Familial Dysbetalipoproteinemia

ABNORMAL BINDING OF MUTANT APOPROTEIN E TO LOW DENSITY LIPOPROTEIN RECEPTORS OF HUMAN FIBROBLASTS AND MEMBRANES FROM LIVER AND ADRENAL OF RATS, RABBITS, AND COWS

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ABSTRACT Patients with familial dysbetalipoproteinemia (F. Dys.), also called familial type 3 hyperlipoproteinemia, are homozygous for a mutant allele, E^d, that specifies an abnormal form of apoprotein (apo) E, a prominent constituent of remnant lipoproteins derived from very low density lipoproteins (VLDL) and chylomicrons. Apo E is thought to mediate the removal of remnant lipoproteins from the plasma by virtue of its ability to bind to hepatic lipoprotein receptors. In F. Dys. patients, remnant-like lipoproteins accumulate, apparently because of delayed clearance by the liver.

In the current studies, we show that the abnormal protein specified by the E^d allele (apo E-D) from some, but not all, patients with F. Dys. has a markedly deficient ability to bind to low density lipoprotein (LDL) receptors. Apo E was isolated from eight control subjects and nine patients with F. Dys. and incorporated into phospholipid complexes. The complexes were tested for their ability to compete with human ^{125}I-LDL or rabbit ^{125}I-β-VLDL for binding to LDL receptors in four assay systems: cultured human fibroblasts, solubilized receptors from bovine adrenal cortex, liver

membranes from rats treated with 17α-ethinyl estradiol, and liver membranes from normal rabbits. The apo E-D from six of the nine patients with F. Dys. showed binding affinities for LDL receptors that were reduced by >98% in all receptor assays (group 1 patients). All of these group 1 patients were unequivocally of phenotype apo E-D/D by the criterion of isoelectric focussing. The apo E from the three other F. Dys. patients showed a near normal binding ability in all four of the receptor assays (group 2 patients). One of these group 2 patients appeared to have the apo E-D/D phenotype by isoelectric focussing. In the other two patients in group 2, apo E-D was the predominant protein (phenotype, apo E-D/D), but traces of protein in the region corresponding to normal apo E (apo E-N) were also present. The difference between group 1 and group 2 patients was also apparent when the apo E was iodinated and tested directly for binding to liver membranes from rats treated with 17α-ethinyl estradiol. The ^{125}I-labeled apo E from a group 2 patient, but not a group 1 patient, showed enhanced uptake when perfused through the liver of an estradiol-treated rate, indicating that the receptor binding ability of apo E correlated with uptake in the intact liver.

The current studies allow the subdivision of patients with F. Dys. into two groups. In group 1, the elevated plasma level of remnants appears to be due to a diminished receptor binding activity of the abnormal pro-

tein specified by the E^d allele; in group 2 patients, the cause of the elevated plasma level of remnants remains to be explained.

INTRODUCTION

In familial dysbetalipoproteinemia (F. Dys.)[1], also called familial type 3 hyperlipoproteinemia, abnormal very low density lipoproteins (VLDL) and intermediate density lipoproteins accumulate in plasma, producing hypercholesterolemia and hypertriglyceridemia (1, 2). In comparison with VLDL from normal subjects, the VLDL particles from patients with F. Dys. are enriched in cholesteryl esters relative to triglyceride and exhibit β-mobility, rather than pre-β mobility, on electrophoresis. These "β-VLDL" particles are believed to be remnants derived from the partial catabolism of the triglyceride-rich lipoproteins, chylomicrons and VLDL. In animals, and probably in man, such remnant lipoproteins are rapidly removed from the circulation by means of receptor-mediated endocytosis in the liver. As a result, in normal subjects only small amounts of remnant lipoproteins are found in the circulation (1).

Studies in rats, dogs, and rabbits have shown that the rapid hepatic uptake of remnants is due to the binding of the lipoproteins to receptors on the hepatocyte surface (3). These hepatic receptors resemble the low density lipoprotein (LDL) receptors that have been characterized in extrahepatic cells, such as cultured human fibroblasts and the adrenal cortex of various animal species. The hepatic and extrahepatic receptors share the following properties: (a) they bind apoprotein (apo) E with higher affinity than apo B; (b) binding of apo B and E is dependent on Ca^{++} and is inhibited by EDTA; and (c) the receptors are subject to metabolic regulation by a variety of hormonal, nutritional, and pharmacologic factors (3–9). Although remnant lipoproteins contain both apo B and apo E, it is generally believed that the apo E component confers high affinity for the hepatic receptors (1, 3, 6).

Genetic studies in humans by Utermann (10–12) and by Zannis and Breslow (13, 14) have shown that apo E is specified by a polymorphic genetic locus with three alleles, here designated apo E^n, E^4, and E^d.[2] These three alleles give rise to six genotypes: apo E^n/E^n, E^4/E^4, E^d/E^d, E^n/E^4, E^n/E^d, and E^4/E^d. The proteins specified by each allele are called isoforms.[2] Current evidence

suggests that the apo E isoforms specified by alleles E^n and E^4 are functionally normal, whereas the apo E specified by allele E^d may be functionally abnormal (1). The protein specified by the E^d allele has one less positive charge than the protein specified by the E^n allele and two less positive charges than the protein specified by the E^4 allele (10–14). Approximately 1% of individuals in the population are homozygous for the E^d allele (10–14).

Virtually all patients with classic F. Dys. so far studied have been homozygous for the E^d allele (1, 11, 13). However, most subjects who are homozygous for the E^d allele do not have elevated cholesterol or triglyceride levels. These subjects have only small amounts of β-VLDL in their plasma and they do not appear to have accelerated atherosclerosis or the other clinical features of F. Dys. (10–12). For this reason Utermann has proposed that the full expression of F. Dys. requires at least two factors: (a) homozygosity for the E^d allele (i.e., genotype E^d/E^d); and (b) a triggering factor such as hypothyroidism, or the independent inheritance of another form of hyperlipoproteinemia such as familial combined hyperlipoproteinemia or familial hypercholesterolemia (11).

