Transthyretin in high density lipoproteins: association with apolipoprotein A-I

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Abstract **Previous studies have revealed the presence of transthyretin (TTR) on lipoproteins. To further address this issue, we fractionated plasma lipoproteins from 9 normal individuals, 10 familial amyloidotic polyneuropathy (FAP) patients, and 19 hyperlipidemic subjects using gel filtration. In the majority of the subjects, as well as in 9 of the 10 FAP patients and 14 of the 19 patients with hyperlipidemia, TTR was detected by ELISA in the high density lipoprotein (HDL) fraction. The presence of TTR in HDL was confirmed by direct sequencing and by immunoblotting; using non-reducing conditions, TTR was found by immunoblotting in a high molecular weight complex, which reacted also for apolipoprotein A-I (apoA-I). The amount of TTR present in HDL (HDL-TTR), as quantified by ELISA corresponded to 1–2% of total plasma TTR. However, no detectable TTR levels were found in HDL fraction from 6 of the hyperlipidemic subjects. No correlation was found between the lack of TTR in HDL and plasma levels of total, LDL-, or HDL-associated cholesterol as well as levels of apoA-I and total plasma TTR. Ligand binding experiments showed that radiolabeled TTR binds to the HDL fraction of individuals with HDL-TTR but not to the corresponding fractions of individuals devoid of HDL-TTR, suggesting that HDL composition may interfere with TTR binding. The component(s) to which TTR binds in the HDL fraction were investigated.** **Polyclonal antibody against apoA-I was able to block the interaction of TTR with HDL, suggesting that the interaction of TTR with the HDL particle occurs via apoA-I. This hypothesis was further demonstrated by showing the formation of a complex of TTR with HDL and apoA-I by crosslinking experiments. Furthermore, anti-apoA-I immunoblot under native conditions suggested the existence of differences in HDL particle properties and/or stability between individuals with and without HDL-TTR.**—Sousa, M. M., L. Berglund, and M. J. Saraiva. **Transthyretin in high density lipoproteins: association with apolipoprotein A-I.** ***J. Lipid Res.*** **2000.** 41: **58–65.**

Supplementary key words amyloidosis • high density lipoproteins • transthyretin • apolipoprotein A-I

Transthyretin, TTR, is a serum tetrameric protein consisting of four identical subunits of approximate molecular mass 14 kDa and is highly conserved in evolution (1). It is synthesized early in development in particular by the liver and the choroid plexus of the brain, although other tissues have been reported to express TTR, but to a much lesser extent (2). Plasma TTR derives mostly from the liver and acts as a transport protein for thyroxine (T_4) and also retinol (vitamin A) (3); in the latter case this is accomplished by the formation of a 75 kDa complex with retinol-binding protein (RBP) (4). Human RBP is a single polypeptide chain with a molecular mass of ~21 kDa and a single binding site for one molecule of retinol. Holo-RBP strongly interacts with TTR and normally circulates as a 1:1 molar TTR-RBP complex. Several point mutations in TTR have been described, the most common being a Val for Met substitution at position 30 (5). Most of these TTR mutations are associated with familial amyloidotic polyneuropathy (FAP). FAP is an autosomal dominant disease characterized by the extracellular deposition of TTR amyloid fibrils in various tissues, primarily in the peripheral nervous system (6).

A number of studies have revealed that apolipoprotein E (apoE) is present in all types of amyloidosis (7) and that other apolipoproteins are also important constituents of amyloid fibrils. For instance, apolipoprotein B (apoB) in addition to apoE has been found in amyloid fibrils in the brain of patients with Alzheimer's disease (8). In this amyloid-related disorder, the allele apoE-4 is now used as a genetic risk factor (9). Variant forms of apolipoprotein A-I (apoA-I) are also related with familial amyloidosis (10), characterized by the deposition of N-terminal fragments of this protein.

In previous studies in Japanese FAP patients carrying a TTR Met30 mutation, TTR was found to be associated with lipoproteins (11). In particular, both the HDL and

Abbreviations: ABTS, 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid); CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; DMSO, dimethylsulphoxide; DTSP, 3,3′-dithio-bis (propionic acid N-hydroxysuccinimide ester); FAP, familial amyloidotic polyneuropathy; HDL-TTR, TTR carried in high density lipoprotein; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; PVDF, polyvinylidene difluoride; RBP, retinol binding protein; TFA, trifluoroacetic acid; TTR, transthyretin; T_4, thyroxine.

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LDL fractions were reported to contain TTR (11). TTR was present in the HDL fraction to the same extent both in normal individuals and FAP Met30 patients, whereas the LDL fraction from the FAP Met30 patients showed higher amounts of TTR than normal individuals. However, virtually nothing was known about the nature of the association between TTR and lipoproteins or about its possible significance. To further investigate the relationship between lipoproteins and TTR, we addressed the molecular basis for this association in normal and hyperlipidemic individuals, as well as in FAP patients representing several different TTR mutations.

