Cholesterol Efflux From Normal and Tangier Disease Fibroblasts Into Normal, High-Density Lipoprotein–Deficient, and Apolipoprotein E–Deficient Plasmas

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Tangier disease (TD) fibroblasts have defective cholesterol release in the presence of lipid-free apolipoproteins. We compared normolipidemic probands and patients with apolipoprotein A-I (apoA-I) deficiency, apoE deficiency, or TD in terms of the plasma capacity to induce the efflux of [^{3}H]-cholesterol from normal and TD fibroblasts and to esterify this cell-derived cholesterol. Compared with normal fibroblasts, TD fibroblasts released a significantly smaller fraction of [^{3}H]-cholesterol into normal, high-density lipoprotein (HDL)-deficient, and apoE-deficient plasmas. Supplementation of apoE-deficient plasma with exogenous apoE normalized the cholesterol efflux from normal cells but did not fully restore the reduced cholesterol efflux from TD fibroblasts. Compared with control plasma, HDL- and apoE-deficient plasmas had a significantly reduced activity to esterify cell-derived cholesterol. Cholesterol derived from TD fibroblasts was less available for esterification in either patient or normal plasmas than cholesterol derived from normal cells. The esterification defect of TD cell-derived cholesterol was more pronounced in patient plasmas than in control plasma. We conclude that (1) apoA-I and, to a lesser degree, apoE are important determinants of the cholesterol efflux and esterification capacity of plasma, (2) TD fibroblasts have a reduced capacity to release cholesterol into the plasma, and (3) TD cell-derived cholesterol is less available for esterification in plasma than cholesterol from normal fibroblasts. The absence of distinct apoA-I– or apoE-containing subclasses aggravates the defective efflux and esterification of cholesterol derived from TD cells.

THE ANTIATHEROGENIC EFFECT of high-density lipoproteins (HDLs) is widely attributed to their ability to mediate the flux of excess cholesterol from peripheral cells to the liver.[1] An essential step in this reverse cholesterol transport is the efflux of unesterified cholesterol from plasma membranes into the extracellular space.[2-4] Different pathways in cholesterol efflux have been differentiated: (1) Lipid-rich HDL (ie, α-HDL) mediate a bidirectional, slow, and unspecific cholesterol efflux that has been found in all cell types investigated thus far.[2-4] Subsequent extracellular esterification of cell-derived cholesterol by lecithin:cholesterol acyltransferase (LCAT) causes net cholesterol efflux.[1-3] (2) Lipid-poor lipoproteins (ie, preβ-HDL) or lipid-free apolipoproteins mediate a LCAT-independent, fast, and saturable cholesterol efflux from fibroblasts, macrophages, and smooth muscle cell–derived foam cells.[1-4]

The importance of cell-specificity for the 2 kinds of cholesterol efflux is highlighted by the observations on cholesterol efflux from fibroblasts of patients with Tangier disease (TD). TD is a familial HDL deficiency syndrome due to genetic defects in the adenosine triphosphate binding cassette 1 transporter (ABC1), which is associated with foam-cell formation in the reticuloendothelial system and Schwann cells.[5-8] Compared with normal fibroblasts, cholesterol efflux from TD fibroblasts is half-normal in the presence of HDL but reduced to zero in the presence of lipid-free apolipoproteins.[9-12] It has been suggested that the failure of lipid-free apolipoprotein A-I (apoA-I) to pick up phospholipids and cholesterol from TD cells is the basis of both the HDL maturation defect and foam-cell formation in TD.[4,11,12]

To investigate the relevance of the cholesterol efflux defect of TD cells under more physiological conditions, we compared the release of [^{3}H]-cholesterol from normal and TD fibroblasts into the plasma of normal probands and the plasma of patients with deficiencies of distinct HDL subclasses. We used plasma from patients with apoA-I deficiency, which does not contain either lipid-poor preβ-LpA-I or lipid-rich α-LpA-I, and plasma from TD patients, which contains lipid-poor preβ-LpA-I but not lipid-rich α-LpA-I.[13] Moreover, since we and others have previously proposed that the cholesterol efflux capacity of plasma is also determined by apoE-containing HDLs, which are either lipid-poor (ie, γ-LpE) or lipid-rich (ie, α-LpA-I,E),[2,13-18] we also investigated the cholesterol efflux into the plasma of an apoE-deficient patient. To discriminate fast efflux to lipid-poor particles and slow cholesterol efflux to lipid-rich particles, we analyzed cholesterol efflux during plasma incubation with cells for 1 minute and 1 hour.[1,13-15,18] Finally, we addressed the question of whether cholesterol released from normal or TD fibroblasts is equally available for esterification in plasma.

SUBJECTS AND METHODS

Subjects

Three patients with TD, 1 patient with apoA-I deficiency, 1 patient with apoE deficiency, and 2 normolipidemic subjects participated in this study after provision of informed consent and in accordance with the ethical standards in the relevant version of the 1964 Declaration of Helsinki. The cases and molecular defects of the 3 TD patients,[6-7,19-21] the apoA-I–deficient patient,[22] and the apoE-deficient patient[23] have

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been previously reported. Serum concentrations of lipids, lipoproteins, and apolipoproteins are listed in Table 1.

