Tangier disease: Defective recombination of a specific Tangier apolipoprotein A-I isoform (pro-apo A-I) with high density lipoproteins
(protein-lipid interactions/cholesterol metabolism/lipid storage disease/hypocholesterolemia/cholesterol esters)

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ABSTRACT Isomers of apolipoprotein A-I (apo A-I) from subjects with Tangier disease were characterized, and their ability to recombine with normal high density lipoproteins (HDL) was studied. In contrast to normal serum, in which isoprotein 4 is the dominant species [79 ± 1.8% (mean ± SD)], the Tangier serum contained much less total apo A-I (∼1% of that in normal serum), and isoproteins 2 and 4 were present in roughly equivalent amounts (35.3 ± 2.5% and 42.7 ± 3.6%, respectively). The Tangier isoprotein 2 was shown to correspond to apo A-I, having a six-amino acid amino-terminal extension with the sequence: Arg-His-Trp-Gln-Gln-Gln. The Tangier isoprotein 4 had the same amino-terminal sequence as normal circulating plasma apo A-I. Its association with normal HDL (70%) was similar to the association of normal apo A-I with HDL (50–90%) in recombination experiments. In marked contrast to this behavior, very little (<1%) of Tangier isoprotein 2 (pro-apo A-I) associated with HDL in recombination experiments. These results suggest that the underlying defect in Tangier disease may be a faulty conversion of pro-apo A-I to mature apo A-I, either due to a defect in the converting enzyme activity or to a further specific structural defect in Tangier apo A-I. The failure of Tangier pro-apo A-I to associate with HDL may be at least partially responsible for the HDL deficiency in Tangier subjects.

Tangier disease is a rare, autosomal recessive disorder characterized by the absence of high density lipoproteins (HDL) in the plasma and an increase in the amount of cholesteryl esters stored in several organs. Patients are characterized clinically by tissular hypertrophy, splenomegaly, and transient peripheral neuropathy (1–4). Cholesteryl ester storage is limited to macrophages, Schwann cells, and intestinal smooth muscle cells (4, 5). Tangier homozgyotes have hypercholesterolemia, moderate hypertriglyceridemia, and no detectable α band (HDL) on agarose electrophoresis (1, 4). In obligate Tangier heterozygotes, HDL levels are ∼50% of normal (6).

The major apoproteins of normal HDL are apolipoprotein A-I (apo A-I) and apolipoprotein A-II (apo A-II) (7). In Tangier subjects, serum concentrations of these apoproteins are <1% and 5–10% of normal, respectively (8, 9). In spite of the diminished concentration of apo A-I in serum, small intestinal epithelial cells from Tangier patients contain normal amounts of apo A-I, as demonstrated by immunofluorescence (10, 11) and intestinal organ cultures (12, 13). Tangier HDL, isolated by a combination of ultracentrifugation and column chromatography (14), have been shown to contain apo A-II as their sole protein constituent. The biochemical origin of the HDL deficiency in Tangier disease is not yet understood. Metabolic studies have indicated that the decreased concentration of apo A-I in Tangier patients is due to the enhanced catabolism of this apoprotein (9, 15, 16).

It has been demonstrated recently that Tangier apo A-I, similar to normal apo A-I, occurs as several electrophoretically separable isomers (13). The isoprotein pattern of newly synthesized Tangier apo A-I obtained from cultured cells was indistinguishable from the newly synthesized apo A-I isoprotein pattern of normal individuals. However, the relative distribution of the Tangier apo A-I isomers in plasma was completely different (13). The basis for this discrepancy in the plasma distribution of apo A-I isoforms between Tangier patients and normal individuals is not clear at present.

The primary translation product of either rat (17) or human (18–20) apo A-I is a pre-pro-apoprotein: the 24-amino acid amino-terminal extension consists of an 18-amino acid presegment and a hexapeptide prosegment, followed by the "mature" plasma apoprotein. The hexapeptide prosegment of human apo A-I has been shown to have the sequence: Arg-His-Phe-Trp-Gln-Gln (19, 20). In the present study, it will be demonstrated that the most alkaline isoform of apo A-I from Tangier subjects represents pro-apo A-I and that this isoform associates poorly with HDL.