METHODS

Subjects

A total of 38 Portuguese subjects, 9 normal individuals, 19 hyperlipidemic subjects, and 10 FAP patients with different TTR mutations participated in the study. For the purpose of this study, the normal subjects had total cholesterol levels ≤200 mg/dL, and hyperlipidemic subjects were classified as total cholesterol >200 mg/dL. One of the hyperlipidemic subjects had cirrhosis of the liver. The FAP patients consisted of 6 heterozygous and 2 homozygous for the TTR Met30 mutation and 1 heterozygotic carrier for each of the TTR mutations Pro52 and Arg50. TTR mutations in the FAP patients were verified by single-strand conformation polymorphism (SSCP), followed by exon sequencing, as described previously (12). All FAP patients presented the characteristic clinical picture of FAP (13). The study was conducted following the guidelines of the Declaration of Helsinki.

Lipoprotein isolation and analysis

Blood samples were obtained after an overnight fast and were drawn into EDTA-containing tubes. Plasma was obtained after centrifugation of the tubes at 3,000 rpm for 20 min at 4°C. Total cholesterol and triglyceride levels were measured using standard enzymatic procedures (Boehringer Mannheim, GmbH, and Sentinel, respectively). HDL cholesterol levels were measured after precipitation of apoB-containing lipoproteins using phosphotungstic acid (14) and LDL cholesterol levels were measured using polyvinylsulfate (15) (Boehringer Mannheim, GmbH). ApoA-I levels were measured by radial immunodiffusion (The Binding Site) (16). Plasma samples were applied diluted 1:10 in phosphate-buffered saline (PBS) and calibrators for the standard curve ranged from 27 to 270 mg/dL. To separate lipoproteins, 250 μL of each plasma sample was fractionated by gel filtration on a 150 mL column of Superose 12 Prepgrade (1.6 cm × 10 cm) (Pharmacia Biotech) equilibrated with PBS. The chromatography was performed at 20 mL/h and fractions of 1 mL were collected. Identification of lipoprotein fractions was performed by quantification of cholesterol in the eluted fractions (cholesterol enzyme reagent-Human, GmbH) (17). For some of the studies, plasma lipoproteins were also isolated by vertical rotor ultracentrifugation (Beckman). The LDL fraction was isolated in the d 1.019–1.063 g/mL density range, and the HDL fraction in the d 1.063–1.21 g/mL density range (18). Bottom fractions (delipidated plasma) were dialyzed overnight against PBS for subsequent studies. When indicated, HDL were delipidated as described by Menzel and Uterman (19).

TTR measurements

Plasma TTR levels were determined using radial immunodiffusion (The Binding Site). TTR presence in the eluted fractions was assessed by a quantitative enzyme-linked immunoreactive assay (ELISA). Ninety-six well plates (Maxisorp-Nunc) were coated overnight at 4°C with 1:500 diluted polyclonal anti-rabbit TTR (Dako A/S) and blocked with 5% skim milk in PBS. Each gel filtration sample, diluted 1:10 in PBS, was applied to the wells for 1 h at room temperature. Development was performed with peroxidase-conjugated anti-human TTR (The Binding Site) 1:500 diluted in PBS–0.05% Tween20 (PBS-T) and 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS)/H_2O_2. TTR concentration in the fractions was calculated from a standard curve ranging from 5 to 200 ng/mL.

Transthyretin iodination

TTR was isolated from human serum by preparative gel electrophoresis after ion exchange chromatography according to Almeida et al. (20). TTR was iodinated following the iodogen method. Briefly, to reaction tubes coated with 10 μg iodogen (Sigma), 1 mCi, 37 MBq Na ^{125}I (Amersham Life Science) was added, followed by 20 μg TTR in PBS. The reaction was allowed to proceed in a ice bath for 10 min. The iodination mix was finally transferred to a 5 mL Sephadex G50 column (Pharmacia) to separate labeled protein from free iodide.

Electrophoresis and blotting

Proteins were separated using a continuous gradient (5–20%) SDS-PAGE unless stated otherwise or native conditions for 2 h at 150 V. Gels were stained with Coomassie or silver nitrate. Proteins were transferred to nitrocellulose (Schleicher & Schüll, 0.45 μm). For TTR, apoA-I and apoA-II immunodetection, blots were saturated in 5% skim milk in PBS 1 h at room temperature followed by incubation 1 h with 1:500 diluted polyclonal rabbit anti-TTR (Dako A/S), polyclonal rabbit anti-apoA-I (Calbiochem) or anti-apoA-II (Calbiochem), respectively. For detection of apoA-I after separation in native gels, blots were incubated with a monoclonal antibody against residues 25 to 82 of mature apoA-I (Sanofi Inc.). TTR, apoA-I and/or apoA-II were visualized using peroxidase-conjugated goat anti-rabbit Ig (Amersham Life Science) or peroxidase-conjugated anti-mouse Ig and 4-chloronaphthol/H_2O_2.

Ligand blotting experiments

HDLs either isolated by gel filtration or by ultracentrifugation were immobilized in nitrocellulose under vacuum. Membranes were saturated as above and then incubated for 3 h at room temperature with 500,000 cpm iodinated TTR in PBS-T. Membranes were extensively washed in PBS-T and exposed overnight at −70°C for autoradiography. Immobilized albumin was used as a negative control of the ligand binding experiments. For inhibition experiments, HDLs were incubated for 1 h at 37°C with either polyclonal rabbit anti-apoA-I (Calbiochem) or anti-apoA-II (Calbiochem) diluted 1:100 in PBS, prior to immobilization in the membrane.

