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HUMAN PROAPOLIPOPROTEIN A-I: DEVELOPMENT OF AN ANTIBODY TO THE PROPEPTIDE AS A PROBE OF APOLIPOPROTEIN A-I BIOSYNTHESIS AND PROCESSING

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SUMMARY: In human plasma, apolipoprotein A-I is present as pro and mature isoproteins. The development of a highly specific antibody to the pro isoprotein of apoA-I has been difficult due to the close structural similarity between the pro and mature isoforms of apoA-I. To sermount this difficulty, a peptide was synthesized by the solid phase method which corresponded to the amino acid sequence present in the pro region of apoA-I. The synthetic peptide was coupled to serum albumin and the conjugate utilized to immunize rabbits for antibody production. Immumoblot analysis of purified proapoA-I and mature apoA-I revealed that the antibody was specific for the propeptide of apoA-I. Analysis of apoA-I in the plasma from a Tangier disease patient and newly secreted apoA-I from HepG2 cells clearly demonstrated the isoforms which contained the proisoprotein. The proapoA-I specific antibody should prove to be a useful tool in developing a radioimmunoassay for quantitation of the proisoprotein in plasma, isolation of proapoA-I from normal and dyslipoproteinemic subjects by immunoaffinity chromatography and in studies related to the synthesis and processing of apoA-I.

Human plasma apolipoprotein A-I is the principal protein constituent of high density lipoproteins. Several epidemiological studies have demonstrated an inverse correlation between plasma HDL levels and the risk of atherosclerosis (1-3). However, the regulatory factors involved in the synthesis and secretion of HDL are not yet fully understood. The roles of apoA-I and HDL are of major interest in lipid metabolism and has been the subject of intense investigation.

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The abbreviations used are: apo, apolipoprotein; HDL, high density lipoproteins; HEPES, N-2-hydroxyethylpiperazine-N-2-ethanesulfuric acid; BSA, bovine serum albumin; PBS, phosphate buffered saline.

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In plasma, apoA-I is polymorphic (4-6). ApoA-I is synthesized as a preproapolipoprotein, and the 18 amino acid presegment is cotranslationally cleaved inside the cell and the proapoA-I is released into the plasma (7-9). The prohexapeptide of apoA-I is then cleaved by a specific calcium dependent plasma protease and converted into mature apoA-I (10,11). Several mutants of apoA-I have been identified in patients with lipid disorders (12-15) however the relationship of these mutants to the dyslipoproteinemia has not been definitely established. The processing of proapoA-I to mature apoA-I has been shown to be different in Tangier disease. These subjects have very low plasma levels of apoA-I as well as HDL, and have an increase in the ratio of the proisoprotein to mature isoprotein when compared to normal subjects (12, 16).

Synthetic peptides as antigens provide a new method for the development of antibodies of defined specificity (17). The present study describes utilization of this approach to develop an antibody specific to the prohexapeptide of the apoA-I protein. The proapoA-I antibody was used as a probe to study the synthesis and processing of apoA-I in HepG2 cells grown in culture and in the plasma of Tangier disease subjects.

MATERIALS AND METHODS

Peptide Synthesis and Purification: A 13 residue peptide (RHFWQQRHFWQQC) designated MDB-13 was synthesized by the solid phase method (18) utilizing the Beckman 990B peptide synthesizer. After completion of the synthesis, the peptide was cleaved from the resin by hydrogen flouride at 0°C in the presence of scavangers. The crude MDB-13 peptide was initially chromatographed on a Bio Gel P-2 column (2x85 cm) equilibrated with 0.1N acetic acid. The MDB-13 peptide was purified by reversed phase HPLC on a C_{18} uBondapack column using a programmed binary solvent gradient of aqueous trifluoroacetic acid (0.1%) and acetonitrile (15-70%). The purified peptide utilized in the present study eluted as a single peak and was characterized by amino acid and sequence analysis.

