Changes of Genetic Apolipoprotein Phenotypes Caused by Liver Transplantation

Implications for Apolipoprotein Synthesis

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Abstract

Liver transplantation provides a unique opportunity to investigate the contribution in vivo of the liver to the synthesis and degradation of genetically polymorphic plasma proteins. We have determined the genetic polymorphisms of apo A-IV, apo E, and of the Lp(a) glycoprotein (apo (a)) in the plasma of subjects undergoing liver transplantation and in respective organ donors. The results show that in humans, > 90% of the plasma apo E and virtually all apo (a) are liver derived, whereas this organ does not significantly contribute to plasma apo A-IV levels.

Introduction

Apolipoproteins are important programmers of lipoprotein metabolism (1). Most apolipoproteins are synthesized by the liver and the intestine and are secreted into the plasma or into intestinal lymph as so-called nascent lipoproteins (2). A notable exception is apo E, which is synthesized not only by the liver, but also in a variety of other tissues of rats, guinea pigs, and primates including kidney, adrenal gland, ovary, testis, macrophages, and astrocytes of the brain (3–9). In many of these tissues, however, apo E is synthesized only by specific cell types (10). Surprisingly, no apo E synthesis was detected in the small intestine (5–9). Little information on the site(s) of synthesis of Lp(a) lipoprotein is presently available. Insights into the sites of synthesis of apolipoproteins have been gained by various techniques e.g., organ perfusion studies in experimental animals, in vitro tissue culture, cell culture of primary cells, and established cell lines combined with pulse-chase experiments and immunohistology (for review, see references 1 and 11). More recently, dot blot and Northern blot analysis of mRNA levels in tissues and organs of different species have been applied (5–9, 12, 13). These studies have provided clear evidence as to where certain apolipoproteins are synthesized and have provided indirect evidence for the major sites of human plasma apolipoprotein synthesis. However, direct evidence for the quantitative contribution of the various sites of biosynthesis of plasma lipoproteins in humans is still lacking.

Recent studies have demonstrated the potential of liver transplantation as a model for studying the role of this organ in the synthesis or catabolism of macromolecules (14–16).

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Received for publication 18 March 1988 and in revised form 1 August 1988.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/89/01/0137/06 $2.00
Volume 83, January 1989, 137–142

In this study, we have used liver transplantation in humans as a model to assess more directly the in vivo significance of the liver for the synthesis of apo A-IV and E and the Lp(a) glycoprotein (apo (a)). These apolipoproteins are polymorphic in humans (17–21) and therefore permit us to detect changes in genetic phenotypes due to organ transplantation and moreover, to estimate the quantitative contribution of the liver compared with other possible sites of synthesis. Our studies demonstrate that the majority of circulating Lp(a) and apo E is derived from the liver. In sharp contrast, apo A-IV is not produced in significant amounts by this organ in humans.

Methods

Deep frozen plasma (−30°C) of 18 patients who had undergone liver transplantation at the transplantation center of the University Clinics of Innsbruck (R. Margreiter, head) were analyzed before and after transplantation. In Table I the patient's initials, sex, age, and disease that led to the organ transplantation are listed. In six cases it was possible to get sera of the respective organ donors. One patient (O.M.) underwent two liver transplantations. All patients had a combination of cyclosporin A (to maintain blood levels between 300 and 500 ng/ml) and azathioprin and prednisolone for immunosuppression. Serum samples were taken when the patients were in stable clinical conditions.

Electrophoretic procedures. The apo E phenotypes were determined by a Western blotting technique essentially as described by Menzel et al. (22). Very briefly, 10 μl of serum were delipidated with 2.5 ml ethanol/ether (3:1 vol/vol). The protein precipitate was dissolved in 200 μl of 0.1 M Tris-HCl, pH 10.0, 6 M urea and 1% SDS and 10 μl β-mercaptoethanol. Electrofocusing was performed in 8% polyacrylamide gels (total acrylamide concentration = 8.1%, percentage of *N,N'*-methylene bisacryl = 1.3%) using carrier ampholytes pH 4–6 from LKB Instruments (Bromma, Sweden) in an electrophoresis apparatus (SE 600; Hoefer Scientific Instruments, San Francisco, CA). After focusing for 16 h at 3 W and for 1 h at 1,000 V, the gels were electroblotted as described by Towbin et al. (23). The immunologic detection was performed using a double-antibody procedure that used a polyclonal affinity-purified anti–apo E antibody as the first antibody. As second antibody we used goat anti–rabbit IgG (Bioyeda, Rehovot, Israel) that was labeled with colloidal gold according to Lin and Langenberg (24).

