Evaluation of Lipoproteins and Apolipoproteins in Serum of a Tangier Patient by Micro-Scale Two-Dimensional Electrophoresis

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We examined lipoproteins and apolipoproteins in serum of a Tangier-disease patient. We used three different techniques of micro-scale two-dimensional electrophoresis: (a) no denaturants; (b) with sodium dodecyl sulfate (SDS) used only in the slab gel electrophoresis; and (c) with urea and a detergent used in isoelectric focusing and with SDS in slab gel electrophoresis. By technique a, an extremely low concentration of high-density lipoproteins (HDL) in the Tangier serum was seen, and lipoproteins that cannot form HDL complexes were detected as multiple spots in the acidic (pI 4–5) and relatively low apparent molecular mass (20 000–80 000) region. By technique b, Tangier low-molecular-mass lipoproteins were dissociated into their constituent apolipoproteins, and we observed a higher proportion of apoC-III, together with lower proportions of apoA-I and apoA-II, than in the normal HDL fraction. Technique c showed the total content of apolipoproteins in the whole Tangier serum, as several workers have reported. The presence of low-molecular-mass lipoproteins and a high concentration of apoC-III in this lipoprotein fraction characterized the Tangier serum.

Tangier disease is a rare hereditary disorder characterized by a marked deficiency of high-density lipoproteins (HDL) in the plasma of the patients (1–4). In normal subjects, plasma HDL are composed of apolipoproteins A-I, A-II, A-IV, C-II, C-III, and E (5), the first two being predominant (90%). In plasma from Tangier homozygotes, the concentrations of apoA-I and apoA-II were approximately 2 and 10% of normal, respectively (6). An increased fractional catabolism of purified apoA-I, but not apoA-II, was observed (6, 7), suggesting a kinetic abnormality in apoA-I isolated from Tangier patients.

Two-dimensional electrophoresis revealed that plasma apoA-I from Tangier patients has an isoformal pattern different from that of normal subjects (8–11), prompting the proposal that there is defective conversion of proapoA-I to mature apoA-I due to a structural mutation (8). However, the proapoA-I isolated from a Tangier disease subject had the same amino-terminal sequence as normal proapoA-I (11). The complete mRNA sequence encoding proapoA-I was determined, and there was no major structural defect in Tangier proapoA-I (12). Further, Bojanovski et al. (13) found that in vitro conversion of proapoA-I to mature apoA-I in Tangier was normal. Thus the low apoA-I concentration in the serum from Tangier patients should be due to the markedly accelerated catabolism of the apolipoprotein, which may in turn reflect a post-translational defect in its metabolism.

We assessed the lipoproteins and apolipoproteins in the serum of a Tangier patient by two-dimensional electrophoresis and compared them with those in normal subjects, looking for differences other than in the quantities of apoA-I and apoA-II. We used a technique of micro-scale two-dimensional electrophoresis in the absence of denaturing agents (14), the better to learn the properties of serum lipoproteins in physiological conditions. The apolipoprotein composition in the Tangier lipoproteins was examined by a micro-scale two-dimensional electrophoretic technique in which sodium dodecyl sulfate (SDS) is used only in the second-dimension electrophoresis (15, 16). The apolipoprotein composition in the whole serum was examined by modifying the technique of Anderson and Anderson (17) to a micro gel version. Using these three different types of micro two-dimensional electrophoresis, we demonstrated the characteristics of the lipoproteins and apolipoproteins in the Tangier serum.

Materials and Methods

Tangier-disease patient. In this 37-year-old man, typical morphological characteristics of Tangier disease were observed, and the values for plasma cholesterol (1.34 mmol/L), triglycerides (2.62 mmol/L), and HDL cholesterol (0.05 mmol/L) were compatible with the diagnosis (18). The detailed description of the homozygous Tangier patient and his obligate heterozygous relatives will be reported elsewhere (M. Clerc et al., in preparation).

Samples. Blood was collected from the homozygous Tangier patient and 10 healthy donors. The blood was allowed to clot for 30 min at room temperature, and serum was obtained by centrifugation (3000 × g, 20 min, 4 °C). Sucrose was added to the serum samples to give a concentration of 400 g/L, and they were stored at −20 °C.

