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APOLIPOPROTEIN C-II DEFICIENCY: IDENTIFICATION OF A STRUCTURAL VARIANT ApoC-II$_{PADOVA}$

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SUMMARY: Apolipoprotein(apo) C-II DNA, RNA and protein from a patient with a familial deficiency of apoC-II were evaluated and compared to normal individuals. No major defect of the apoC-II gene could be detected by Southern blot hybridization. Northern and slot blot analyses of total liver RNA documented normal levels of a normal sized apoC-II mRNA. Immunohistochemical studies of the liver of the apoC-II deficient patient revealed a normal to slightly elevated intracellular content of the C-II apolipoprotein. Plasma apoC-II was 3 to 5% of normal apoC-II levels and exhibited abnormal electrophoretic mobility on two dimensional gel electrophoresis and immunoblotting. We postulate that at the molecular level, the deficiency of apoC-II in the plasma of this patient results from a structural defect in the coding portion of the apoC-II gene leading to either defective secretion of cellular apoC-II or increased catabolism of a structurally defective apoC-II in plasma.

Familial deficiency of apoC-II is a rare disease inherited as an autosomal recessive trait. Patients homozygous for the disease may present with lipemia retinales, eruptive xanthomas, and pancreatitis (1-7). The syndrome is also characterized by elevated fasting triglycerides and chylomicrons, decreased LDL and HDL concentrations, and a type I lipoprotein phenotype. The diagnosis of apoC-II deficiency is confirmed by finding reduced or absent levels of apoC-II and the absence of post-heparin plasma lipoprotein lipase activity which may be corrected by the addition of normal apoC-II containing plasma. Patients with near normal levels of a nonfunctional apoC-II varient are given the same diagnosis. Infusions of normal plasma (1,3,7), isolated apoC-II fractions (5), or synthetic apoC-II fragments (7) result in transient normalization of plasma triglycerides and lipoproteins in these patients. The marked aberration of triglyceride metabolism evident in patients with a deficiency of apoC-II has served to underscore the importance of apoC-II as the physiological activator of lipoprotein lipase.

Of the various families with a defect in apoC-II that have been described, an abnormal plasma apoC-II variant has been isolated and the protein defect

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determined in two kindreds. In apoC-II$_{Toronto}$, the nonfunctional apoC-II protein is present at approximately normal levels and has a different amino acid sequence at positions 69-74 as well as loss of amino acids 75-79 when compared to normal apoC-II (8). ApoC-II$_{St. Michael}$ contains a proline instead of a glutamine at position 70 and the abnormal apoC-II sequence is extended 17 residues past the normal carboxyl-terminal amino acid (9). These abnormalities are most consistent with a base deletion for apoC-II$_{Toronto}$ and a base insertion for apoC-II$_{St. Michael}$ resulting in a subsequent shift of the translation reading frame. The nature of the molecular defect in the other apoC-II deficient families is as yet unknown but it is likely that the syndrome will be found to be a heterogeneous disorder at the molecular level.

In the present study we evaluate the molecular defect in one of the apoC-II deficient probands of the kindred from Padova (7).

METHODS

Experimental Subject:

The apoC-II deficient patient (SF) is 1 of 2 affected individuals from the Padova kindred described previously (7).

RNA Preparation:

Liver tissue was obtained from the patient during open abdominal surgery for cholecystectomy, and from control subjects at the time of organ donation. Tissue was stored at -70°C until used. RNA was isolated utilizing the guanidine thiocyanate method as previously described (10).

Complementary DNA (cDNA) Probes:

A 354 base pair AluI restriction fragment of an apoC-II cDNA clone (11) was utilized for our studies. For positive controls, an 850 base pair MspI restriction fragment of an apoA-I clone (12) and a 412 base pair fragment from a DraI and RsaI double digest of a 3'-untranslated β-actin cDNA clone (13) were used. The β-actin cDNA clone was kindly provided by Dr. Lawrence Kedes.