Considered together, the above information raised the possibility that the accumulation of β-VLDL in F. Dys. patients is due to a failure of apo E-D, the protein specified by the E^d allele, to bind to hepatic receptors. Evidence in favor of this hypothesis has appeared in two recent studies. First, Havel and associates isolated apo E from normal subjects and patients with F. Dys. and labeled them with ^{125}I (15). Complexes of the ^{125}I-labeled apo E with phospholipid were perfused through the livers of rats that had been treated with 17α-

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trophoresis, Zannis and Breslow have proposed that isoforms β-IV (corresponding to E-2), β-III (corresponding to E-3), and β-II (corresponding to E-4) are each specified by one of three alleles at a single locus and that minor isoforms, including those corresponding to E-1, occur as a result of posttranslational glycosylation of one of the three major isoforms (13, 14). The following two lines of evidence indicate that isoforms E-2, E-3, and E-4 (Utermann nomenclature) correspond to isoforms β-IV, β-III, and β-II (Zannis-Breslow nomenclature). First, when apo E was treated with neuraminidase and then subjected to one-dimensional isoelectric focusing, we observed a simplified isoform pattern that was comparable to that observed with two-dimensional electrophoresis (35). Second, direct comparison of the one-dimensional and two-dimensional electrophoretic patterns of the same apo E preparations showed that isoforms E-2, E-3, and E-4 correspond to isoforms β-IV, β-III, and β-II, respectively (36). For purposes of terminology in the current paper, the E^n allele specifies predominantly the E-3 or E-N isoform, the E^d allele specifies predominantly the E-2 or E-D isoform, and the E^4 allele specifies predominantly the E-4 isoform. Thus,

alleles: E^n, E^d, E^4
phenotypes: E-N/N, D/D, 4/4, N/D, N/4, D/4
apoproteins: apo E-N, E-D, E-4

ethinyl estradiol to increase the number of hepatic lipoprotein receptors. The uptake of the ^{125}I-labeled apo E-D was markedly reduced compared with normal ^{125}I-labeled apo E. Second, Gregg et al. infused ^{125}I-labeled apo E from F. Dys. subjects into normal subjects and observed a reduced rate of clearance as compared with normal apo E (16). Patients with F. Dys. cleared normal apo E at a normal rate, but they cleared the abnormal apo E slowly.

The above two physiologic studies suggest strongly that the apo E specified by the E^d allele may not bind normally to lipoprotein receptors. The current studies were designed to test this hypothesis directly by measuring the ability of apo E from control subjects and patients with F. Dys. to bind to lipoprotein receptors in several different assay systems. These included monolayers of cultured human fibroblasts, solubilized lipoprotein receptors from bovine adrenal cortex, liver membranes from normal rabbits, and liver membranes from rats treated with 17α-ethinyl estradiol. The resulting in vitro binding data have been correlated with measurements of the uptake of these apoproteins in perfused rat livers. The results indicate that the mutant apo E-D isolated from some, but not all, F. Dys. patients binds abnormally to lipoprotein receptors.

METHODS

Materials

Sodium [^{125}I]iodide (carrier-free) was purchased from Amersham Corp., Arlington Heights, Ill. Bovine serum albumin and dimyristoylphosphatidylcholine were obtained from Sigma Chemical Co., St. Louis, Mo. Egg phosphatidylcholine was from Avanti PolarLipids Birmingham, Ala. or Sigma Chemical Co. Other materials were obtained from sources as previously reported (4, 5).

Lipoproteins and apoproteins

Human VLDL ($d < 1.006$ g/ml) and LDL (d, 1.019–1.063 g/ml) were prepared from plasma or serum of fasting subjects by ultracentrifugation (17). Rabbit β-VLDL ($d < 1.006$ g/ml) was isolated from animals fed a cholesterol-corn oil diet as previously described (9). The concentrations of lipoproteins were expressed in terms of their protein content.

Apo E was isolated from VLDL obtained from nine patients with F. Dys. (Table I) and eight control subjects with primary hyperlipoproteinemia and a type 4 or type 5 lipoprotein pattern. All of the control subjects had normal apo E (phenotype, E-N/N, N/4, or 4/4) as judged by the isoelectric focusing in polyacrylamide gels.

Human LDL and rabbit β-VLDL were radiolabeled with ^{125}I by the iodine monochloride method (18). Apo E was radiolabeled with ^{125}I by a modification (19) of the iodine monochloride method.

Formation of apo E/phospholipid complexes

Three methods were used to prepare soluble apo E/phospholipid complexes. The method used for each experiment is indicated in the legends to Figs. 1–5 and in Table II. The concentration of the apo E/phospholipid complexes is given in terms of the protein content. All apo E/phospholipid complexes were stored at 4°C and used within 2 wk.

Method A. Apo E was isolated by delipidation of VLDL with acetone/ethanol (1:1) followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis as described by Utermann (20). The detergent-dialysis procedure described by Helenius et al. (21) was modified for incorporation of apo E into phospholipid vesicles. Lyophilized apo E (0.5–2.0 mg protein) was dissolved at room temperature in 0.5–2 ml of buffer containing 50 mM Tris-chloride, 0.3 M NaCl, and 25 mM octyl-β-D-glucoside (pH 7.4) and incubated for 16 h at 4°C. Undissolved material was removed by centrifugation (5 min, 4°C, 4000 g). The resultant concentration of soluble protein was 0.5–0.9 mg/ml. Egg phosphatidylcholine (two times the weight of apo E) in ethanol and octyl-β-D-glucoside (four times the weight of phosphatidylcholine) in acetone were dried together in a glass tube under a stream of nitrogen, taken up in ether, and dried again twice so as to form a film covering the walls of the tube. The lipid-detergent film was then dissolved at room temperature by addition of the apo E solution under slight agitation. This mixture was dialyzed at room temperature against three changes of 1,000 vol of buffer containing 4 mM Tris-chloride, 3 mM MgCl₂, 0.1 mM dithiothreitol, and 2 mM Na₃N (pH 7.4) for a total of 24 h.