Blood Samples

Blood samples were taken after the subjects fasted overnight. The blood was immediately placed on ice. EDTA plasma and sera were obtained by centrifugation at 4°C (2,000× *g* for 15 minutes), divided into aliquots, and stored at −70°C. Samples from the apoE-deficient patient were sent on dry ice to the laboratory in Münster.

Determination of Lipids, Lipoproteins, Apolipoproteins, and Lipid Transfer Proteins

Serum concentrations of triglycerides and cholesterol were quantified with an autoanalyzer (Hitachi/Boehringer, Mannheim, Germany). The HDL cholesterol level was measured in the supernatant obtained by precipitation of apoB-containing lipoproteins with phosphotungstic acid/$MgCl_2$ (Boehringer). Low-density lipoprotein (LDL) cholesterol was estimated by the Friedewald formula.[24] ApoA-I was quantified with a modified commercially available turbidimetric immunoassay (Boehringer)[25] and apoE with Hydragel LpE particle gels (Sebia, France) as described by the supplier. LCAT was determined as the plasma activity to esterify radiolabeled cholesterol in exogenous reconstituted HDL.[26,27] Cholesteryl ester transfer protein (CETP) activity was determined as the transfer of radiolabeled cholesteryl oleate from reconstituted HDL to LDL.[27]

Preparation of ApoE

Recombinant apoE3 was produced in the baculovirus system following the protocol of Gretch et al[28] using the baculovirus transfer vector pAc-E3 (provided by Dr. Alan D. Attie, Department of Biochemistry, University of Wisconsin, Madison, WI), a derivative of pAcYMI.[29] Cotransfection of BaculoGold baculovirus DNA (PharMingen, San Diego, CA) and pAc-E3 was performed using *N*-[1-(2,3-dioleoyloxy)-propyl]-*N,N,N*-trimethylammonium methylsulfate (DOTAP; Boehringer) as a transfection reagent. To produce recombinant apoE on a large scale, 2×10^6 Sf21 cells/mL were seeded in a spinner flask and infected with a high-titer stock solution of recombinant baculovirus. The multiplicity of infection was 10. After incubation at 27°C for 72 to 96 hours, the apoE-containing supernatant was collected by centrifugation. Prior to storage (−80°C) or purification, a protease inhibitor cocktail was added to produce a final concentration of 32 mg/L benzamidine HCl, 20 mg/L aprotinin, 10 mg/L leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. The apoE solution with 25 mmol/L NH_4HCO_3 was applied to a heparin Sepharose 4B column (2 × 20 cm). After washing with 15 vol 25-mmol/L NH_4HCO_3 and 10 vol 300-mmol/L NH_4HCO_3 apoE was eluted with 700 mmol/L NH_4HCO_3, dialyzed against 25 mmol/L NH_4HCO_3, and rechromatographed under identical conditions. ApoE-deficient plasma was incubated with different concentrations of exogenous apoE3 for 1 hour at 37°C. Afterward, the plasma was used directly for agarose gel electrophoresis or diluted with fetal calf serum (FCS)-free Dulbecco's modified Eagle's medium (DMEM) (1:10 vol/vol) and used for cell culture experiments.

Electrophoretic Techniques

The distribution of apoA-I–containing and apoE-containing lipoproteins (LpA-I and LpE) was analyzed by nondenaturing 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE) of the plasma as described previously.[13,30,31] ApoE-deficient plasma, which was supplemented with different concentrations of exogenous apoE3, was analyzed by agarose gel electrophoresis in Titan Gels (Helena Laboratories, Sunderland, UK) as recommended by the supplier. ApoE was immunodetected with the biotinylated anti-apoE antibodies and streptavidin biotinylated horseradish peroxidase. The immunoreaction was visualized with BM chemiluminescence blotting substrate (Boehringer Mannheim) and a photo imaging system (BAS 1500; Fuji, Tokyo, Japan).[18]

Determination of [^{3}H]-Cholesterol Efflux From Fibroblasts Into Plasma

Fibroblasts were cultivated from skin biopsies obtained from the hip of 2 unrelated healthy normolipidemic subjects (N1 and N2) and 3 patients with TD (T1 to T3) as described previously.[32] The cells were grown and maintained in DMEM that contained 10% FCS and 1% ciprobay. The experiments were performed with cells that were 3 to 10 passages old. The capacity of the plasma from controls and patients to mediate cholesterol efflux from radiolabeled fibroblasts and to esterify cell-derived cholesterol was determined as previously described.[13-15,30,31] Briefly, at about 70% to 80% confluence, cells were labeled in the presence of FCS with 0.5 mCi [1,2-^{3}H]-cholesterol (51.7 Ci/mmol; New England Nuclear, Boston, MA) for 72 hours at 37°C. After 6 washes with phosphate-buffered saline (pH 7.4), the specific radioactivity was $5.0 \pm 1.2 \times 10^6$ cpm/mg protein. To measure the cholesterol efflux capacity and esterification activity of the total plasma, radiolabeled fibroblasts were incubated for 1 minute or 1 hour with plasma diluted in DMEM to a final concentration of 10% (vol/vol). After incubation, the medium was removed and the cell debris was pelleted by centrifugation at 30,000 rpm for 15 minutes at 4°C. After removal of the supernatant, radioactivity in a 50-μL aliquot was determined directly by scintillation spectrometry. Lipids from a 375-μL aliquot were extracted with chloroform/methanol (2:1 vol/vol) for 72 hours, and unesterified cholesterol (UC) and cholesteryl esters (CE)