Protein sequence analysis

Protein separated by SDS-PAGE was transferred to a polyvinylidene difluoride (PVDF) membrane in 10% methanol, 10 mm 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) buffer. Sequence analysis was performed in an Applied Biosystems model 470A gas-phase sequencer equipped with a 120A PTH analyser.

High performance liquid chromatography (HPLC)

TTR was separated from other proteins present in the HDL-rich region by reverse phase HPLC. About 6 pools of the HDL fraction were loaded on a Resource 15RPC 1 mL column (Pharmacia Biotech), in a Gilson HPLC system in 0.05% trifluoroacetic acid (TFA) containing 10% acetonitrile; elution was carried out on a 10–60% acetonitrile gradient as previously described (5).

TABLE 1. Total and HDL-associated TTR levels in individuals with normal cholesterol levels

Subjects	TTR	HDL-TTR	Total Chol	HDL-Chol
		mg/dL		
Normal				
1	25.7	0.32	169	58
2	27.3	0.24	181	29
3	28.3	0.5	184	32
4	29.2	0.33	200	31
5	28.5	0.23	139	21
6	18.5	0.34	137	26
7	26.0	0.41	184	20
8	27.5	0.33	173	25
9	26.0	0.32	183	26
Mean ± SE	26.4 ± 2.9	0.34 ± 0.08	172 ± 19	31 ± 11
Met30 het				
1	17.2	0.42	191	79
2	22.3	0.34	153	84
3	15.6	0.32	174	74
4	23.9	0.41	176	71
5	14.8	0.31	124	21
6	14.1	0.34	156	29
Mean ± SE	18.0 ± 0.8	0.35 ± 0.04	162 ± 21	68 ± 40
Met30hm2	17.4	0.32	171	31
Arg50 het	20.7	0.31	156	29
Pro52 het	23.9	0.33	164	39

Met30 het, Arg50 het, and Pro52 het refer to heterozygous FAP patients carrying these mutations, whereas Met30hm2 refers to a homozygous carrier; Chol, cholesterol.

Crosslinking of ^{125}I-TTR to HDL

^{125}I-labeled TTR (50,000 cpm) was incubated 1 h at room temperature with 1 μg of HDL or 1 μg of apoA-I (Calbiochem) in the presence of 1 mm of 3,3′-dithio-bis (propionic acid N-hydroxysuccinimide ester) (DTSP) (Sigma). DTSP was prepared 100-fold concentrated in dimethylsulfoxide (DMSO). The crosslinked products were separated on a 12% SDS-PAGE and visualized by autoradiography after overnight exposition at −70°C.

RESULTS

TTR and serum lipid quantification

As seen in **Table 1** and **Table 2**, the mean plasma TTR levels of the healthy and the hyperlipidemic subjects (26.4 ± 2.9 mg/dL and 26.8 ± 2.7 mg/dL, respectively) were in the same range as previously established for normal subjects (21). For the FAP patients heterozygous for the Met30 mutation, TTR levels were lower (18.0 ± 0.8 mg/dL) and in good agreement with earlier studies on FAP patients (21). Total plasma cholesterol levels reflected the recruitment of normal and hyperlipidemic subjects, being <200 mg/dl in the normal subjects (Table 1) and >200 mg/dl in the hyperlipidemic subjects (Table 2). In the latter group, HDL cholesterol levels ranged from 16 to 113 mg/dl and 7 of the 19 hyperlipidemic subjects had triglyceride levels >200 mg/dl. For all FAP patients studied, total cholesterol levels were similar to normals, while mean HDL cholesterol levels were higher (Table 1).

TTR in HDL

Plasma samples from all 38 subjects were fractionated by gel filtration as described in the previous section. The lipoprotein-rich region was identified by cholesterol measurements in the eluted fractions, and as seen in **Fig. 1**, two major cholesterol peaks, corresponding to LDL and

TABLE 2. Total TTR and apoA-I levels in hypercholesterolemic individuals

Subjects	TTR	HDL-TTR	Total Chol	HDL	TG	ApoA-I
			mg/dL			
Hypercholesterolemic individuals with HDL-TTR						
2	27.2	0.24	211	79	92	122
4	30.9	0.42	306	26	239	214
5	26.6	0.51	306	26	239	160
6	26.9	0.34	255	37	94	250
7	26.1	0.32	292	37	101	156
8	28.4	0.41	262	26	102	215
9	27.2	0.25	262	41	40	182
11	25.0	0.34	291	30	147	214
12	27.3	0.29	282	28	62	193
13	18.3	0.24	326	113	108	181
15	29.5	0.33	244	63	106	219
16	27.5	0.23	236	74	166	232
17	27.5	0.33	232	76	70	114
18	26.5	0.24	225	27	199	158
Mean ± SE	26.8 ± 2.7	0.32 ± 0.08	266 ± 34	49 ± 26	126 ± 61	186 ± 39
Hypercholesterolemic individuals devoid of HDL-TTR						
1	25.3	0	279	34	220	196
3	28.7	0	294	30	227	278
10	28.3	0	282	32	218	218
14	30.5	0	320	66	158	186
Mean ± SE	28.2 ± 2.2	0	249 ± 18	40 ± 6	206 ± 32	220 ± 41
Cyrrhotic	12.4	0	414	16	363	150
Met30hm1	15.2	0	252	26	784	129

Met30hm1, homozygous carrier of TTR Met 30 mutation; Chol, cholesterol.