Method B. Apo E was isolated by delipidation of VLDL with ethanol/ether (3:1) followed by gel-filtration chromatography of the proteins in 6 M urea as described by Fainaru et al. (22). Unilamellar liposomes of egg phosphatidylcholine were prepared with a French pressure cell (23) and incubated with apo E as previously described (15). The apo E/phosphatidylcholine complexes were isolated on Bio-Gel A-0.5 m (10% agarose, Bio-Rad Laboratories, Richmond, Calif.) (15).

Method C. Apo E was isolated by delipidation of VLDL with tetramethylurea followed by gel chromatography in 4 M guanidine as described by Weisgraber et al. (24). Apo E was incorporated into dimyristoylphosphatidylcholine vesicles as described by Innerarity et al. (25). The apo E/dimyristoylphosphatidylcholine complexes were isolated by ultracentrifugation (25).

Lipoprotein binding assays

The binding of human ^{125}I-LDL, human ^{125}I-apo E/phosphatidylcholine complexes, and rabbit ^{125}I-β-VLDL to lipoprotein receptors was studied in three assay systems, as indicated below. All assays were carried out in Dallas except those in Fig. 1B, which were carried out at the Gladstone Foundation Laboratories in San Francisco.

Cultured human fibroblasts. Binding of ^{125}I-LDL to monolayers of normal human fibroblasts was carried out at 4°C as previously described (26).

Liver membranes from animals. Binding of ^{125}I-LDL, ^{125}I-apo E/phosphatidylcholine complexes, and ^{125}I-β-VLDL to liver membranes was carried out by the Airfuge ultracentrifugation assay of Basu et al. (27) as modified by Kovanen et al. (4). Membranes (fraction sedimenting between 8,000 and 100,000 g) were prepared from the livers of male rats treated with 17α-ethinyl estradiol (4, 5) and from the livers of normal male New Zealand White rabbits (9).

Partially purified LDL receptors from solubilized bovine adrenal cortex. Binding of ^{125}I-LDL to partially purified LDL receptors was measured by a microporous filter assay as previously described (28). LDL receptors were solubilized from bovine adrenal cortex with octyl-β-D-glucoside, partially purified by DEAE-cellulose chromatography, and precipitated with acetone in the presence of phosphatidylcholine (29).

Uptake of ^{125}I-Apo E/phospholipid complexes by perfused livers

The uptake of ^{125}I-apo E/phosphatidylcholine complexes was measured in the isolated perfused liver from ·normal rats and rats treated with 17α-ethinyl estradiol as previously described (15). Single-pass perfusions were conducted at a rate of 12 ml/min with Krebs-Henseleit buffer/10% rat erythrocytes. At various intervals, the concentration of ^{125}I in the infusate and in the effluent were measured, and the difference was taken as a measure of the amount of ^{125}I-apo E removed by the liver.

Other assays

The protein content of lipoproteins, apo E, and cell extracts was measured by the method of Lowry et al. (30) or by radioimmunoassay (31). The distribution of isoforms of apo E in VLDL was determined by analytical isoelectric focusing on polyacrylamide gels followed by quantitative densitometric analysis. These analyses were carried out either in Marburg (11) or in San Francisco at the Cardiovascular Research Institute (31). The presence or absence of β-VLDL was determined by inspection of agarose electrophoretograms that were overloaded with the $d < 1.006$ fraction of plasma (1, 2). The content of cholesterol and triglycerides in serum and lipoproteins was measured as described (11, 32).

RESULTS

Each of the nine patients in the current study exhibited the classic clinical syndrome of F. Dys. (1, 2), including elevations in the total plasma cholesterol and/or triglyceride level, an elevated ratio of cholesterol to triglyceride in VLDL, and electrophoretic evidence for β-VLDL (Table 1). Four of the patients had planar or tuberous xanthomas, and six had clinical signs of atherosclerosis. Isoelectric focusing of total VLDL proteins showed the presence only of the apo E-D protein in seven of the nine patients (phenotype, apo E-D/D). In another patient J.T., isoform E-D was the major protein band,[2] but a faint band was also seen in the region of the gel that is normally occupied by isoform E-N (see legend to Table I). The apo E-D protein was estimated by visual inspection to constitute ~95% of the total apo E in this patient. In patient E.S., isoform E-D was also the predominant protein band. However, this patient also had a well-defined band in the isoform E-N region, which accounted for 9% of the apo E as estimated by densitometry. None of the patients had an E-4 isoform.

Control apo E was obtained from eight individuals who had elevated VLDL levels and a type 4 or type 5 lipoprotein pattern. Isoelectric focusing showed that their apo E corresponded to phenotypes apo E-N/N, N/4, or 4/4.

Apo E from the $d < 1.006$ g/ml fraction of control subjects and F. Dys. patients was isolated and reconstituted into phospholipid complexes by three different methods as described in Methods. Complexes prepared by the three different methods showed similar binding activities. In each of the legends to the Figures and Tables, the method for preparation of the apo E complexes is stated.

Four different assay systems were used to assess the binding activity of apo E from control and F. Dys. patients (Table II). These included monolayers of intact human fibroblasts, solubilized receptors from bovine adrenal cortex, liver membranes from estradiol-treated rats, and liver membranes from normal rabbits. In each system we measured the ability of increasing concentrations of unlabeled apo E/phospholipid complexes to compete with ^{125}I-lipoproteins for binding to the LDL receptor. In three systems (human fibroblasts, bovine adrenal, and rat liver), human ^{125}I-LDL was used as the labeled ligand; in the rabbit liver membrane system rabbit ^{125}I-β-VLDL was used. As shown in Table II, apo E from control subjects produced 50% inhibition of ^{125}I-lipoprotein binding at concentrations ranging from 0.03–0.13 μg/ml (human fibroblasts) to 2–6 μg/ml (rabbit liver membranes).