Table 1. Lipid, Lipoprotein, and Apolipoprotein Concentrations in Plasma From Normolipoproteinemic, TD, ApoA-I–Deficient, and ApoE-Deficient Patients

Parameter	Normal		TD			ApoA-I Deficiency	ApoE Deficiency
	N1	N2	TD1	TD2	TD3		
Sex	M	M	M	F	M	F	M
Age (yr)	37	37	65	67	51	35	32
Cholesterol (mg/dL)	170	225	51	109	82	129	443
Triglycerides (mg/dL)	38	214	213	293	366	40	329
HDL cholesterol (mg/dL)	64	44	<1	<1	<1	<1	42
LDL cholesterol (mg/dL)	98	138	7	50	8	118	124
ApoA-I (mg/dL)	167	134	<1	<1	3	<1	129
ApoE (mg/dL)	9.5	3.7	1.3	2.6	3.4	4.6	<1
ApoE phenotype	3/3	3/3	3/3	3/3	3/2	3/3	ApoE deficiency
LCAT (nmol/h/mL)	20.4	19.5	3.9	8.8	6.1	5.5	19.6
CETP (nmol/h/mL)	104	110	135	149	121	96	149

Abbreviations: M, males; F, females.

were separated by thin-layer chromatography in silica gel plates (Merck, Darmstadt, Germany) using hexane:ether (6:4 vol/vol) as the mobile phase. The cells were lysed with 1.5 mL 0.5-mol/L NaOH, and the lipids were extracted with hexane:isopropanol (3:2, vol/vol). The associated radioactivity was counted by scintillation spectrometry. Fractional cholesterol efflux was calculated as $cpm_{medium}/(cpm_{medium} + cpm_{cells}) \times 100\%$. The fractional esterification rate was calculated as $cpm_{CE}/(cpm_{CE} + cpm_{UC})$. The cholesterol efflux capacity of apoE-deficient plasma was also analyzed after supplementation of the apoE-deficient plasma with 0.2, 2, and 20 μg apoE3/mL plasma.

Statistics

All experiments were performed in duplicate and in parallel on normal and patient plasma using normal cells and TD cells. Because of the high interassay variation in cholesterol efflux and esterification capacity, data on cholesterol efflux and esterification capacity are also presented as a percent of the control. The level of significance for differences between different cell types or plasma was calculated by ANOVA. All calculations were made with Excel (Microsoft, Redmond, WA) and an add-in program for Excel (Astute, 1994 Software, The University of Leeds, Leeds, UK).

RESULTS

Lipids, Apolipoproteins, and Lipoproteins

Table 1 summarizes data on lipid metabolism for the 4 patients with TD, 1 apoA-I–deficient patient, 1 apoE-deficient patient, and 2 normolipidemic controls. Compared with normal plasma, plasma from the apoA-I–deficient and TD patients was characterized by the absence or severely reduced levels of HDL cholesterol and apoA-I and reduced LCAT activity. Serum concentrations of apoE were slightly reduced in these groups as compared with normal control plasma (Table 1). By contrast, the apoE-deficient patient had normal plasma levels of HDL cholesterol, apoA-I, and LCAT, but apoE was undetectable in the serum.

2D-PAGE of the plasma and subsequent anti-apoA-I or anti-apoE immunoblotting identified no apoA-I–containing lipoproteins (LpA-I) in the apoA-I–deficient plasma. However, apoE-containing particles (LpE) with preβ- and γ-mobility were found in apoA-I deficiency (data not shown; see von Eckardstein et al[13]). Plasma from the 4 TD patients contained preβ-LpA-I but no α-LpA-I and an anti-apoE–immunoreactive particle with γ-, β-, and preβ-mobility rather than α-mobility (data not shown; see von Eckardstein et al[13]). As expected, no apoE-containing lipoproteins were detectable in the apoE-deficient plasma. However, apoA-I–containing particles with preβ- and α-mobility were present in the apoE-deficient plasma (Fig 1).

After incubation of apoE-deficient plasma for 1 hour at 37°C with 2, 20, or 50 μg/mL plasma and subsequent separation by agarose gel electrophoresis, apoE-containing lipoproteins became detectable by anti-apoE immunoblotting (Figs 2 and 3). These particles had preβ- and α-mobility and did not differ from preβ- and α-LpE in normal plasma. However, it is noteworthy that no γ-mobile particle became immunodetectable.