The detection of the apo A-IV phenotypes was also done using an Immunoblotting technique as recently described by Menzel et al. (25). In brief, 1 μl of serum or plasma was incubated with 50 μl of 0.01 M Tris-HCl, pH 8.2, containing 1% decylsulfate, 2% ampholytes (pH 4–6), 10% β-mercaptoethanol and 10 μl 80% sucrose for 1 h before electrofocusing. 7 μl of the mixture were applied to the gel. The electrophoretic and blotting procedures were essentially the same as described for apo E. The immunostaining procedure used a rabbit anti–apo A-IV antiserum as the first antibody and the gold-labeled goat anti-rabbit IgG.

The phenotypes of apo (a) were determined by SDS-PAGE followed by Western blotting essentially as described by Utermann et al. (21). As the first antibody, an affinity-purified anti–Lp(a) antibody was used, followed by the gold-labeled anti–rabbit IgG. As an internal standard, a serum with the S1/S2 phenotype was used in each run.

Analytical procedures. The determination of Lp(a) lipoprotein concentration in serum or plasma was done by an electroimmunodif-

Table I. Clinical Diagnosis in the Transplanted Patients

Patient	Sex	Age	Diagnosis
P.V.	M	50	Cirrhosis, non-A non-B infection
W.V.	M	15	Hepatoblastoma
O.M.	F	33	Cryptogenic cirrhosis
L.A.	F	17	α1-Antitrypsin deficiency, cirrhosis
K.P.	M	1	Biliary atresia
S.P.	M	41	Cirrhosis, non-A non-B infection
K.H.	F	43	Bile duct carcinoma
St.M.	F	33	Cirrhosis, autoimmune hepatitis
R.R.	M	43	Cirrhosis, hepatitis B
S.L.	M	27	Hemangioendotheliosarcoma
H.A.	M	62	Amine-precursor uptake decarboxylation-oma
C.E.	W	54	Primary biliary cirrhosis
H.K.	M	54	α1-Antitrypsin deficiency, cirrhosis
Z.H.	W	49	Primary biliary cirrhosis
N.T.	M	39	Cirrhosis, hepatitis B
S.C.	W	29	Primary biliary cirrhosis
M.H.	M	43	Hematochromatosis
O.K.	M	56	Cirrhosis

fusion assay essentially as described by Krempler et al. (25), using affinity-purified polyclonal rabbit anti-Lp(a) and a human Lp(a) lipoprotein reference standard from Immuno AG (Vienna, Austria).

Results

In a retrospective study, plasma samples from 18 patients that had undergone liver transplantation were investigated for genetic apolipoprotein polymorphisms. In six cases, we also were able to obtain plasma samples from the respective organ donors. From all patients, a pretransplantation plasma sample (days −60–0) was obtained. Samples taken after transplantation were obtained between 16 and 275 d after surgery. Lp(a) phenotypes were determined in all and apo A-IV and E phenotypes in 17 of the patients. In all three investigated genetic apolipoprotein systems (apo A-IV, apo E, and Lp(a)), we were able to detect changes in the genetic patterns.

Apo A-IV polymorphism. None of the 17 patients exhibited a change in apo A-IV isoforms upon liver transplantation. 16 had the most common apo A-IV 1-1 type before and after transplantation. One (Z.H.) had the heterozygous A-IV 2-1 type. His apo A-IV phenotype remained unchanged by the transplantation. Although it seems unlikely, this could be because the untyped donor was also a phenotype 2-1. However, two of the organ donors (donor K.H. and donor O.K., Table II) happened to have the heterozygous A-IV 2-1 phenotype. Still no change occurred in the transplanted patients (Fig. 1). Densitometric scans of the blots showed that following transplantation < 1% of immunoreactivity was in the position of apo A IV-2. This demonstrates that most if not all apo A-IV in plasma is derived from tissues other than the liver.