Apo E from six of the nine F. Dys. patients was severely defective in its ability to bind to lipoprotein receptors in each of the systems tested. These patients are hereafter referred to as group 1 patients (Table II). Patient A.G. was the most extensively studied patient in this group. Different preparations of her apo E failed to compete significantly for ^{125}I-LDL binding at concentrations as high as 100 μg/ml in the three systems tested (human fibroblasts, bovine adrenal, and rat liver). This was true whether the apo E/phospholipid complex was prepared by method A or B. Apo E from patients B.K., A.H., S.J., I.K., and D.R. also failed to compete for the binding of ^{125}I-LDL or ^{125}I-β-VLDL at concentrations 50-fold higher than those at which control apo E competed. Apo E from three group 1 patients (A.G., B.K., and I.K.) was tested in more than one receptor system and in each case the binding deficit was similar (Table II).

The apo E from three of the F. Dys. patients (designated Group 2 patients) was distinctly different from those in group 1 (Table II). W.McC. was the most extensively studied patient in this group. In all four assay systems, his apo E produced 50% inhibition of ^{125}I-lipoprotein binding at concentrations similar to those at which control apo E was effective. This was true whether the apo E/phospholipid complexes were made by methods B or C. Apo E from the two other group 2 patients (E.S. and J.T.) also showed a potent, albeit slightly reduced, ability to compete for ^{125}I-LDL binding to human fibroblasts. The concentrations of apo E required for 50% competition ranged from 0.3–1.6 μg/ml.

Examples of competition experiments are shown in Figs. 1–3. Each panel presents the results of a single binding experiment carried out on a single day. Fig. 1A

TABLE I

Clinical Data on Patients with Familial Dysbetalipoproteinemia

Initials of patients	Sex	Age	Plasma lipid levels								VLDL Ratio C/TG	Apo E isoforms in VLDL[e]	Xanthomas	Clinical atherosclerosis	Other features	Medications at the time of preparation of Apo E	Functional group[g]
			Total cholesterol (C)	Trigly- cerides (TG)	VLDL		LDL		HDL								
					C	TG	C	TG	C	TG							
			mg/dl														
I.K.[a]	F	63	353	282	92	115	183	102	73	47	0.80	E-D	None	Coronary	None	Clofibrate	1
B.K.[a]	M	60	310	380	152	224	—	—	28	—	0.68	E-D	None	Coronary, peripheral	Diabetes, hypertension	None	1
S.J.[a]	F	64	368	361	198	282	135	41	35	38	0.70	E-D	Planar	Coronary, peripheral	Diabetes	Benzafibrate	1
A.H.[b]	M	57	290	341	159	315	204	15	31	9	0.50	E-D	None	None	Hypertension	Hydrochloro- thiazide, Propanolol, Aldomet	1
A.G.[b]	F	59	571	830	338	640	157	107	41	32	0.53	E-D	None	Coronary, peripheral	Diabetes	Clofibrate, nicotinic acid	1
D.R.[c]	M	49	725	670	465	510	195	114	30	16	0.91	E-D	Planar, tuberous	Peripheral	None	Clofibrate, nicotinic acid	1
W.McC.[b]	M	51	250	224	155	194	45	9	28	16	0.80	E-D	Planar, tuberous	None	Black Race, hyperuricemia	Clofibrate, Probenecid	2
J.T.[c,f]	M	42	287	208	86	157	166	—	35	—	0.55	E-D/trace E-N	None	Coronary, cerebral	Black Race	None	2
E.S.[d,f]	F	52	513	970	471	900	131	100	26	24	0.52	E-D/trace E-N	Planar, tuberous	None	Treated hypothy- roidism	L-Thyroxine	2

The $d < 1.006$ g/ml fraction from all patients contained β-VLDL. All patients had pedigree evidence of vertical transmission of the abnormal apo E^d allele except patients A.G. and J.T. in whom family studies were not performed.

[a] Patients of G. Utermann.

[b] Patients of R. J. Havel.

[c] Patients of R. W. Mahley.

[d] Patient of D. W. Bilheimer.

[e] The isoforms of apo E were determined by isoelectric focussing of apo VLDL and assessed by visual inspection of overloaded gels or by densitometry (see Footnote 2).

[f] Patient J.T. had small amounts of the E-N isoform ($<5\%$ of total apo E) when his isolated apo E was subjected to isoelectric focussing, but no E-N isoform was detected in his apo VLDL. Patient E.S. had small amounts of the E-N isoform ($\sim 9\%$ of total apo E) in her apo VLDL and isolated apo E (see Footnote 2).

[g] Patients were classified into functional groups based on the ability of their apo E to bind to lipoprotein receptors. Binding ability was estimated from the concentration of apo E required to competitively reduce by 50% the binding of ^{125}I-LDL or ^{125}I-β-VLDL to lipoprotein receptors. Group 1 = greatly reduced binding. Group 2 = normal-to-slightly abnormal binding.