Northern and Slot Blot Hybridization Analyses of RNA:

Gels for Northern blot analysis were prepared with 1% agarose in the presence of 6% formaldehyde, electrophoresed at 25 volts for 16 hours, and transferred to nitrocellulose filters (Schleicher and Schuell, Inc., Keene, N.H.) as described previously (14). Eight µg of total RNA were analyzed and gels were stained with ethidium bromide to confirm that equivalent quantities of RNA were loaded in each lane.

For slot blot analysis, serial dilutions of total RNA, (3.0, 2.0, and 1.0 µg) were loaded in duplicate onto nylon filters (Gene Screen Plus, NEN/Dupont, Boston, MA) using a slot blot apparatus (Bethesda Research Labs, Gaithersburg, MD.). Baking, prehybridization, and hybridization conditions were as previously described except that hybridization was performed at 42° for 24 hrs (15). Filters were autoradiographed and the blots were quantitated using a laser densitometer (Ultrascan XL, LKB Instruments, Bromma, Sweden). The absorbancy values were normalized with the mean of the values for normal RNA being assigned a value of 1. For re-use, filters were stripped of radiolabelled probe by incubation in 0.1 x SSC (30mM citrate, 0.3M NaCl, pH 7.0)/1% NaDodSO$_4$ at 90°C for one hour.

In Situ Hybridization:

Frozen sections were prepared as described for immunohistochemical studies. An AluI cDNA apoC-II fragment was ligated into the SmaI cloning site of PGem 3 vector DNA (Promega, Madison, WI.), and radiolabelled sense and antisense strands were synthesized by using T7 and SP6 DNA polymerases (Promega, Madison, WI.), respectively (16). In situ RNA hybridization was performed as previously described (16).

Immunohistochemistry:

Frozen sections (8μm thick) were prepared from liver biopsies of normal and apoC-II deficient individuals. The liver tissue was embedded in OCT compound, (Miles Scientific, Naperville, Il.) sectioned and stored at -70°C until used. Specimens were incubated at room temperature for 30 minutes with anti-apoC-II mouse monoclonal antibodies or an irrelevant monoclonal antibody derived from a neuroblastoma (HSAN 1.2, a gift of C Patrick Reynolds, Naval Medical Research Institute) followed by incubation with the colloidal gold linked secondary antibody (Auro Probe 1M LM, Jansen Life Sciences Products, Olen, Belguim) at room temperature for 30 minutes. Silver enhancement was as described by the manufacturer (Intergrated Separation Systems, Hyde Park, MA). Following counter staining with Hanes' haematoylin, the slides were evaluated for staining with brown or black pigment indicative of a positive reaction.

Characterization of the Plasma ApoC-II Protein:

Two-dimensional gel electrophoresis consisting of isoelectric focusing followed by $NaDodSO_4$ gel electrophoresis was performed as described previously (17). The gels were stained by the silver stain method (18,19). The proteins separated by $NaDodSO_4$ gel electrophoresis were transferred to nitrocellulose paper at 80V for 1 hr. (20). ApoC-II was detected by utilizing monospecific rabbit apoC-II antisera as the first antibody and visualized by indirect immunoperoxidase assay on the nitrocellulose paper as described by the manufacturer's (BioRad, Inc. Richmond, CA.) instruction. Plasma apoC-II concentrations were kindly determined by Dr. Moti Kashyap.

RESULTS

Previous Southern blot analyses of the DNA from this apoC-II deficient proband have demonstrated that there are no major rearrangements of the apoC-II gene (11,12). Northern blot analysis of total liver RNA revealed the apoC-II mRNA of the patient to be of normal size and apparent quantity when compared to total RNA from normal subjects (Fig 1C). Stripping and rehybridization of the blot, with nick translated apoA-I and β-actin cDNA probes followed by autoradiography revealed that nearly equal levels of both of these mRNAs were present in hepatocytes from both the apoC-II deficient patient and normal subjects (Fig. 1B, 1A). Ethidium bromide staining of the gel confirmed that equivalent amounts of nucleic acid had been loaded in all lanes (data not shown).