Apo E. Of the 17 patients, 13 were apo E 3/3 homozygous, three were E-3/2 heterozygous, and one was E-4/3 heterozygous. Upon transplantation, several patients changed phenotype from a E-3/3 to a 4/4, 4/3, or 3/2 phenotype, respectively. The 4/3 heterozygous changed to a 3/3 homozygous. Two of the E-3/2 heterozygotes changed to a homozygous E-3/3 type (Fig. 2) and one to a 4/3 phenotype. In five of the patients, (see Table II) that changed apo E phenotype, we could demonstrate that the new phenotype corresponded to that of the organ donor. In view of the apo E phenotype frequencies in the population, these results are not unexpected. They clearly indicate that the liver is a major source of apo E in plasma.

Table II. Apo A-IV and apo E Phenotypes in Patients before and after Liver Transplantation and in Organ Donors

Patient/donor initials		Days before (−) and after (+) transplantation	Apo A-IV phenotype	Apo E phenotype
P.V.		−1	1-1	3/3 F
		+16	1-1	3/3 F
W.V.		0	1-1	3/3 F
		+31	1-1	3/2 F/S
		+91	1-1	3/2 F/S
O.M.	I	−3	1-1	3/3 F
		+275	1-1	3/3 F
	II	−201	1-1	3/3 F
		+189	1-1	4/4 F
L.A.		−2	1-1	3/2 F/S
		+93	1-1	3/3 F
K.P.		−1	1-1	3/3 F
		+18	1-1	3/3 F
K.H.		−1	1-1	3/3 F
		+9	1-1	3/3 F
		+17	1-1	3/3 F
Donor K.H.		−1	2-1	3/3 F
St.M.		−60	1-1	3/2 F/S
		+43	1-1	3/3 F
Donor St.M.		−1	1-1	3/3 F
N.T.		−6	1-1	3/3 F
		+27	1-1	4/3 F
Donor N.T.		−1	1-1	4/3 F
S.C.		−60	1-1	4/3 F
		+23	1-1	3/3 F
Donor S.C.		−1	1-1	3/3 F
M.H.		−28	1-1	3/3 F
		+40	1-1	3/2 F/S
Donor M.H.		−1	1-1	3/2 F/S
O.K.		−55	1-1	3/3 F
		+17	1-1	4/3 F
Donor O.K.		−1	2-1	4/3 F
R.R.		−30	1-1	3/3 F
		+78	1-1	3/3 F
S.L.		−1	1-1	3/3 F
		+47	1-1	3/3 F
H.A.		−1	1-1	3/3 F
		+46	1-1	3/3 F
C.E.		−58	1-1	3/3 F
		+22	1-1	4/3 F
H.K.		−4	1-1	3/3 F
		+32	1-1	3/3 F
Z.H.		−1	2-1	3/2 F/S
		+91	2-1	4/3 F

F, common isoforms E3 and E4 with apparent molecular mass of 34 kD. S, common E2 (Arg 158 → Cys) isoform with apparent molecular mass 35 kD.

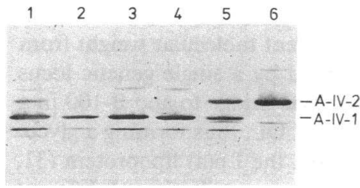

Figure 1. Immunoblot of serum apo A-IV from patient K.H., the respective organ donor and controls. Delipidated serum samples were subjected to electrofocusing in a pH gradient from 4–6 and proteins were blotted into nitrocellulose. Apo A-IV was detected with a polyclonal rabbit anti–apo A-IV antibody followed by gold labeled goat anti rabbit IgG. Lanes *1* and *2*, sera from apo A-IV 1-1 controls; lane *3*, serum from patient K.H. before transplantation; lane *4*, patient K.H. after transplantation; lane *5*, serum from organ donor; lane *6*, apo A-IV 2-2 control.

