Free Cholesterol Deposition in the Cornea of Human Apolipoprotein A-II Transgenic Mice With Functional Lecithin:Cholesterol Acyltransferase Deficiency

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We have developed several lines of transgenic animals that overexpress different levels of human apolipoprotein A-II (apoA-II). The 11.1 transgenic line has human apoA-II in plasma at threefold the level in normolipidemic humans and a functional lecithin:cholesterol acyltransferase (LCAT) deficiency. The latter is a biochemical phenotype similar to that of fish-eye disease (FED), which is characterized by free cholesterol (FC) and phospholipid accumulation in the cornea, leading to opacity and impaired vision. To assess whether the metabolic alterations in these mice also lead to lipid accumulation in the cornea, we fed them on a long-term regular chow or high-fat/high-cholesterol (HF/HC) diet. The 11.1 transgenic mice showed a moderate accumulation of FC in the cornea, but only when fed the regular chow diet. This FC accumulation was less severe than the accumulation described in FED, which may explain the lack of corneal opacity in these mice. Electron microscopy and immunoblotting analysis of the cornea of 11.1 transgenic mice in comparison to control mice showed (1) a mild but nevertheless more intense intracytoplasmatic lipid particle deposition in the epithelial cells and (2) a decrease of immunoreactive apoA-I in the area of Bowman's layer and at the superficial stroma. The serum capacity to cause cholesterol efflux from rat fibroblasts was decreased in 11.1 transgenic mice, but only in those fed a regular chow diet. We conclude that 11.1 human apoA-II transgenic mice may be a useful model for studies of early lipid deposition in the cornea and its possible prevention.

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THERE ARE TWO MAIN FORMS of lipid accumulation in the cornea, both associated with alterations in lipoprotein metabolism. One is arcus lipoides, which occurs in the periphery of the cornea, does not interfere with vision, and is associated with an elevation of plasma apolipoprotein B (apoB)-containing lipoproteins.[1] The second form, known as corneal opacity, is characterized by a diffuse cloudiness of the corneal stroma and interferes with vision.[1] Corneal opacities are usually associated with inherited high-density lipoprotein (HDL) deficiency. One such disorder is due to a total or partial deficiency of the enzyme lecithin:cholesterol acyltransferase (LCAT),[1-4] which is responsible for catalyzing the esterification of plasma cholesterol using a fatty acid from phosphatidylcholine as a source.[2-4] Impaired LCAT activity results in a failure to esterify cholesterol in plasma, HDL deficiency, and an accumulation of free cholesterol (FC) and phospholipids in the tissues.[2-4] The magnitude and site of lipid deposition differs depending on the form of inherited LCAT deficiency. One form is a complete familial LCAT deficiency (FLD) and the other is a partial LCAT deficiency, also known as fish-eye disease (FED). Both FLD and FED have a similar molecular basis and are characterized by lipid deposition in the cornea, but FLD has clinically important depositions in the red blood cells and kidney.[2-4]

The lack of animal models for LCAT deficiency has precluded investigation into the deposition of lipids in the cornea and other tissues. We have reported previously that transgenic mice overexpressing human apoA-II show functional LCAT deficiency related to a decreased plasma apoA-I concentration[5] and, as such, represent a phenotype similar to that of FED patients.[2-4] The present study was undertaken to determine whether these mice develop corneal opacities and/or FC accumulation over a protracted time course, and to gain insight into the molecular mechanisms that may be implicated in the process.

MATERIALS AND METHODS

Animals

The C57BL/6 human apoA-II transgenic mice used in these studies have been described previously.[5,6] Transgenic and control mice used for the corneal studies were fed ad libitum with regular chow diet or a high-fat/high-cholesterol (HF/HC) diet. The animals were 3 months old when introduced into the study, and then spent either 16 months on a regular chow diet or 9 months on a HF/HC diet.