TABLE II

Ability of Apo E/Phospholipid Complexes from Control Subjects and Patients with F. Dys. to Compete for the Binding of ^{125}I-LDL or ^{125}I-β-VLDL to the LDL Receptor of Various Systems

Receptor system	^{125}I-Lipoprotein ligand	Concentration of Apo E needed to give 50% competition (µg/ml)									
		Control subjects	Group 1 patients						Group 2 patients		
			A.G.	B.K.	A.H.	S.J.	I.K.	D.R.	W.McC.	E.S.*	J.T.
Human fibroblast monolayers	Human ^{125}I-LDL, 2 or 3 µg/ml	0.03–0.13[a,b,c]†	>100[b]			>100[a]		10–46[c]	0.1[b], 0.3[c]	0.3[c]	0.5–1.6[c]
Bovine adrenal cortex (solubilized)	Human ^{125}I-LDL, 12.5 µg/ml	0.8[a]	>100[a,b]		>100[a]				0.8[b]		
Rat liver membranes (estradiol-treated)	Human ^{125}I-LDL, 25 µg/ml	1[a], 5[a], 0.5[b]	>100[b]	>100[a]			60[a]		2[b]		
Rabbit liver membranes (normal)	Rabbit ^{125}I-β-VLDL, 0.75 µg/ml	2[a], 4[b], 6[b]		>100[a]			>100[a]		2[b]		

These data were obtained from competitive binding experiments carried out as described in the legends to Figs. 1–3. The method for preparing each apo E/phospholipid complex is indicated by the superscript a, b, or c (a = method A; b = method B; c = method C). The data for control apo E in the human fibroblast system represents the range of values observed in 16 different experiments using apo E from eight different subjects. The data for patients D.R. and J.T. represents the range of values observed in six and five different experiments, respectively.

* In previous studies, it was concluded that the E apoprotein from one F. Dys. patient had a near normal binding activity in cultured human fibroblasts (25). The apo E from E.S. was used in these earlier studies.

† Apo E/phospholipid complexes from the eight control subjects were prepared as follows: method A was used for three subjects; method B for one subject; method C for two subjects; and both methods A and B were used for two subjects.
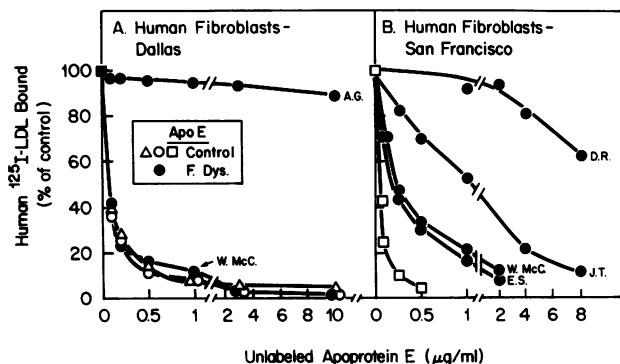

FIGURE 1 Ability of apo E/phospholipid complexes from control subjects (○, △, □) and F. Dys. subjects (●) to compete for the binding of human ^{125}I-LDL to monolayers of human fibroblasts at 4°C. The binding experiments were performed in semi-confluent cells (35-mm petri dishes) that had been incubated for the preceeding 48 h in 10% human lipoprotein-deficient serum (26). Experiment A (Dallas): each monolayer received 0.6 ml of ice-cold growth medium containing 10% human lipoprotein-deficient serum, 3 µg/ml of ^{125}I-LDL (316 cpm/ng protein), and the indicated concentration of unlabeled apo E/phosphatidylcholine complexes prepared by method B. After incubation for 2 h at 4°C, the cells were washed extensively, and the total amount of ^{125}I-LDL bound to the cells was determined (26). The "100% of control" value was 103 ng/mg protein. Experiment B (San Francisco): each monolayer received 0.6 ml of ice-cold growth medium containing 10% human lipoprotein-deficient serum, 2 µg/ml of ^{125}I-LDL (345 cpm/ng protein), and the indicated concentration of unlabeled apo E/dimyristoylphosphatidylcholine complexes prepared by method C. After incubation for 2 h at 0°C, the cells were washed extensively and the total amount of ^{125}I-LDL bound to the cells was determined (25). The "100% of control" value was 157 ng/mg protein.

shows the striking difference in competition for binding of human ^{125}I-LDL to monolayers of human fibroblasts by apo E from one group 1 patient (A.G.) as compared with one group 2 patient (W.McC.) and two control subjects. Fig. 1B shows a similar fibroblast experiment in which the apo E from one group 1 patient (D.R.) and three group 2 patients (E.S., W.McC., and J.T.) were tested. Apo E from D.R. failed to achieve 50% competition at the highest concentration tested (8 µg/ml), which was >100-fold higher than the concentration of control apo E that gave 50% competition. The three group 2 patients showed 50% competition in the range of 1 µg/ml or less. Comparison of Figs. 1A and 1B also shows that the apo E from one group 2 patient (W.McC.), prepared by two different methods and tested in two different laboratories, gave similar results.

Experiments with LDL receptors solubilized from bovine adrenal cortex membranes (Fig. 2) gave results that were similar to those obtained with intact human fibroblasts. Thus, apo E from two group 1 patients (A.G. and A.H.) failed to compete significantly with human ^{125}I-LDL (Fig. 2). The results with patient A.G. were similar whether the apo E was prepared by method A or B. On the other hand, apo E from a group 2 patient (W.McC.) was indistinguishable from control apo E in competing for ^{125}I-LDL binding.