To avoid misinterpretation of phenotypes that might occur due to the presence of sialilated apo E isoforms (18), we also used SDS-PAGE to distinguish between apo E-4 (Cys 112 → Arg) and apo E 3 on the one hand, apo E 2 (Arg 158 → Cys), and sialilated forms (27). In this system, apo E 4 and E 3 have a lower apparent molecular mass (~ 34 kD = F type) than apo E-2 (Arg 158 - Cys) (~ 35 kD = S type), whereas sialilated forms of apo E have an even higher apparent molecular weight and exhibit a diffuse band (27). This method not only confirmed the changes in apo E phenotypes in the patients with apo E2 isoforms (see Fig. 2) but also allowed for a crude quantitative estimate of the relative amount of apo E that is due to synthesis by the liver. In patients L.A. and St.M., whose apo E types changed from E-3/2 to E-3/3, there was < 5% of apo E in the E2-position following transplantation. Even assuming that all this material is apo E 2 produced by the one $\epsilon 2$ allele in the nonhepatic tissues of these patients the contribution of these tissues to total apo E concentration in plasma is only ~ 10% or less.

Lp(a) lipoprotein. The Lp(a) lipoprotein exhibits a quantitative genetic polymorphism (28, 29) and the Lp(a) specific glycoprotein (apo (a)) shows a qualitative genetic polymorphism (21). 14 of the 18 patients showed significant changes (defined as at least twofold) in Lp(a) lipoprotein plasma concentration (Table III). Notably, these changes were in both directions, from high to low (six patients) as well as from low concentration before to high concentration after the transplantation of the new organ (eight patients).

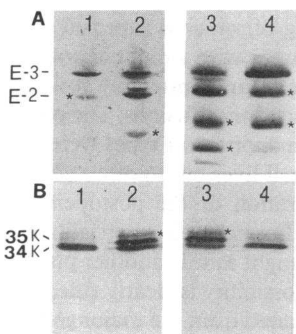

Figure 2. (*A* and *B*) Immunoblots of serum apo E from patients W.V. and L.A. before (b.t.) and after transplantation (a.t.). Delipidated serum samples were subjected to electrofocusing in a pH gradient from (*A*) 4–6 or to (*B*) SDS-PAGE and proteins were transferred to nitrocellulose by electroblotting. Apo E was detected with an affinity-purified polyclonal rabbit anti–apo E antibody followed by gold-labeled goat anti–rabbit IgG. Serum from patient W.V. b.t. (lane *1*) and a.t. (lane *2*) and from patient L.A. b.t. (lane *3*) and a.t. (lane *4*). The a.t. samples were from 31 d (W.V.) and 93 d (L.A.) after surgery. Note change from an apo E 3/3 (F) to an apo E 3/2 (F/S) phenotype in patient W.V. and from apo E 3/2 (F/S) to apo E 3/3 (F) in patient L.A. The 34 kD and 35 kD bands are referred to as F (fast) and S (slow) in Table II and the text. Stars indicate sialilated apo E isoforms.

Apo (a) phenotypes were reversed in 14 patients (Table III). Three principle types of changes were observed (see Fig. 3). Some patients (N.T., C.E., O.M., S.C., and M.H.) changed from a null type (no detectable Lp(a) glycoprotein band) to a single- or double-band type. A second category (K.P., H.K., and O.K.) changed from a single-band type to a null type and in the third group (patients W.V., S.L., S.P., R.R., H.A., and Z.H.), one single or double band phenotype was converted into another one after transplantation (Fig. 3, Table 3). In informative patients of the two latter categories, virtually no pretransplantation Lp(a) species were seen in the posttransplantation plasma samples (Fig. 3). In view of the known individual constancy of Lp(a) lipoprotein concentration and the genetic determination of Lp(a) glycoprotein phenotypes, these findings firmly establish the role of the liver in plasma Lp(a) synthesis.

