Overexpression of human apolipoprotein A-I in transgenic rats and the hyperlipoproteinemia associated with experimental nephrosis

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Abstract Hyperlipoproteinemia contributes both to kidney disease progression and the development of atherosclerosis. Elevated high density lipoprotein cholesterol and apolipoprotein A-I (apoA-I) serum levels are independent factors protective against the atherosclerotic process. We examined the effects in a transgenic rat model of human apoA-I expression on the hyperlipoproteinemia and edema after puromycin aminonucleoside-induced nephrosis in three groups of animals: low line ($TgR[hAI]_{low}$, human plasma apoA-I = 16.0 mg/dl); high line ($TgR[hAI]_{high}$, 284 mg/dl); and non-transgenic litter mates ($TgR[hAI]_{non}$). Nephrosis increased total plasma apoA-I levels 2-fold in $TgR[hAI]_{non}$ rats (75 vs. 162 mg/dl) and 4-fold in the $TgR[hAI]_{low}$ (97 vs. 458 mg/dl) and $TgR[hAI]_{high}$ rats (356 vs. 1,346 mg/dl). In both transgenic lines, this increase was due mainly to elevations of serum human apoA-I. The hepatic steady-state levels of rat apoA-I mRNA increased 5- to 7-fold in all three groups, while human apoA-I mRNA levels increased 21- and 65-fold in the low and high expressing groups, respectively, indicating a different degree of responsiveness of the rat and human genes. While nephrotic $TgR[hAI]_{non}$ and $TgR[hAI]_{low}$ rats showed severe hyperlipoproteinemia and edema, much lower levels of edema and of serum triglycerides, phospholipids, and cholesterol were seen in the $TgR[hAI]_{high}$ group. Urinary excretion of apoA-I, phospholipids, and cholesterol was significantly increased in the $TgR[hAI]_{high}$ group, indicating this as one possible mechanism for the relatively lower serum levels of these lipids. ■ We conclude that the human apoA-I gene is responsive to nephrosis and that human apoA-I-transgenic rats with this syndrome provide an animal model for the study of human high density lipoprotein and apoA-I metabolism.—**Burkey, B. F., D. France, H. Wang, X. Ma, B. Brand, C. Abuhani, M. R. Diffenderfer, J. B. Marsh, J. R. Paterniti, Jr., and E. A. Fisher.** Overexpression of human apolipoprotein A-I in transgenic rats and the hyperlipoproteinemia associated with experimental nephrosis. *J. Lipid Res.* 1995. **36:** 1463–1473.

Supplementary key words high density lipoprotein • kidney disease • puromycin aminonucleoside • hyperlipidemia • proteinuria

Hyperlipoproteinemia is a common feature of the nephrotic syndrome and other glomerular diseases in both humans and experimental animals (1–4). While, in the general population, hyperlipoproteinemia is a strong risk factor for coronary artery disease, in those with renal disease its potential for adverse consequences may be further increased by contributing to the progression of glomerular dysfunction (5).

In previous studies of non-transgenic rats with experimental nephrotic syndrome, a hallmark of the hyperlipoproteinemia has been the overproduction by the liver of apolipoprotein A-I (apoA-I) (4). Thus, in spite of urinary losses of high density lipoprotein (HDL) (6), serum levels of HDL cholesterol typically increase, contributing to the hyperlipidemic state. The overproduction of hepatic apoA-I has been shown to result from increased apoA-I synthesis (7), and in more recent studies this was attributed to increased apoA-I gene expression (8–10).

The availability of transgenic rats expressing human apoA-I (h-apoA-I) (11) allowed us to determine whether the human gene also responds to the nephrotic state. In addition, by studying lines with different quantitative levels of basal h-apoA-I expression (low and high), we were able to explore relationships among the degree of h-apoA-I production, the serum levels of apoA-I and apoB-containing lipoproteins, and the severity of the nephrotic state.

Abbreviations: TgR, transgenic rat; FPLC, fast protein liquid chromatography; TBS, Tris-buffered saline; PAN, puromycin aminonucleoside; h-apoA-I, human apolipoprotein A-I.

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The results demonstrate that the h-apoA-I gene responded to the nephrotic syndrome in each transgenic group (low and high basal expressors). Furthermore, when nephrosis was induced in the group with high basal expression, compared to non-transgenic controls and low expressors, there was suppression of edema and less hyperlipoproteinemia, associated with increased urinary excretion of apoA-I, cholesterol, and phospholipids. Thus, in addition to the value of these two groups of transgenic rats to studies of lipid and apolipoprotein metabolism, their differing responses to nephrosis could potentially be used to test the effects of plasma lipid and lipoprotein levels on the progression of glomerular dysfunction in experimental renal disease.

MATERIALS AND METHODS

Induction of experimental nephrosis

Transgenic rats were generated by microinjection of a 13 kbp DNA fragment containing the human apoA-I gene plus 10 kbp of 5′ and 1 kbp of 3′ flanking sequence. Two established transgenic rat lines, TgR[0hAI]7 and TgR[0hAI]2, express moderate and high levels of h-apoA-I, respectively. Both lines were originally made using OFA rats (Oncins France Strain A derived from the Sprague-Dawley strain) and subsequently rederived to a Sprague-Dawley background (11). TgR[0hAI]7 will be referred to as $TgR[hAI]_{low}$, TgR[0hAI]2 will be referred to as $TgR[hAI]_{high}$, and non-transgenic litermates will be referred to as $TgR[hAI]_{non}$. Male rats, 10 per group, age 7–11 weeks were used. In each group, experimental nephrosis was induced in one-half of the rats by bolus intraperitoneal injections of puromycin aminonucleoside (PAN) at 65 mg/kg body weight (No P-7103; Sigma Chemical Co., St. Louis, MO) in saline, on 2 consecutive days. All other animals were injected with equivalent volumes of saline. Within 8 days, the response to PAN treatment was readily apparent as peritoneal edema, which could be clinically graded (by a blinded observer) over a range of slight to severe. Animals were killed 8 days after the initial injection of PAN or saline. Whole blood for serum isolation and livers were collected at the time of killing. In a separate experiment, urine was collected from four $TgR[hAI]_{non}$ and four $TgR[hAI]_{high}$ rats by placing them in metabolic cages 20 h prior to killing. All animal studies were performed under a protocol approved by the Animal Care Committee of the Medical College of Pennsylvania and the Sandoz Animal Care and Use Committee.