Effects of HDL and ApoE Deficiency on Cholesterol Efflux Capacity of Plasma

To determine their cholesterol efflux capacities, normal, TD, apoA-I–deficient, and apoE-deficient plasmas were incubated for 1 minute and 1 hour with control and TD human skin fibroblasts radiolabeled with [^{3}H]-cholesterol. Compared with normal plasma, plasma from patients with TD, apoA-I deficiency, and apoE deficiency had a significantly reduced capacity to release [^{3}H]-cholesterol from normal or TD fibroblasts during either 1 minute or 1 hour of incubation (Tables 2 and 3). The decrease was more pronounced after 1 minute of incubation versus 1 hour of incubation with the cells. Independently of incubation time or cell type, apoA-I–deficient plasma had the greatest defect, TD plasma an intermediate defect, and apoE-deficient plasma the least pronounced defect in cholesterol efflux. The differences in the cholesterol efflux capacity of apoA-I– versus apoE-deficient plasma were statistically significant at 3 of 4 conditions ($P < .01$, ANOVA). The efflux capacity

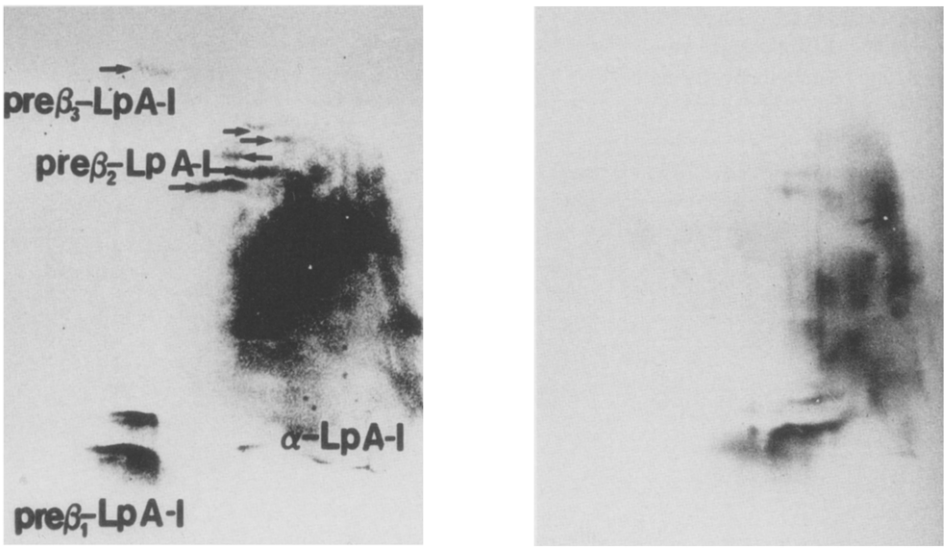

Fig 1. Nondenaturing 2D-PAGE and immunoblotting of apoA-I–containing lipoproteins in the plasma of normolipidemic probands and in apoE-deficient plasma. 2D-PAGE was performed in the sequence agarose gel electrophoresis → nondenaturing PAGE. After electroblotting to a nitrocellulose membrane, apoA-I–containing lipoproteins were detected with polyclonal sheep antisera against human apoA-I and horseradish peroxidase–conjugated antibodies from rabbit to sheep immunoglobulin G (IgG). Note that apoA-I–containing lipoproteins in apoE-deficient plasma do not significantly differ from those in normolipidemic plasma.

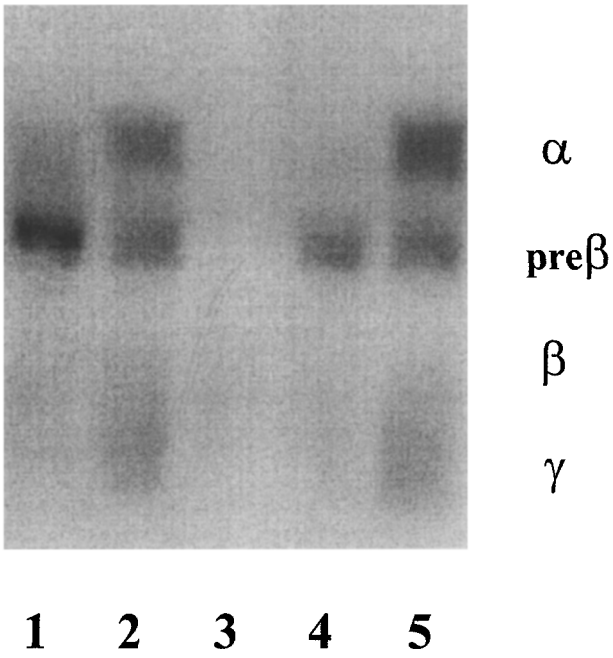

Fig 2. Agarose gel electrophoresis and immunoblotting of apoE-containing lipoproteins of normolipidemic plasma and apoE-deficient plasma. ApoE-deficient plasma was incubated with exogenous apoE3 for 1 hour at 37°C. Lipoproteins separated by agarose gel electrophoresis were electroblotted to a nitrocellulose membrane. ApoE-containing lipoproteins were detected with biotinylated γ-globulins of a goat antiserum against human apoE and streptavidin-biotinylated horseradish peroxidase. Lanes 1 and 4, 2 μg apoE/mL apoE-deficient plasma; lanes 2 and 5, normal plasma; lane 3, native apoE-deficient plasma.

of apoA-I–deficient and TD plasma was significantly different at 1 of 4 conditions, and the efflux capacity of TD and apoE-deficient plasma showed significant differences at 2 of 4 conditions (Tables 2 and 3).