Reagents. Ampholytes (Ampholines, pH 3.5–10 and 3.5–5) were obtained from LKB Produkter, Bromma, Sweden. Acrylamide and N,N'-methylenbisacrylamide (both "special grade for electrophoresis"), glycine, Tris base, ammonium persulfate, urea, and reagents for silver staining were all from Wako Pure Chemical Industries, Tokyo, Japan. N,N,N',N'-Tetramethylethylenediamine and Coomassie Brilliant Blue R-250 were from Nakarai Chemicals, Kyoto, Japan. Nonidet P-40 (NP-40) detergent was from BDH Chemicals, Poole, England. Rabbit antiserum against human plasma proteins were obtained from Behring, Marburg, F.R.G. Goat antiserum against human apoA-I, apoA-II, apoC-II, and apoC-III were kindly provided by Prof. J. C. Fruchart (Pasteur Institute, Lille, France). Peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated anti-goat IgG were from Miles Laboratories, Naperville, IL. Nitrocellulose sheets, 0.45 μm pore size, were from Schleicher & Schuell, Dassel, F.R.G.

Micro two-dimensional electrophoresis. For micro-scale two-dimensional electrophoresis, we used three different combinations of the first- and the second-dimension electrophoresis...
phoresis, namely, (a) without denaturants in either dimension (b) without denaturants in the first dimension but with sodium dodecyl sulfate (SDS) in the second, and (c) with urea and NP-40 in the first dimension and SDS in the second. The gel size was the same for all three techniques, because the same apparatus was used for preparing the gels and for electrophoresis (14).

The first type of micro two-dimensional electrophoresis was performed basically as described previously (14). We subjected the serum samples (2 μL) to isoelectric focusing in the absence of denaturant. For this we used capillary gels (1.3 mm i.d. × 35 m) that had been prepared from a mixture that contained, per liter, 40 g of acrylamide, 2 g of the bisacrylamide, 20 g of Ampholine pH 3.5–10, and 5 g of Ampholine pH 3.5–5, by adding N,N,N',N'-tetramethylethylenediamine and ammonium persulfate to give final concentrations of 29 μL/L and 0.5 g/L, respectively. Isoelectric focusing was run at 0.1 mA per tube constant current until the voltage reached 300 V (about 30 min), then at 300 V constant voltage for 40 min; the focusing gels were set on the second-dimension slab gels without equilibration, and electrophoresis was run in micro gradient (4–17% acrylamide and 0.2–0.85% of the bisacrylamide linear gradient, 38 mm × 38 mm × 1 mm) slab gels, without denaturants, at 10 mA per slab constant current for 30 min.

The second type of micro two-dimensional electrophoresis was run as described previously (16). Capillary gels were prepared and sample treatment and isoelectric focusing were done as described above for type a, except that 1 μL of each sample was applied; the focusing gels were equilibrated by setting them for 20 min in the molds of the second-dimension slab gel, which had been filled with a buffer containing 2 g of Tris, 9.6 g of glycine, and 20 g of SDS per liter. The electrophoresis was run in micro gradient (4–17%) slab gels, which contained 10 g of SDS per liter in addition to the constituents of the type a slab gels, at 10 mA per slab (constant current) for 40 min.

The third type of micro two-dimensional electrophoresis was performed by a modification of the method of Anderson and Anderson (17). A 20-μL serum sample that contained 400 g of sucrose per liter was mixed with 20 μL of a "detergent solution" containing, per liter, 40 g of SDS and 50 mL of 2-mercaptoethanol. The mixture was heated at 95 °C for 5 min, then centrifuged at 10,000 × g for 3 min. We applied 0.5 μL of the mixture and isoelectric focusing was run in capillary gels, which contained 5 μl of urea and 20 g of NP-40 per liter in addition to the gel constituents of the type a focusing gels, at 0.1 mA per tube constant current until the voltage reached 300 V (about 40 min), then at 300 V constant voltage for 80 min. The focusing gels were equilibrated for 10 min in the molds of the second-dimension slab gel, which had been filled with a buffer containing 2 g of Tris, 20 g of SDS, 50 mL of 2-mercaptoethanol, and 100 mL of glycerol per liter and pH adjusted to 6.8 with HCl. The second-dimension electrophoresis was run in micro gradient (8–17%) slab gels that contained 10 g of SDS per liter, at 10 mA per slab constant current for 40 min.

Staining. Polyacrylamide slab gels were stained with Coomassie Brilliant Blue R250 (1 g/L) in acetic acid/methanol/water (7/50/43 by vol) for 15 min and destained in acetic acid/methanol/water (7/20/73 by vol) for 2 h. SDS-containing gels were washed in acetic acid/methanol/water (7/50/43 by vol) for 15 min before staining. Silver staining was done according to Oakley et al. (19).

Electrophoretic transfer was performed as described previ-

ously, with the apparatus constructed for blotting from micro slab gels to nitrocellulose sheets (20). Immunochi-

mical staining of lipoproteins and apolipoproteins on the blots

was done according to the method of Towbin et al. (21), with

some modifications (22).

Results

As the first step in analyzing lipoproteins in the Tangier
erum, we ran micro two-dimensional electrophoresis type a
(no denaturing agents). Because the serum samples are not

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with detergents, the chylomicrons and very-low-
density lipoproteins (VLDL) in serum, with their extremely

large molecular size, cannot move into the focusing gels.