Serum lipid analysis

One part of serum was diluted with four parts of distilled water for the analysis of total serum cholesterol, triglyceride, and phospholipid. In a 96-well microplate (Nunc-Immuno Plate MaxiSorb, InterMed) 25 μL of diluted serum was assayed for its lipid content by the addition of 200 μL cholesterol reagent (No. 352-1000, Cholesterol 1000, Sigma), or 200 μL triglyceride reagent (No. 450032; Triglycerides/GB, Boehringer Mannheim Co., Indianapolis, IN), or 200 μL phospholipid reagent (No. 996-54001; Phospholipids B, Wako Pure Chemical Industries, Osaka Japan) as specified by the manufacturers. Samples were developed by incubating for 30 min at room temperature and absorbance was determined at 490 nm. Equivalent dilutions of calibrated lipid standards (No. C 0534, Cholesterol Calibrator Set, Sigma) and calibrated controls (No. L 1008, Lipid Control-E and No. L 2008, Lipid Control-N, Sigma) were used as reference standards. Fractionation of serum lipoproteins by Superose-6 gel permeation chromatography was performed with a robotic FPLC system as previously described (12). Briefly, the column matrix was equilibrated with Tris-buffered saline (TBS, 50 mM Tris, pH 7.4, 0.15 M NaCl) containing 0.01% sodium azide at 0.5 mL per min. Serum, 200 μL, was injected and 40 0.5-mL fractions were collected at a flow rate of 0.5 mL/min. The cholesterol profile was determined on an 80-μL fraction with 120 μL of cholesterol reagent (No. 81423, Fast Cholesterol, Sclavo, Wayne, NJ). After incubation at room temperature for 30 min, absorbance was determined at 490 nm.

Urine lipid analysis

Rats were placed in individual metabolic cages and urine was collected overnight. Aliquots were analyzed for cholesterol and phospholipids. Proteins were first precipitated with 10% trichloracetic acid and the precipitates were extracted twice at room temperature with 1% trichloracetic acid in ethanol. The precipitates were dissolved in 0.1 N NaOH and protein concentrations were measured by the micro-biuret method (13).

The supernatant solution was treated with 3 volumes of ether to precipitate the acid–alcohol-soluble protein. The protein was dissolved in 0.1 N NaOH and its concentration was determined (13). The solvents were removed from the lipid extract, which was then re-dissolved in chloroform–methanol 2:1. Water-soluble substances were removed by the method of Folch, Lees, and Sloane Stanley (14). Cholesterol and phospholipid in the chloroform phase of the Folch extract were determined by the methods of Zlatkis, Zak, and Boyle (15) and Sokoloff and Rothblat (16), respectively.

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Nondenaturing gradient gel electrophoresis of serum lipoproteins

Total lipoproteins from 200 μL serum, adjusted to d 1.23 g/mL with potassium bromide, were isolated by a 12-h (42,000 rpm) single-spin density ultracentrifugation in a Ti42.2 rotor (Beckman Instruments, Palo Alto, CA). Two parts of lipoprotein fraction (the upper 60 μL sample from the spin) were mixed with one part of Sudan Black B (7 mg/mL in polyethylene glycol) and incubated at room temperature for 2 h. Lipoproteins from 20 μL were loaded onto a nondenaturing polyacrylamide gradient gel (PAA 2/16; Pharmacia LKB, Uppsala, Sweden) and resolved by electrophoresis at 125 V for 16 h in 89 mM Tris, pH 8.3, 89 mM boric acid, and 2.6 mM EDTA (No. SA 100033, TBE Seprabuff, Integrated Separation Systems, Natick, MA). After lipid-containing bands were identified, apolipoproteins and molecular weight markers were visualized by staining with Coomassie Brilliant Blue (No. B-8647, Brilliant Blue R, Sigma).

Quantitation of serum apoA-I

Levels of human and rat apoA-I were determined by competition ELISA. For the h-apoA-I assay, microplate wells (Nunc-Immuno Plate MaxiSorb, Baxter Scientific, Edison, NJ) were coated overnight at 4°C with 0.4 μg per well of purified h-apoA-I (No. A-9284, Sigma), then blocked for 2 h at room temperature in TBS containing 5% bovine serum albumin (BSA). Assay plates were incubated overnight at room temperature with rat serum diluted 1:20 in TBS containing 4% Tween-20 (preheated to 52°C, 1 h) and goat anti-human apoA-I immune serum diluted 1:10,000 in TBS containing 4% Tween-20. Anti-human apoA-I immune serum was generated by immunizing a goat with h-apoA-I isolated from human HDL_3 and purified by electroelution of a single band from an SDS polyacrylamide gel (17). After washing, bound anti-human apoA-I antibodies were tagged with alkaline phosphatase-conjugated rabbit anti-goat IgG (No. A-7650, Sigma) diluted 1:1,000 in TBS containing 2% BSA and detected using 200 μL of a 1 mg/mL phosphatase substrate (No. 104-0, Sigma) dissolved in 2% diethanolamine. After incubation at room temperature for 30 min, the absorbance was determined at 405 nm. Standard curves were made by serial dilution of human serum containing known amounts of h-apoA-I. There was no detectable cross-reactivity (by either immunoblot or ELISA format) of the anti-human apoA-I immune serum for rat apoA-I. The assay was linear over the range of 1.5–24 μg apoA-I per well.

The rat apoA-I ELISA was identical to the above ELISA, except 800 ng of rat apoA-I, purified from rat HDL by preparative SDS PAGE (17), was used to coat each well of the microtiter plate. Diluted rat serum samples were not preheated, and goat anti-rat apoA-I immune serum was diluted 1:7,500. Anti-rat apoA-I immune serum was generated by immunizing a goat with rat apoA-I isolated from rat HDL_3 and purified by electroelution of a single band from an SDS polyacrylamide gel (17). Standard curves were made by dilution of rat serum containing known amounts of rat apoA-I. Minor cross-reactivity of anti-rat apoA-I antiserum for h-apoA-I was removed by affinity chromatography over a human serum affinity column. The assay was linear in the range of 0.9–15 μg of apoA-I per well.

Non-reducing SDS-PAGE analysis of serum and urine proteins

Rat serum or urine (3 μL) was mixed into Tris-SDS sample solubilization buffer (No. SA100051, Tris-SDS SepraSOL, Integrated Separation Systems) to yield a 50 μL final volume. Samples were heat-denatured at 80°C for 15 min, and loaded on an 11% SDS polyacrylamide gel. Proteins, resolved by electrophoresis at 75 V for 6 h, were visualized by staining with Coomassie Brilliant Blue.

Density gradient ultracentrifugation of total serum lipoproteins

Pools of serum (1 mL) from each treatment group were density adjusted to 1.23 g/mL with potassium bromide in 14 × 95 mm polyallomer centrifuge tubes (No. 331374, Beckman). Four potassium bromide solutions of descending density from 1.15 to 1.01 g/mL were layered over the serum sample and ultracentrifuged in a Beckman SW40Ti rotor at 35,000 rpm for 36 h at 4°C. Lipoproteins were collected by pumping a 1.30 g/mL potassium bromide solution at a rate of 0.4 mL/min through a 20-gauge needle inserted through the tube wall at the bottom of the centrifuge tube. Fractions of 0.2 mL were collected for analysis of total cholesterol, h-apoA-I and rat apoA-I.

