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APOLIPOPROTEIN CII (APO CII) GENE EXPRESSION DEFECT IN AN INDIVIDUAL WITH FAMILIAL APO CII DEFICIENCY

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We have examined the expression of the apolipoprotein CII (apo CII) gene in an individual with familial apo CII (apo CII) deficiency. Total RNA was prepared from this patient's liver tissue and analysed in Slot Blot and Northern Blot experiments using a cloned apo CII cDNA as a probe. In this patient, there is at least a four-fold decrease in the level of apo CII mRNA, when compared to liver tissue from a control individual. The residual apo CII mRNA detected in this patient is of normal length. These results suggest that the failure to detect apo CII protein in this patient's serum is not due to a failure to transcribe or process apo CII mRNA, but probably to a defect in the translation of the apo CII message. This defect results in partial degradation of the apo CII message leading to the much reduced levels which we have observed.

There are now at least seven independent families who show familial deficiency of apolipoprotein CII (apo CII),(1-7). Patients with this disorder are characterized by high fasting levels of triglycerides, associated with a complete absence of apo CII in circulating lipoproteins. This arises because apo CII is an essential co-factor for lipoprotein lipase, which is responsible for hydrolysing the triglycerides of VLDL and chylomicrons (8,9). Apo CII deficiency is transmitted as an autosomal recessive trait (5, 10, 11,) with obligate heterozygous individuals having reduced levels of apo CII but without hypertriglyceridaemia.

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Several of these families have now been studied at the DNA level. In all patients so far examined, the structure of the apo CII gene is identical by restriction enzyme mapping, to the normal gene (6, 12, 13,). Apo CII deficiency could therefore be caused by small deletions, or by a single base mutation which reduces transcription of the gene, altering the correct splicing of the RNA or causing premature chain termination during translation. Mutations in the coding sequence of the gene may also result in a protein with altered function such as reduced lipid binding, or lipoprotein lipase activation. In several of the patients, very low levels of apo CII have been detected, (6,14,15). In some patients the protein has an altered size or charge (1, 15) and the amino acid sequence of one of these variants (apo CII Toronto) has recently been reported (16).

It is evident that, at the molecular level, apo CII deficiency is a heterogenous disorder. It is therefore possible that in some patients the defect may not be in the apo CII gene itself, but in a second gene that is involved in the correct processing or glycosylation of the protein. We have previously reported on one kindred with apo CII defiency where it was possible to use a DNA polymorphism to follow the inheritance of the apo CII gene (12). The pattern of inheritance observed strongly suggested that, in this family, the deficiency is due to a defect within, or close to the apo CII gene itself. We now report on the levels and size of the apo CII mRNA in the liver tissue of one of the affected members of this kindred.

MATERIALS AND METHODS

Tissue samples and cell lines

Liver tissue from the apo CII deficient individual was obtained at surgery and immediately frozen in liquid nitrogen and stored at -70^{0} C. Surgery was performed because of stenosis of the bile duct as a result of recurrent pancreatitis. On the

day of surgery, serum triglycerides were 17.6 m mol/l (1558 mg/dl). Control liver samples were obtained from a donor in a hepatic transplant and treated similarly.

The tumour cell lines U937 and Jurkat have been described previously (17). U937 is a human monocyte/macrophage cell line and Jurkat is an immature, but committed T cell line.

Isolation of RNA

Tissue samples: Frozen liver tissue was ground to a powder in dry ice and homogenized in Guinidium Isothiocyanate/2-Mercaptoethanol (GITC) using a hand held homogenizer. 5 ml volumes of the resulting lysate were layered onto a cushion of 5.7 M CsCl/0.1 mM EDTA and centrifuged at 28K for 48 hr in a Sorvall AH629 rotor at 20^{0}C as previously described (18). The pelleted RNA was washed in 70% Ethanol and resuspended in 500 µl of H_2O. The concentration of RNA was determined by spectrophotometry and adjusted to 5.0 µg/50 µl in H_2O.

Cell Lines: Cells were grown using standard cell culture conditions and harvested by centrifugation. Cell pellets containing 10^8 cells were dispersed directly into GITC and the resulting lysate treated as for the tissue samples.

DNA probes

The apo CII DNA probe consists of a 440 bp cDNA insert (19) cloned into the Pst I site of pKT218 (a derivative of pBR 322: 20). The apo E cDNA clone pEB4 and the mouse muscle actin cDNA probe have been previously described (21, 22). For each probe the cDNA insert was excised and labelled by the random oligonucleotide primer method (23).