To investigate whether exogenous apoE repairs the cholesterol efflux defect of apoE-deficient plasma, we supplemented this plasma with different amounts of exogenous apoE3 before incubation with the cells. Maximal cholesterol efflux from control and TD cells was found after supplementation with 2 μg apoE3/mL plasma (Fig 4). Therefore, we used apoE-deficient plasma supplemented with 2 μg apoE3/mL for all subsequently described experiments. Although it was less pronounced versus the experiments already described, the cholesterol efflux capacity of apoE-deficient plasma was significantly reduced compared with normal plasma ($P < .01$ for 1 minute and 1 hour of incubation with normal cells and $P < .05$ for 1 minute of incubation with TD cells). Supplementation of apoE-deficient plasma with 2 μg/mL apoE3 increased the cholesterol efflux capacity to values similar to those in normal plasma (Table 4). By contrast, supplementation of normal plasma with exogenous apoE3 did not further increase the cholesterol efflux capacity of native normal plasma.

Effect of Cell Type on Cholesterol Efflux Capacity of Plasma

Compared with normal fibroblasts, TD cells released a significantly smaller fraction of [^{3}H]-cholesterol into either normal, apoA-I–deficient, TD, or apoE-deficient plasma (Tables 2 and 3). It is important to note that the cholesterol efflux defect of TD cells into the α-HDL–deficient plasma of patients with apoA-I deficiency or TD was more pronounced than the defect of normolipidemic and apoE-deficient plasmas, at least during 1-minute incubations. Cholesterol efflux from TD cells into both TD and apoA-I–deficient plasmas was significantly reduced compared with apoE-deficient plasmas ($P < .01$); however, we did not find significant differences between cholesterol efflux into TD and apoA-I–deficient plasmas.

Supplementation of apoE-deficient plasma with exogenous apoE3 increased cholesterol efflux from TD fibroblasts (Fig 4 and Table 4). However, in contrast to the cholesterol efflux from normal cells into apoE-deficient plasma that was normalized by supplementation with apoE3, exogenous apoE did not fully restore the reduced cholesterol efflux from TD cells into apoE-deficient plasma (Table 4).

Esterification of Cell-Derived Cholesterol in HDL- and ApoE-Deficient Plasma

We compared the esterification of cell-derived unesterified [^{3}H]-cholesterol (UC) after incubation of radiolabeled normal and TD fibroblasts with normal, apoA-I–deficient, TD, and apoE-deficient plasma for 1 hour (Table 5). Independently of the cell type used as the cholesterol donor, the esterification of cell-derived cholesterol was significantly reduced in plasma from apoA-I–deficient, TD, and apoE-deficient patients compared with normal plasma. The decrease was most pronounced in apoA-I deficiency ($P < .05$ *v* TD or apoE deficiency).

In addition to the cholesterol efflux defect of TD cells, cholesterol derived from TD fibroblasts was less available for esterification than cholesterol derived from normal cells (Table 5). Again, the absence of HDL led to the most pronounced

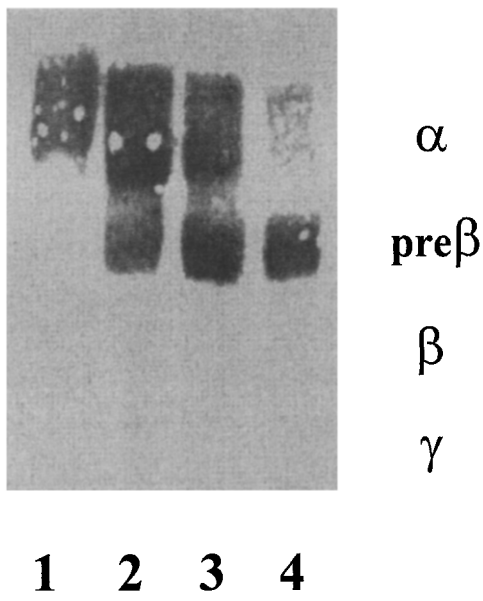

Fig 3. Agarose gel electrophoresis and immunoblotting of apoE-containing lipoproteins of apoE-deficient plasma supplemented with different amounts of exogenous apoE3. For methods and further details, see Fig 2. Lane 1, 20 μg apoE3/mL water; lane 2, 50 μg apoE3/mL apoE-deficient plasma; lane 3, 20 μg apoE3/mL apoE-deficient plasma; lane 4, 2 μg apoE/mL apoE-deficient plasma.