Coupling of Peptide to Carrier Protein and Immunization Protocol: The carrier protein BSA (5mg) was dissolved in HEPES buffer (HEPES 0.15M, EDTA 1mM, pH 7.6) and initially coupled to a heterobifunctional reagent m-maleimido-benzoyl-N-hydroxy succinimide ester (19). The excess reagent was removed by passing through a PD-10 desalting column. HPLC

purified MDB-13 containing a cysteine residue was added to the modified BSA and coupled for 3h at room temperature. The peptide-BSA conjugate was dialyzed against phosphate buffered saline (pH 7.4) to remove unconjugated peptides. Rabbits were immunized with 500 µg of peptide conjugate in complete Freud's adjuvant followed by three injections at 10 day intervals in incomplete Freud's adjuvant.

Purification of Antibodies: Antibodies were initially purified by ammonium sulfate precipitation (33% saturation) followed by protein A Sepharose column chromatography. The antibodies were then passed through an affinity column of CNBr activated Sepharose 4B coupled to the synthetic peptide. The bound antibodies were eluted with 0.1M glycine HCl buffer at pH 3.0 and the pH of the eluant was adjusted to 7.0 by addition of 0.5M Tris solution. The antibodies were stored in PBS containing 0.001% merthiolate at 4°C.

Polyacrylomide Gel Electrophoresis and Immunoblot Analysis: Isoelectrofocusing and analytical two dimensional gel electrophoresis was performed as previously described (20). After gel electrophoresis, the protein samples were electrophoretically transferred to nitrocellulose paper. After blocking the remaining active sites with gelatin (3%), the nitrocellulose paper was reacted with the first antibody (1:300 dil) for 18h at 4°C. The second antibody used was GAR-HRP conjugate (1:300 dil). Color was developed by using the chromogenic substrate 4-chlor-1-naphthol.

RESULTS AND DISCUSSION

In plasma apoA-I exhibits polymorphism. The functional significance of different isoforms of apoA-I is as yet not established. In order to understand the synthesis and processing of apoA-I, an antibody recognizing only the proform of apoA-I could be an extremely useful tool. To develop a site specific apoA-I antibody has been difficult due to the close structural similarity between the pro and mature isoprotein of apoA-I. In the present study a proapoA-I antibody was prepared using a synthetic propeptide segment of apoA-I as an antigen. For this purpose, a 13 residue peptide, designated MDB-13 (RHFWQQRHFWQQC) was synthesized by the solid phase procedure. The design of the peptide included a repeat of the hexapeptide (RHFWQQ) to increase the length of the peptide to enhance antigencity and the addition of a cysteine residue for coupling of the synthetic peptide MDB-13 to the carrier protein BSA. The MDB-13 peptide purified by HPLC was coupled to BSA employing the heterobifunctional reagent m-maleimidobenzoyl-N-hydroxysuccinimide and antibodies were raised in

