Failure of Antioxidant Therapy to Attenuate Interstitial Disease in Rats with Reversible Nephrotic Syndrome

ALFRED DRUKKER* and ALLISON A. EDDY,†
WITH THE TECHNICAL ASSISTANCE OF LORI MCCULLOCH† AND ELAINE LIU†
Shaare Zedek Medical Centre, Hebrew University Medical School, Jerusalem, Israel; and †The Hospital for Sick Children and the University of Toronto, Toronto, Ontario, Canada.

Abstract. The present two studies were designed to determine whether oxidized LDL contributes to the tubulointerstitial changes seen in rats during the acute phase of acute puromycin aminonucleoside nephrosis (PAN). In the single-dose study, rats were given one injection of puromycin aminonucleoside (PA; 15 mg/100 g body wt) and killed 1, 2, or 3 wk thereafter. The four animal groups were saline controls, PAN controls, PAN plus probucol, and PAN plus lovastatin. This study showed that the addition of probucol significantly reduced the mean levels of serum cholesterol and renal lipid-peroxidation products, an effect not seen with lovastatin therapy. Compared with saline controls, PAN controls had a significant increase in total kidney collagen (7.9 ± 1.2 *versus* 5.9 ± 0.6 mg/kidney at 3 wk). Neither probucol nor lovastatin therapy attenuated the interstitial inflammation or fibrosis. In the multidose study, rats were given the same initial PA dose and were uninephrectomized on day 12. They were killed on day 35 after two smaller PA doses were given on days 16 and 23. Animal groups were saline controls, PAN controls, PAN plus probucol, and PAN plus vitamin E. Hepatic lipid-peroxidation products were significantly lower in the probucol-treated, but not in the vitamin E-treated, PAN groups when compared with the PAN controls. Neither probucol nor vitamin E prevented the increase in total kidney collagen that was observed in the PAN control group (7.4 ± 0.7, 10.1 ± 2.6, and 9.3 ± 1.8 mg of collagen/kidney, respectively, *versus* 5.4 ± 0.5 mg/kidney for the saline controls). Renal cortical mRNA levels for matrix-encoding genes and protease inhibitors were similar in the three nephrotic groups. Transforming growth factor-β1 mRNA levels were highly variable within each group and not significantly different at day 35, but showed a significant positive correlation with the degree of albuminuria ($r = 0.70$). The present results demonstrate that the treatment of acutely nephrotic rats with antioxidant therapy did not attenuate interstitial inflammation or fibrosis. We speculate that other factors, possibly a consequence of proteinuria itself, are the predominant pathogenetic mediators of the tubulointerstitial damage in acute nephrotic syndrome. (J Am Soc Nephrol 9: 243–251, 1998)

Changes in the metabolism of lipoproteins, as seen in the nephrotic syndrome, have been implicated in progressive renal disease as one of the mediators (1). In the past, glomerulosclerosis has been described as analogous to atherosclerosis. In both processes, the oxidation of LDL and the infiltration of the tissue with macrophages are the critical initiating events associated with hypercholesterolemia. (2). Although there is overwhelming evidence that chronic damage to the tubulointerstitium is an essential feature of chronic and progressive glomerulonephritis, the role of hypercholesterolemia in interstitial disease has not been extensively investigated. Rats exposed to prolonged periods of hypercholesterolemia develop more severe interstitial fibrosis than animals without hypercholesterolemia (3–7). We (A. Eddy, Interstitial fibrosis in hypercholesterolemic rats: Role of oxidation, matrix synthesis, and proteolytic cascades, submitted for publication) recently observed that uninephrectomized rats with diet-induced hypercholesterolemia have markedly attenuated interstitial fibrosis at 12 wk if they are treated with antioxidants. Vitamin E therapy has been reported to reduce the severity of interstitial fibrosis in rats 12 wk after induction of the nephrotic syndrome (8). Vitamin E or probucol therapy significantly reduced interstitial disease in rats with puromycin aminonucleoside-induced nephrosis (PAN) of 32 wk duration, whether the animals were fed a standard or high-cholesterol diet (9). In another study (10) of rats with chronic PAN of 98 d duration, probucol preserved renal function and glomerular architecture, although the specific effect of the antioxidant on tubulointerstitial histology was not stated.