Table 2. One-Minute Cholesterol Efflux From Normal or TD Fibroblasts Into Normal, TD, ApoA-I–Deficient, or ApoE-Deficient Plasma

Plasma	Cholesterol Efflux Capacity, % Cellular [^{3}H]-Cholesterol (% cholesterol efflux of normal plasma and normal cells)		*P* (ANOVA)
	Normal Cells	TD Cells	
A. Normal	1.65 ± 0.43 (100)	1.24 ± 0.45 (60 ± 23)	.001
B. TD	0.63 ± 0.19 (38 ± 6)	0.38 ± 0.11 (27 ± 15)	.01
C. ApoA-I–deficient	0.57 ± 0.12 (35 ± 4)	0.33 ± 0.12 (24 ± 15)	.001
D. ApoE-deficient	0.71 ± 0.24 (42 ± 6)	0.55 ± 0.03 (36 ± 9)	.001
	P (ANOVA)		
A *v* B	.001	.001	
A *v* C	.001	.001	
A *v* D	.001	.001	
B *v* C	NS	NS	
B *v* D	NS	.01	
C *v* D	.01	.01	

NOTE. Radiolabeled normal and TD human skin fibroblasts were incubated with FCS-free DMEM containing 10% normal, TD, apoA-I–deficient, or apoE-deficient plasma. After 1 minute of incubation, 100 µL medium was removed, and after 1 hour of incubation, the residual 900 µL medium and washed cells were removed. Radioactivity levels of the media and cells were measured. Cholesterol efflux was calculated as follows: efflux 1 min = cpm medium 1 min/(cpm medium 1 h + cpm cells) in %. Data are from 3 independent experiments in duplicate and show % cholesterol efflux capacity of normal plasma. Statistical analyses for data of normal and HDL-deficient or apoE-deficient plasma were calculated with ANOVA.

Abbreviation: NS, not significant.

defect in apoA-I–deficient plasma. However, compared with normal plasma, TD plasma and apoE-deficient plasma had a significantly reduced capacity to esterify cell-derived cholesterol.

DISCUSSION

The properties of both the cell and the acceptor particle are important determinants of cholesterol efflux from cells.[2-4] This is highlighted by the previous observation of acceptor-specific defects in cholesterol efflux from TD cells.[11,12] TD fibroblasts were 100% effective in releasing cholesterol in the presence of cyclodextrins and phospholipid vesicles, 50% effective in the presence of native or reconstituted HDL, and completely ineffective in the presence of lipid-free apolipoproteins. The aim of this study was to analyze the determinants of cholesterol efflux and esterification capacity under more physiological conditions. Therefore, we incubated [^{3}H]-cholesterol-labeled fibroblasts from TD patients or normal donors with normolipidemic, apoA-I–deficient, TD, and apoE-deficient human plasma. Our data show that apoA-I and, to a lesser degree, apoE determine the cholesterol efflux and esterification capacity of plasma. TD fibroblasts showed a reduced capacity to release cholesterol into the plasma. The absence of distinct apoA-I– or apoE-containing subclasses aggravated this defect, especially during short incubations. Cholesterol released from TD cells was less available for esterification in the plasma than cholesterol from normal fibroblasts.

Both 1-minute and 1-hour incubations led to significantly reduced cholesterol efflux into apoA-I–deficient, TD, and apoE-deficient plasma compared with normal plasma from normal or TD fibroblasts (Tables 2 and 3). These data confirm our previous data on impaired cholesterol efflux into the plasma

Table 3. One-Hour Cholesterol Efflux From Normal or TD Fibroblasts Into Normal, TD, ApoA-I–Deficient, or ApoE-Deficient Plasma

Plasma	Cholesterol Efflux Capacity, % Cellular [^{3}H]-Cholesterol (% cholesterol efflux of normal plasma and normal cells)		*P* (ANOVA)
	Normal Cells	TD Cells	
A. Normal	9.73 ± 1.14 (100)	6.69 ± 0.39 (63 ± 19)	.001
B. TD	6.32 ± 0.83 (65 ± 6)	5.10 ± 0.43 (42 ± 18)	.01
C. ApoA-I–deficient	6.55 ± 1.07 (67 ± 13)	4.47 ± 1.35 (38 ± 22)	.001
D. ApoE-deficient	7.80 ± 0.95 (78 ± 10)	5.29 ± 0.68 (44 ± 20)	.001
	P (ANOVA)		
A *v* B	.001	.001	
A *v* C	.001	.001	
A *v* D	.001	.001	
B *v* C	NS	NS	
B *v* D	NS	.01	
C *v* D	.01	.01	

NOTE. Radiolabeled normal and TD human skin fibroblasts were incubated with FCS-free DMEM containing 10% normal, TD, apoA-I–deficient, or apoE-deficient plasma. After 1 minute of incubation, 100 µL medium was removed, and after 1 hour of incubation, the residual 900 µL medium and washed cells were removed. Radioactivity levels of the media and cells were measured. Cholesterol efflux was calculated as follows: efflux 1 h = cpm medium 1 h/(cpm medium 1 h + cpm cells) in %. Data are from 3 independent experiments in duplicate and show % cholesterol efflux capacity of normal plasma. Statistical analyses for the data of normal and HDL-deficient or apoE-deficient plasma were calculated with ANOVA.

Abbreviation: NS, not significant.