Isolation and analysis of hepatic RNA

Total RNA was isolated from rat liver by a modified guanidine salt-based procedure previously described (18). Ten-μg aliquots of RNA were mixed in loading buffer (50% formamide, 16% formaldehyde, 50 mM MOPS, 2 mM EDTA, pH 7.0), heated to 56°C for 30 min, rapidly chilled on ice, and electrophoresed at 50 V for 3–5 h on a 1% agarose gel containing 17.75% formaldehyde, 50 mM MOPS, 2 mM EDTA, pH 7.0. Resolved RNA was transferred to a nylon membrane (ZetaProbe, Bio-Rad, Melville, NY) by passive capillary transfer in 50 mM NaOH and UV crosslinked. Oligonucleotide probes complementary to the rat and human apoA-I mRNAs were labeled by the T4 polynucleotide kinase technique (19) with [γ^{32}P]ATP (3000 Ci/mmol, New England Nu-

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clear, Boston, MA). All blots were prehybridized for 4–5 h at 65°C in 5 × SSC, 20 mM sodium phosphate, pH 7.0, 10 × Denhardt's solution, 7% SDS, with 100 μg/mL denatured salmon sperm DNA. Heat-denatured probes were hybridized to the immobilized RNA at 65°C in hybridization buffer (the same as prehybridization buffer, but with the addition of 10% dextran sulfate). The blots were washed twice with 0.5 × SSC, 0.1% SDS at room temperature, then once with 0.1 × SSC, 0.1% SDS at 65°C. Blots were then exposed to a phosphor screen overnight and scanned on a Molecular Dynamics Phosphoimager. The intensity of the hybridization signal was normalized to elongation factor 1 α, as described by Lu and Werner (20), and expressed as relative to this control.

Values are given as the mean ± standard error of the mean. Statistical differences between treatment groups were sought by using Student's *t* test.

RESULTS

Rat and human apoA-I protein and mRNA levels

As previously reported (21, 22), total serum apoA-I increased after the induction of nephrosis (**Table 1**). However, a more pronounced total apoA-I elevation occurred in TgR[hAI]$_{low}$ and especially in TgR[hAI]$_{high}$ nephrotic rats, where apoA-I replaced albumin as the major serum protein (see Fig. 4A). The increase in total apoA-I in the TgR[hAI]$_{low}$ and TgR[hAI]$_{high}$ nephrotic rats was primarily due to increased h-apoA-I levels. Rat apoA-I actually decreased in the nephrotic TgR[hAI]$_{high}$ group.

TABLE 1. Rat and human apoA-I serum levels 8 days after PAN (nephrotic) or saline (control) injection

Group	Serum ApoA-I Levels		
	Rat ApoA-I	Human ApoA-I	Total ApoA-I
		mg/dL	
TgR[hAI]$_{non}$			
Control	75.2 ± 10.0		75.2 ± 10.0
Nephrotic	162.4 ± 26.6		162.4 ± 26.6
N/C	2.16[a]		2.16[a]
TgR[hAI]$_{low}$			
Control	81.2 ± 10.4	16.0 ± 4.7	97.2 ± 8.7
Nephrotic	127.4 ± 9.1	330.8 ± 51.7	458.2 ± 44.0
N/C	1.57[a]	20.68[b]	4.71[b]
TgR[hAI]$_{high}$			
Control	71.8 ± 17.7	284.2 ± 113.4	356.0 ± 96.1
Nephrotic	38.3 ± 5.9	1307.5 ± 186.0	1345.8 ± 191.3
N/C	0.53[c]	4.60[b]	3.78[b]

Human and rat apoA-I were measured by species-specific competition ELISA. Values are given as mean ± SEM, n = 5; total apoA-I is the sum of rat and human values. The change from control in each group is expressed as the ratio of N/C (nephrotic/control).

[a]$P < 0.05$; [b]$P < 0.005$; [c]no significant difference, where the two groups are statistically different as determined by Student's *t*-test.

We have shown previously that the genetic construct used to produce TgR[hAI]$_{low}$ and TgR[hAI]$_{high}$ rats drove the expression of h-apoA-I in the liver but not in the intestine (11). Hepatic rat and human apoA-I mRNA levels were measured by Northern blot hybridization in control and nephrotic rats (**Fig. 1** and **Table 2**). Similar to previous reports (8–10), the induction of experimental nephrosis significantly increased the steady-state levels of rat apoA-I mRNA (5- to 7-fold) over the controls. By contrast, h-apoA-I mRNA levels were elevated 21-fold in TgR[hAI]$_{low}$ rats and 65-fold in TgR[hAI]$_{high}$ rats. These data demonstrate that rat and h-apoA-I mRNA and protein were differentially elevated by the induction of experimental nephrosis.

Effects of human apoA-I on PAN-induced hyperlipoproteinemia

In control TgR[hAI]$_{high}$ rats, expression of h-apoA-I was associated with raised circulating levels of cholesterol (51.2%) and phospholipid (66.5%) compared with TgR[hAI]$_{non}$ rats, while triglycerides were unchanged. The increased serum levels of cholesterol and phospholipid in TgR[hAI]$_{high}$ rats reflected expansion of the HDL pool, evident in the FPLC lipoprotein cholesterol profile of Fig. 2A, and were consistent with our previous report (11). The minor contribution of h-apoA-I in the control TgR[hAI]$_{low}$ rats was not enough to alter total serum lipid and lipoprotein profiles.

Consistent with the model of experimental nephrosis (4), marked elevations of total cholesterol (+460%), phospholipid (+212%), and triglyceride (+627%) occurred in TgR[hAI]$_{non}$ rats after PAN induction. The degree of lipid elevation in nephrotic TgR[hAI]$_{non}$ rats and nephrotic TgR[hAI]$_{low}$ rats was virtually identical (**Table 3**). By contrast, the induction of experimental nephrosis in TgR[hAI]$_{high}$ animals produced only a modest elevation (+52%) of total serum cholesterol and actually decreased phospholipid levels (Table 3). Total serum triglyceride increased 219% (control: 70.1 mg/dL vs. nephrotic: 223.7 mg/dL). However, this elevation was less than one-half of the response of nephrotic TgR[hAI]$_{non}$ or TgR[hAI]$_{low}$ rats, and was not statistically significant ($P = 0.092$). Thus, overexpression of h-apoA-I in the TgR[hAI]$_{high}$ rats suppressed the hyperlipoproteinemia associated with experimental nephrosis.