Further quantitation of the level of apoC-II mRNA in the apoC-II deficient patient's liver was obtained by slot blot and *in situ* RNA hybridization. Slot blot analysis was performed on duplicate samples of 3 different quantities of RNA. As illustrated in Fig. 2, the level of the hepatic apoC-II mRNA from the patient was within the range observed in the mRNA present in control subjects. The levels of apoA-I and β-actin mRNAs were similar in both the apoC-II deficient patient and the normal subjects, confirming the results obtained with Northern blot analysis (data not shown). Similar levels of apoC-II message detected as black grains by *in situ* hybridization are present in normal liver (Fig. 3B) and the liver of the apoC-II deficient patient (Fig. 3C) when hybridized with the apoC-II antisense RNA. Figure 3A illustrates normal liver hybridized with the apoC-II sense RNA as a negative control.

Immunohistochemistry was performed using an apoC-II monoclonal antibody and the immunocolloidal gold procedure. Hepatic tissue from the apoC-II patient as

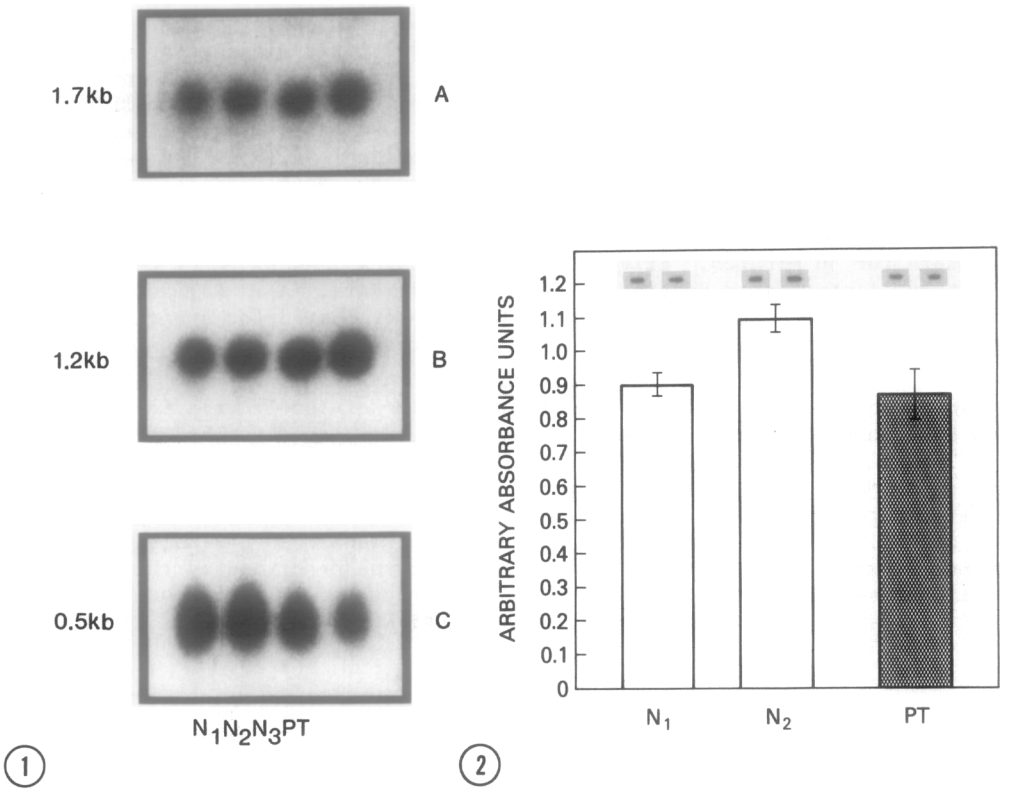

Figure 1: Northern blot analyses of total RNA from normal (N1, N2, N3) and patient (PT) liver hybridized with apoC-II(C), apoA-I(B) or β-actin (A) cDNA probes.