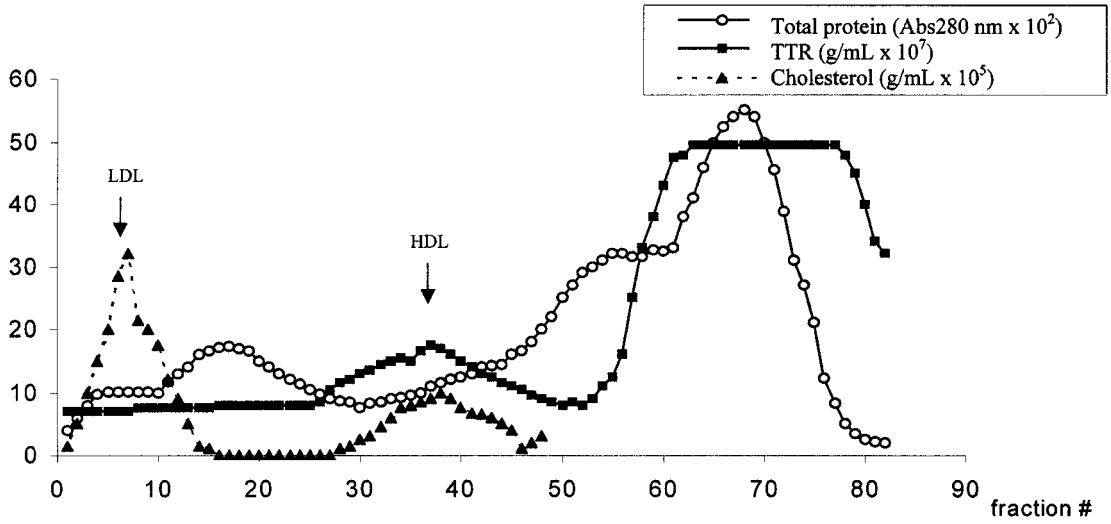

Fig. 1. Representative profiles of total protein, TTR, and cholesterol of total plasma fractionated by gel filtration from individuals with HDL-TTR.

HDL, respectively, could be identified in all subjects. The same fractions were also analyzed for TTR content by quantitative ELISA measurements, and two peaks of TTR were observed, a major peak eluting in fractions 55–80, corresponding to free TTR, and a smaller peak, co-eluting with the HDL fraction (Fig. 1). It has been reported that a small proportion of TTR circulates as a free form of 56 kDa while the majority of TTR circulates as part of a 1:1 molar complex with RBP (75 kDa) (4). Using the present gel filtration conditions, with an optimal separation of the different lipoprotein fractions, we were unable to separate these two non-lipoprotein TTR forms which together constituted the major TTR peak. However, the presence of an HDL-associated TTR peak was found in all the 9 normal individuals, in the FAP patients (except for one homozygous Met 30 patient), and in 14 hypercholesterolemic individuals, the majority of the subjects. The TTR level of this peak (HDL-TTR) ranged from 0.24 to 0.51 mg/dl and the total HDL-TTR content corresponded to about 1–2% of the plasma TTR levels (Table 1). Using one of the above plasma samples but devoid of lipoproteins (bottom fraction) with the same gel filtration conditions, the TTR peak previously observed in the HDL region was now absent. This evidence strongly suggested that this TTR fraction in fact circulates bound to HDL. Interestingly, as seen in Fig. 1, no TTR was present in the LDL fractions. In 6 of the subjects, we could not detect any TTR associated with HDL (Table 2). These subjects were all hyperlipidemic (5 hyperlipidemic patients and 1 FAP patient homozygous for the Met 30 mutation who also was hyperlipidemic). An example of a lipoprotein profile lacking TTR in the HDL region is shown in **Fig. 2**. Though individuals lacking HDL-TTR belong to a hypercholesterolemic population, this was not related either with total cholesterol or with LDL/HDL-associated cholesterol levels: individuals with similar cholesterol profiles were found in the hyperlipidemic population that contained HDL-TTR. As apoA-I is the main apolipoprotein present in HDL, we analyzed apoA-I levels in all the subjects. There was no correlation between apoA-I levels and presence or absence of HDL-TTR. Further, there was no correlation between total TTR levels and HDL-TTR levels in all the groups studied. Also, the absence of TTR from HDL observed in some of the individuals was not related to total TTR levels as these were within the normal range (28.2 ± 2.2 mg/dL) (see Table 2).