To obtain lipoprotein profiles, serum samples were fractionated by FPLC gel permeation chromatography and lipoprotein peaks were determined by elution volume and cholesterol content. In control TgR[hAI]$_{non}$ rats, HDL was the predominant lipoprotein species (Fig. 2A). Induction of experimental nephrosis resulted in

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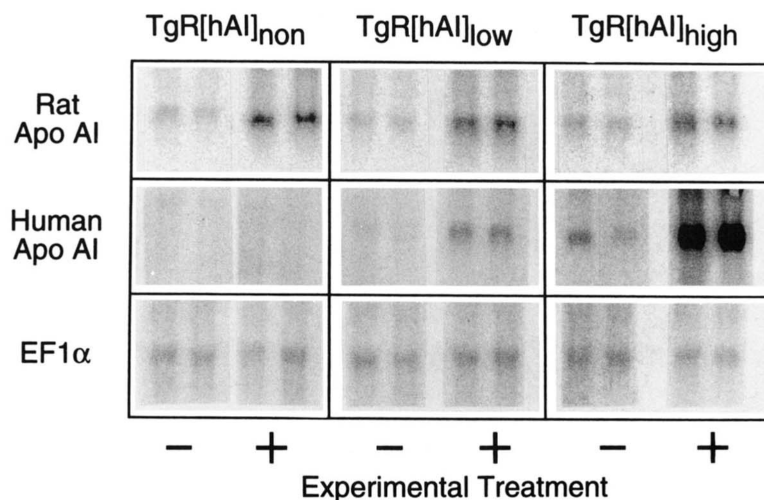

Fig. 1. Northern blot analysis of hepatic rat apoA-I, human apoA-I, and rat EF1α. Total RNA was isolated from livers of $TgR[hAI]_{non}$, $TgR[hAI]_{low}$, and $TgR[hAI]_{high}$ rats killed 8 days after PAN or saline injection. Ten micrograms per lane of total RNA was resolved using a formaldahyde-denaturing gel, transferred to nitrocellulose, and hybridized with rat-specific apoA-I, human-specific apoA-I, or EF1α radiolabeled probes. Each experimental group, PAN (+) or saline (–) injected rats, is represented by two individuals within each group.

hyperlipoproteinemia evident as increases of all lipoprotein classes. Lipoprotein profiles of $TgR[hAI]_{low}$ control and nephrotic rats (data not shown) were virtually identical to the corresponding profiles of $TgR[hAI]_{non}$ rats shown in Fig. 2A. Overexpression of h-apoA-I in $TgR[hAI]_{high}$ control rats expanded the HDL cholesterol pool (compare Figs. 2A and 2B). While some increase in non-HDL cholesterol fractions occurred in $TgR[hAI]_{high}$ rats with experimental nephrosis, hyperbetalipoproteinemia was decreased (compare Figs. 2A and 2B). Surprisingly, in the face of large differences of total serum apoA-I, HDL cholesterol levels in $TgR[hAI]_{high}$ rats were unchanged in controls (142.4 ± 33.4 mg/dL) versus in nephrotics (140.5 ± 21.2 mg/dL). However, HDL particles isolated from $TgR[hAI]_{high}$ nephrotic animals tended to elute ahead of control HDL on FPLC, indicating an increased particle size.

The HDL particle size distribution was further examined by non-denaturing gradient gel electrophosis (**Fig. 3**). In control $TgR[hAI]_{non}$ and $TgR[hAI]_{low}$ rats, a single HDL_2 species was present (23). Control $TgR[hAI]_{high}$ rat HDL was polydisperse with distinct HDL_1-like, small HDL_2, and very small HDL_3 species. In $TgR[hAI]_{non}$ and $TgR[hAI]_{low}$ rats with experimental nephrosis, two distinct HDL species, comparable to human HDL_2 and HDL_3, were observed. Nephrotic $TgR[hAI]_{high}$ rat serum contained only large particles that displayed size heterogeneity among individual rats. Smaller HDL particle classes were absent in these animals.

TABLE 2. Levels of hepatic human and rat apoA-I mRNA 8 days after PAN (nephrotic) or saline (control) injection as determined by Northern blot analysis

Group	Hepatic ApoA-I mRNA	
	Rat ApoA-I	Human ApoA-I
$TgR[hAI]_{non}$		
Control	0.41 ± 0.04 (3)	
Nephrotic	2.49 (2.28, 2.70)	
N/C	6.1	
$TgR[hAI]_{low}$		
Control	0.45 ± 0.15 (4)	0.15 ± 0.06 (4)
Nephrotic	3.24 ± 0.25 (4)	3.16 ± 0.27 (4)
N/C	7.2	21.1
$TgR[hAI]_{high}$		
Control	0.35 ± 0.06 (4)	1.19 ± 0.80 (4)
Nephrotic	1.79 (1.91, 1.67)	77.03 (80.4, 73.7)
N/C	5.1	64.7

Rat and human apoA-I mRNA levels in livers were determined using species-specific oligonucleotide probes and are expressed as phosphorimager units normalized to constitutively expressed Elongation Factor 1 alpha (EF1 α). Each value is the mean ± SEM, with the number (n) in parentheses, except where the n was less than 3, when the average of two determinations (values in parentheses) is given. The change from the control in each group is expressed as the ratio of nephrotic/control (N/C).

Effect of overexpression of human apoA-I on albuminemia, edema, and urine composition in PAN-treated rats

Eight days after the initial injection of PAN, rats were killed and serum proteins were resolved on a non-reducing SDS polyacrylamide gel (**Fig. 4A**). Hypoalbuminemia was evident in all three PAN-treated groups. Additionally, upon killing, all rats receiving PAN displayed peritoneal edema while their saline-treated counterparts appeared normal. The edema in all $TgR[hAI]_{high}$ nephrotic rats, however, was clinically less severe than that observed in nephrotic $TgR[hAI]_{low}$ and $TgR[hAI]_{non}$ animals. Urine from control (saline-treated) and nephrotic $TgR[hAI]_{non}$ and $TgR[hAI]_{high}$ rats was collected for a 20-h period prior to killing. Urine protein excretion was low from two saline-treated $TgR[hAI]_{non}$ rats (0.54 and 0.55 mg/h) as well as from two saline-treated $TgR[hAI]_{high}$ rats (0.77 and 0.59 mg/h). As expected, significant proteinuria was detected in two PAN-treated $TgR[hAI]_{non}$ rats, 5.18 and