An unresolved question is whether the short-term hypercholesterolemia encountered in reversible nephrotic syndrome causes damage to the tubulointerstitium. In the rat model of PAN, glomerular histologic changes are initially reminiscent of minimal lesion nephrotic syndrome. However, these PAN animals also develop interstitial nephritis that parallels in intensity the proteinuria, reversing as it resolves (11). As a sequela, foci of interstitial fibrosis develop within 3 wk (12). Whether the hypercholesterolemia of acute PAN contributes to interstitial fibrogenesis is unknown. Oxidized LDL has monocyte chemotactic activity *in vitro* (13). Oxidized LDL also has

Received April 30, 1997. Accepted August 11, 1997.
Correspondence to Dr. Allison Eddy, Division of Nephrology, Children's Hospital and Regional Medical Centre, 4800 Sand Point Way N.E., P.O. Box 5371/CH-46, Seattle, Washington 98105-0371.
1046-6673/0902-0243$03.00/0
Journal of the American Society of Nephrology

indirect chemoattractive effects: It stimulates the synthesis of chemokines such as monocyte chemoattractant protein-1 (MCP-1) (14). In several organ systems, macrophages are thought to play a role in mediating fibrosis because of their ability to produce fibrogenic growth factors, protease inhibitors, and even matrix proteins (15). Recent evidence suggests that oxidized LDL itself also has direct fibrosis-promoting effects. For example, oxidized LDL stimulates the production of transforming growth factor beta-1 (TGF-β1) and fibronectin in human glomerular epithelial cells (16). Oxidized LDL has also been shown to induce collagen-gene expression in human mesangial cells (17). The present study was designed to determine whether antioxidant therapy could attenuate interstitial inflammation or fibrosis in rats with acute puromycin aminonucleoside (PA)-induced nephrotic syndrome.

Materials and Methods

Experimental Design

Sixty-eight female Sprague-Dawley rats weighing 100 to 120 g were purchased from Charles River Breeding Laboratories (Wilmington, MA) and divided into two study groups: single-dose and multidose PAN. The single-dose study was performed first. The results of the initial study led us to design a second study to determine whether acute nephrotic syndrome of a longer duration, *i.e.*, 5 rather than 3 wk, would respond differently to antioxidant therapy. We deliberately designed both of these studies to evaluate a time course of nephrotic syndrome that is reminiscent of minimal lesion nephrotic syndrome in humans. All animal experimentation was conducted according to the guidelines established by the Canadian Council on Animal Care. All animals were fed standard rat chow and given water *ad libitum*. The overall study design is summarized in Table 1.

Single-Dose Study. In the single-dose study, nephrotic syndrome was induced by one intraperitoneal injection of PA (15 mg/100 g body wt). Four groups of nine to 12 animals each were studied: (*1*) saline controls that received an equal-volume intraperitoneal injection of saline; (*2*) PAN controls that received PA and no further treatment; (*3*) PAN plus lovastatin animals that received PA and daily subcutaneous injections of 4 mg/kg lovastatin (kindly provided by Merck, Sharp, and Dohme, Kirkland, Quebec, Canada); and (*4*) PAN plus probucol animals that received PA plus 2% probucol (kindly provided by Marion Merrell Dow, Cincinnati, OH) added to freshly ground chow (wt/wt) that was prepared daily.

Table 1. Summary of investigations[a]

Parameter	Single-Dose Study	Multidose Study
Urinary albumin	1, 2, 3 wk	8, 21 d
Serum cholesterol	1, 2, 3 wk	35 d
Renal TBARS	2, 3 wk	12 d
Hepatic TBARS	ND	35 d
Total kidney collagen	1, 2, 3 wk	35 d
Renal mRNA levels		
TGF-β1	ND	12, 35 d
matrix/protease inhibitors	ND	35 d
Renal immunostaining		
macrophages	1, 2, 3 wk	35 d
matrix proteins	3 wk	35 d

[a] TBARS, thiobarbituric acid assay; ND, not done; TGF-β1, transforming growth factor-β1.

Three or four rats from each group were killed at 1, 2, and 3 wk after the administration of PA by inhalation of nitrous oxide, oxygen, and enflurane. Both kidneys were quickly procured, and the renal capsules were removed. The kidneys were carefully weighed (wet weight). The left kidney was divided in half longitudinally, and the cortex of one half was dissected from the medulla and stored for RNA extraction. The second half was divided into two equal pieces. One piece was reweighed, snap-frozen, and stored at −70°C for measurement of lipid peroxidation products. Pieces of cortex from the remaining portion were snap-frozen in liquid nitrogen and stored at −70°C for subsequent immunostaining. The right kidney was frozen in liquid nitrogen and stored at −70°C for measurement of total kidney collagen.

Multidose Study. In the multidose study, nephrotic syndrome was induced by a single intraperitoneal injection of PA (15 mg/100 g body wt) on day 0. On day 12, all animals underwent a left nephrectomy under general anesthesia (nitrous oxide and enflurane) through a flank incision. Subsequent intraperitoneal injections of PA (2.5 mg/100 g body wt) were given to the nephrotic animals on days 16 and 23. Saline controls received equal volumes of intraperitoneal saline on days 0, 16, and 23.