Slot Blot Analysis

5 µg of RNA in 50 µl H_2O was adjusted to 6 x SSC and 20% formaldehyde in a volume of 100 µl and denatured by heating at 65^{0}C for 15 min. Doubling dilutions were carried out serially in 15 x SSC in a microtiter plate and 100 µl volumes of each dilution were applied to a Hybond-N filter (Amersham) using a Schleicher and Schuell Slot Blot manifold under vacuum from a water pump. Filters were then hybridized with radioactive probes in 50% formamide at 45^{0}C overnight. After washing at 0.2 x SSC, filters were exposed to X-ray film and examined by autoradiography. Scanning of autoradiographs was carried out on a Joyce-Loebl Chromoscan 3 scanning densitometer.

Northern Blotting

10-20 µg of total RNA was electrophoresed in a 1% formaldehyde denaturing agarose gel and transferred in 20 x SSC to a Hybond-N membrane (24, 25). DNA markers were denatured as for the RNA samples and electrophoresed in parallel with the samples in each denaturing gel. Before transfer, the gel track containing the markers was cut out and fixed in 10% TCA for 20 minutes, then rinsed in water. Markers were then stained with Ethidium Bromide and photographed under ultraviolet illumination.

RESULTS

Total RNA was isolated from tissues and cell lines as described in Materials and Methods and used in Slot Blot experiments with a number of DNA probes. Figure 1 shows the results of these experiments.

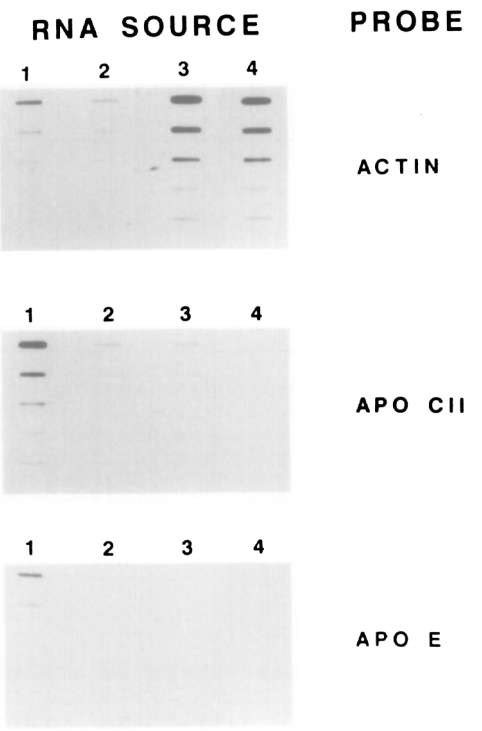

Figure 1: RNA from the indicated sources was applied to Hybond filters as described in materials and methods, and hybridised to DNA probes for Actin, Apo CII and Apo E.
RNA sources were as follows;
1. Control human liver tissue.
2. Liver tissue from the apo CII deficient patient.
3. U937 tumour cell line.
4. Jurkat tumour cell line.

In order to estimate the relative amounts of RNA from each source which were applied to the Slot Blot filters, one filter was hybridized with a DNA probe for Actin. Actin is required in large amounts by all cell types and can be regarded as an indicator of overall cell mRNA synthesis. Figure 1 shows that this actin probe hybridized to all the RNA samples. We estimated from this result that RNA from the individual in column 1 (normal with respect to apo CII) was present at approximatly twice the concentration of the RNA from the apo CII deficient patient (column 2). The signal detected using RNA derived from the tumour cell lines U937 and Jurkat, (columns 3 and 4), is particularly strong suggesting that, in these

TABLE 1

RNA SOURCE	PROBE	TOTAL SIGNAL (Linear range)	CII+/CII- RATIO (Corrected)
CII+	ACTIN	7376	1.0
CII-	STANDARD	2987	/
CII+	APO CII	14256	5.7
CII-		1007	/
CII+	APO E	12092	1.7
CII-		2867	/

The values obtained by scanning densitometry of the slot blots in figure 1 are shown. They are derived by summing the values for those peaks in which the autoradiographic signal falls in the linear range of the x-ray film used . For the Actin probe which is used as a standard, the correction factor of 2.47 is derived. This correction factor is applied to values obtained with subsequent probes.

actively dividing cell types, there is a higher proportion of actin mRNA than in the liver cells. In order to quantitate these mRNA levels, the Slot Blots were analysed by scanning densitometry, and values for actin mRNA signal strength are shown in Table 1.