Figure 2: Quantitation of total hepatic apoC-II mRNA from two normal controls (N1, N2), compared to the apoC-II deficient patient (PT) by slot blot analysis. All values were normalized to the mean of the control values being equal to one. Error bars are standard deviation of the respective mean. Representative autoradiographs of the slot blot studies are included at the top of the figure.

well as normal controls were studied. Normal liver, when incubated with an irrelevant monoclonal antibody (Fig. 4A) showed no cytoplasmic staining. When the apoC-II monoclonal antibody was used as the primary antibody on normal liver, significant cytoplasmic staining of hepatocytes was noted (Fig. 4B). The liver specimen from the apoC-II deficient patient contained diffuse cytoplasmic brown staining of apoC-II protein which was normal to slightly more intense than that observed in normal hepatocytes (Fig. 4C). Specificity of the procedure was also demonstrated by the lack of staining within connective tissue which is not expected to contain the C-II apolipoprotein (Fig. 4B).

Analysis of plasma for apoC-II was performed by two dimensional gel electrophoresis and immunoblotting with a monospecific rabbit anti-apoC-II polyclonal antibody. Fig. 5A and 5B illustrate the pattern of apolipoproteins present in the plasma, as detected by silver staining of normal and apoC-II deficient subjects, respectively. The absence of electrophoretically normal

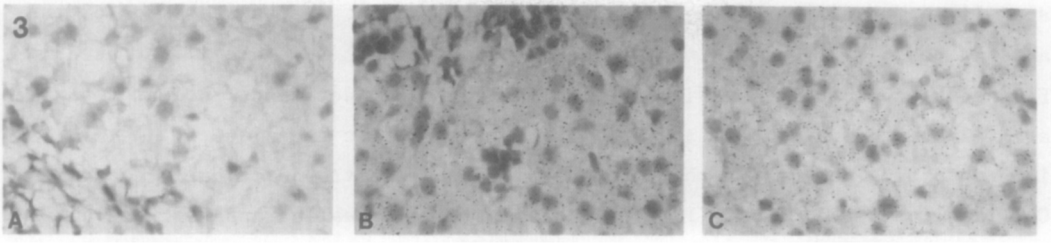

Figure 3: In situ RNA hybridizations of normal liver (B) and liver of the apoC-II deficient patient (C) hybridized with the apoC-II antisense RNA probe. Normal liver hybridized with a apoC-II sense RNA probe (A) is included as a negative control.

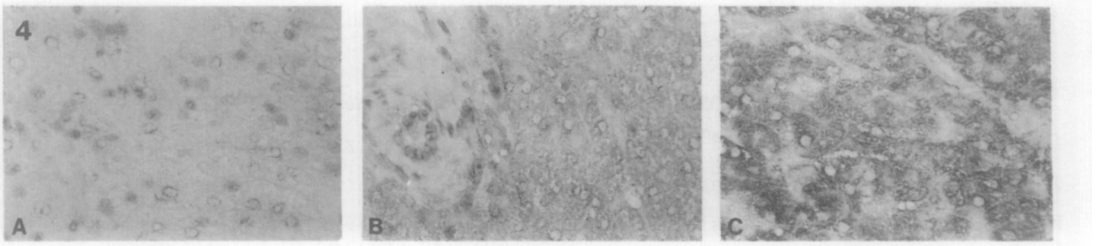

Figure 4: Immunohistochemical analysis of normal liver incubated with irrelevant antibody (A) and with an anti-apoC-II monoclonal antibody (B). Normal or slightly increased cytoplasmic staining is present in the liver of the apoC-II deficient patient following incubation with the anti-apoC-II monoclonal antibody (C).