There were several reasons for the unilateral nephrectomy; although the specific time point of 12 d was somewhat arbitrary, it was selected as a point when the ascites were resolving, making the surgery itself easier from a technical perspective. In the original study of PAN-induced chronic nephrosis by Glasser and colleagues (18), uninephrectomy was shown to accelerate the severity of glomerulosclerosis. Although the specific mechanism was not elucidated, uninephrectomy caused a significant increase in proteinuria but no difference in BP. In our previous study of chronic PAN-induced nephrosis in uninephrectomized rats (19), animals examined at 37 d were found to have significant interstitial fibrosis, which is why the day 35 end point was chosen for the second study. Lipid peroxidation products were measured in the day 12 nephrectomy specimens (see below) to confirm the results of the single-dose study.

Four groups of five to seven rats each were studied: (*1*) saline controls; (*2*) PAN controls that received PA and no further treatment; (*3*) PAN plus probucol animals that received PA plus 2% probucol in their diet, as outlined above in the single-dose study; and (*4*) PAN plus vitamin E animals that received PA plus 75 mg (100 IU) of alpha tocopherol acetate (Sigma Chemical, St. Louis, MO) added to fresh 30-g batches of chow each day. In this second study, we chose to evaluate the efficacy of vitamin E rather than lovastatin due to recent reports of the protective effects of vitamin E in chronic models of PAN (8, 9).

Each rat was housed in a separate metabolic cage on days 8 and 21 to collect spontaneously voided urine over a 24-h period. All animals were killed by exsanguination under general anesthesia on day 35. The day 12 nephrectomy specimen was carefully weighed and divided in half. From one half, the cortex was carefully dissected, frozen in liquid nitrogen, and stored at −70°C for RNA extraction. The second half was divided into two pieces. One piece was weighed and frozen before measurement of the lipid peroxidation products. The remaining piece was snap-frozen and stored at −70°C for immunostaining. The right kidney was removed at the time of death; it was carefully weighed after removal of the capsule and divided in half longitudinally. One half was reweighed and stored for measurement of the total collagen. The cortex was dissected from the second half, and the pieces were snap-frozen and stored at −70°C for subsequent immu-

nostaining and RNA isolation. At the time of death, a piece of the liver (approximately 1 g) was also snap-frozen and stored for measurement of lipid peroxidation products.

Biochemical Studies

Urinary albumin concentrations were measured by radial immunodiffusion, according to our previously described methods (11). Plasma total cholesterol levels were measured on individual samples of heparinized blood obtained when the rats were exsanguinated.

Total kidney collagen was calculated as described previously (20) based on measurements of the hydroxyproline concentration in kidney homogenates, according to the technique of Kivirikko *et al.* (21). Collagen was assumed to contain 12.7% hydroxyproline by weight. The final results, based on the wet weight of the kidney, were expressed as milligrams of collagen per kidney. Renal peroxidation products were assessed with the thiobarbituric acid assay to measure the renal malondialdehyde content, according to the methods of Ohkawa *et al.* (22). Because there was not an adequate amount of kidney tissue available on day 35, we used the thiobarbituric acid assay to measure hepatic levels of lipid peroxidation products as done by Trachtman *et al.* (8). The hepatic protein concentration was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Final results were expressed as nmol malondialdehyde/μg protein.

Renal Immunofluorescence Studies

The number of interstitial macrophages expressing the ED-1 cytoplasmic marker (Serotec, Oxford, United Kingdom) (23) was determined on 3-μm-thick acetone-fixed kidney cryosections with the dual fluorochrome labeling and enumeration technique we have described elsewhere (11).

The accumulation of interstitial matrix proteins in the renal interstitium was assessed semiquantitatively, as described previously (19). The primary antibodies used were goat antihuman collagen III (Southern Biotechnology Associates, Birmingham, AL) and a murine monoclonal antibody to the human extracellular domain A (ED-A fibronectin [24], an alternatively spliced form of fibronectin [gift from Dr. L. Zardi, Instituto Nazionale per la Ricerca sol Cancro, Genova, Italy]). The secondary antisera were FITC-conjugated rabbit anti-goat IgG antisera (Chemicon International, Temecula, CA) and FITC-conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA). The FITC-conjugated antisera were absorbed with normal rat plasma; lack of staining indicated that they were nonreactive with the control kidney sections. Code numbers were used to blind the investigator to the animal group in each case.

We isolated renal cortical RNA according to the guanidinium isothiocyanate–cesium chloride method of Chirgwin *et al.* (25). Ten-microgram samples from each rat were separated by electrophoresis in a 1% formaldehyde agarose gel. The gel was then transferred to a nylon membrane (Hybond N, Amersham Life Sciences, Oakville, Ontario, Canada) and incubated with ^{32}P-dCTP-labeled complementary DNA probes. We obtained autoradiographs and quantified the bands by laser densitometry. The density reading of each band on the autoradiograph was adjusted for any RNA loading inequality, as described previously (19). The studies of renal gene expression were limited to the day 12 and day 35 kidneys that were obtained from the second study group.