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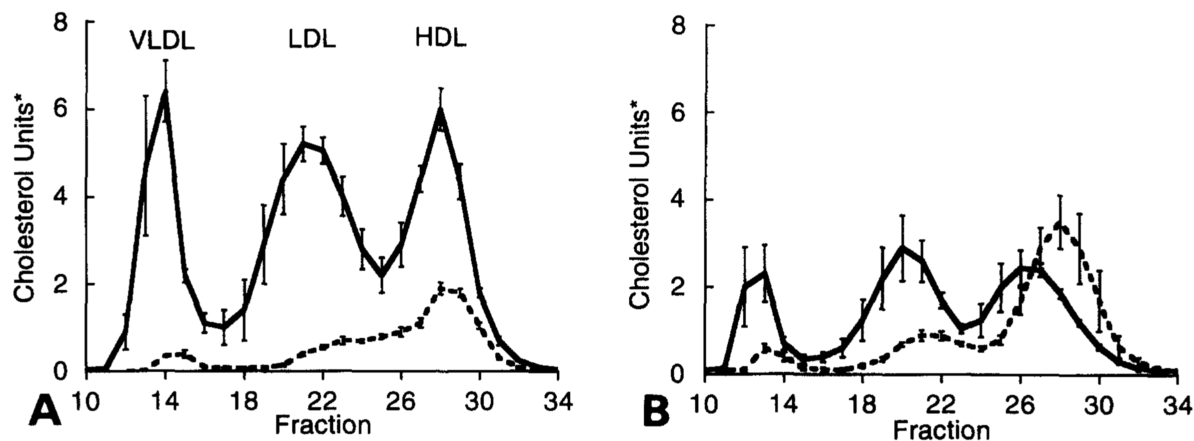

Fig. 2. Analysis of lipoprotein profiles from $TgR[hAI]_{low}$ (A) and $TgR[hAI]_{high}$ (B) rat sera by gel filtration chromatography. Individual rat serum samples were fractionated by FPLC over a Superose 6 sizing column into 40 fractions. Total cholesterol from each fraction was assessed enzymatically, the absorbance at 490 nm* was assigned whole-number values using a linear relation and the profiles were plotted. Dashed lines (–) are saline-injected control rats and solid lines (___) are PAN-injected nephrotic rats. Each profile is the mean of five individual profiles, with error bars representing the standard error of the mean. The elution positions of human VLDL, LDL and HDL are indicated at the top of each profile.

11.42 mg/h. However, the proteinuria was considerably greater in two PAN-treated $TgR[hAI]_{high}$ rats, 20.85 and 27.36 mg/h. Analysis of the urine proteins by non-reducing SDS PAGE (Fig. 4B) shows that much of the protein eliminated in the urine of PAN-treated $TgR[hAI]_{non}$ rats is albumin, whereas in PAN-treated $TgR[hAI]_{high}$ rats, the albumin band has reduced intensity and the apoA-I band becomes more prominent. In a separate set of urine samples, lipid excretion was measured. As shown in **Table 4,** the urine concentrations of both phospholipid and cholesterol were significantly increased in the PAN-treated $TgR[hAI]_{high}$ rats relative to those in the PAN-treated $TgR[hAI]_{non}$ rats.

TABLE 3. Effect of human apoA-I overexpression on levels of triglyceride, phospholipid, and cholesterol in serum from rats 8 days after PAN (nephrotic) or saline (control) injection

Group	Serum Lipid Levels		
	Triglyceride	Phospholipid	Cholesterol
		mg/dL	
$TgR[hAI]_{non}$			
Control	78.3 ± 11.5	201.4 ± 6.7	116.9 ± 5.0
Nephrotic	568.9 ± 49.1	627.8 ± 49.3	654.9 ± 52.3
N/C	7.27[a]	3.12[a]	5.60[a]
$TgR[hAI]_{low}$			
Control	70.0 ± 11.4	186.3 ± 9.0	110.6 ± 7.2
Nephrotic	505.7 ± 54.8	536.2 ± 45.7	569.3 ± 71.0
N/C	7.22[a]	2.88[a]	5.15[a]
$TgR[hAI]_{high}$			
Control	70.1 ± 10.7	304.4 ± 43.8	194.5 ± 35.6
Nephrotic	223.7 ± 68.9	202.4 ± 56.1	296.1 ± 41.4
N/C	3.19[b]	0.67[b]	1.52[b]

Values given as mean ± SEM, n = 5. The change from control in each group is expressed as the ratio of nephrotic/control (N/C).
[a]$P < 0.001$; [b]no significant difference, when the two groups are statistically different by Student's *t*-test.

Effect of human apoA-I overexpression on cholesterol and apolipoprotein density class distribution

To better define the circulating form of apoA-I, serum lipoproteins were resolved by continuous gradient density centrifugation. Rat and human apoA-I distributions were determined across gradient fractions by ELISA. In saline-treated $TgR[hAI]_{non}$ rats, rat apoA-I was found exclusively in the HDL density range (1.063–1.21 g/mL). This pattern persisted after the induction of nephrosis (data not shown). In saline-treated $TgR[hAI]_{low}$ rats, both the rat and human apoA-I were found in the HDL density range (**Fig. 5A**). After the induction of nephrosis in $TgR[hAI]_{low}$ rats, the human apoA-I partitioned mainly into HDL (Fig. 5B), but approximately one-half of the rat apoA-I was now associated with density > 1.21 g/mL fraction. This distribution of human and rat apoA-I was also observed in $TgR[hAI]_{high}$ control rats (Fig. 5C). One possibility suggested by these results is that with sufficiently high levels of expression of h-apoA-I (nephrotic $TgR[hAI]_{low}$ or non-nephrotic $TgR[hAI]_{high}$ rats), rat and human apoA-I compete for the available lipid pool, and that h-apoA-I may be bound tighter to HDL particles than rat apoA-I, allowing for dissociation from HDL particles upon centrifugation. In nephrotic $TgR[hAI]_{high}$ rats, with the greatest expression of h-apoA-I (circulating levels > 1300 mg/dL, Table 1), the h-apoA-I was distributed across three peaks (Fig. 5D). The first peak corresponded to a

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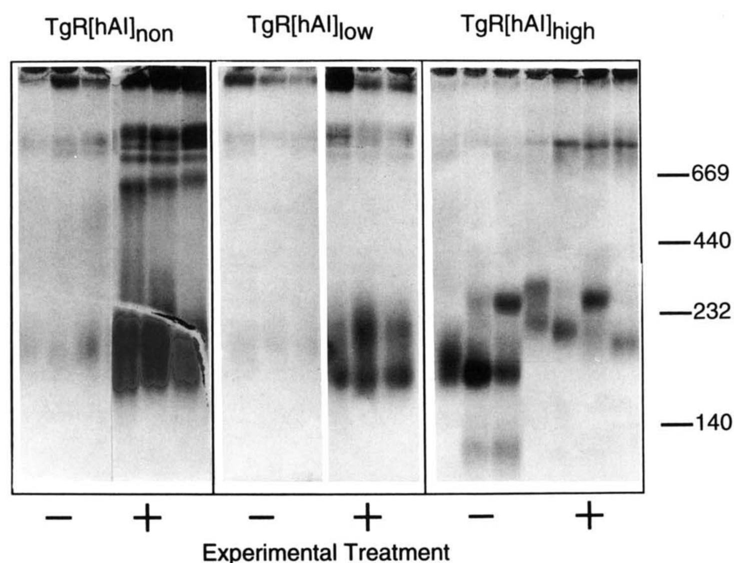