Lipid Measurements in Plasma and Isolated Lipoproteins and LCAT Activity

Lipoproteins from pooled plasma were isolated by sequential ultracentrifugation as previously described.[5] Total cholesterol, FC, and triglyceride (corrected for free glycerol) levels were measured colorimetrically using commercial kits (Boehringer, Mannheim, Germany). Plasma human apoA-II concentrations were determined using a commercial immunoassay (Immuno, Vienna, Austria). Mouse apoA-I was determined by radial immunodiffusion.[7] Endogenous plasma LCAT activity and LCAT activity for an exogenous substrate were measured as previously described.[5]

Lipid Extraction and Determination of Cholesterol in the Cornea

The mice were killed and the eyes excised. A corneal-scleral button was cut from the eye, and the corneal tissue was removed from any remaining sclera and stored at −80°C for lipid analysis. Pooled samples

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Table 1. Lipid Parameters in Plasma and Lipoproteins Isolated by Sequential Ultracentrifugation and Apolipoproteins and LCAT Activity in Plasma of Control Mice and Human ApoA-II Transgenic Mice Fed Either a Regular Chow Diet or HF/HC Diet

Parameter	Regular Chow Diet			HF/HC Diet		
	Control (n = 11)	25.3 (n = 11)	11.1 (n = 12)	Control (n = 12)	25.3 (n = 11)	11.1 (n = 9)
Lipids†						
Plasma						
TC (mg/dL)	78.9	74.7	42.3	125.0	161.7	320.3
% FC	20.5	15.0	41.8	23.7	20.5	28.6
Triglycerides (mg/dL)	14.2	15.0	48.7	13.3	11.5	70.8
VLDL						
TC (mg/dL)	1.5	1.8	1.92	35.6	63.9	136.6
% FC	75.0	74.0	88.0	22.7	19.3	28.0
IDL						
TC (mg/dL)	0.8	0.4	3.1	25.0	33.9	70.1
% FC	24.1	49.3	50.0	23.1	21.6	25.3
LDL						
TC (mg/dL)	10.4	6.5	15.8	24.2	21.6	81.2
% FC	22.2	17.7	34.8	28.5	28.5	34.1
HDL						
TC (mg/dL)	55.0	50.0	12.9	26.9	26.6	19.9
% FC	7.0	7.2	24.0	12.6	10.8	56.0
LDP						
TC (mg/dL)	4.6	4.6	3.1	7.3	3.0	3.1
% FC	83.3	83.3	97.4	79.4	89.4	80.7
Apolipoproteins and LCAT						
Plasma						
Mouse apoA-I (mg/dL)	128.3 ± 16.6	110.6 ± 14.7	23.6 ± 11.1*	157.6 ± 9.5	133.7 ± 33.6	39.3 ± 4.0*
Human apoA-II (mg/dL)	0.0 ± 0.0	28.4 ± 5.4*	84.7 ± 7.3*	0.0 ± 0.0	25.4 ± 5.4*	100.7 ± 7.3*
Endogenous LCAT (nmol CE/mL/h)	145.0 ± 47.6	125.9 ± 34.7	16.8 ± 12.8*	176.2 ± 62.3	ND	43.6 ± 20.4*
Exogenous LCAT (nmol CE/mL/h)	4.67 ± 2.74	4.00 ± 1.5	3.31 ± 1.3	5.43 ± 4.4	ND	4.15 ± 3.0

Abbreviations: TC, total cholesterol; LDP, lipoprotein-deficient plasma; ND, not determined; CE, cholesteryl esters.

*$P < .05$ *v* control.

†Each result represents 1 measurement of a pool of samples that included all mice.

of up to three to four corneas from mice of each transgenic and control line were finely minced, and the lipids were extracted as previously described.[8] The measurement of FC was made by enzymatic conversion of cholesterol to cholest-4-ene-3-one by cholesterol oxidase followed by analysis of the sample by reverse-phase high-performance liquid chromatography (HPLC).[9] The within- and between-assay imprecision for corneal FC determinations was 7.1% and 11.8%, respectively.

Immunoblot Analysis of Corneal ApoA-I

The corneas were processed as described previously.[10] After protein extraction, samples were centrifuged at 10,000 × *g*, delipidated, and concentrated by precipitation with chloroform-methanol according to the procedure described by Wessel and Flügge.[11] The precipitates were redissolved in solubilization buffer containing 0.01% bromophenol blue, subjected to electrophoresis, blotted, and probed with rabbit immunoglobulin G (IgG) polyclonal antibodies with specificity against apoA-I and/or with monoclonal antibodies to mouse IgG.[12] The amount of IgG in the corneal extracts was used to obtain a relative assessment of the amount of corneal apoA-I.