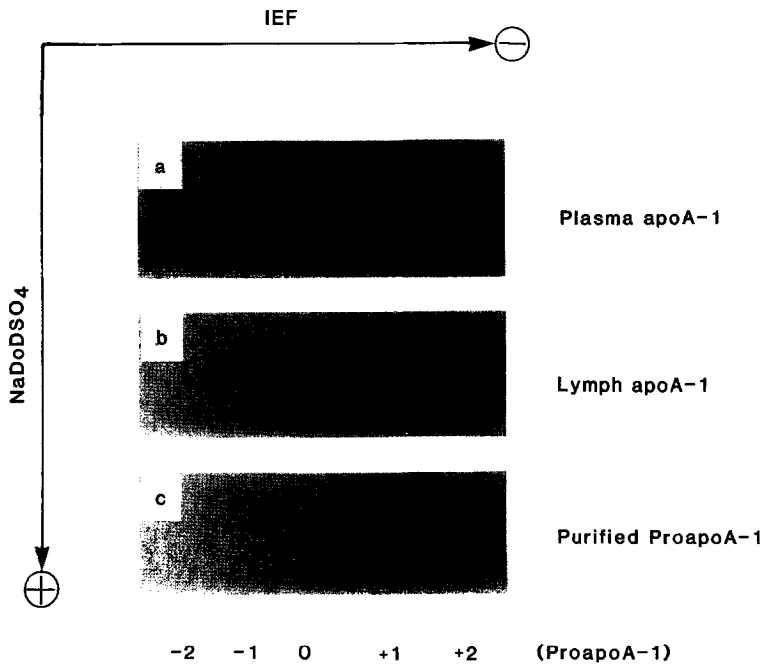

Figure 1. Two dimensional gel electrophoresis of human apoA-I. The mature isoform is specified as apoA-I_o and the proisoprotein as apoA-I_{+2}. The minor isoforms apoA-I_{+1} and apoA-I_{-1}/apoA-I_{-2} are primarily deamidated proapoA-I and mature apoA-I respectively.

rabbits. Antibodies were purified by a protein A column followed by immunoaffinity chromatography using MDB-13 as the ligand.

Fig. 1 illustrates the pattern of the major and minor isoforms of human apoA-I present in plasma HDL and in the thoracic duct lymph ($d<1.006$ g/ml) of a subject after fat feeding. The two major forms of apoA-I in normal plasma are the mature forms designated apoA-I_o and apoA-I_{-1}, (Fig. 1a). $ApoA_{+2}$ (pro) becomes a major form in lymph after fat feeding (Fig 1b). The previous studies conducted in our laboratory (20,21) have established that apoA-I_o, apoA-I_{-1} and apoA-I_{-2} are all mature isoforms of apoA-I and apoA-I_{+2} represent the proform of apoA-I. Fig. 1c indicates the position of the proisoform, apoA-I_{+2} in two dimensional gel system.

The specificity of the proapoA-I antibody was evaluated by immunoblot analysis of purified pro and mature isoforms of apoA-I. The isolated apoA-I isoproteins were initially separated on a