Duplicate Slot Blot filters were then hybridized with a DNA probe for apo CII. Figure 1 shows the result of this hybridization and demonstrates quite clearly that the signal detected in the RNA from the apo CII deficient patient is at least four times weaker than the corresponding signal from the normal liver sample, and the corrected values, (normalized to actin mRNA levels), are shown in Table 1. As a further internal control, we hybridized another duplicate filter with a DNA probe for apo E. We expected that the corrected signal strength detected with this probe would be the same in both our apo CII^{+} control and our apo CII deficient samples. Figure 1 and table 1 show this to be the case. As expected, the signal detected with both the apo CII and apo E probes in RNA from the tumour cell lines U937 and Jurkat is very low (when adjusted

for the quantity of RNA on the filter) compared to that detected using the liver samples. This result confirms the ability of the Slot Blot technique to discriminate between low and high copy numbers of different mRNA species.

Northern Blot Studies

The results shown in Figure 1 allowed us to quantitate the expression of these genes by measuring mRNA levels. However they must be interpreted with caution since the signals detected could be due to small amounts of DNA which might be present in the RNA samples. This is unlikely since our Jurkat controls were negative for all probes except actin. In addition, our liver RNA samples produced different signal strengths for different probes which would not be expected if the signal was due to contaminating DNA. In order to measure the molecular weight of the apo CII mRNA detected in the Slot Blot experiments, total liver RNA from the apo CII deficient patient was subjected to Northern Blot analysis using apo CII and apo E as DNA probes. The results of this analysis are shown in Figure 2. For apo CII, a mRNA of 600 bp was detected and for apo E, of 1.2 kb. Both these values correspond well with the published molecular weights for apo CII and apo E mRNA (19,21). As expected, the signal detected with the apo E probe is much stronger than the signal detected with the apo CII probe, in agreement with the results of the Slot Blot analysis shown in Figure 1.

DISCUSSION

In the plasma of the patient that we have examined, it has been shown that circulating levels of apo CII are reduced almost to undetectable levels. It is therefore surprising that,

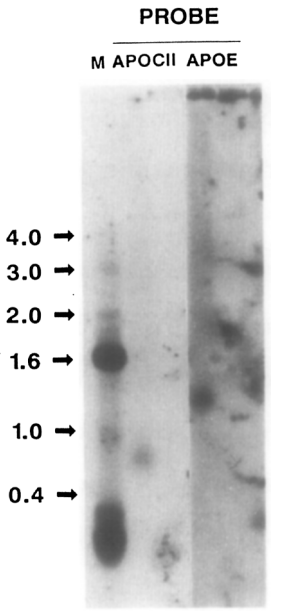

Figure 2: Total RNA from the apo CII deficient patient was electrophoresed on a 1% denaturing gel, blotted onto a Hybond membrane and hybridised with the apo CII and apo E DNA probes. The sizes of the hybridising RNA was estimated by comparison with marker fragments of known size run in parallel tracks (track M), and are given in kilobase pairs (kb).

in the liver of the patient, normal sized apo CII mRNA is detectable. By comparison with the normal liver sample, the level of apo CII mRNA is reduced by roughly 75%.

It is known that apolipoprotein mRNA levels in the liver can be altered by various hormones and that certain hormone levels can fluctuate in response to dietary changes (26). These effects have also been demonstrated in cultured hepatocytes and enterocytes *in-vitro* (26). Both the patient and control liver samples were removed at surgery after at least an overnight fast, but it is possible that other factors such as long term drug therapy might be affecting the absolute levels of apolipoprotein production in both the patient and the control sample. The probes used in these experiments however, provide internal controls of mRNA levels in both the patient and control liver samples. Thus although, in our patient, a very

marked difference in apo CII mRNA levels compared to normal liver is apparent, this is not the case for apo E.

The precise nature of the defect in the patient is not yet known. The data that we present here shows that the defect is probably not primarily at the level of gene transcription. One possibility is that, in this individual, normal ammounts of apo CII mRNA are transcribed and transported to the cytoplasm of the liver cell, but that this mRNA is not translated normally and is rapidly turned over in the cytoplasm, thus having a short half life. This may occur if the mutation introduces a stop codon in the coding region of the gene, causing premature termination of translation. The 3' portion of the mRNA unprotected by ribosomes may then be rapidly degraded, as has been reported for some gene defects causing β^0 thalassaemia (27). The shorter, partially degraded mRNA may be rapidly turned over and may not, therefore, be detected on Northern blots. This hypothesis would predict that any immunologically detectable apo CII in the patient would be smaller than the normal protein and might itself be degraded in the liver or in the plasma.

To resolve these questions, the apo CII gene from this patient is being isolated from a genomic library. The sequences of the apo CII alleles should then allow the complete analysis of this interesting defect.