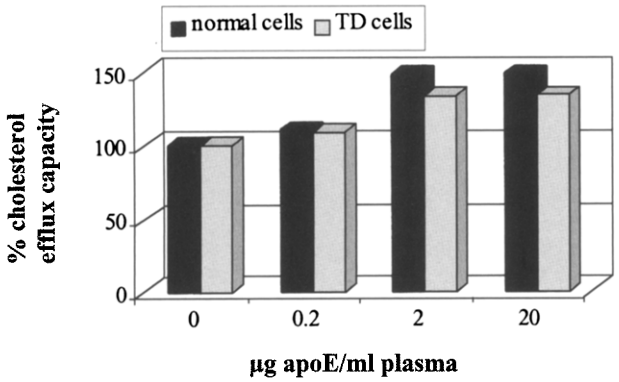

Fig 4. Effect of supplementation of apoE-deficient plasma with different amounts of apoE3 on cholesterol efflux from normal and TD fibroblasts during a 1-minute incubation. Cell culture was performed as described in Table 2. Afterward, normal and TD human skin fibroblasts were incubated for 1 minute with DMEM containing 10% human apoE-deficient plasma supplemented with 0, 0.2, 2, and 20 µg/mL exogenous apoE3. Cholesterol efflux was determined as described in Table 2. Data are the percent cholesterol efflux capacity of apoE-deficient plasma without apoE.

of patients with different forms of familial HDL deficiency,[13] and suggest that apoA-I and, to a lesser degree, apoE are important determinants of the cholesterol efflux capacity of plasma. Our findings on cholesterol efflux in apoA-I and apoE deficiency indicate that the bulk of HDL does not play an exclusive role in reverse cholesterol transport but that quantitatively minor plasma subfractions, eg, preβ1-LpA-I and γ-LpE, are important contributors to the release of cellular cholesterol into the plasma.

The 36% to 65% reduction of the cholesterol efflux capacity of apoE-deficient plasma is in agreement with the results of our previous studies on apoE-deficient mouse plasma.[15,18] This cholesterol efflux defect could be corrected by the addition of small amounts of apoE, which increased the apoE level in apoE-deficient plasma to less than 10% of the value in normal plasma. Likewise, we have previously demonstrated that macrophage-specific expression of a human apoE transgene in apoE-deficient mice, which results in less than 10% of the normal apoE plasma level and does not correct remnant hyperlipidemia, restores the cholesterol efflux defect of apoE-deficient mouse plasma.[18] Obviously, very small amounts of apoE are sufficient to optimize the cholesterol efflux capacity of plasma. However, it is also important to note that other investigators reported that the absence of apoE from plasma does not impair the cholesterol efflux capacity.[33] Possibly, methodological differences are responsible for the discrepancies in the results.

Compared with normal fibroblasts, TD fibroblasts, which have a defective ABC1 transporter,[6-7] released significantly smaller fractions of [^{3}H]-cholesterol into normolipidemic, HDL-deficient, and apoE-deficient plasmas (Tables 2 and 3). It has been previously shown that cholesterol efflux from TD fibroblasts is half-normal in the presence of HDL but is reduced to zero in the presence of the lipid-free apolipoproteins A-I, A-II, A-IV, Cs, and E.[9-12] The acceptor-specific defect of cholesterol efflux from TD cells was explained by the different mechanisms of cholesterol efflux that are induced by HDL or lipid-free apolipoproteins. HDLs have been suggested to release cholesterol by 2 distinct mechanisms, namely unspecific aqueous diffusion from the plasma membrane onto lipoproteins and activation of a brefeldin-sensitive translocation of cholesterol from intracellular compartments.[11] However, cholesterol efflux by lipid-free apolipoproteins fully depends on the brefeldin-sensitive and ABC1-dependent pathway. In TD cells, the brefeldin-sensitive and ABC1-dependent cholesterol mobilization by lipid-free apolipoproteins is impaired, but not the diffusion out of the membrane onto HDL. Moreover, the extraction of cholesterol from plasma membranes by lipid-free apolipoproteins or lipid-poor particles like preβ-HDL has been shown to be more rapid than the diffusion of plasma membrane cholesterol onto lipid-rich lipoproteins such as α-HDL or even LDL. In agreement with a specific defect of lipid-free apolipoprotein–induced efflux from TD cells, at least during 1-minute incubations, the cholesterol efflux defect of TD cells into α-HDL–deficient plasma of patients with apoA-I deficiency or TD was more pronounced than this defect of normolipidemic and apoE-deficient plasma, which both contain α-HDL.