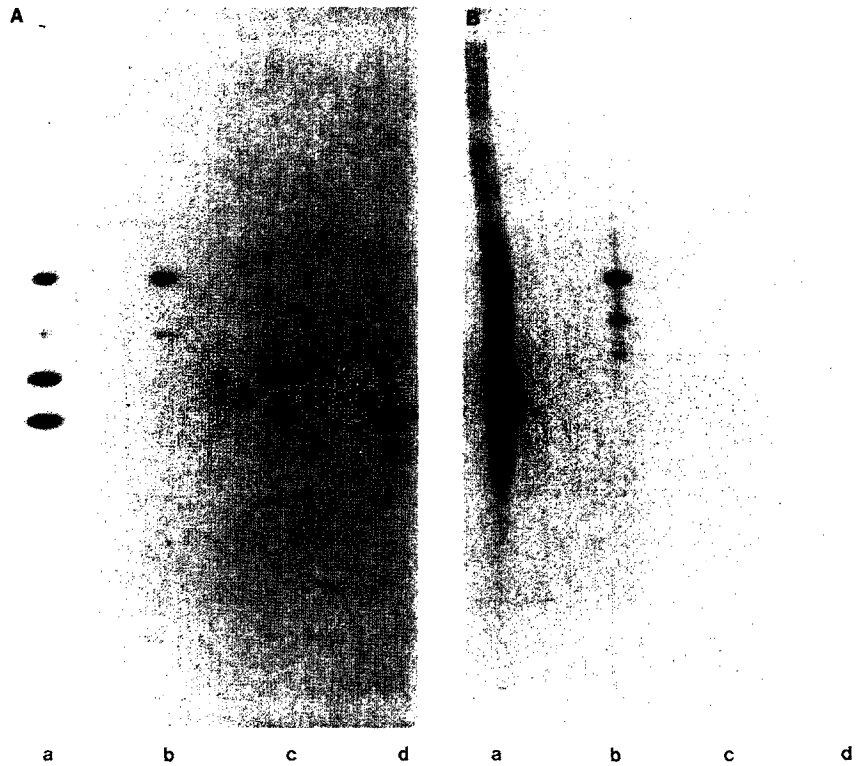

Figure 2. Isoelectrofocusing and immunoblot analysis of isolated pro and mature isoproteins of apoA-I. Lane a, mixture of pro and mature isoproteins of apoA-I (2 µg each); Lane b, proapoA-I (2 µg); Lanes c and d, mature isoproteins of apoA-I (2 µg). A. Isoelectrofocusing of apoA-I isoproteins stained with Coomassie blue. B. Immunoblot analysis of apoA-I isoproteins: Lane a, analysis with polyclonal apoA-I antibody; lanes b, c and d, with the proapoA-I antibody.

isoelectrofocusing slab gel and transferred to nitrocellulose paper (Fig. 2). In Fig 2A, each of the isolated isoforms of apoA-I stained with Coomassie blue showed additional bands. These additional bands are attributed to the deamidation or carbamylation of apoA-I during its isolation or storage (22). On immunoblot analysis as illustrated in Fig. 2B, the polyclonal antibody recognized all the isoforms of apoA-I (Fig 2B, lane a). In contrast, the proapoA-I antibody recognized only the proapoA-I (Fig 2B, lane b) and did not react with the mature isoproteins of apoA-I (Fig 2B, lanes c, d). Additional studies have also indicated that the reactivity of the proapoA-I antibody can be completely abolished by preincubating with the synthetic MDB-13 peptide (data not

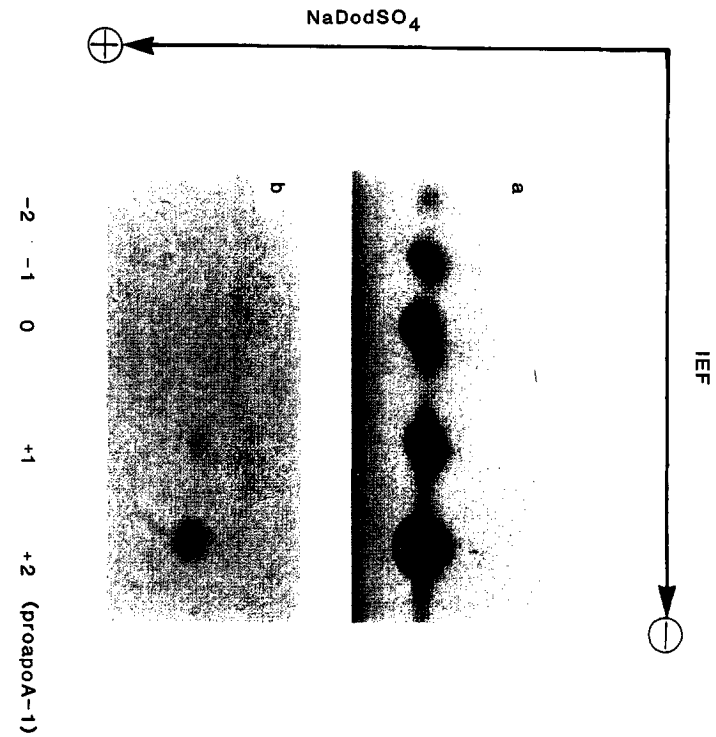

Figure 3. Immunoblot analysis of newly secreted isoforms from human HepG2 cells cultured in vitro. The apoA-I isoproteins were isolated by ultracentrifugation of the media at density 1.21 g/ml and delipidated. a. Stained with Coomassie blue. b. Immunoblot analysis using the proapoA-I antibody.

shown). Taken together, these results clearly demonstrate that the proapoA-I antibody recognizes specifically the prohexapeptide sequence of apoA-I.