Therefore, HDL and low-density lipoproteins (LDL) can

be analyzed by this technique. Figure 1 (A and B) shows

the protein patterns of a normal serum and the Tangier serum,

respectively. When the gels were stained with Coomassie

Brilliant Blue R-250, HDL in normal sera were reproducibly

observed as broad bands with isoelectric pH at 4.5–5.5 and

apparent relative molecular mass of 70 000–500 000 (Figure

1A). The locations of various serum proteins, including

HDL, have been determined by blotting-immunochi-

mical staining (22). In case of the Tangier serum, the broad
gobserved bands of normal HDL were not observed by dye

staining (Figure 1B). LDL were reproducibly detected at pl

about 6 and had an apparent relative molecular mass of about

1 000 000, both for normal and Tangier sera. Several spots not
detected in the patterns of normal sera were observed in the pattern

of the Tangier serum. Figure 2, A and B, illustrate enlarged

sections of the gels shown in Figure 1, A and B, respectively.

The spots arrowed in Figure 2B were normally not observed,

Fig. 1. Micro two-dimensional electrophoretic patterns of human serum proteins separated in the absence of denaturing agents.

Gels stained with Coomassie Blue. A, serum from a normal subject; B, serum from the Tangier patient. HDL was not observed in B.

Fig. 2. Enlarged sections of the patterns of human serum proteins.

The region of acidic and low-molecular-mass proteins are shown. A, serum from a normal subject; B, serum from the Tangier patient. Arrows in B indicate the protein spots characteristic of the Tangier serum.

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but were clearly detectable in the Tangier serum. To identify these proteins, we used the technique of electrophoretic transfer followed by immunochemical staining.

Figure 3A shows the pattern for HDL in normal serum. Serum proteins separated two-dimensionally were transferred to a nitrocellulose sheet and the HDL were immunochemically stained. HDL was distributed from pI 4.5 to 5.5 and apparent relative molecular mass 70 000 to 500 000. When Tangier serum proteins were immunochemically stained by using anti-HDL, the positions corresponding to normal HDL were only faintly stained and about 10 spots appeared at pI 4.5–5 and apparent relative molecular mass between 20 000 and 80 000 (Figure 3B). These results suggested that, in the Tangier serum, high-molecular-mass lipoprotein complexes of HDL cannot be constructed because of the abnormally low concentrations of apoA-I and apoA-II (6) and can only be present as the low-molecular-mass lipoproteins. Because the antiserum to HDL showed high reactivity to apoA-I and apoA-II, but not to apoC, the pattern in Figure 3B corresponds to the distribution of apoA-I plus apoA-II in the Tangier serum. We examined the distribution of apoC-II and apoC-III, using specific antisera.

Figure 4 shows the locations of lipoproteins that contain apoC-II and apoC-III, stained immunochemically after blotting. In normal serum, apoC-II and apoC-III were detected as broad bands, proving that they are the constituents of HDL (Figure 4A, B), although they are localized at the relatively acidic part of HDL complexes. Some part of apoC-III was also present as spots at pI 4–5 and apparent relative molecular mass about 60 000. In the Tangier serum, both apoC-II and apoC-III were not present at the position of normal HDL. ApoC-II was detected as two spots at pI 4.2–4.5, apparent relative molecular mass about 60 000. ApoC-III was present as multiple spots in the region of pI 3.8–4.8 and apparent relative molecular mass 20 000–80 000. The four spots of apoC-III arrowed in Figure 4D overlapped with the four spots in Figure 2B, diagonally distributed from pI 3.8 to 4.5.

We analyzed the apolipoprotein composition in the Tangier low-molecular-mass lipoproteins by micro two-dimensional electrophoresis type b (above), using SDS in the second dimension. Figure 5 (A and B) show the dye-stained patterns for normal and Tangier serum proteins, respectively. Because the same conditions were used for isoelectric focusing as in the gels shown in Figure 1, the pI distribution of the serum proteins is the same as in Figure 1. When SDS was present only in the second dimension, then lipoprotein complexes such as HDL dissociated into their constituent apolipoproteins. Figure 5 summarizes results of immunochemical identification of apolipoproteins, some of which are shown in Figures 6 and 7. In the case of normal serum, apolipoproteins A-I, A-II, C-II, and C-III were mainly distributed at pI 4.5–5.5, showing that they constitute HDL. However, apolipoproteins in the Tangier serum showed discrete pI distribution: apoA-I was at pI 5, apoA-II mainly at pI 4.7, apoC-II at pI 4.5, and apoC-III mainly at pI 4.2–4.4. These results are in harmony with those (Figures 3 and 4) showing that apolipoproteins in the Tangier serum do not form HDL complexes but are present as separate spots in the acidic and low-molecular-mass region. Low relative quantities of apoA-I and apoA-II at the pI range of normal HDL were obvious in the pattern of the Tangier serum. ApoC-III was more abundant in the Tangier serum than in normal sera. Apolipoproteins B and H, which are not
shown in Figure 8 make visible all the apolipoproteins in serum lipoproteins. Low proportions of apoA-I and apoA-II, together with a high proportion of apoC-III, are seen for the Tangier serum. These results are consistent with those of the apolipoprotein composition of the fraction of Tangier low-molecular-mass lipoproteins (Figure 5).