MATERIAL AND METHODS

Patients. Plasma from the following patients was utilized in the studies: (patient I) a 52-year-old homozygous Tangier patient (female) with typical symptoms, as described elsewhere (4, 8, 14, 15) (triglycerides, 2.94–4.89 mmol/liter; cholesterol, 2.02–2.67 mmol/liter); (patient II) a 49-year-old homozygous Tangier patient (male), who is the brother of patient I (4, 8, 14, 15) (triglycerides, 1.58–2.24 mmol/liter; cholesterol, 1.16–1.50 mmol/liter); (patient III) a 33-year-old homozygous Tangier patient (male), who is unrelated to patients I and II, born in Pakistan (not previously described) (triglycerides, 3.42–3.88 mmol/liter; cholesterol, 1.50–2.02 mmol/liter).

apo A-I Purification. To prepare apo A-I, either 50 μl of normal serum or 5,000 μl of Tangier serum was mixed with 700 μl of a commercially available apo A-I antibody (Boehringer-Mannheim, batch no. 3540) (21) and incubated for 24 hr at room temperature. After incubation, the mixtures were centrifuged for 10 min at 5,000 × g in conical glass tubes. The precipitates were washed twice with 1 ml of saline by vortexing and subsequent centrifugation. The immunochemical identity of the apo A-I preparation was confirmed by immuno-diffusion (22).

Abbreviations: apo A-I and apo A-II, apolipoprotein (or apoprotein) A-I and A-II, respectively; HDL, high density lipoprotein(s).

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apo A-I from the immunoprecipitates was performed as follows: 100 μl of distilled water was added to the immunoprecipitate. Aliquots (25 μl) were dissolved in 70 μl of sample buffer containing 2% amylomphates (LKBI, pH 3–7), 1% decylisulate (Eastman Kodak), and 5 μl of 2-mercaptoethanol in freshly deionized 6 M urea. The samples were incubated in the electrophoresis buffer for 2–3 hr before application to 2-mm vertical isoelectric focusing polyacrylamide gels (pH 3–7). Isoelectric focusing was performed in a Bio-Rad model 220 flatbed electrophoresis cell. Polyacrylamide gel preparation and selection of buffers were done according to described methods (23). After isoelectric focusing, one of the sample tracks was sliced and stained with Coomassie blue to detect the apoprotein bands. The polymeric forms of apo A-I were then sliced from the gels by using the stained gel pattern for reference, washed in 0.05 M Tris-HCl buffer (pH 8.6) containing 6 M urea, and eluted from the gel by pressing through Seraclear tubes (Technicon). The extracted apoproteins were dialyzed against 0.001 M ammonium bicarbonate containing 0.01% NaCl (pH 8.6). Proteins were measured by the method of Lowry et al. (24) prior to labeling efficiency of 30–35%. After incubation, the unreacted ester was inactivated by the addition of 500 μl of labeled ester reacted per mol of apoprotein with a range was 0.9 mol of labeled ester reacted per mol of apoprotein with a 0.1 M Tris-HCl/0.15 M NaCl/0.01% EDTA, pH 7.4, and then were dialyzed twice with ethanol/diethyl ether, 3:1 (vol/vol), at −20°C and once with pure diethyl ether (26). The apoprotein mixtures were separated by preparative isoelectric focusing (pH 4.6) on Ultrodex gels (LKB) (27). apo A-II was eluted from the granulated gel with 6 M urea, refocused (pH 4.5–5.5), and reeled. Similar procedures, as described for apo A-I, were used for the subsequent isolation of apo A-II.

Radioiodination of apo A-I and apo A-II. Borate buffer (10 μl, 0.1 M at pH 8.5) was added to 10–20 μg of the purified, lyophilized apoproteins. The apoprotein solution was then added to a solution of Bolton–Hunter reagent reconstituted in 0.1 M Tris-Cl, 0.15 M NaCl, 0.01% EDTA (pH 7.4), to remove unreacted ester. The radioiodinated apo A-I and apo A-II preparations were monitored for purity (after addition of unlabeled apoprotein standards) on NaDodSO₄/polyacrylamide gels (28). Only preparations with >85% of total activity in the apo A-I or apo A-II bands were used for the recombination experiments.

Recombination Studies. A total radioactivity of 270–350 μCi of the labeled apoproteins (specific activity, 50–70 μCi/μg of protein) was added to either 1.8 ml of normal serum or 500 μl of ultracentrifugally isolated HDLs (2 mg of protein per ml in 0.1 M Tris-HCl/0.15 M NaCl/0.01% EDTA, pH 7.4) derived from normal or diabetic donors. These HDLs had a typical apoprotein profile, with a ratio of A-I to A-II of about 4:1. After a 30-min incubation at 4°C, the samples were then subjected to zonal ultracentrifugation. The recombination experiments with normal serum and isolated HDL fractions were repeated four times (with similar results), each time with freshly isolated apoprotein preparations from different Tangier patients.