Fig. 3. Non-denaturing gradient gel analysis of total lipoproteins isolated from TgR[hAI]$_{non}$, TgR[hAI]$_{low}$, and TgR[hAI]$_{high}$ rat serum by single spin 1.23 g/mL density centrifugation. Lipoprotein fractions, preincubated 2 h with Sudan black 7B to stain neutral lipids, were resolved on PPA 2/16 nondenaturing gradient gels. The prestained lipoprotein profiles were photographed and the gels were then stained and destained in Coomassie Brilliant Blue to develop the molecular weight standards, shown at the right as $M_r \times 10^3$. Each experimental treatment group, PAN (+) and saline (−) injected, is represented by three individuals, except the TgR[hAI] Pan-treated group that contains four.

buoyant HDL population that co-eluted with a major cholesterol peak, the second one, a denser HDL population co-eluting with a minor cholesterol peak, and the third, a lipid-free or poor population with density > 1.21 g/mL. Rat apoA-I was now found mainly in the density range > 1.21 g/mL. Thus, the induction of experimental nephrosis in TgR[hAI]$_{high}$ rats resulted in significant proportions of the serum pools of both rat and human apoA-I appearing in the non-lipoprotein density fraction.

DISCUSSION

Effects of overexpression of human apoA-I on edema in experimental nephrosis

The mechanisms underlying fluid volume expansion in the nephrotic syndrome have been studied extensively (24). Hypoalbuminemia decreases plasma oncotic pressure, antagonizing forces in the venular capillary that counteract hydrostatic pressure. Fluid balance is further deranged by changes in capillary permeability

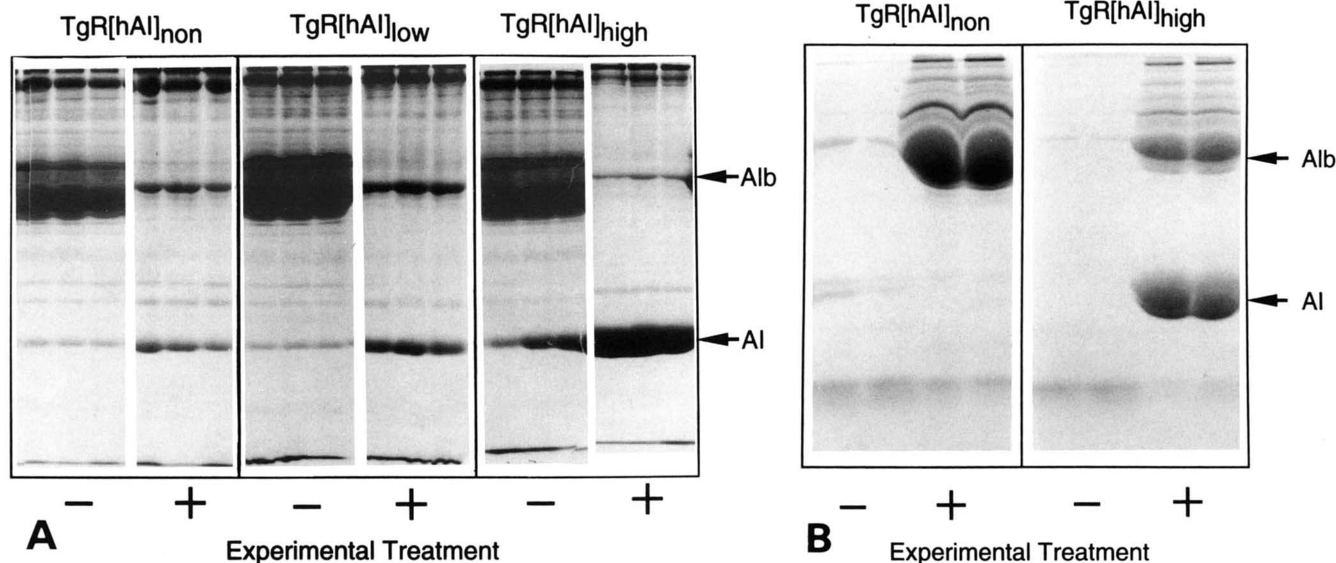

Fig. 4. Analysis of total serum and urine proteins by non-reducing SDS-PAGE. Total proteins from 3 μL of serum (A) or 5 μL of urine (B) per rat were resolved on an 11% non-reducing SDS polyacrylamide gel. The resolved proteins were visualized with Coomassie Brilliant Blue R-250. The locations of albumin and apoA-I bands are indicated to the right. Each experimental group (PAN (+) or saline (−) injected) is represented by three serum samples (panel A) and two urine samples (panel B) from individuals within each group.

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TABLE 4. Urine lipid concentrations in nephrotic rats

Group	Urine Lipid Levels	
	Phospholipid	Cholesterol
	μg/mL	
$TgR[hAI]_{non}$ Nephrotic (n = 3)	235.0 ± 18.7	162.0 ± 14.3
$TgR[hAI]_{high}$ Nephrotic (n = 6)	870.0 ± 152.0	348.0 ± 43.2

Experimental nephrotic syndrome was induced in $TgR[hAI]_{non}$ and $TgR[hAI]_{high}$ rats and urine samples were collected in metabolic cages. Total proteins were precipitated and quantitated. Lipids were extracted by the Folch procedure and cholesterol and phospholipid contents of the extracts were determined. Values are given as mean ± SEM. The urine protein concentrations averaged 43 ± 1.09 and 33 ± 2.87 mg/mL in the $TgR[hAI]_{non}$ and $TgR[hAI]_{high}$ groups, respectively. $P < 0.03$ for the group differences in cholesterol and phospholipid.

and an increased natriuretic peptide activity (25). Intravenous administration of albumin or other plasma expander macromolecules will decrease edema, at least temporarily (26). Earlier work in humans with nephrotic syndrome has shown that plasma lipid levels will also decline when plasma expanders are given (27).