In order to further ascertain that TTR immunoreactivity in the HDL region was in fact part of the HDL particle, continuous gradient SDS-PAGE of the isolated HDL fraction was performed both under non-reducing and reducing conditions (**Fig. 3A**). TTR was not detected by protein staining but was visualized by anti-TTR immunostaining. Under non-reducing conditions, a high molecular mass complex of ~200 kDa staining for anti-TTR was observed (Fig. 3A, panel a, lane 1). This complex reacted also with apoA-I, the main apolipoprotein component of HDL (Fig. 3A, panel b, lane 1) and apoA-II (Fig. 3A, panel c, lane 1), thus showing that this 200 kDa complex is HDL-related. As a control for the experiment, isolated TTR was also run on a continuous gradient SDS-PAGE under non-reducing conditions and only the TTR monomer of 14 kDa was observed (not shown). When the complex was delipidated and analyzed under non-reducing conditions, TTR, apoA-I, and apoA-II presented the molecular masses corresponding to the isolated proteins (14, 28 and 17 kDa, respectively) (Fig. 3A, lane 2 of each panel). Therefore, under conditions that disassemble the HDL vesicle, such as delipidation, TTR is released as well as the other apolipoprotein components. The same experiment with delipidated HDL, but under reducing conditions, showed that only apoA-II is reduced to its monomeric form (Fig. 3A, panel c, lane 3), whereas TTR and apoA-I present the same molecular mass as the corresponding isolated proteins (Fig. 3A, panels a and b, lane 3, respectively). These data dem-

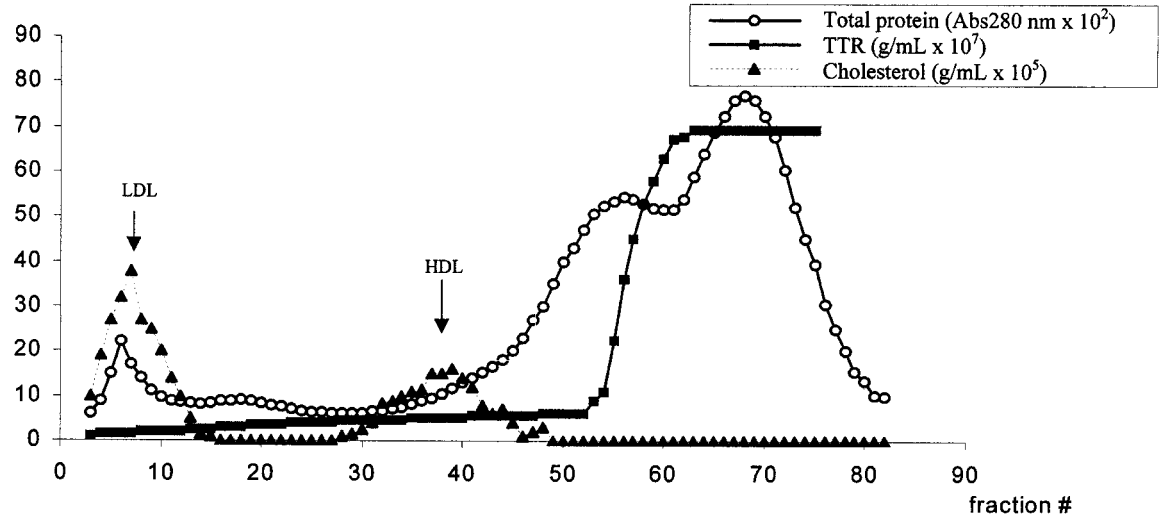

Fig. 2. Representative profiles of total protein, TTR, and cholesterol of total plasma fractionated by gel filtration from individuals devoid of HDL-TTR.

onstrated that the association between TTR and lipoproteins occurs non-covalently. To further verify the presence of TTR in HDL, HDL fractions isolated by sequential ultracentrifugation were subjected to TTR immunoblotting. Essentially the same results were obtained (data not shown).

HDLs from individuals with and without HDL-TTR isolated by gel filtration were further compared by anti-apoA-I immunoblot under native conditions. Differences in migration were observed between both groups of individuals (Fig. 3B) suggesting the existence of differences between the corresponding HDL. In individuals with HDL-TTR, the HDL vesicle, assessed by anti-apoA-I immunostaining, shows a faster migration (Fig. 3B, lanes 1 and 4) than the corresponding HDL of individuals devoid of HDL-TTR (Fig. 3B, lanes 2 and 3). The stability of the HDL vesicle in individuals devoid of HDL-TTR seems to be decreased with apoA-I being released under the native electrophoretic conditions used (Fig. 3B, lanes 2 and 3, lower arrow).

To further characterize TTR in HDL, HDL protein components from a pool of HDL fractions isolated by gel filtration were separated by reverse phase HPLC. A protein peak corresponding to the position where TTR normally elutes (5) was analyzed on SDS-PAGE and a band of 14 kDa was electroblotted and subjected to N-terminal se-

