64 of cerebral hemorrhage and his mother at age 68 of myocardial infarction. The patient's 13-yr-old daughter had normal plasma lipid levels and was in good health. The patient had no lymphadenopathy and no hepato- or splenomegaly. The tonsils had a yellow-gray coloration. A radiograph of the chest and a plain radiograph of the abdomen were both normal. Urinalysis, serum protein electrophoresis, glucose, uric acid, hemoglobin, white cell count, serum glutamic oxaloacetic and pyruvic transaminases, alkaline phosphatase, sedimentation rate, blood-urea nitrogen, and prothrombin time were normal. The platelet count was 190,000. Histological and ultrastructural examination of tissue specimens demonstrated the presence of a large number of foam cells in the tonsils and lipid storage in Schwann's cells in peripheral nerves.

The plasma cholesterol, triglycerides, HDL cholesterol, apo A-I and apolipoprotein B values were compatible with the diagnosis of Tangier disease (Table I). Agarose gel electrophoresis of plasma performed according to the procedure of Noble (11), showed a broad band with pre- β mobility and no α -migrating band, (Fig. 1). Normal and Tangier plasma were also analyzed by column chromatography. On the basis of cholesterol determination of the column eluates (Fig. 2), Tangier plasma exhibited a bimodal very low density lipoprotein ($d < 1.006$ g/ml; VLDL) peak, a small low density lipoprotein ($d = 1.012$ – 1.063 g/ml; LDL) peak, and no detectable HDL. Sequential ultracentrifugation of Tangier plasma resulted in the following cholesterol distribution: 46.5 mg/dl for VLDL + IDL ($d < 1.019$ g/ml), 47.8 mg/dl for LDL ($d = 1.012$ – 1.063 g/ml), 3.1 mg/dl for HDL ($d = 1.063$ – 1.21 g/ml), and 0.4 mg/dl for the bottom fraction of $d > 1.21$ g/ml. After ultracentrifugation of plasma at $d = 1.21$ g/ml, 3% of apo A-I was in the top 1-ml fraction and 97% was in the bottom. By isoelectric focusing, HDL (after delipidation [12]) exhibited apolipoprotein A-II (apo A-II) as its major band but no detectable apo A-I (Fig. 3).

Measurements of pro-apo A-I converting activity in Tangier and normal plasma

ISOLATION OF ^3H -PRO-APO A-I. HepG2 cells were pulse labeled with [^3H]proline or [^3H]phenylalanine for 2 h and pro-apo A-I was purified from the culture medium as previously described (4).

Table I. Lipid* and Apo A-I Distribution in Tangier and Normal Plasma

	Tangier	Normal†
	mg/dl	mg/dl
Total cholesterol	98	170±26
Triglycerides	355	87±35
HDL-Cholesterol	1–3	45±11
Apo AI	2	130±16
Apo B	108	100±10

* Plasma cholesterol, triglycerides, and HDL cholesterol were determined enzymatically (Boehringer and Sons, Mannheim, Federal Republic of Germany). HDL cholesterol was quantitated after precipitation of apo B-containing lipoproteins by the magnesium chloride/phosphotungstic acid method (8) and by ultracentrifugation (9). Apo A-I and apo B levels in the plasma were determined by the Laurell method (10) using rabbit anti-human apo A-I and anti-human apo B serum, respectively.

† Values are means±SD in 40 subjects.

Figure 1. Agarose gel electrophoresis of Tangier (T) and normal (N) plasma. After electrophoresis, the gels were stained with fat red 7B and dried. o, origin of sample application.

DETERMINATION OF PRO-APO A-I CONVERTING ENZYME ACTIVITY. For the assays, the blood was collected from fasting normolipidemic subjects and from the Tangier patient in lithium heparin-coated tubes. The plasma was recovered after centrifugation (13) and phenylmethylsulfonyl fluoride (PMSF) was added to bring the solution to 2 mM. Radiolabeled pro-apo A-I (30,000–50,000 dpm) was incubated from 1 to 16 h at 37°C with normal or Tangier plasma. The control incubations in a total volume of 50 μl contained radiolabeled pro-apo A-I with either 0.15 M NaCl, pH 7.0, or plasma preincubated

Figure 2. Column chromatography of normal (A) and Tangier plasma (B). A 2.6×95 cm column was packed with Biogel A-15m and was equilibrated with 0.15 M NaCl, 0.01 M Tris-HCl, 0.01% wt/vol EDTA, pH 7.4. The sample (3 ml) was eluted at 4°C with the same buffer at a flow rate of 20 ml/h. Cholesterol content was determined in each fraction and plotted as a function of fraction number. The void volume is indicated by VV.

Figure 3. Isoelectric focusing of Tangier (T) and normal (N) apolipoprotein. T-VLDL, ($d < 1.029$), LDL ($d = 1.012-1.063$), and HDL ($d = 1.063-1.21$ g/ml) were delipidated and the protein precipitate was dissolved in 6 M urea. The pH gradient was from 4 (bottom) to 6 (top). V, VLDL; L, LDL; H, HDL.

with 5 mM EDTA, an inhibitor of the converting enzyme (6). The conversion of [^3H]pro-apo A-I to mature [^3H]apo A-I was determined by the three following independent methods.