Zonal Ultracentrifugation. Zonal ultracentrifugation was performed with a Beckman L8-70 ultracentrifuge and a Beckman Z-60 rotor at 250,000 × g for 18 hr at 4°C. A nonlinear three-step NaBr gradient was used for the separation of lipoproteins (29). With this procedure, lipoproteins of ρ < 1.063 g/ml emerged in the first 30–40 ml of the rotor effluent, while the HDL range was maximally expanded. The samples were adjusted to ρ = 1.4 g/ml with NaBr, and either 1.5–2.0 ml of serum or 500 μl of HDL was injected into the rotor, followed by 20 ml of ρ = 1.4 g/ml NaBr solution. After centrifugation, the rotor was unloaded by displacing the contents with ρ = 1.4 g/ml NaBr solution at a flow rate of 10 ml/min. The effluent was continuously monitored by absorbance at 280 nm and collected in 5-ml fractions. The density was calculated as described (29). The fractions were pooled as indicated in the figures and analyzed for total radioactivity. Radioactivity specifically contained within apo A-I and apo A-II was monitored by immunoprecipitation or polyacrylamide gel electrophoresis.

Cyanogen Bromide Cleavage of apo A-I Isoforms. The CNBr cleavage was performed by solubilizing the samples in 70% formic acid and incubating them with a 400-fold molar excess of CNBr (Sigma) for 6–10 hr at 30°C (30).

Apoprotein Sequence Analysis. Sequence analyses were performed on a Beckman 890C Sequencer equipped with a cold-trap accessory. The lyophilized samples were dissolved in 0.5 ml of 50% acetic acid and applied along with 2 mg of Polybrene (Sigma). A standard 0.1 M Quadrupole program (no. 129974) was used. After conversion in 1 M HCl at 80°C for 10 min and extraction with ethyl acetate, phenylthiohydantoin amino acids were identified and quantified on a Beckman model 332 liquid chromatography system, which was equipped with a CR1A integrator recorder. The mobile phase, chromatography parameters, and criteria for identification have been described (31). Other Techniques. Lipid analyses were performed on a Technicon SMAC autoanalyzer by enzymatic methods. Triglycerides were corrected for free glycerol. The HDL cholesterol was measured enzymatically from the supernatant after phosphotungstic acid/MgCl₂ precipitation (21). The relative mass distribution of the apo A-I polymorphic forms was determined with a Shimadzu CS 910 densitometer at a wavelength of 590 nm. Crossed immunoelectrophoresis and two-dimensional electrophoresis of apo A-I and apo A-II were performed after separation in the first dimension by isoelectric focusing as described elsewhere (8, 25).

RESULTS

To characterize apo A-I from normal subjects and from subjects with Tangier disease, isoforms were purified by preparative isoelectric focusing from apo A-I immunoprecipitated from serum. As shown in Fig. 1, four major bands (bands 2–5) were visualized, all of which exhibited lines of identity by crossed immunoelectrophoresis with monospecific A-I antibodies (data not shown). In agreement with previous results (12, 13), the distribution of the polymorphic forms of the apo A-I from normal subjects differed from that of the apo A-I from Tangier patients. In normal subjects, it was found that isoform band 4 was the major form [79.0 ± 1.8% (mean ± SD)], whereas the other forms were minor components (band 2, 3.7 ± 1.5%; band 3, 0.9 ± 0.5%; and band 5, 16.4 ± 5%). By contrast, in the A-I immunoprecipitates derived from Tangier subjects, band 2 (35.3 ± 2.5%) and band 4 (42.7 ± 3.6%) accounted for almost equal quantities of the isoforms, whereas band 3 (8.7 ± 1.8%) and band 5 (13.4 ± 0.5%) were minor components.
Asp-Glu-Pro-Pro-Gln-

of this analysis as compared to difference observed from humans A-I in pro-apo Tangier propeptide previously reported the terminal isoprotein cusing in of the 2 isoprotein alkalin extension-i.

Electrophoretic apo Tangier of the results indicated apo A-I (Fig. 1). The control and three homozygous Tangier patients (I, II, and III) (pH range, 3.5–7.0). The four polymorphic forms (isoproteins 2–5) of apo A-I are indicated.