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REFERENCES

1. Little, J.A., Cox, D.W., Breckenridge, W.C., and McGuire, V.M. (1979) in Fifth International Symposium on Atherosclerosis, Eds. Gotto, A.M., Smith, L. & Allen, B. (Springer, New York) pp 671-674.
2. Yamamura, I., Sudo, H., Ishikawa, K. & Yamamoto, A. (1979) Atherosclerosis 34, 53-65

3. Capurso, A., Pace, L., Bonomo, L., Catapano, A., Schiliro, G., LaRosa, M. & Assman, G. (1980), Lancet i, 268.
4. Miller, N.E., Rao, S.N., Alaupovic, P., Noble, N., Slack, J., Brunzell, J.D. & Lewis, B. (1981) Eur. J. Clin. Invest. 11, 69-76
5. Stalenhoef, A.F.H., Casparie, A.F., Denmacker, P.N.M., Stouten, J.T.J., Lutterman, J.A., & van't Laar, A. (1981) Metabolism 30, 919-926
6. Fojo, S.S., Law, S.W., Sprecher, D.L., Gregg, R.E., Baggio, G. & Brewer, H.B., Jr. (1984) Biochem. Biophys. Res. Commun. 124, 308-313
7. Saku, K., Cedres, C., McDonald, B., Hynd, B.A., Liu, B.W., Srivasteva, L.S. & Kashyap, M.L. (1984) Am. J. Med. 77, 457-462
8. Havel, R.J., Shore, V.G., Shore, B. & Bier, D.M. (1970) Circ. Res. 27, 595-600
9. LaRosa, J.C., Levy, R.I., Herbert, P., Lux, S.E. & Fredrickson, D.S. (1970) Biochem. Biophys. Res. Commun. 41, 57-62
10. Cox, D.W., Breckenridge, W.C. & Little, J.A. (1978) N. Engl. J. Med. 299, 1421-1424
11. Breckenridge, W.C., Little, J.A., Steiner, G., Chow, A. & Poapst, M. (1978) N. Engl. J. Med. 298, 1265-1273
12. Humphries, S.E., Williams, L., Myklebost, O., Stalenhoef, A.F.H., Demacker, P.N.M., Baggio, G., Crepaldi, G., Galton, D.J., Williamson, R. (1984) Hum. Genet 67, 151-155
13. Hayden, M.R., Vergani, C., Humphries, S.E., Kirby, L., Shukin, R., McLeod, R. (1986) Lipoprotein Deficiency Syndrome, Edited by Aubie Angel and Jiri Frohlich (Plenum Publishing Corportion, 1986).
14. Fullmer, C.S. (1984) Anal. Biochem. 142, 336-339
15. Maguire, G.F., Little, J.A., Kakis, G & Breckenridge, W.C. (1984) Can. J. Biochem. Cell Biol. 62, 847-852
16. Connelly, P.W., Maguire, G.F., Hofmann, T., & Little J.A. (1987) Proc. Natl. Acad. Sci. 84, 270-273.
17. Sundstrom, C. and Nilsson, K. (1976) Int. J. Cancer, 17, 565-577.
18. Glison, V., Crkvenjakov, R & Byus, C. (1974) Biochemistry 13, 2633
19. Myklebost, O., Williamson, R.,, Markham, A.F., Myklebost, S., Rogers, J., Woods, D.E., Humphries, S.E. (1984) J. Biol. Chem. 259, 4401-4404
20. Talmadge, K., Stahl, S., Gilbert, W. (1980) Proc. Natl. Acad. Sci, U.S.A. 77, 3369-3373
21. Wallis, S.C., Rogne, S., Gill, L., Markham, A., Edge, M., Woods, D., Williamson, R. & Humphries, S.E. (1983) EMBO Journal 2, 2369-2373
22. Humphries, S.E., Whittall, R., Minty, A., Buckingham, M. and Williamson, R., (1981) Nucl. Acid Res. 9, 4895-4908.
23. Feinberg, A.P. and Vogelstein, B. (1984) Anal. Biochem. 137, 266-267.
24. Lehrak, H., Diamond, D., Wozney, J.M. and Boedther, H. (1977) Biochemistry 16, 4743.
25. Maniatis T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual, Cold Spring Harbour.
26. Elshourbagy, N.A., Boguski, M.S., Liao, W.S.L., Jefferson, L.S., Gordon, J.I. and Taylor, J.M. (1985) Proc. Natl. Acad. Sci, 82, 8242-8246.
27. Antonarakis, S.E., Hazazain, H.H., and Orkin, S.H. (1985) Hum. Genetics 69, 1-14