The functional application of this site directed antibody was further investigated by two dimensional polyacrylamide gel electrophoresis of apoA-I present in HepG2 cell culture media and the plasma of a Tangier disease subject. Figure 3a illustrates the newly secreted isoforms of apoA-I in the medium of HepG2 cell culture. The proapoA-I specific antibody recognized two isoproteins present at the position of apoA-I_{+1}, and apoA-I_{+2} (Fig. 3b). These results indicate that HepG2 cells secrete both the pro and mature isoproteins of apoA-I. It also confirms that both apoA-I_{+1} and apoA$_{+2}$ are proisoforms and apoA-I_{+2} is the precursor for apoA-I_{+1} probably due to deamidation. It is consistent with the earlier observation that apoA-I_o is the precursor

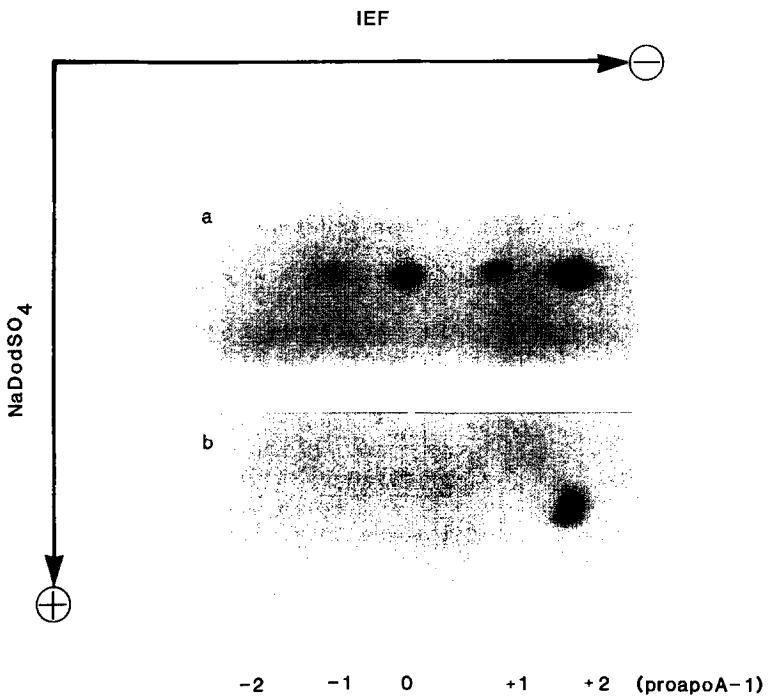

Figure 4. Immunoblot analysis of plasma (3 µl) from a Tangier disease patient. a. Probed with polyclonal apoA-I antibody; b. Probed with the proapoA-I antibody.

for other mature isoforms apoA-I_{-1} and ApoA-I_{-2} and apoA-I_{+1} is generated from the precursor proisoform apoA-I_{+2}. Since HepG2 cell medium contains mature isoforms of apoA-I, it is likely that these cells synthesize the proapoA-I peptidase which converts pro apoA-I to mature apoA-I. It also raises the interesting possibility that the proapoA-I peptidase activity observed in plasma may be synthesized in the liver.

The immunoblot analysis of plasma (3µl) from a Tangier disease subject is illustrated in Figure 4. The polyclonal apoA-I antibody recognized both the pro and mature isoforms of apoA-I (Fig. 4a) whereas the proapoA-I antisera reacted only with the two proforms of apoA-I (Fig 4b).

These studies illustrate the usefulness of proapoA-I antibody in the analysis of apoA-I biosynthesis and provide the opportunity for a radioimmunoassay for the quantitation of proapoA-I in plasma samples. The monospecific proapoA-I antibody also now permits the isolation of

proapoA-I from the plasma of normal and dyslipoproteinemic subjects by immunoaffinity chromatography. In general, the approach described here can be applied to the development of propeptide specific antibody for other secretory proteins. The site directed antibody for proapoA-I should prove to be a valuable tool in understanding the physiological importance of the propeptide and whether the propeptide provides a signal for intracellular targeting and processing of specific apolipoproteins.

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