Previous studies have shown that rabbit β-VLDL binds to the LDL receptor of rabbit liver membranes and that this lipoprotein provides a sensitive ligand for assessing LDL receptor activity (9, 33). Apo E from a control subject competed with rabbit ^{125}I-β-VLDL
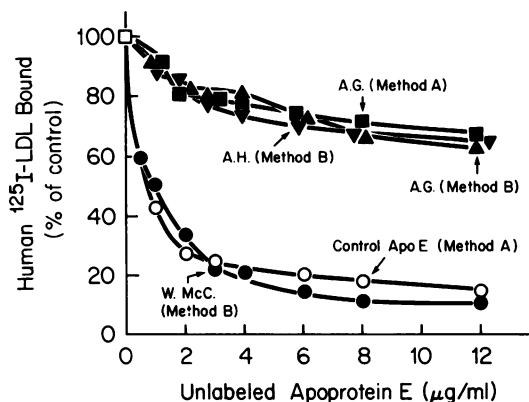
FIGURE 2 Ability of apo E/phosphatidylcholine complexes from a control subject (O) and patients with F. Dys. (■, ▼, ▲, ●) to compete for the binding of human ¹²⁵I-LDL to partially purified LDL receptors from solubilized bovine adrenal membranes. Each assay tube contained the following concentrations of components at pH 8 in a volume of 80 μl: 60 mM Tris-chloride, 2 mM CaCl₂, 25 mM NaCl, 20 mg/ml bovine serum albumin, 14 μg of partially purified receptor protein, and the indicated concentration of unlabeled apo E/phosphatidylcholine complexes. The LDL binding reaction was initiated by the addition of 12.5 μg/ml of human ¹²⁵I-LDL (336 cpm/ng protein). The tubes were incubated for 1 h at 4°C. The data shown represent values for specific binding, which were calculated by subtracting the values in the presence of EDTA (nonspecific binding) from the values in the absence of EDTA (total binding). The "100% of control" value was 4.9 μg of ¹²⁵I-LDL bound/mg protein. The nonspecific binding averaged 0.3 μg/mg. Apo E from all patients was isolated by delipidation of VLDL with ethanol/ether followed by gel filtration in 6 M urea as described in method B. The apo E/phosphatidylcholine complexes were prepared by method A or B as indicated.
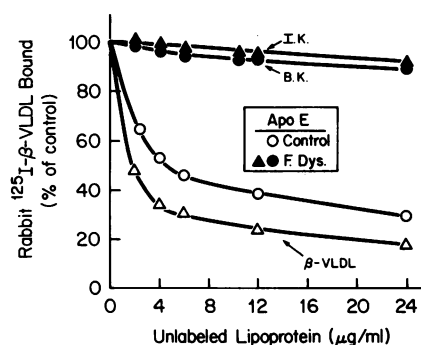

FIGURE 3 Ability of apo E/phosphatidylcholine complexes from a control subject (O) and patients with F. Dys. (●, ▲) to compete for the binding of rabbit ¹²⁵I-β-VLDL to normal rabbit liver membranes. Each assay tube contained the following concentrations of components at pH 8 in a volume of 80 μl: 50 mM Tris-chloride, 0.5 mM CaCl₂, 25 mM NaCl, 20 mg/ml of bovine serum albumin, 100 μg of rabbit liver membrane protein, and the indicated concentration of one of the following unlabeled lipoproteins: rabbit β-VLDL (△) or apo E/phosphatidylcholine complexes (prepared by method A) from either a control subject (O) or the indicated patients with F. Dys. (▲, ●). The binding reaction was initiated by the addition of 0.75 μg/ml of rabbit ¹²⁵I-β-VLDL (333 cpm/ng protein). The tubes were incubated for 1 h at 4°C. The data shown represent total binding values uncorrected for nonspecific binding. The "100% of control" value was 112 ng of ¹²⁵I-β-VLDL bound/mg protein.

for binding to rabbit liver membranes (Fig. 3), 50% competition occurring at a concentration of 4 μg/ml. Apo E from two group 1 patients (B.K. and I.K.) failed to compete significantly at concentrations as high as 24 μg/ml.

The differences in receptor binding activity of apo E from a group 1 patient and a group 2 patient were also apparent when the apoproteins were radioiodinated and incubated with liver membranes from estradiol-treated rats (Fig. 4). When subjected to Scatchard plot analysis (34), the ¹²⁵I-apo E binding data from the group 2 patient (W.McC.) showed an apparent dissociation contant (K_d) of 0.6 μg/ml and maximal binding (B_max) of 225 μg/ml protein. When ¹²⁵I-apo E from the group 1 patient (A.G.) was used, the amount of receptor-specific binding (i.e., binding reduced by EDTA) was only slightly above the value for nonspecific binding (i.e., binding in the presence of EDTA). The receptor-specific binding in A.G. was too low to permit accurate calculation of an apparent K_d or B_max value by the Scatchard method. A similar difference in the binding properties of ¹²⁵I-apo E from W.McC. and A.G. was

observed when the apoprotein/phospholipid complexes were incubated with solubilized bovine adrenal receptors (data not shown).

When the same two preparations of ¹²⁵I-labeled apo E used in the in vitro binding experiments shown in Fig. 4 were perfused through isolated rat livers, a similar difference in behavior between W.McC. and A.G. was observed (Fig. 5). With both preparations of apo E, no uptake could be detected in perfusions of livers from normal rats. However, uptake of apo E from W.McC. (group 2 patient) was greatly stimulated in livers from estradiol-treated rats (Fig. 5B), whereas no stimulation was observed with apo E from A.G. (group 1 patient) (Fig. 5A). The uptake of apo E from W.McC. in control and estradiol-treated rats was similar to that observed with complexes of apo E from control subjects (15).