Ultrastructural Analysis of the Cornea

The corneas were dissected and removed at the limbus level using the appropriate corneal microsurgery forceps and scissors. Specimens were immediately fixed in 0.1% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) solution (pH 7.4) for at least 12 hours at 4°C. They were then rinsed in PBS and divided into four pieces under a dissecting microscope. Half the specimens were placed in 2.5% glutaraldehyde and 4% paraformaldehyde in PBS and stored at 4°C for conventional ultrastructural microscopy, and the remainder were prepared for immunocytochemistry analysis. For both embedding techniques, the corneas were placed and oriented to obtain ultrathin transverse sections of the polymerized blocks.

For conventional ultrastructural microscopy, specimens were post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate-buffered (PB) solution (pH 7.4) for 1 hour, dehydrated in increasing concentrations of acetone, and then embedded in resin (Spurr technique) for polymerization at 60°C. Ultrathin sections (50 to 75 nm) were obtained by conventional ultramicrotomy (OmU2; Reichert-Jung, Vienna, Austria), placed on copper grids (200 mesh), and then stained with uranyl acetate and lead citrate solution for conventional transmission electron microscopy (TEM) (600 AB; Hitachi, Tokyo, Japan). For lipid detection on TEM, the osmophilic thiocarbohydrazide/osmium tetroxide[13] (OTO) method was used. For immunocytochemistry procedures, the specimens were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in PB solution for at least 12 hours at 4°C and then rinsed in PBS and treated with 0.15 mol/L ammonium chloride in PBS solution. The corneas were then progressively dehydrated and embedded in the hydrophilic resin Lowicryl K4M (Chemiche Werke Lowi, Waldkraiburg, Germany) for polymerization at −35°C. Consecutive serial ultrathin sections (60 to 85 nm) were obtained by conventional ultramicrotomy, placed on gold grids (200 mesh), and Formvar-coated for conventional TEM. The grids were hydrated in 0.1 mol/L glycine plus 0.1 mol/L PBS (PBSG) solution and blocked in 2% ovalbumin in PBSG–0.005% Tween 20 solution for 30 minutes at room temperature, and then incubated with rabbit polyclonal antibodies to apoA-I (diluted 1:200) in 1% ovalbumin in PBSG solution for 2 hours at room temperature in a humidified

Table 2. Total Cholesterol and FC Measurements in the Cornea of Control Mice and Human ApoA-II Transgenic Mice Fed Either a Regular Chow Diet or a HF/HC Diet

Parameter	Control	25.3 Transgenic	11.1 Transgenic
Chow diet			
No. of animals	11	8	7
TC (μg/mg)	3.2 ± 0.4	3.6 ± 0.4	3.9 ± 0.0*
FC (μg/mg)	2.5 ± 0.3	2.9 ± 0.2	3.5 ± 0.2*†
% FC	78.4 ± 2.5	79.9 ± 4.0	91.6 ± 4.5*†
HF/HC diet			
No. of animals	7	8	9
TC (μg/mg)	3.0 ± 0.6	3.0 ± 0.4	3.2 ± 0.3
FC (μg/mg)	2.4 ± 0.7	2.4 ± 0.5	2.8 ± 0.5
% FC	80.0 ± 7.1	78.1 ± 9.2	84.3 ± 9.4

Abbreviation: TC, total cholesterol.
*$P < .05$ *v* control.
†$P < .05$ *v* 25.3 transgenic.

chamber. After several washes in PBSG solution, the sections were incubated with conjugated colloidal gold goat IgG (Ig-Au 15 nm) against rabbit IgG (British BioCell Research Laboratory, Cardiff, UK) for 1 hour at room temperature in a humidified chamber. After several washes, first in PBS and then in redistilled water, the grids were contrasted with 2% uranyl acetate solution and lead citrate. Control experiments were performed in parallel but with the primary antibody omitted. Electron micrographs were obtained on a Hitachi 600 AB.