To characterize the origin of the charge differences between Tangier apo A-I isoproteins 2 and 4, CNBr cleavage was performed. Electrophoretic analysis suggested that CNBr fragments of isoprotein 2 containing the amino-terminal sequence demonstrated altered mobility on isoelectric focusing gels, focusing in a more alkaline position compared to the same peptides of isoprotein 4 (not shown). Because of this observation, isoprotein 2 was examined further. Approximately 50 μg (1.7 nmol) of isolated isoprotein 2 (patient I) was subjected to aminoterminal sequence analysis (Table 1). The results indicated that isoprotein 2 has a six-amino acid amino-terminal extension—i.e., the propeptide previously reported for rat apo A-I (17) and human apo A-I (18–20). The sequence of the propeptide of the Tangier pro-apo A-I was identical to that reported for pro-apo A-I from humans (19, 20) but differed in at least one position (residue 2) from that of rat pro-apo A-I. The two positive charges in the propeptide (due to the occurrence of arginine and histidine at residues 1 and 2) were consistent with the two-charge difference observed on isoelectric focusing for isoprotein 2 as compared to isoprotein 4 (19). Amino-terminal sequence analysis of ≈50 μg of Tangier isoprotein 4 showed the same sequence as that determined for mature plasma apo A-I—i.e., Asp-Glu-Pro-Pro-Gln-. Because the electrophoretic behavior of this isoprotein was shown to be identical to the major apo A-I isoprotein from normal plasma (Fig. 1), this was not an unexpected finding.

The abilities of normal apo A-I and Tangier apo A-I or isolated Tangier isoproteins 2 and 4 to recombine with whole serum or HDL were compared. The apoproteins were iodinated and their ability to recombine was analyzed by zonal ultracentrifugation. As shown in Fig. 2 Upper Left and Upper Right, normal apo A-I readily recombined with the HDL. As calculated from the distribution of the radiolabeled apo A-I (and also monitored by gel electrophoresis and slicing the gels), 98–99% of the normal apo A-I was found in the HDL subfractions. The remainder appeared in the ρ < 1.063 g/ml fraction (4–8%) and the ρ > 1.21 g/ml infranant fraction (6–11% of the radiolabeled apo A-I). By contrast, <30% of Tangier apo A-I (using the mixture of isoproteins 2–5) was found associated with the normal HDL subfractions after centrifugation, and the major part (>60%) of the radioactively labeled Tangier apo A-I appeared in the ρ > 1.21 g/ml infranant (Fig. 2 Lower Left and Lower Right). Tangier apo A-I and normal apo A-I associated with ρ < 1.063 g/ml lipoproteins to a similar extent (3–9%).

Because total Tangier apo A-I associated with HDL less efficiently than normal apo A-I and because it was known that the Tangier apo A-I included pro-apo A-I as a major component, it became necessary to examine the reconstitution behavior of the individual Tangier apo A-I isoforms. The recombination of the isolated Tangier isoproteins 2 and 4 with HDL revealed a striking difference in the behavior of these two isoforms. As shown in Fig. 3, <10% of the Tangier apo A-I isoprotein 2 (the pro-apo A-I) was found associated with the HDL subfractions, whereas as much as 70% of the Tangier isoprotein 4 (the mature apo A-I) recombined with the HDL.

In addition to the recombination studies carried out with the apo A-I, studies using the apo A-II from normal subjects and a Tangier subject (patient I) were performed by using the same protocol as that used in the apo A-I studies. Very similar results were obtained with either apo A-II from normal subjects or apo A-II from the Tangier subject (data not shown). Approximately 50–60% of the apo A-II was incorporated into the HDL in either case.

**DISCUSSION**

Our studies have demonstrated differences in the reconstitution behavior of Tangier apo A-I isoproteins 2 and 4 (Fig. 3). Isoform 2, corresponding to pro-apo A-I, failed to associate readily in vitro with normal HDL, whereas the reconstitution properties of Tangier apo A-I isoform 4 proved to be almost indis-
A-I in Tangier A-I, of this Tangier A-I to terminal pro-apo A-I and the additional defect affects only the reconstitution properties of Tangier pro-apo A-I (isoprotein 4) and not mature Tangier apo A-I (isoprotein 4).