Apo A-I isoform analyses. Plasma and lipoprotein subfractions were analyzed by two-dimensional polyacrylamide gel electrophoresis according to the methods of O'Farrel (14) as modified by Lester et al. (15). Gels were stained and the ^3H -labeled proteins were subjected to fluorography (4).

NH_2 -terminal sequence analysis. Automated sequential Edman degradation of [^3H]proline-labeled-substrate contained in the reaction mixture was performed by using a 0.33 M Quadrol program and a Beckman 890 C sequenator (Beckman Instruments, Inc., Fullerton, CA) (4).

Trichloroacetic acid (TCA) assay. [^3H]phenylalanine-labeled pro-apo A-I obtained from the HepG2 cell medium was used as a substrate (4). Since the sequence of the hexapeptide prosegment (5) is Arg-His-Phe-Trp-Gln-Gln, radiolabeled phenylalanine is a convenient marker

for detection of the cleaved prosegment. For the assay, a mixture of plasma and [^3H]phenylalanine-pro-apo A-I incubated for 16 h at 37°C was mixed for 1 h at room temperature with an equal volume of 10% TCA. The solution was spun at 10,000 g and the supernatant as well as a 10% TCA wash of the pellet were pooled and counted in 10 ml of Budget Solve (Research Products International Corp., Mt. Prospect, IL). The percent conversion of pro-apo A-I to apo A-I was calculated from the ratio of the radioactivity in the supernatant to that in the initial incubation mixture. Since the hexapeptide contains one of the seven phenylalanine residues present in pro-apo A-I, the ratio was multiplied by seven on the assumption that the tritium label was uniformly distributed among all the phenylalanine residues.

Results

Converting enzyme activity of normal and Tangier plasma.

Converting activity in Tangier plasma was initially surveyed by isoform analyses of the radiolabeled normal pro-apo A-I substrate. The isoforms 2 and 3 of pro-apo A-I are more basic than the isoforms 4 and 5 of mature apo A-I (4, 5). Tangier plasma produced a slow, time-dependent conversion of basic to acidic isoforms (Fig. 4), indicating that some converting activity was present. Isoform conversion did not occur when plasma was omitted from the reaction mixture. To quantitate the extent and accuracy of polypeptide processing, [^3H]proline-labeled pro-apo A-I was incubated with unlabeled

Figure 4. Two-dimensional gel fluorographs of incubated mixtures of radiolabeled pro-apo A-I and Tangier plasma. Tangier plasma (40 μl , 3 mg plasma proteins) was incubated with 50,000 cpm [^3H]proline pro-apo A-I at 37°C for 1, 3, and 16 h in the presence of 2 mM PMSF. The reaction was stopped with lysis buffer (14) and subjected to electrophoresis. The gels were stained with Coomassie Blue and subsequently fluorographed. Pro-apo A-I refers to [^3H]proline pro-apo A-I that had been incubated in 0.15 M NaCl, pH 7.0, alone. A Coomassie Blue-stained gel of purified mature apo A-I is shown (16).

normal or Tangier plasma for 16 h and the reaction mixtures were subjected to automated sequential Edman degradation. Identical amounts of total plasma protein (4 mg) were included in the separate incubations. Pro-apo A-I contains proline residues at positions 9 and 10. Mature apo A-I has proline residues at positions 3, 4, and 7 (4, 5). On the basis of distribution of radioactive peaks obtained after NH₂-terminal sequencing (Fig. 5), we were able to determine that accurate pro-apo A-I processing at a Gln-Asp bond occurred with both normal and Tangier plasma. The extent of the processing after

Figure 5. NH₂-terminal sequence of [³H]proline-labeled pro-apo A-I after incubation with Tangier and normal plasma. The substrate was incubated at 37°C for 16 h with Tangier or normal plasma (4 mg plasma protein) in the presence and absence of 5 mM EDTA and the reaction mixture was subjected to NH₂-terminal sequence analyses. The previously reported NH₂-terminal sequence of purified apo A-I (17) and pro-apo A-I (5, 18) are given as references at the top of the figure. Incubations of Tangier and normal plasma contained 33,750 dpm of [³H]pro-apo A-I.

16 h was quite comparable: 41% with Tangier plasma, 49% with normal plasma. In both cases, cleavage of normal pro-apo A-I was blocked by 5 mM EDTA (Fig. 5). These assays were cumbersome and time consuming. A rapid, sensitive assay for propolypeptide processing was developed based on the different predicted solubilities of the hexapeptide prosegment and mature apo A-I molecule in 10% TCA. Although we did not know the molecular mechanism of prosegment removal, this predicted partitioning should occur whether or not the hexapeptide prosegment was cleaved uniquely after the Gln-Asp bond or was removed by other mechanisms. We included PMSF in the incubations to reduce nonspecific cleavage of [³H-Phe]pro-apo A-I by serine proteases present in plasma. The results are shown in Table II. Under identical experimental conditions using the TCA assay, we estimated that 12% of pro-apo A-I was converted to apo A-I per milligram of Tangier plasma protein after 16 h of incubation. This value is comparable to that exhibited by normal plasma (11.7%) and to that obtained by sequential Edman degradation i.e., 10% per mg of Tangier plasma. In addition, the level of converting activity was similar in Tangier and normal plasma when measured with different dilutions of plasma (Table II). Moreover, both normal and Tangier plasma were inhibited by a reagent specific for metal-dependent proteases, 1,10-phenanthroline and by the reducing agent dithiothreitol. EDTA (final concentration 5 mM) resulted in a dramatic reduction in the amount of TCA-soluble radioactivity released from the [³H]phenylalanine-labeled substrate. The degree of inhibition was comparable to that shown with [³H]proline pro-apo A-I by Edman degradation (Fig. 5).