Cell Culture and Cholesterol Efflux

The capacity of serum to promote cholesterol efflux from normal rat kidney (NRK) fibroblasts in culture was quantified using the recently published method of de la Llera Moya et al.[14] All samples from an individual animal were processed in the same assay. A pool of serum was used as a control. The intraassay and interassay coefficients of variation were 2.27% and 3.75%, respectively.

Statistical Analysis

Results are expressed as the mean ± SEM. Comparisons were performed using the Mann-Whitney *U* test, and *P* values less than .05 were considered significant.

RESULTS

Plasma Lipid and Lipoprotein Profile and LCAT Activity

In the present study, experiments were performed using the higher- and lower-expressor human apoA-II transgenic lines (11.1 and 25.3, respectively) fed either a regular chow or a HF/HC diet. On regular chow, the plasma of 11.1 mice showed hypertriglyceridemia, hypocholesterolemia, hypoalphalipoproteinemia, and an increased percentage of FC, whereas the plasma of the 25.3 line showed a lipoprotein profile similar to that of control mice. On the HF/HC diet, plasma cholesterol levels were increased, depending on the line, 1.6- to 7.6-fold relative to the levels on regular chow. On this HF/HC diet, the ratio of cholesterol in apoB-containing lipoproteins to HDL cholesterol increased progressively from control to 25.3 to 11.1 transgenic mice, although HDL cholesterol was only moderately lower in 11.1 mice versus 25.3 and control mice (Table 1).

On regular chow, the mouse apoA-I plasma concentration in 11.1 transgenic mice was 18.4% of the level in controls, and although exogenous LCAT activity in 11.1 transgenic mice was 70.8% of the level in control mice, endogenous LCAT activity was only 11.6% of the level in the controls. All of these parameters were similar in 25.3 and control mice. On the HF/HC diet, the mouse apoA-I plasma concentration in transgenic mice was 24.9% of the level in the controls, and again, even though LCAT exogenous activity in 11.1 transgenic mice was 76.4% of that in control mice, endogenous LCAT activity was only 24.8% of the level in the controls. The plasma concentration of human apoA-II was similar on both diets (Table 1).

Cornea Analyses

None of the mice developed corneal opacities when examined by an expert ophthalmologist (Dr Duch, Department of Ophthalmology, Hospital de la Santa Creu i Sant Pau). Cornea cholesterol, FC, and the percentage of FC were significantly elevated in 11.1 mice versus control or 25.3 mice, but only on the regular chow (Table 2). Immunoblot analysis of the corneas showed that the apoA-I immunoreactive band was slightly

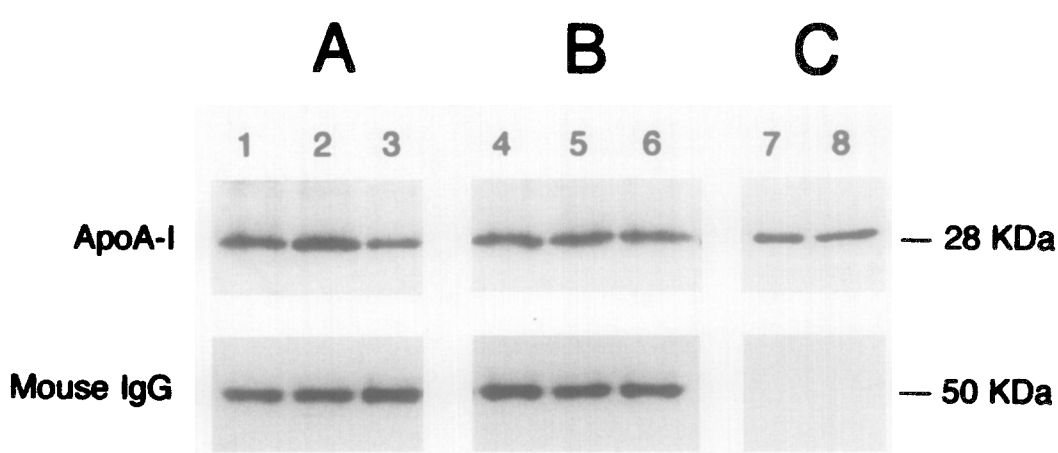

Fig 1. Representative immunoblots of corneal apoA-I and IgG. (A) Animals fed regular chow: lane 1, control; lane 2, 25.3 transgenic mice; lane 3, 11.1 transgenic mice. (B) Animals fed a HF/HC diet: lane 4, control; lane 5, 25.3 transgenic mice; lane 6, 11.1 transgenic mice. (C) ApoA-I standards prepared from mouse HDL (lanes 7 and 8).

decreased in 11.1 mice versus control and 25.3 transgenic mice, especially when the mice were fed regular chow. In contrast, the IgG immunoreactive band in the 11.1 transgenic mice cornea was not decreased on either diet in comparison to 25.3 transgenic mice or control mice (Fig 1).