The esterification of cell-derived cholesterol from either

Table 4. Effect of ApoE Supplementation on Cholesterol Efflux Capacity of Normal and ApoE-Deficient Plasma

	% Cholesterol Efflux of Normal Plasma and Normal Cells			
	1-Minute Cholesterol Efflux		1-Hour Cholesterol Efflux	
Plasma	Normal Cells	TD Cells	Normal Cells	TD Cells
A. Normal	100	88 ± 37	100	80 ± 13
B. Normal + 2 µg/mL apoE3	97 ± 5	81 ± 9	93 ± 6	79 ± 3
C. ApoE-deficient	65 ± 6	60 ± 5	85 ± 16	68 ± 12
D. ApoE-deficient + 2 µg/mL apoE3	97 ± 12	73 ± 4	103 ± 29	74 ± 5
	P (ANOVA)			
A *v* B	NS	NS	NS	NS
A *v* C	.01	.05	.01	NS
A *v* D	.001	NS	NS	NS

NOTE. Normal and TD cells were incubated with normal plasma, normal plasma supplemented with 2 µg/mL apoE3, apoE-deficient plasma, and apoE-deficient plasma supplemented with 2 µg/mL apoE3. For further details, see Table 2. Data are the median and (ranges) from 2 independent experiments in duplicate and show the % efflux obtained at the indicated condition relative to efflux obtained with normal plasma in the presence of normal cells. *P* was calculated by ANOVA.

Abbreviation: NS, not significant.

Table 5. Esterification of Cholesterol From Normal and TD Fibroblasts by Normal, ApoAI-Deficient, TD, and ApoE-Deficient Plasma

Plasma	Fractional Esterification Rate, % [^{3}H]-Cholesteryl Esters of [^{3}H]-Cholesterol in the Medium (% fractional esterification of normal plasma and normal cells)		*P* (ANOVA)
	Normal Cells	TD Cells	
A. Normal	3.13 ± 0.34 (100)	2.04 ± 0.18 (65 ± 4)	.001
B. TD	2.56 ± 0.54 (82 ± 17)	1.65 ± 0.08 (49 ± 17)	.01
C. ApoA-I–deficient	2.05 ± 0.33 (65 ± 8)	1.40 ± 0.22 (44 ± 7)	.001
D. ApoE-deficient	2.57 ± 0.56 (82 ± 15)	1.71 ± 0.28 (54 ± 10)	.001
	P (ANOVA)		
A *v* B	.001	.001	
A *v* C	.001	.001	
A *v* D	.001	.001	
B *v* C	NS	NS	
B *v* D	NS	.01	
C *v* D	.01	.01	

NOTE. Radiolabeled normal and TD human skin fibroblasts were incubated for 1 hour with FCS-free DMEM containing 10% normal, TD, apoA-I–deficient, or apoE-deficient plasma. Unesterified [^{3}H]-cholesterol and [^{3}H]-cholesteryl esters in the medium were separated by thin-layer chromatography. Fractional cholesterol esterification rate was calculated as [^{3}H]-cholesteryl esters in the medium/[^{3}H]-cholesteryl esters in the medium + [^{3}H]-unesterified cholesterol in the medium × 100%. Data are the median and (range) from 3 independent experiments in duplicate. *P* was calculated by ANOVA.

Abbreviation: NS, not significant.

normal or TD cells was significantly reduced in the plasma of apoA-I–deficient, TD, and apoE-deficient patients compared with normal subjects (Table 5). This is in agreement with our previous finding of defective esterification of cell-derived cholesterol in the plasma of normal, apoA-I–deficient, and apoE-deficient mice.[15] Since the majority of cell-derived cholesterol is esterified in α-LpA-I[30] and since LCAT activity is reduced in HDL deficiency (Table 1), the reduced esterification of cell-derived cholesterol in apoA-I–deficient plasma and TD plasma is not surprising. Possible reasons for the reduced cholesterol esterification rate in apoE-deficient plasma despite its normal LCAT activity include the lack of apoE as a LCAT activator[34,35] and the absence of lipoproteins that are important for effective cholesterol esterification.[18]

Most interestingly, TD cell-derived cholesterol was less available for esterification in plasma than cholesterol from normal fibroblasts (Table 5). In conjunction with the aforementioned efflux defect of TD cells, this indicates that cellular cholesterol is released into various plasma pools which differ in esterification capacity. It is probable that one of these pathways is not used if cholesterol is released from TD cells. In general, cell-derived cholesterol appears to be esterified either in preβ$_3$-LpA-I, a particle containing apoA-I, apoB, LCAT, and CETP, or in α-LpA-I. It has been suggested that preβ$_3$-LpA-I esterifies cholesterol that is taken up by preβ$_1$-LpA-I, whereas α-LpA-I esterifies cholesterol that is taken up by this particle either directly from cells or from LDL.[30,36] The coincidence of absent efflux from TD cells in the presence of lipid-free apoA-I and the reduced availability of TD cell-derived cholesterol for esterification in plasma supports the model specifying that cellular cholesterol taken up by preβ1-HDL is esterified in preβ$_3$-HDL.[1,30,36]

In conclusion, the present studies have confirmed the partial defects of both HDL- and apoE-deficient plasmas to release cholesterol from cells and to esterify it. Thus, both apoA-I and, albeit to a lesser degree, apoE are important determinants of the cholesterol efflux and esterification capacity of plasma. Moreover, the reduced cholesterol efflux from TD fibroblasts into plasma provides evidence that the previously demonstrated cholesterol efflux defect in the presence of HDL or lipid-free apolipoproteins has physiological relevance. Most notably, cholesterol released from TD cells is less available for esterification in plasma than cholesterol released from normal cells. This esterification defect may further impair reverse cholesterol transport in TD.

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