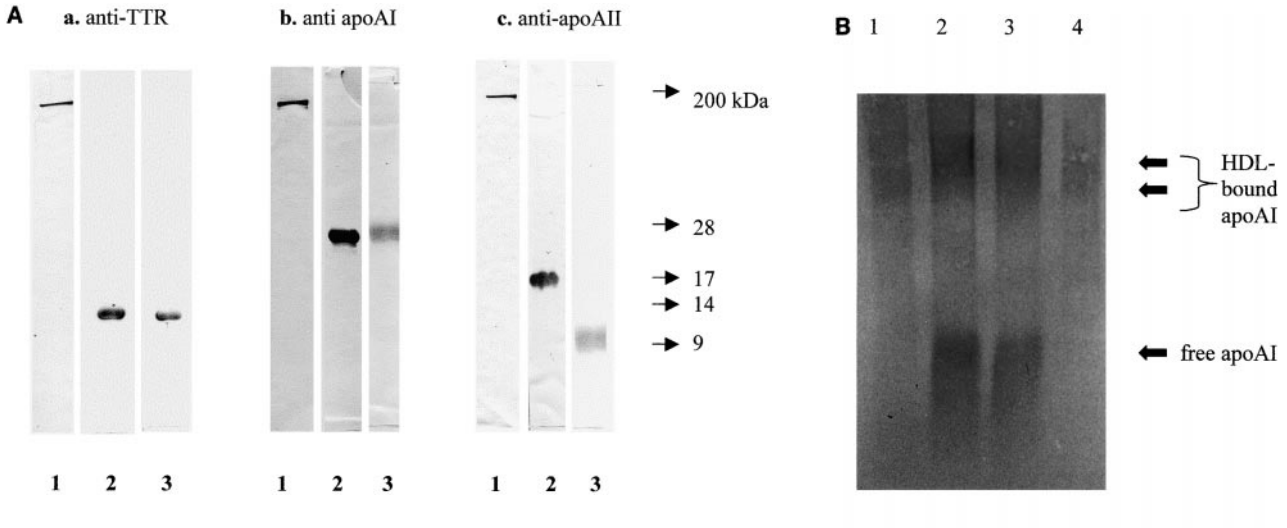

Fig. 3. A: Immunoblot analysis on continuous gradient (5–20%) SDS-PAGE of the HDL-rich region from an individual with HDL-TTR. Panel a. anti-TTR; panel b. anti-apoA-I; panel c. anti-apoA-II. 1. Intact HDL vesicle under non-reducing conditions; 2. delipidated HDL under non-reducing conditions; and 3. delipidated HDL under reducing conditions. B: Anti-apoA-I immunoblot analysis under native conditions of the HDL-rich fraction isolated by gel filtration from 2 individuals devoid of HDL-TTR (lanes 2 and 3) and 2 individuals with HDL-TTR (lanes 1 and 4). The upper arrows indicate HDL bound apoA-I presenting a faster migration in individuals with HDL-TTR (lanes 1 and 4). The lower arrow indicates free apoA-I partially released in individuals devoid of HDL-TTR (lanes 2 and 3).

quencing; the sequence obtained, XPTGTGESK, corresponds to the N-terminal sequence of TTR, definitively proving the presence of TTR in HDL.

Ligand binding of ^{125}I-labeled TTR to immobilized HDL

Ligand binding studies were performed in individuals either having or lacking HDL-TTR. As seen in **Fig. 4.1 A**, binding of ^{125}I-labeled TTR to membrane-immobilized samples from the HDL fraction isolated by gel filtration was observed in individuals containing HDL-TTR. In contrast, no binding was seen to the HDL-rich region of plasmas devoid of HDL-TTR (Fig. 4.1 B). Control experiments using samples from the LDL fraction showed no binding to ^{125}I-labeled TTR (data not shown), further demonstrating the inability of TTR to bind to LDL. As apoA-I is the major apolipoprotein in HDLs and as this apolipoprotein seems to be released from HDL more easily in individuals devoid of HDL-TTR (Fig. 3B), we investigated next whether TTR binding to HDL occurs through this protein. Ligand binding assays were performed using different concentrations of HDLs isolated by ultracentrifugation with and without prior incubation with polyclonal rabbit anti-apoA-I (Fig. 4.2). Anti-apoA-I was able to block ^{125}I-labeled TTR binding to HDLs thus suggesting that the interaction of TTR with HDL occurs via apoA-I. The same experiment was performed with anti-apoA-II in order to rule out the possibility that binding inhibition occurred due to steric hindrance caused by the antibody. However, anti-apoA-II did not inhibit TTR binding to HDL (not shown).