In nephrotic animals, overexpression of human apoA-I did not prevent hypoalbuminemia (Fig. 1) or albuminuria (Fig. 4B). Extreme overproduction of h-apoA-I in the PAN-treated $TgR[hAI]_{high}$ animals was associated with decreased edema in the nephrotic state. This can be most simply explained by the support of plasma oncotic pressure by the high serum levels of apoA-I. In other words, in spite of the urinary losses of apoA-I (Fig. 4B and ref. 28), hepatic apoA-I production was so increased that the serum level reached 1346 mg/dL. Given the relative molecular weights of albumin (67 kD) and apoA-I (28 kD), this level of apoA-I would exert an oncotic pressure corresponding to 3.2 g/dL of albumin, which is within the normal plasma range. Though this calculation assumes that all apoA-I was in the free or lipid-poor state (d > 1.21) and this was not strictly the

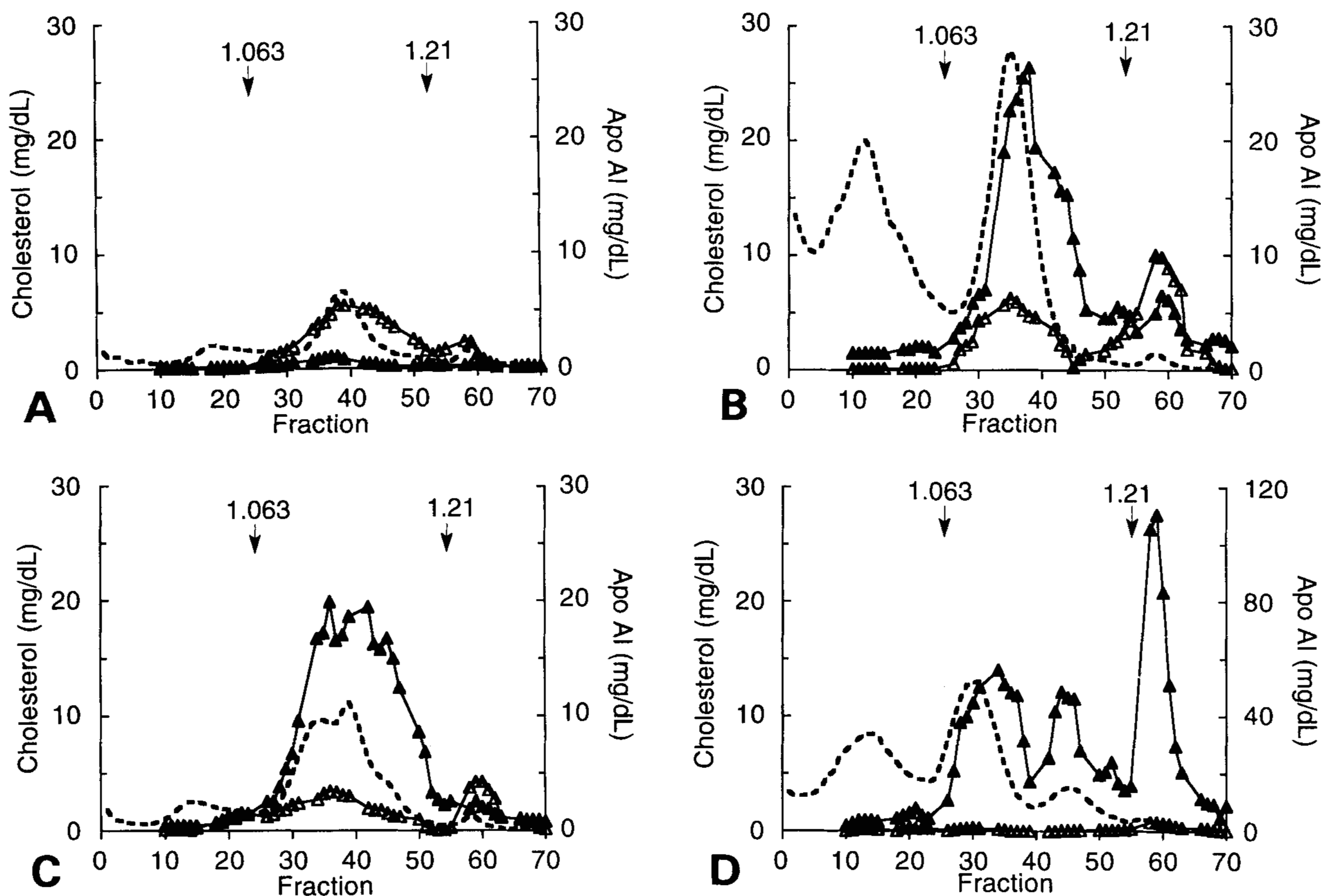

Fig. 5. Distribution of apoA-I and cholesterol by density gradient ultracentrifugation. Pooled sera from A) $TgR[hAI]_{low}$ saline-injected, B) $TgR[hAI]_{low}$ PAN-injected, C) $TgR[hAI]_{high}$ saline-injected, and D) $TgR[hAI]_{high}$ PAN-injected groups were subjected to density gradient ultracentrifugation. Profiles were fractionated for the analysis of total cholesterol (—), rat apoA-I (-△-), and human apoA-I (-▲-). The densities of the fractions were determined and reference densities 1.063 g/mL and 1.21 g/mL are shown above each profile.

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case, nonetheless, a large fraction of apoA-I in nephrotic $TgR[hAI]_{high}$ rats was indeed found in the d > 1.21 fraction of serum (Fig. 5D). In contrast, while nephrotic $TgR[hAI]_{low}$ rats expressed over 450 mg/dL of apoA-I, almost all of it was associated with HDL particles (Fig. 5B). Thus, because of both the lower concentration of serum apoA-I and its presence predominantly in a form (HDL) that contributes little to oncotic pressure, it is not surprising that in contrast to nephrotic $TgR[hAI]_{high}$ rats, these animals showed the same degree of edema as the non-transgenic nephrotic controls.

Effects of experimental nephrosis on apoA-I gene expression

Although the detailed molecular mechanisms underlying the hepatic response to the nephrotic syndrome are unknown, there is an increased steady-state level of apoA-I mRNA upon the induction of nephrosis in rats (8–10). As the apoA-I mRNA level is a critical determinant of apoA-I production rate (29, 30), an increased level of apoA-I mRNA most likely explains the elevated synthesis and secretion of hepatic apoA-I observed in nephrotic rats (7).

In the present study, non-nephrotic animals in all three lines had essentially identical levels of rat hepatic apoA-I mRNA (Table 2). In agreement with the literature (8–10), after the induction of nephrosis, these levels rose significantly (5- to 7-fold) in all the groups. There were also effects of nephrosis on the expression of the h-apoA-I mRNA. PAN treatment resulted in elevations of h-apoA-I mRNA of 21-fold in $TgR[hAI]_{low}$ and 65-fold in $TgR[hAI]_{high}$ animals. Thus, both transgenic lines showed a striking differential response of rat and human apoA-I mRNA levels to experimental nephrosis, with increased sensitivity of the human mRNA.