To compare the compartmentalization of converting enzyme activity in plasma, Tangier and normolipidemic samples were adjusted to $d = 1.21$ g/ml with solid KBr and spun for

Table II. TCA Assay of Pro-Apo A-I Converting Activity in Tangier and Normal Plasma

	Percent conversion*	
	Tangier	Normal†
Plasma§	12.3	11.7±1.5
Plasma (diluted 1:1)§	10.7	10.0±1.2
Plasma (diluted 1:3)§	8.3	7.2±0.9
Plasma + EDTA (5 mM)	0.4	0.8±0.1
Plasma + DTT (1 mM)	~0	~0
Plasma + 1,10 Phenanthroline (10 mM)	0.6	0.8±0.1

* The percent conversion is expressed as the amount of radioactivity recovered in the TCA supernatants (see Methods) per milligram of plasma protein.

† Values are means±SD in eight normolipidemic subjects.

§ 45 µl of undiluted plasma or diluted plasma in 50 µl of reaction mixture.

24 h at 59,000 rpm in a 50.3 Beckman Instruments, Inc. rotor. Tubes were equally divided into top $d = 1.219$ g/ml, middle $d = 1.225$ g/ml and $d = 1.376$ g/ml bottom fractions. In normolipidemic plasma, the specific activity of the converting enzyme was equal in the top (LDL, VLDL, and HDL) and bottom fractions. The specific activity in the middle fraction was 20-fold less (data not shown). In Tangier plasma, the specific activity in the middle fraction was 10-fold higher than in the top or bottom fraction. The converting activity present in this middle fraction of Tangier plasma correctly processed [^3H]proline-labeled pro-apo A-I as determined by NH_2 -terminal sequence analyses (data not shown).

Discussion

We have here reported a case of a male adult subject with clinical presentation and biochemical and immunological findings typical of those described in Tangier disease (1–3, 19) and have shown that his plasma was able to convert pro-apo A-I to apo A-I as efficiently as normal plasma. Several lines of evidence point to the validity of this conclusion. In both cases amino terminal sequence analyses showed the cleavage to occur at the Gln-Asp bond, which is known to link the carboxyl-terminal residue of the hexapeptide prosegment to the amino-terminal residue of mature pro-apo A-I (4, 5, 18). Also, both specimens exhibited a comparable degree of conversion at different dilutions of plasma. Moreover, both activities appeared to be metal dependent and inhibited by reducing agents (Table II). However, there was an interesting difference between normal and Tangier plasma. In the normal plasma the highest specific activity of the converting enzyme was in the floating HDL at $d = 1.21$ g/ml (6). In contrast, the Tangier plasma exhibited its highest specific activity in the middle fraction. This phenomenon may be due to the low levels of normal HDL in Tangier plasma, or the presence of a heavier lipoprotein fraction in the subject under study.

From previous results, mainly based on isoform analyses, we (6) and others (7) suggested that a defective converting enzyme activity could account for the relative high ratio of pro-apo A-I to apo A-I in Tangier plasma. The data presented here on our Tangier patient invite consideration of alternative possibilities. It is noteworthy that plasma levels of pro-apo A-I are in the same range in normal and Tangier subjects (7). Moreover, no change in either the sequence of the prosegment or the amino acids flanking the posttranslational processing site has been reported (20, 21). On the other hand, Kay et al. (22) have shown amino acid composition differences between normal and Tangier mature apo A-I. It follows that structural differences between normal and Tangier apo A-I may exist in the mature apolipoprotein. A structural change in the mature apo A-I domain could have several effects. By inducing a conformational change in pro-apo A-I, the cleavage site might be masked or, because of reduced binding to lipoproteins (21), the propolypeptide might not be efficiently cleaved by a

lipoprotein-affiliated converting enzyme. A structural change in the mature domain could affect the catabolism of apo A-I. The normal absolute steady state pro-apo A-I levels and the reduced apo A-I concentration found in Tangier patients may simply be due to accelerated catabolism of apo A-I. This notion is supported by the in vivo studies of Schaefer et al. (2, 3). It is also possible that Tangier disease may reflect several different lesions.

With the availability of the rapid assay described here, it should be possible to quantitate pro-apo A-I converting enzyme activity in a variety of lipoprotein disorders and thereby gain insights about the function of this protease and its substrate in normal lipoprotein metabolism.

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