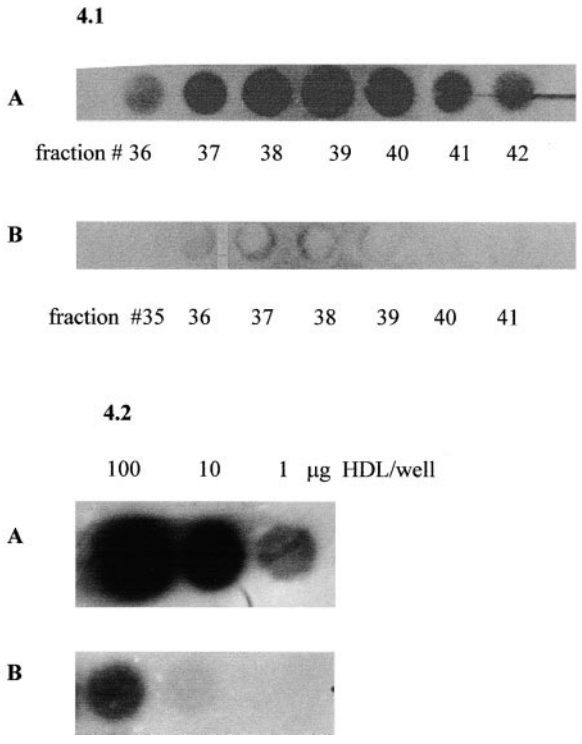

Fig. 4. Ligand binding of ^{125}I-labeled TTR to membrane immobilized HDL. 4.1: Fractions isolated by gel filtration. A, individual with HDL-TTR (fractions 36–42 from Fig. 1); B, individual devoid of HDL-TTR (fractions 35–41 from Fig. 2). 4.2: A, HDL isolated by ultracentrifugation (100, 10, and 1 μg/well); B, HDL isolated by ultracentrifugation (100, 10, and 1 μg/well) blotted after prior incubation with polyclonal anti-apoA-I.

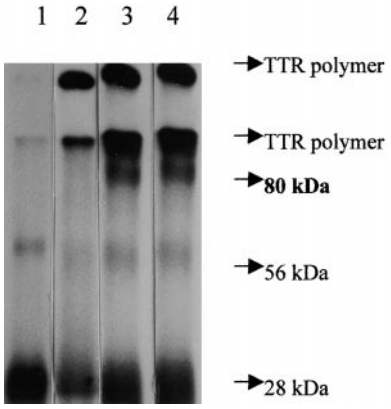

Fig. 5. Crosslinking of TTR to HDL and apoA-I; autoradiography of 12% SDS-PAGE of lane 1, ^{125}I-labeled TTR; lane 2, ^{125}I-labeled TTR crosslinked with DTSP; lane 3, ^{125}I-labeled TTR and HDL crosslinked with DTSP; lane 4, ^{125}I-labeled TTR and apoA-I crosslinked with DTSP.

Crosslinking of ^{125}I-labeled TTR to isolated HDL and apoA-I

In order to further demonstrate that the interaction of TTR with HDL is through binding to apoA-I, ^{125}I-labeled TTR was crosslinked to both isolated HDL and apoA-I using DTSP (**Fig. 5**). DTSP is expected to conjugate existing interactions between proteins. This crosslinker, also known as Lomant's reagent, is a homofunctional N-hydroxysuccinimide ester (NHS-ester) with a spacer arm length of 12 Å. Primary amines are the principal targets for NHS-esters. As the α-amine groups on the N-termini of peptides are seldom available, the reaction with the ξ-amines of lysines is the principal target of this crosslinker. As a control to the experiment, ^{125}I-labeled TTR (Fig. 5, lane 1), and ^{125}I-labeled TTR crosslinked (Fig. 5, lane 2) are shown. Upon crosslinking of ^{125}I-labeled TTR alone, TTR polymers are formed (Fig. 5, lane 2) and still both dimeric (28 kDa) and tetrameric (56 kDa) forms can be observed. A complex of ~80 kDa (Fig. 5, lane 3) corresponding to TTR bound to HDL was formed, showing the interaction of TTR with the HDL vesicle. The size of this complex is compatible with crosslinking of tetrameric TTR to HDL via apoA-I. To investigate this hypothesis, similar crosslinking experiments were performed using ^{125}I-labeled TTR and isolated apoAI (Fig. 5, lane 4). Again, a complex of ~80 kDa was formed showing that TTR interaction with HDL occurs through apoA-I. In a parallel experiment, TTR did not crosslink to apoA-II, further corroborating the specificity of TTR-apoA-I binding.

DISCUSSION

The main finding of the present study was the association of TTR with HDL through interaction with apoA-I.

Human plasma lipoproteins comprise a spectrum of macromolecules that exhibit a high range of particle size, hydrated density, molecular mass, and a broad diversity of lipid and protein composition (22). The HDL range includes a highly heterogeneous class of lipoprotein particles that interact with a wide variety of cells and although reverse cholesterol transport has been associated with the HDL fraction, other functions have also been suggested (23–25). However, it is likely that HDL might mediate several still poorly defined physiological functions.

The major protein constituents of the HDL fraction are apoA-I and apoA-II, although apoA-IV, C, D and E have also been found in association with this fraction (26). The apoA-I content has been associated with the anti-atherogenic properties of HDL, as HDL particles carrying apoA-I, in contrast to particles carrying both apoA-I and apoA-II, have been shown to be negative risk factors that are associated with cardioprotection (26–28). In addition, several other proteins have been associated with HDL, some of which have enzymatic function (25). Recently, interest has focused on paraoxonase, an enzyme associated with HDL, also suggested to be a negative risk factor associated with cardioprotection (25). These studies demonstrate that the protein content of HDL is complex and suggest that a number of proteins may be carried in this fraction. As the functional specificity of plasma lipoproteins resides mostly in the apolipoprotein moieties, it is of importance to clearly identify proteins bound to lipoprotein fractions. Previous studies on patients with TTR mutations such as the FAP Met30 mutation have suggested apolipoprotein abnormalities in lipoproteins, such as a decreased apoA-II/apoA-I ratio, possibly due to changed affinity of apoA-II to the HDL fraction (11). Although the role of apoA-II in HDL metabolism and atherogenicity has not been clarified, the results suggest that variations in lipoproteins and/or their apolipoprotein content may participate in an altered lipid metabolism and/or amyloid formation in patients with amyloidosis. Previous studies on Japanese FAP patients (11) showed TTR presence in HDLs but no information was given relating to the percentage of the total TTR pool that circulates bound to this lipoprotein nor on the nature of this association.