Prior studies in the PAN nephrosis model (8), the Heymann nephritis model, and the Nagase analbuminemic rat (10) indicated that elevation of rat hepatic apoA-I mRNA was associated with an increased transcriptional activity of the apoA-I gene. This is most likely also to be the basis for the increase in the h-apoA-I mRNA in the transgenic livers, as preliminary experiments (L. Sambucetti and E. A. Fisher, unpublished observations) indicate that the stability of the human apoA-I message was not changed in nephrosis. Why the human gene may be more sensitive than the rat gene to transcriptional activation is currently under study. Hypotheses include activation of additional transcription units in a high-copy cluster of tandemly repeated human apoA-I genes, increased efficacy of rodent factors to *trans*-activate the heterologous human *cis*-elements, and regulatory information in the 5′-flanking sequence of the human gene not present in the rat gene. Nonetheless, despite the need for additional studies to elucidate molecular mechanisms, the present results clearly demonstrate for the first time that the human apoA-I gene responds in the experimental nephrotic syndrome.

Effects of apoA-I overexpression on apoA-I and lipoprotein metabolism in nephrotic rats

Rubin et al. (31) have reported that in transgenic mice expressing human apoA-I there is a decrease in the serum level of mouse apoA-I. They hypothesized that HDL containing both species of apoA-I are unstable, and this instability leads to the selective loss of mouse apoA-I. In this study, $TgR[hAI]_{high}$ control rats displayed an inverse relationship between circulating levels of rat and human apoA-I ($r^2 = -0.97$, $P < 0.005$, $n = 5$). This line of rats normally displays a significant decrease of rat apoA-I levels when h-apoA-I becomes greater than 200 mg/dl (unpublished data). $TgR[hAI]_{low}$ rats rendered nephrotic had circulating levels of h-apoA-I in excess of 300 mg/dl, yet rat apoA-I levels did not decrease, and actually increased significantly (Table 1). However, $TgR[hAI]_{high}$ rats rendered nephrotic had massive increases in h-apoA-I serum levels and a corresponding decrease (47%) in the level of serum rat apoA-I (Table 1). In the experimental nephrosis model, higher levels of h-apoA-I (> 1,300 mg/dl) are needed to observe a decrease in circulating levels of rat apoA-I, possibly reflecting a greater stability of HDL containing rat and human apoA-I as compared to HDL containing the mouse and human proteins. Other potential explanations include differences in the metabolic parameters that impact the forces responsible for displacement of rat apoA-I from HDL, such as the amount of phospholipid associated with apoA-I, the fractional catabolic rate of rat and h-apoA-I, the structure of the nascent apoA-I particle produced by the liver, and alterations in plasma lipase activity associated with nephrosis. Such changes may result in displacement from HDL of rat apoA-I by the large pool of h-apoA-I, with subsequent loss in the urine, or the formation of HDL species, containing rat apoA-I, small enough to be lost in the urine, resulting in the excretion of cholesterol and phospholipids in addition to the apoA-I. Both of these explanations are supported by the gel analysis of urine (Fig. 4), the urine composition analysis (Table 3), and the analyses of serum by non-denaturing gradient gels (Fig. 3) and FPLC (Fig. 5). Associated with the nephrotic state in $TgR[hAI]_{high}$ animals were large urinary losses of apoA-I, cholesterol, and phospholipids and the disappearance from the serum of a small HDL_3-like lipoprotein species. This disappearance could be explained by increased loss of this small particle into the urine. Finally, the FPLC profile demonstrated that a large fraction of the rat apoA-I had a buoyant density > 1.21, not

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associated with HDL and therefore in a form that might be readily lost in the urine.

Nephrotic TgR[hAI]$_{non}$ and TgR[hAI]$_{low}$ rats showed the expected increases in all lipoprotein classes, including triglyceride-rich fractions. However, the results in the TgR[hAI]$_{high}$ rats were dramatically different; although overexpression of h-apoA-I in non-nephrotic TgR[hAI]$_{high}$ rats produced an increase in HDL cholesterol and serum phospholipid (compared to non-nephrotic animals of the other two groups), no further increase in HDL cholesterol was observed after PAN treatment (Table 2). In addition, triglyceride and non-HDL cholesterol levels showed only modest increases with nephrosis, and the associated lipoprotein peaks were blunted (Table 3 and Fig. 3).

Reasoning similar to that just discussed above (i.e., increased urinary losses of HDL or its components) may also explain these results. Alternatively, it is also possible that they reflect a smaller than expected increase in the hepatic production of lipoproteins in nephrotic TgR[hAI]$_{high}$ rats. One potential explanation for this is found in the work of Appel et al. (32), who have shown a significant inverse relationship between plasma cholesterol and oncotic pressure in patients with nephrotic syndrome. This relationship probably reflects a regulatory role of oncotic pressure on hepatic lipoprotein production (10). As the overproduction of apoA-I reduced edema in TgR[hAI]$_{high}$ nephrotic rats, it is reasonable to assume that this was due to the support of oncotic pressure in these animals, perhaps reducing the hepatic stimulation of lipoprotein production by this factor. Another contributing factor is implied by studies by Verkade et al. (33) demonstrating that when newly synthesized phospholipid becomes limiting, there is decreased hepatic secretion of apoB-containing triglyceride-rich lipoproteins. Thus, if the massive overproduction of apoA-I in the nephrotic TgR[hAI]$_{high}$ resulted in a metabolic diversion of phospholipid due to HDL formation, the assembly and secretion of non-HDL lipoproteins may suffer as a consequence. Finally, the overutilization of the protein synthetic machinery, such as ribosomes and translocation channels, by the highest levels of apoA-I production may directly interfere with the translation and processing of other proteins, such as apoB. Though in this section we have offered a number of plausible explanations, many of the issues raised by the urine and serum data, particularly in the nephrotic TgR[hAI]$_{high}$ rats, such as the attribution of results to changes in clearance versus production, are currently being investigated.

Concluding remarks

The induction of the nephrotic syndrome in transgenic rats has demonstrated that the expression of the human apoA-I gene can be significantly stimulated. Also, a number of effects of this stimulated expression on the metabolism of both HDL and non-HDL lipoproteins were observed. Further investigation of the mechanisms responsible for the effects of nephrosis in transgenic rats expressing h-apoA-I are likely to lead to new insights concerning the regulation of rodent and human apoA-I gene expression as well as the formation and catabolism of lipoproteins. In addition, these animals also represent a potentially useful model for the study of progressive renal disease and its amelioration. For example, we have presented preliminary evidence that apoA-I overexpression in rats decreased smooth muscle cell accumulation in the aorta after balloon injury (34) and Rubin et al. (31) have demonstrated that the overexpression of h-apoA-I in transgenic mice suppressed the formation of aortic foam cells in animals fed an atherogenic diet. Given that a major feature of many models of the atherosclerotic lesion (for example, see ref. 35) is proliferation of smooth muscle cells and accumulation of macrophages, similar to the effects in the aorta, perhaps overexpression of apoA-I in the nephrotic syndrome could also inhibit those changes in glomerular lesions (36) that are hallmarks of the progressive deterioration in renal function.

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