DISCUSSION

The current results demonstrate that apo E isolated from the plasma of some, but not all, patients with clinically symptomatic F. Dys. is markedly defective in binding to LDL receptors. On the basis of this difference in binding ability, the F. Dys. patients could be divided into two groups. In group 1 patients, who comprised six of the nine patients studied, the apo E failed to compete with ¹²⁵I-LDL or ¹²⁵I-β-VLDL for binding to receptors at concentrations that were 50- to
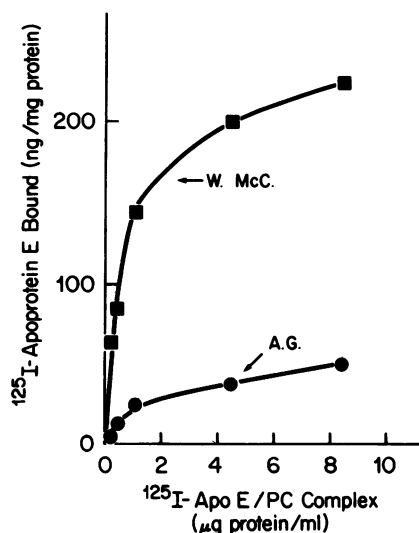
FIGURE 4 Binding of ^{125}I-apo E/phosphatidylcholine complexes from two subjects with F. Dys. to membranes from livers of rats treated with 17α-ethinyl estradiol. Apo E from patients W.McC. (■) and A.G. (●) were radiolabeled with ^{125}I and incorporated into phosphatidylcholine complexes by method B. The specific activities of the complexes were 897 and 366 cpm/ng protein for W.McC. and A.G., respectively. Each assay tube contained the following concentrations of components at pH 8 in a volume of 80 μl: 50 mM Tris-chloride, 0.5 mM CaCl₂, 100 mM NaCl, 20 mg/ml of bovine serum albumin, 100 μg of rat liver membrane protein, and the indicated concentration of ^{125}I-apo E/phosphatidylcholine complexes in the absence or presence of 10 mM EDTA. The tubes were incubated for 1 h at 4°C. The data shown represent values for specific binding, determined by subtracting the values in the presence of EDTA (nonspecific binding) from the values in the absence of EDTA (total binding). PC, phosphatidylcholine.

1000-fold higher than those at which control apo E showed competition. In the other three patients studied (group 2), the isolated apo E showed a near-normal ability to compete for the binding of ^{125}I-LDL.

The differences between the apo E of group 1 and group 2 patients were apparent whether the LDL receptors were obtained from human fibroblasts, bovine adrenal, normal rabbit liver, or estradiol-treated rat liver. The findings were consistent whether the assays were performed with intact monolayers of cells, isolated membranes, or solubilized receptors. The findings were also reproducible when apo E was isolated from the same patient on repeated occasions and studied on different days. The different binding activities of the two groups were not due to differences in methods of preparation of the apo E/phospholipid complexes: similar results were obtained with each of the three methods. The failure of the apo E-D from group 1 patients to bind to receptors was not due to a failure to be incorporated into phospholipid complexes. When the

complexes were re-isolated by gel filtration, similar quantitative amounts of apo E from control subjects and group 1 and 2 patients were found to be incorporated (data not shown). Moreover, by electron microscopy the apo E/phospholipid complexes (Method B) had a similar disc-like appearance (15) whether prepared from a control subject, from A.G. (a group 1 patient), or from W.McC. (a group 2 patient) (unpublished observations). These findings rule out a gross difference in phospholipid binding between the apo E produced by group 1 and group 2 patients, but they cannot rule out a subtle difference in phospholipid binding that might contribute to the altered receptor binding of the apo E-D from group 1 patients.

The difference between the group 1 and group 2 patients that was disclosed by the competition studies was borne out by direct binding studies using ^{125}I-labeled apo E. Moreover, the lack of binding of ^{125}I-apo E from a group 1 patient to isolated hepatic membranes was associated with a marked reduction in the uptake of this lipoprotein by the perfused liver of an estradiol-treated rat. Conversely, the ^{125}I-apo E from a group 2 patient, which bound to liver membranes in a relatively normal fashion, was taken up at a normal rate (15) by the perfused rat liver.

In order to integrate all of the known aspects of F. Dys., the relationship between four variables must be elucidated: (a) the genotype at the apo E locus; (b) the phenotype of the apo E protein; (c) the ability of apo E to bind to lipoprotein receptors; and (d) the level of β-VLDL in plasma. From the present data, it is evident that these variables are related to each other in a complex manner.

The current data show a general but not absolute correlation between apo E phenotype and apo E binding activity. All of the patients in group 1 have the E-D/D phenotype, as determined by the sole presence of the E-D protein on isoelectric focussing and by family studies where available. On the other hand, one of the patients in Group 2 (W.McC.) also appears to have the E-D/D phenotype by the same criteria. His father has phenotype E-D/D with symptomatic dysbetalipoproteinemia; his mother has phenotype E-N/D and has hyperlipidemia without β-VLDL (unpublished data). The situation in the other two patients in group 2 (J.T. and E.S.) is more complex. In these two patients the predominant form of apo E appears to be apo E-D. Nevertheless, they both have a trace of protein detectable in the apo E-N region by isoelectric focussing. In both patients genetic studies are compatible with the possibility that their genotype is E^d/E^d (the E-N/D phenotype is present in both maternal and paternal relatives). If E.S. and J.T. are of genotype E^d/E^d, then the material in the apo E-N region on electrophoresis must represent contaminating protein or the product of some posttranslational modification of the apo E-D
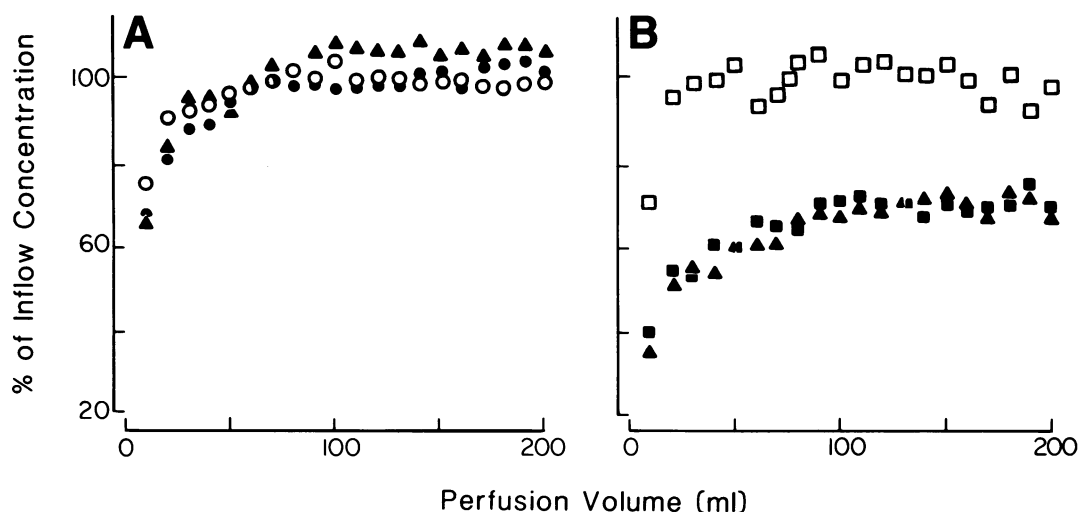
FIGURE 5 Uptake of ^{125}I-apo E/phosphatidylcholine complexes from two subjects with F. Dys. into livers of normal and estradiol-treated rats perfused in a single-pass system at a rate of 12 ml/min. The ^{125}I-apo E/phosphatidylcholine complexes were the same as those used in Fig. 4. Medium containing no complexes was replaced by medium containing ^{125}I-labeled complexes at the beginning of the collection periods shown, and all perfusate was collected in 10-ml portions during the next 17 min. The ordinate shows the concentration of ^{125}I-apo E in the effluent fluid expressed as a percentage of the concentration in the inflow fluid. A value of 100% signifies no removal of ^{125}I-apo E by the liver. Panel A: complexes of apo E from A.G. (0.10 µg apo E per ml perfusate). Panel B: complexes of apo E from W.McC. (0.18 µg apo E/ml perfusate). With each preparation of apo E, one liver from a control rat (open symbols) and two livers from estradiol-treated rats (filled symbols) were perfused.