In HDL isolated by gel filtration, our study demonstrates that in most individuals a fraction of TTR identified by immunochemical and sequencing methods, constituting approximately 1%–2% of the total TTR plasma concentration, circulates bound to the HDL fraction. The association with TTR appeared to be specific to HDL, as we did not detect any presence of TTR in other lipoprotein fractions. The possibility of coelution of a higher molecular weight form of TTR and HDL without interaction between them was ruled out as we performed HDL isolation by ultracentrifugation and identified TTR also in this particle. Furthermore, in delipidated plasma, TTR was not detected in the fraction corresponding to elution of HDL in complete plasma. This evidence also excludes the possibility of circulation of aggregated high molecular mass TTR. As TTR has high affinity binding sites for RBP, we also tested the HDL-rich fraction containing TTR for presence of RBP. However, RBP was not detected (data not shown), demonstrating that TTR is associated with HDL in a non-RBP bound form.

In all individuals with normal cholesterol levels, TTR was present in the HDL fraction. In subjects with high cholesterol levels, we identified 6 subjects (5 subjects with hypercholesterolemia and 1 dyslipidemic FAP patient homozygous for the Met30 TTR mutation) where the HDL fraction was devoid of TTR. However, there was no correlation between the absence of TTR in the HDL fraction on one hand and total TTR or cholesterol levels on the other hand, and we did detect the presence of TTR in other subjects with high cholesterol levels. A potential difference in HDL structure or composition in subjects with and without HDL-TTR was not related to cholesterol content or apoA-I levels. Further studies are needed to clarify the underlying mechanisms resulting in absence of HDL-TTR in some hypercholesterolemic subjects. However, our finding that HDL in subjects with HDL-TTR presents a faster migration under native conditions than HDL from individuals devoid of HDL-TTR, and that in the latter apoA-I is partially released from the HDL particle, suggested the existence of a difference in HDL particle properties and/or stability between the groups of individuals. In Japanese FAP patients it had been shown (11) that HDLs presented an increased negative charge, suggesting that in these individuals the nature of HDL itself may be changed. The ability of HDL to bind ^{125}I-labeled TTR as assessed by blotting experiments on the HDL-rich region and HDL isolated by ultracentrifugation varied between individuals. In none of the subjects with absence of TTR in the HDL fraction did we detect any binding of ^{125}I-labeled TTR to HDL in vitro, while this was always seen in individuals with TTR present in HDL. These results suggest that lipoprotein composition or metabolism could have impact on TTR binding. No differences between the free and bound pools of TTR were seen with the methods used; upon release from delipidated HDL, TTR has the same migration on SDS gels (Fig. 3A), the same N-terminal sequence and presented the same retention time on the reverse HPLC column as the isolated protein, thus having identical hydrophobic properties as free TTR. In individuals with HDL-TTR, free TTR is able to bind HDL as assessed by ligand binding studies (Fig. 4.1A). The extent of exchange between free and bound TTR in vivo should be addressed by other types of studies.

To further characterize the nature of TTR interaction with HDLs, we attempted successfully to block binding with a polyclonal antibody against apoA-I. Anti-apoA-I was chosen for these experiments not only because apoA-I represents the major apolipoprotein in HDL but also because we observed that under native conditions this protein is partially released from HDL of individuals devoid of HDL-TTR. Blocking TTR binding to HDL by the use of anti-apoA-I was the first evidence of the fact that TTR is binding to HDL via apoA-I. Crosslinking of TTR with HDL and apoA-I showed in both instances the formation of a complex with ~80 kDa corresponding to a molecular complex of TTR and apoA-I. Several reports have suggested

that apolipoproteins play an important role in amyloid formation, and apoE is found in amyloid fibrils in all types of amyloidosis. In Alzheimer's disease, the apoE4 allele is considered to be a genetic risk factor (29). Extensive work has been performed relating to the Alzheimer's amyloid β peptide interaction with the HDL fraction. Studies have shown that the soluble β peptide is associated with lipoprotein particles, in particular to HDL fractions, where it is mainly complexed to apoJ and also to a lesser extent to apoA-I (30). It cannot be excluded that the presence of TTR in HDL could be significant in both TTR metabolism and FAP, though this study demonstrated that the FAP-related mutations, TTR Met 30, Arg 50, and Pro 52, did not interfere with the formation of the HDL-TTR complex. However, further studies are needed to assess the impact of HDL binding on TTR metabolism. The current strain of transgenic mice carrying human TTR Met30 on a TTR null mouse background (31) could be useful to assess the physiological meaning of HDL-TTR. However, preliminary experiments in our laboratory on the plasma of this mouse strain did not detect human TTR in association with mouse HDL. Also, in vitro experiments were unable to show reconstitution of human TTR binding to isolated mouse HDL.

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