Discussion

Tangier disease is characterized by a marked deficiency of HDL. Lux et al. (23) found that HDL isolated from Tangier-disease patients migrated differently from HDL from control subjects when subjected to immunoelectrophoresis, and they designated them HDL, HDL, reported to contain a high proportion of apoA-II to apoA-I, which is distinctly different from that of normal HDL. Assmann et al. (3) isolated HDL particles containing only apoA-II from Tangier plasma by ultracentrifugation followed by column chromatography. These results dealt with Tangier HDL obtained by ultracentrifugation, but HDL can also be analyzed without separating them from the serum, by two-dimensional electrophoresis in the absence of denaturants.

As shown in Figure 3, lipoproteins in the Tangier serum that contained apoA-I and apoA-II differ distinctly from normal HDL in the distribution in molecular size. Analysis of the apolipoprotein content in these lipoproteins (Figure 5, 6) revealed a high ratio of apoA-II to apoA-I. The presence of a lipoprotein species that contains only apoA-II and has a pl value of 4.5 was also detected (Figure 6B). These results coincide with former reports on Tangier HDL.

The presence of low-molecular-mass lipoproteins in the serum of Tangier homozygotes has been suggested (24, 25), because substantial quantities of apoA-I have been detected in the 1.21 g KBr per milliliter infranatant. The low-molecular-mass lipoproteins we detected in the Tangier serum (Figures 3, 4) included not only those that contain apoA-I but also those containing only apoC-II or apoC-III (Figures 5, 7).

It has been proposed (8) that the abnormally low proportion of apoA-I in plasma from Tangier patients is ascribable to a structural mutation of proapoA-I that causes defective conversion to mature apoA-I. However, no structural defect was observed in Tangier proapoA-I (11, 12). The increased rate of apoA-I catabolism in Tangier patients (6, 7) can explain the low proportion of apoA-I. It is noteworthy that more apoC-III was present in the Tangier serum than in normal serum (Figure 8). Because apoC-III is one of the constituents of HDL, the change in the quantity of apoC-III constituents of HDL, showed no marked difference in their pl distribution.

Some examples of the immunochemical identification of apolipoproteins are shown in Figures 6 and 7. Low proportions of apoA-I and apoA-II in the fraction of Tangier low-molecular-mass lipoproteins were seen (Figure 6B). Tangier apoC-III was stained as four spots, two dense and two faint, as shown in Figure 7D. The pl distribution was consistent with the results shown in Figure 4D.

By the two techniques of micro two-dimensional electrophoresis described above, LDL, HDL, and low-molecular-mass lipoproteins could be analyzed. However, chylomicrons and VLDL could not be analyzed because they do not move into the focusing gels under the conditions we used. To obtain information on apolipoproteins in the whole serum, we used two-dimensional electrophoresis type c (denaturants used throughout sample treatment), isoelectric focusing, and gradient gel electrophoresis (17). The patterns
in Tangier serum may cause displacement of apolipoproteins in HDL and then irregular catabolism of HDL.

Multiple spots of Tangier low-molecular-mass lipoproteins can be partly explained by the difference in their apolipoprotein compositions and by the presence of sialylated apolipoprotein isoforms. Detailed identification of each lipoprotein awaits further investigation.

We used here three different types of two-dimensional electrophoresis. The properties of lipoproteins in physiological conditions can be analyzed by technique a (denaturants absent in both dimensions, Figures 1–4). The apolipoprotein constituents in the lipoproteins can be analyzed by technique b (no denaturant in the first dimension isoelectric focusing but SDS in the second dimension gradient gel electrophoresis, Figures 5–7). The total contents of lipoprotein polypeptides can be studied by technique c (urea–NP-40 in the first dimension and SDS in the second dimension, Figure 8). The micro gel system is suited for comparative studies of lipoproteins in multiple serum samples, because electrophoresis time is relatively short and the resolution suffers for the analysis of lipoproteins and apolipoproteins. The combination of the three types of micro two-dimensional electrophoretic technique will be useful for the analysis of the sera of other Tangier patients and of patients with various lipoproteinemia.

References