protein. It seems unlikely that this trace amount of material can account for the near normal binding of the apo E from these patients. In studies not shown, we have found that apo E from an E^n/E^d heterozygote, which contains approximately equal amounts of isoforms specified by the apo E^n and E^d alleles, showed binding activity intermediate between the normal and group 1 values. The VLDL of such individuals is known to contain increased amounts of remnant-like particles (12, 31) and the concentration of apo E in their serum is increased (31).

The most straightforward explanation for the findings in this paper is that one or both of the apo E gene products in each group 2 patient is produced by a mutant allele distinct from the E^d allele of the group 1 patients. This formulation implies that there are two types of mutant gene products, both of which appear in the E-D position on gel electrophoresis and isoelectric focussing. One of these proteins, the product of the classic E^d gene, occurs in homozygous form in the group 1 patients and fails to bind to LDL receptors. The other gene product has the same size and isoelectric point as the E^d gene product, but it retains the ability to bind to LDL receptors. Group 2 patients have one or two copies of this allele. If this formulation is correct, one still must explain why the level of β-VLDL is elevated in the plasma of group 2 patients,

who produce an apo E that is capable of binding to lipoprotein receptors.

A second possible explanation for the difference between the group 1 and group 2 patients is that both groups are homozygous for the same apo E^d allele. However, the group 2 patients, but not the group 1 patients, are able to modify the E-D gene product to a form in which it binds to LDL receptors. The difference between group 1 and 2 patients might be conditioned by a difference at another genetic locus specifying a protein that modifies apo E-D.

The phenotype determined by the apo E locus and the apo E binding activity both must be related to the plasma level of β-VLDL. Here, too, the relations are complex. The original studies of Utermann showed that the apo E-D/D phenotype is necessary but not sufficient to produce a marked elevation in β-VLDL (10–12). Most patients with the E-D/D phenotype have only mild elevations of β-VLDL levels. Grossly elevated β-VLDL levels and F. Dys. develop only when E^d/E^d homozygotes also have an independent factor such as the gene for familial combined hyperlipidemia or familial hypercholesterolemia or some other trait as yet undefined (12). If the product of the apo E^d allele in group 1 patients does not bind to lipoprotein receptors, then why do most patients with the apo E-D/D phenotype (1% of the population) fail to accumulate

large amounts of remnant lipoproteins? On the other hand, if the product of the apparent E^d allele in group 2 patients can bind to lipoprotein receptors, then why are their β-VLDL levels elevated?

From the above discussion, it is clear that unknown factors must regulate the interrelation between genotype at the apo E locus, the apo E phenotype as deduced by isoelectric focusing, the binding activity of apo E for lipoprotein receptors, and the plasma level of β-VLDL. It seems likely that one of these factors may be an agent that mediates the posttranslational modification of apo E so as to regulate its binding activity. There may also be factors that regulate the production of VLDL and its conversion to LDL. Another set of contributory factors may be those that control the number and specificity of hepatic lipoprotein receptors (3).

Recent experiments indicate that dog livers may be capable of producing two types of lipoprotein receptors that are capable of mediating the uptake of remnants (8). One of these receptors is equivalent to the LDL receptor studied in this paper: it binds LDL (apo B) as well as remnants (apo E and B) and is subject to metabolic regulation (3). The other receptor (called the remnant receptor) binds only apo E (8). Dogs produce different amounts of each receptor depending on such variables as age, cholesterol intake, or the demand for bile acids (3, 7, 8). If such a duality of receptors exists in human liver, it seems possible that the apo E-D from group 1 patients may retain its ability to bind to the remnant receptor, although it cannot bind to the LDL receptor. In this case, the differences in plasma β-VLDL levels among patients with the apo E-D/D phenotype may be due to the production of different proportions of remnant receptors and LDL receptors in the liver. The group 1 patients with high β-VLDL levels might be those who produce LDL receptors rather than remnant receptors. In the group 2 patients, a different allelic form of apo E-D may retain its ability to bind to the LDL receptor (as shown in the current studies), but not to the remnant receptor. Patients from group 2 would develop hyperlipidemia if their livers produced remnant receptors, but did not express LDL receptors.

All such explanations are at present speculative. Elucidation of the modulating factors will require the integration of genetic, structural, and functional binding studies of the apo E protein of the type described in the current report.

ACKNOWLEDGMENTS

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