Conventional electron microscopy did not reveal differences between 11.1 transgenic mice and control mice corneas (Fig 2A and B), including the areas of Descemet's membrane and the endothelium (data not shown). In both lines of mice, the corneal epithelium was regular without lipid deposition, whereas the most internal epithelial cell layers were characterized by abundant accumulation of glycogenic granules. Bowman's layer (Fig 2A and B) and the corneal stroma of control mice and 11.1 transgenic mice were also regular and did not present any apparent alteration. Lipid-specific staining by the OTO method showed a limited amount of intracytoplasmic lipid particle deposition in the most internal epithelial layer of the 11.1 transgenic mice cornea, which was almost absent in the same regions of the control mice cornea (Fig 2C and D). No such lipid particles were found in the stromal keratocytes or in the surrounding collagen fibers (Fig 3A and B). An immunocytochemical technique was used to detect apoA-I in corneal tissue.

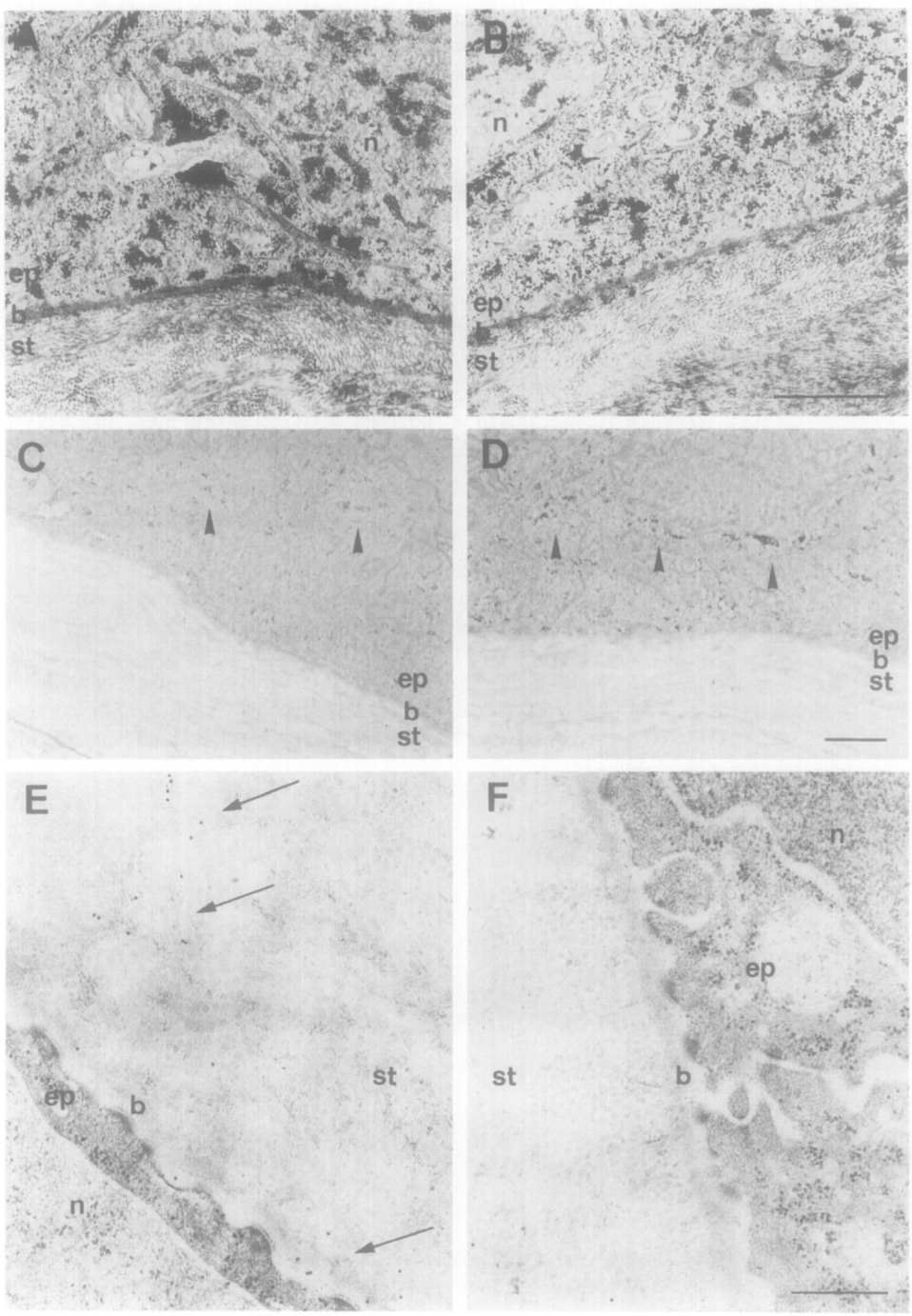

Fig 2. Representative electron micrographs showing Bowman's layer and the bordering epithelium and stroma of corneas from control mice (A, C, and E) or 11.1 transgenic mice (B, D, and F). (A and B) Conventional ultrastructural analysis of the most internal epithelial cell (ep) layer; glycogenic granules are noted as electrodense particles. The location of the basement membrane (b) and superficial stroma (st) is shown. (C and D) Lipid staining by the OTO method. The presence of lipid particles (arrowheads) in the deep epithelium (ep) is shown. (E and F) Location of immunoreactive apoA-I at the superficial stroma (st) is shown by arrows. n, nucleus. Bar = 1 μm for A and B, 1 μm for C and D, and 0.5 μm for E and F.

Labeling was observed mainly in control corneas rather than the corneas of 11.1 transgenic mice, and was especially apparent in the collagen fibers of Bowman's layer, at the superficial stroma level (Fig 2E and F), and at the collagen matrix surrounding the stromal keratocytes (Fig 3C and D). No differences were observed in Descemet's membrane or in the endothelium of control and 11.1 transgenic mice corneas when the OTO method and immunocytochemical techniques were applied (data not shown).