Discussion

In this study, we capitalized on therapeutic liver transplantation in humans as a model to determine the contribution of

Table III. Effects of Liver Transplantation on Lp(a) Phenotype and Concentration in Plasma

Patient	Lp(a) glycoprotein phenotype			Lp(a) lipoprotein concentration		
	b.t.		a.t.	b.t.		a.t.
					mg/dl	
P.V.	S3		S3	8		20
W.V.	S3		S2	21		65
O.M. I	0		0	0		0
II	0		S2/S3	0		20
L.A.	0		0	4		0
K.P.	S2		0	33		0
S.P.	S3		S2	22		10
K.H.	S4		S4*	25		5*
Donor K.H.		0			0	
St.M.	0		0	0		0
Donor St.M.		0			0	
N.T.	0		S2	0		9
Donor N.T.		S2			15	
S.C.	0		S1	0		42
Donor S.C.		S1			27	
M.H.	0		B/S2	0		96
Donor M.H.		B/S2			30	
O.K.	S4		0	18		0
Donor O.K.		0			0	
R.R.	S2/S3		S2	3		5
S.L.	S2/S4		S3	7		11
H.A.	S3		S2/S3	9		36
C.E.	0		S4	0		22
Z.H.	S2/S4		S4	ND		ND
H.K.	S2		0	10		0

Given as milligrams lipoprotein/deciliter; b.t., before transplantation, a.t., after transplantation.
* Measured 9 d after transplantation.

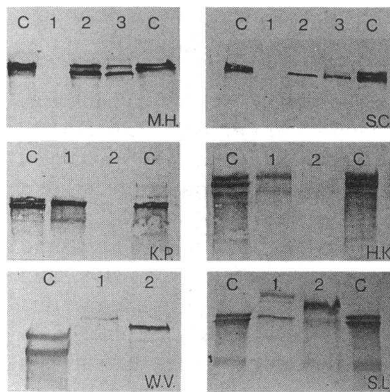

Figure 3. Immunoblots of plasma Lp(a) glycoprotein from six patients before (1) and after (2) transplantation, the respective liver donor (3) and from controls (C). Serum samples were subjected to SDS-PAGE under reducing conditions and proteins were transferred to nitrocellulose by electroblotting. Lp(a) protein was detected with an affinity-purified polyclonal rabbit anti–Lp(a) antibody followed by gold-labeled goat anti-rabbit IgG. Patients' initials are given in the lower right corner.

the liver to the synthesis of plasma apo A-IV, E, and Lp(a). These proteins are polymorphic in humans, thus allowing us to detect and quantify changes in genetic forms of these proteins following transplantation. Apo A-IV is controlled by two common alleles A-IV^1 and A-IV^2 (20, 25, 30). Three frequent alleles, $\epsilon2$, $\epsilon3$, and $\epsilon4$, control apo E polymorphism (17–19) whereas apo(a) is under control of at least seven alleles designated Lp^F, Lp^B, Lp^{S1}, Lp^{S2}, Lp^{S3}, Lp^{S4}, and Lp^o, six of which code for isoforms of different apparent molecular weight, whereas one represents a null allele (21).

Our data show that transplantation of a liver may convert one genetic apo E type into another. This is in general agreement with earlier results from liver transfusion studies in experimental animals and with recent data on apo E mRNA distribution in different organs and tissues of a variety of species both of which have shown that the liver is a major source of plasma apo E (3–9). Using quantitative mRNA measurement Newman et al. (8) estimated that 60–80% of total body apo E mRNA is synthesized by the liver and 20–40% extrahepatically in nonhuman primates. But it is not known, especially not for humans, to what extent the peripheral apo E synthesis contributes to the plasma apo E. Elshourbagy et al. (6) have estimated that ~ 10% of rat and ~ 20% of marmoset apo E in the circulation could be derived from extrahepatic tissues. The concentration of mRNA does not necessarily reflect, however, the amount of protein that is synthesized by the respective tissue or organ. We estimate from the semiquantitative evaluation by scanning densitometry and immunoblotting with ^{125}I-labeled anti–apo E (data not shown) that < 10% of plasma apo E is derived from extrahepatic tissues in the patients studied here. A note of caution is necessary, however. Apo E synthesis in hepatic and extrahepatic cells may be under different control e.g., nutrient intake, and therefore the relative contributions of these sites may vary.

Synthesis of apo A-IV has been postulated to occur primarily in the intestine in humans and to a much lesser extent, if at all, in the liver. Apo A-IV mRNA in human liver was < 2–5% of that found in the small intestine (12, 13). Our results agree with those of Elshourbagy et al. (13) and of Katharanasis (12) and show for the first time on the protein level that in vivo, < 5% of apo A-IV is liver derived.