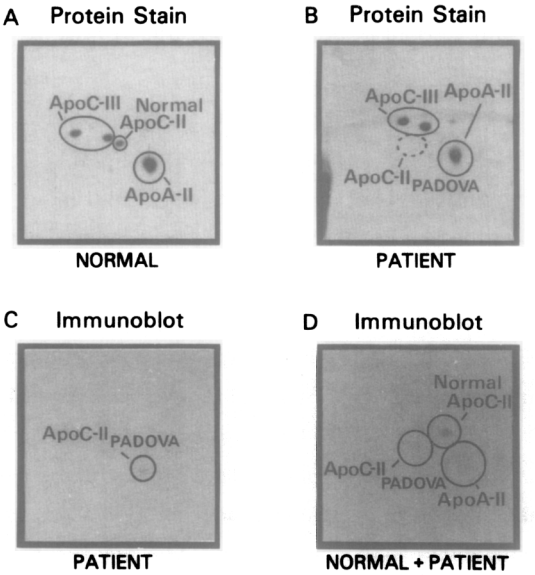

Figure 5: Two-dimensional gel electrophoretograms of plasma from a normal subject or/and the patient with apoC-II deficiency. Electrophoretograms in panels A and B are stained with silver stain, and panels C and D are immunoblots utilizing a monospecific anti-apoC-II antibody. Panel D contains a mixture of plasmas from a normal subject and the apoC-II deficient patient as well as an apoA-II standard to facilitate the comparison of the electrophoretic position of normal and apoC-II$_{Padova}$.

apoC-II in the plasma of the apoC-II deficient patient and the position of the abnormal apoC-II_{Padova} protein indicated by a dotted circle are illustrated in Figure 5B. Immunoblot studies of normal and apoC-II deficient patient's plasma are illustrated in Fig. 5C and 5D. The more sensitive technique of immunoblotting detects in the patients plasma an electrophoretically abnormal C-II apolipoprotein (apoC-II_{Padova}) that exhibits a smaller apparent molecular weight and more acidic pI than normal apoC-II. Mixing experiments of normal and the apoC-II deficient patient's plasma confirmed that the apoC-II_{Padova} protein exhibited a different electrophoretic mobility than the normal apoC-II protein (Fig. 5D). ApoC-II plasma levels by RIA were 0.13 mg/dl with normal values being 5.18 $\pm$ 0.3 mg/dl.

DISCUSSION

We have analyzed the DNA, RNA and protein from the apoC-II deficient proband from a previously described kindred (7). We and others (11,21) have previously identified no major rearrangements of the apoC-II gene by Southern blot hybridization. Northern, slot blot and *in situ* hybridization studies established that the hepatic apoC-II mRNA was of normal size and quantity. Normal to slightly increased levels of apoC-II could be detected by immunohistochemistry in the hepatocytes of this patient. Plasma analysis by 2-dimensional gel electrophoresis and immunoblotting revealed small quantities of an apolipoprotein with aberrant electrophoretic mobility (7). The plasma apoC-II concentration was 3-5% of controls. Thus, this patient has normal quantities of apoC-II mRNA and protein in the liver but markedly reduced concentrations of an abnormal C-II apolipoprotein in plasma.

These findings are most consistent with a DNA mutation in the apoC-II gene that leads to either defective secretion of apoC-II_{Padova} from the hepatocyte followed by intracellular protein degradation or with normal secretion of apoC-II_{Padova} with enhanced catabolism of the abnormal apolipoprotein once secreted into the plasma. Another, less likely possibility is an abnormality in an enzyme important in the processing of the apoC-II protein resulting in a structurally abnormal apoC-II. In all cases, the intrahepatic content of both apoC-II mRNA and protein would, as observed, be normal or slightly elevated, however, plasma apoC-II levels would be decreased resulting in an inability to activate lipoprotein lipase and a type I lipoprotein phenotype. The observation that apoC-II_{Padova} has a smaller apparent molecular weight and more acidic pI than normal apoC-II suggests that the abnormality involves the coding region of the apoC-II gene. Our data clearly rule out a regulatory defect or alteration of the apoC-II gene resulting in a decreased transcription rate as the primary defect in this kindred.

In conclusion, we have analyzed DNA, RNA and protein from a patient with deficiency of an aberrant apoC-II protein. Our results indicate that this

patient has a mutation resulting in either abnormal secretion of cellular apoC-II or increased catabolism of the abnormal plasma apolipoprotein. Further studies are underway to define the precise molecular defect in this family with apoC-II deficiency.

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