Cholesterol Efflux

The potential of human apoA-II transgenic mice and control mice serum to mediate cholesterol efflux was determined in [^{3}H]cholesterol-labeled NRK fibroblasts. The efflux was found to be 40% lower in the serum of 11.1 transgenic versus control or 25.3 transgenic mice on regular chow. No differences were observed between the groups for serum samples from animals on the HF/HC diet (Fig 4A and B).

DISCUSSION

The goal of the present study was to assess whether the hyperexpressor human apoA-II transgenic line (named 11.1) accumulates FC in the cornea and develops corneal opacities like patients with FED and other inherited HDL deficiencies. For this purpose, we also investigated a lower-expressor line of human apoA-II transgenic mice (named 25.3, which does not have LCAT deficiency) and control mice fed for protracted periods on either regular chow or a HF/HC diet. After regular chow, the plasma of 11.1 mice showed a biochemical phenotype consistent with the existence of a functional LCAT deficiency similar to that of our previous report.[5] Moderate overexpression of human apoA-II in the 25.3 line did not cause such dramatic changes (Table 1). After 9 months of the HF/HC diet, the 11.1 mice presented with hypertriglyceridemia and a high percentage of FC in HDL but with a considerably milder HDL cholesterol decrease, probably resulting from a more conserved capacity for cholesterol endogenous esterification. The LCAT concentration in the plasma of 11.1 transgenic mice, inferred from exogenous LCAT activity, was similar on both diets (Table 1).

Cornea cholesterol, FC, and the percentage of FC were elevated in 11.1 mice versus control or 25.3 transgenic mice, but only when the mice were fed regular chow (Table 2). These results support previous suggestions that high-fat diets could partially prevent or delay the manifestation of LCAT deficiency in patients with FED.[2] Needless to say, these diets would not be recommended to these patients because of the associated atherogenic potential.