The major focus of our study was on the site of apo (a) synthesis. This protein occurs in various genetic forms in human plasma that differ in apparent molecular weight from ~ 400,000–700,000 and are coded by a single genetic locus (21). Apo (a) is linked by a disulfide bridge to apo B-100 in a lipoprotein that closely resembles LDL, thus forming a glycoprotein-lipoprotein complex called the Lp(a) lipoprotein (31, 32, 21). No data on the quantitative contribution of different organs and tissues to plasma Lp(a) levels are presently available. Protein and cDNA sequencing have recently demonstrated an extraordinarily high degree of homology of the Lp(a) glycoprotein with plasminogen (33–35). The cDNA used in the study of McLean et al. (34) were derived from a liver library and northern blotting demonstrated mRNA for apo (a) in human liver and the human hepatoma cell line Hep G2 (35). This demonstrated for the first time that Lp(a) glycoprotein is synthesized by the liver. Bersot et al., however, (36) found Lp(a) protein in a fraction of chylomicrons after an oral fat load, suggesting an intestinal origin of Lp(a) protein. Although our studies cannot exclude the possibility that a very minor fraction of Lp(a) is produced by the intestine or other tissues, they clearly demonstrate that plasma Lp(a) is almost exclusively liver derived.

The most definitive and elegant way to prove that the transplanted organ is responsible for the change of a phenotype is to demonstrate that the recipient exhibits the donors phenotype after transplantation. As our study was done retrospectively, determination of the donors' phenotype was not possible in all cases. In all those cases, however, where serum from the donor was available, the situation was very informative. In all cases where the donor and the recipients had a different apo E and/or apo (a) phenotype, the donors' type occurred in the posttransplantation sample. In two cases where the donor and recipient were discordant in the apo A-IV system, the transplantation did not change the recipient's phenotype. If the liver played a significant role in apo A-IV synthesis, one would expect the appearance of the donors apo A-IV type in the recipients' sera. However, we also observed an apparent discrepancy in the Lp(a) system. Whereas patient K.H. had the Lp(a) S4 phenotype before and after transplantation, the donor had no detectable Lp(a) protein (O-phenotype). We believe that this is because of the short interval between transplantation and sampling (only a 9-d posttransplantation sample was available for Lp(a) typing). In this period, Lp(a) plasma levels had dropped from 25 to 5 mg/dl, which is expected after transplantation of a liver from a donor with a null phenotype. The S4 protein detected therefore is most probably residual Lp(a) from the recipient or from blood transfusions. The possibility that the observed changes in apoprotein types were not due to the functioning of the normal liver but to other effects, e.g., the disease state before operation or the postoperative drug regime, has been considered. For example, oversialilation as a consequence of liver disease might mimic another phenotype. For the apo E system, this possibility is clearly ruled out as sialilated forms are clearly separated from the major genetic isoforms in SDS-PAGE (see Fig. 2 B). Moreover, changes were observed in both directions e.g., from E 3/3 to E 3/2 and vice versa. For the Lp(a) system we have shown by neuraminidase treatment that the transplantation-induced changes are not due to changes in the degree of sialilation (data not shown).

One further possible pitfall in our studies might be that patients receive multiple blood transfusions during transplan-

tation. With the only exception discussed above, the time intervals between surgery and determination of apolipoprotein phenotype in the posttransplantation state was in all cases, however, sufficiently long to exclude any possible misinterpretation due to blood transfusion. Half-lives of the proteins under investigation range from 0.37 d for apo E4 (37), to 0.64 d for apo A-IV (38), to 3.32 d for Lp(a) lipoprotein (26). In those patients in whom changes were observed, the changes were manifested in samples taken between 17 (O.K.) and 189 d (O.M.) after transplantation. Hence there is no indication that anything other than the exchange of the organ liver was responsible for the changes in genetic apolipoprotein types.

Acknowledgments

The skillful technical assistance of Linda Fineder and Ulrike Keller is gratefully acknowledged. We thank Prof. Dr. R. Margreiter from the transplantation center of the University Clinics of Innsbruck for providing plasma samples from his patients and Eva Dollinger for typing the manuscript.

This work was supported by grants from the Fonds zur Förderung der wissenschaftlichen Forschung in Österreich to G. Utermann.

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