The failure of Tangier pro-apo A-I to associate with plasma HDL in vitro has profound implications. It is likely that HDL particles in vivo cannot form or remain stable without the presence of apo A-I in the lipid complex. If the association of apo A-I with HDL is dependent on the conversion of pro-apo A-I to mature apo A-I, then the inability to make this conversion may result in either a lack of HDL particle formation or the destruction of unstable HDL particles and could account for the exceedingly low levels of HDL in Tangier subjects. Tangier pro-apo A-I may be rapidly catabolized, which would account for the overall low plasma levels of apo A-I in Tangier subjects. Therefore, the nature of the defect in Tangier subjects may be a deficiency in the enzyme responsible for the conversion of pro-apo A-I to mature apo A-I. However, it is unlikely that this enzyme would be totally defective, because there is detectable mature apo A-I in Tangier serum. Proof of an abnormality in this converting enzyme will require that it be isolated from normal individuals and that it be shown to be defective or diminished in Tangier serum. [The previous studies of Zannis et al. (12, 13) and Gordon et al. (18) have indicated that apo A-I is secreted as pro-apo A-I, implying that conversion takes place extracellularly in the lymph or plasma compartments; therefore, the converting enzyme should be present in serum.]

Alternate explanations of the Tangier defect would not necessarily require that the converting enzyme for pro-apo A-I be abnormal but could still yield the same end result—i.e., the failure of the conversion of pro-apo A-I. First, Tangier pro-apo A-I may not be processed by the converting enzyme because an as yet unknown defect in the Tangier apo A-I polypeptide may interfere with, or mask, the cleavage site of pro-apo A-I. Second, if pro-apo A-I must be lipoprotein-bound to be converted to mature apo A-I, then this would not happen in Tangier subjects because their pro-apo A-I does not readily associate with HDL. This latter possibility implies that the association with lipid is required to make the pro-apo A-I cleavage site available to the converting enzyme. With both of these possibilities it is not necessary that either the apo A-I propieceptide cleavage site sequence or the converting enzyme be abnormal.

Several conditions may explain why Tangier isoprotein 2 fails to associate with HDL, while isoprotein 4 associates normally. First, there is the possibility that Tangier pro-apo A-I does not bind well to lipids. This implies that the addition of six amino acids at the amino terminus of Tangier apo A-I either interferes with the ability of the large amphiphilic region of apo A-I to interact with lipids or somehow destroys the amphiphilic struc-

**Fig. 2.** Recombination studies of normal and Tangier apo A-I (mixture of all isoforms) with normal serum or isolated normal HDL. (Upper Left) Zonal ultracentrifugation pattern of normal 125I-labeled apo A-I (apo A-I) (5 μg of protein; specific activity = 64.3 μCi/μg of protein) after incubation with 1.8 ml of whole normal serum (30 min, 4°C). (Upper Right) Zonal ultracentrifugation pattern of normal 125I-labeled apo A-I (apo A-I) (5 μg of protein; specific activity = 61.5 μCi/μg of protein) after incubation with 500 μl of isolated HDL (1 mg of HDL protein). (Lower Left) Zonal ultracentrifugation pattern of Tangier 125I-labeled apo A-I (apo A-I) (5 μg of protein; specific activity = 59.2 μCi/μg of protein) after incubation with 500 μl of isolated HDL (1 mg of HDL protein). In each case, one representative experiment out of four independent recombination studies is shown. Bold line, absorbance at 280 nm; narrow line, 125I radioactivity. Fractions were pooled as indicated.
tories of apo A-I. Second, Tangier pro-apo A-I may have a greater tendency to associate with itself rather than with lipid, and this self-association could preclude lipid binding or at least make that process less favorable. A third possibility, related to the second, is that Tangier pro-apo A-I may actually recombine with HDL, but with an altered affinity, such that it does not survive the centrifugation step used in this study. It will be important in the future to study the physical chemistry of pro-apo A-I.

The normal function of pro-apo A-I is an open question. Gordon et al. (17) have speculated that pro-apo A-I conversion may have a role in the formation of nascent HDL particles. A corollary to this might be that, although pro-apo A-I may associate poorly with HDL, it might bind well to chylomicrons and function in the packaging of apo A-I into chylomicrons. Conversion of pro-apo A-I could then act as a signal for the transfer of apo A-I from chylomicrons to HDL. It is also possible that because apo A-I is a very lipophilic molecule, the presence of the propeptide may insulate that the protein reaches its destination within the cell so that it can be secreted properly. The propeptide could prevent the protein from binding to intracellular membranes during translocation of apo A-I prior to secretion. Further experimentation on this unusual proprotein from both normal and Tangier subjects will be necessary to delineate the function of pro-apo A-I and its exact relationship to Tangier disease.

Note Added in Proof. In a recent article (32) the amino-terminal sequence of pro-apo A-I from a Tangier subject was reported and is identical to that reported herein.

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