The relative magnitude of cholesterol and FC accumulation in the cornea of 11.1 mice versus control mice is considerably lower than that found in the cornea of human FED patients versus controls.[15] This is probably the reason that no mice developed corneal opacities. The short life span of the mouse may be a relevant factor for the lack of significant FC accumulation and the absence of corneal opacities in these animals; FED patients usually develop corneal opacities later in life. FC accumulation in the cornea of patients with other inherited diseases such as FLD and Schnyder's corneal dystrophy is also considerably higher than that observed in 11.1 transgenic mice.[10]

It is possible that the increased FC found in the cornea of 11.1

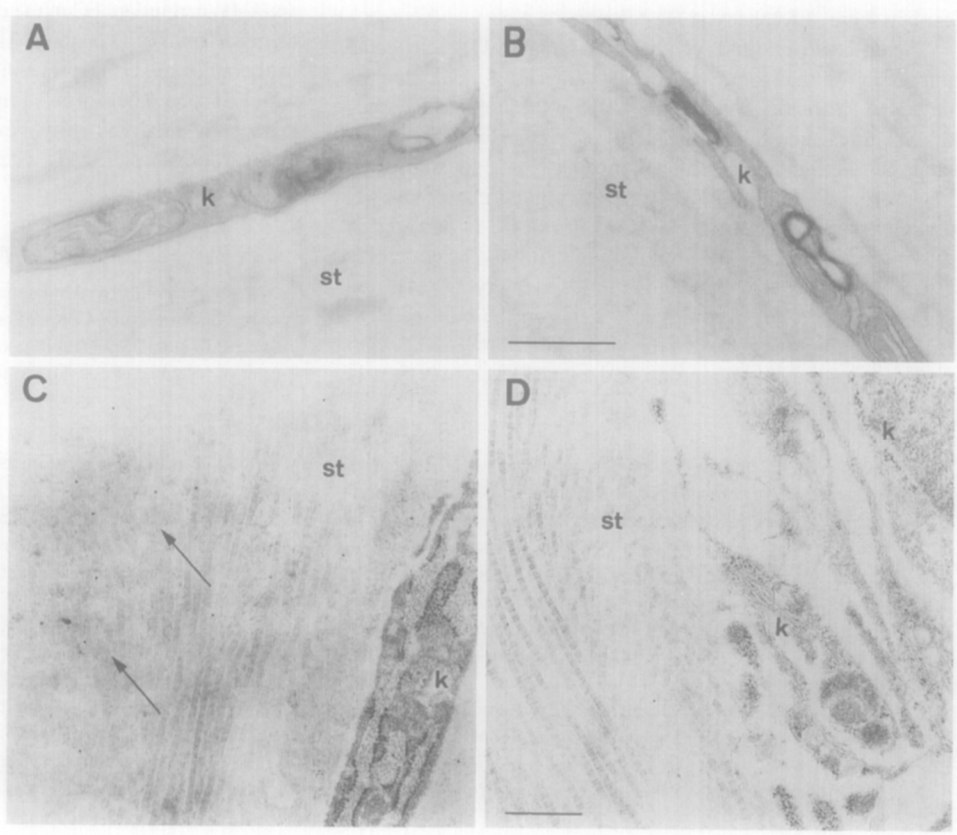

Fig 3. Representative electron micrographs of keratocytes located at the stromal collagen layers of the cornea from control mice (A and C) or 11.1 transgenic mice (B and D). (A and B) Lipid staining by the OTO method showing keratocytes (k) and collagen fibers in the stromal (st) layers. (C and D) Immunoreactive apoA-I (arrows) mainly detected in stromal collagen fibers (st) surrounding keratocytes (k). Bar = 0.5 μm for A and B and 0.5 μm for C and D.

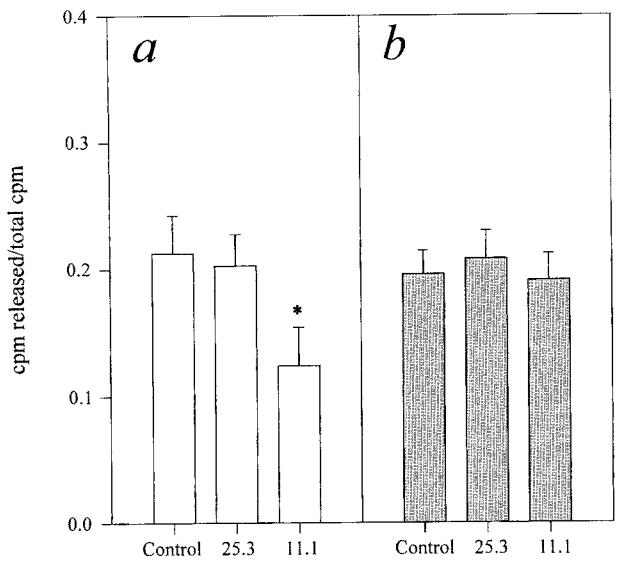

Fig 4. Cellular cholesterol efflux capacity of control and transgenic mice serum using cultures of NRK fibroblasts. The results are the mean ± SEM of triplicate determinations for 10-13 samples per line. *$P < .001$. (a) Mice fed a regular chow diet; (b) mice fed a high-fat/high-carbohydrate diet.

transgenic mice is contained in the intracytoplasmic lipid particles found in the epithelial cells (Fig 2D). Ultrastructural analyses of the cornea of patients with FED demonstrated a notable amount of lipid vesicles in Bowman's layer and the stroma.[16] The different developmental stage of lipid deposition and species-specific differences in corneal structure could explain these differences, at least in part.

The mild corneal lipid accumulation found in 11.1 transgenic mice may be due to an increased synthesis of FC and/or its defective clearance. The decreased apoA-I found in the cornea of 11.1 transgenic mice (Figs 1 to 3) together with the lower cholesterol efflux and esterification capacity of 11.1 transgenic mice serum when the animals were fed regular chow (Fig 4A) strongly suggest that the removal of corneal FC is impaired in these mice. In this regard, it is of note that only a nonspecific efflux of FC has been suggested to function in erythrocytes and cornea.[17] This efflux would then depend on the bulk of HDL (α-HDL) particles rather than the interaction of preβ-HDL with specific receptor(s). Our group and others[6,18] had previously shown that apoA-I preβ-HDL → apoA-I α-HDL conversion is impaired in human apoA-II transgenic mice and in human apoA-I/apoA-II transgenic mice, and as a consequence, apoA-I in preβ-HDL is increased and apoA-I in α-HDL migrating particles is decreased. It is therefore likely that the lower plasma apoA-I containing α-HDL found in 11.1 transgenic mice[6] is a determinant of the decreased cholesterol efflux capacity. This lower efflux combined with a lower LCAT endogenous capacity results in impairment of reverse cholesterol transport sufficient to cause FC accumulation at the cornea level. In contrast, a similar, albeit milder, LCAT impairment such as that observed in 11.1 transgenic mice on a HF/HC diet is not capable of causing FC accumulation in the cornea without a concomitant alteration of cholesterol efflux (Fig 4B).

Two HDL parameters that play a critical role in modulating cell cholesterol efflux are particle size[19] and the amount and type of phospholipids associated with the various HDL particles.[20,21] Variations in these parameters could be involved in the diet-specific decreased efflux observed in 11.1 transgenic mice. It has been reported[19] that the larger the particle, the more efficient the cholesterol efflux capacity, and we have observed an increase toward large-size HDL particle distribution in 11.1 transgenic mice fed a HF/HC diet relative to those on a regular chow diet.[22] Moreover, there was no difference in the concentration of HDL phospholipids (a measurement based on its content in choline) in 11.1 transgenic mice and 25.3 transgenic mice or control mice when fed a HF/HC diet, whereas the concentration of HDL phospholipids in 11.1 transgenic mice was only half the level in 25.3 transgenic mice or control mice when fed regular chow.

In conclusion, 11.1 transgenic mice overexpressing human apoA-II accumulate FC in the cornea, although to a much lower degree than in FED patients. This mouse model could be useful in therapies aimed at preventing corneal opacities in patients with FED and other forms of inherited HDL deficiencies. Other genetically modified mice developed recently, such as those lacking apoA-I or LCAT, would be useful for this purpose as well.[23-25]

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