The cDNA probes used were rat α1(I) procollagen (supplied by Dr. S. Thorgeirsson, National Cancer Institute, Bethesda, MD) (26); murine α1(III) procollagen (supplied by Dr. B. de Crombrugghe, M. D. Anderson Cancer Center, The University of Texas, Houston, TX) (27); fibronectin (supplied by Dr. R. Hynes, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA) (28); murine tissue inhibitor of metalloproteinases-1 (TIMP-1) (supplied by Dr. R. Khokha, London Regional Cancer Centre, London, Ontario, Canada) (29); rat plasminogen activator inhibitor-1 (rat PAI-1) (supplied by Dr. Z. R. Gelehrter, University of Michigan, Ann Arbor, MI) (30); and rat TGF-β1 (supplied by Dr. S. W. Qian, National Cancer Institute, Bethesda, MD) (31).

Statistical Analyses

All results are expressed as the group mean ± 1 SD. Bonferroni's modified *t* test was used to compare the results of each experimental group with the results of the PAN control group. To avoid the need to use the large numbers of rats that would be necessary if comparisons were made between several groups, the study was designed to compare each group with the PAN control group only. A P value of less than 0.05 was considered statistically significant.

Results

Study One: Single-Dose Study

All nephrotic rats developed significant hypercholesterolemia that was maximal at week 1 and declined thereafter as the nephrotic syndrome resolved (Figure 1). In the animals given probucol therapy, serum cholesterol levels were significantly reduced at 2 and 3 wk compared with the untreated nephrotic rats. Compared with saline control animals, renal lipid peroxidation products were significantly increased by 3 wk in the nephrotic animals; this increase was attenuated by probucol, but not lovastatin, therapy (Figure 2). Maximal albuminuria occurred at week 1 and was similar in the three nephrotic groups. The resolution of albuminuria was more rapid in the probucol-treated group, as indicated by significantly lower albumin excretion rates at week 2 (Figure 3). Neither lovastatin nor probucol therapy prevented the interstitial influx of macrophages (Figure 4). In fact, the number of macrophages was even slightly increased at 2 wk in the probucol-treated group.

Compared with rats in the saline group, rats with single-dose PA-induced nephrosis had a significant increase in total kidney collagen (Figure 5). Neither lovastatin nor probucol had a significant effect on the kidney collagen content (Figure 5) or the weight of the kidney (weight data not shown) compared with the PAN control group at the time of death. The similarities in the degree of interstitial fibrosis were confirmed by

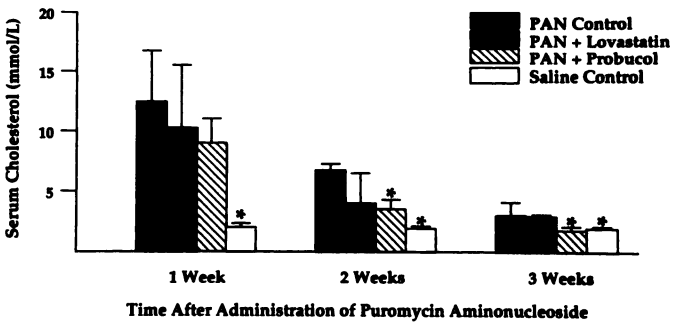

Figure 1. Serum cholesterol levels in rats after a single dose of puromycin aminonucleoside (PA). Results are mean ± 1 SD. *P < 0.05 by Bonferroni's *t* test.

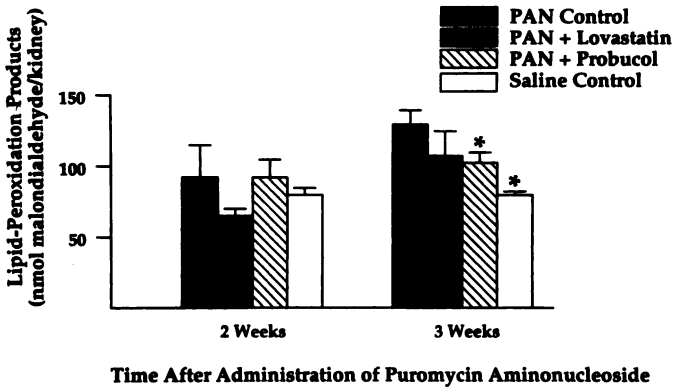

Figure 2. Renal lipid peroxidation products in rats after a single dose of PA. Results are mean ± 1 SD. *$P < 0.05$ by Bonferroni's t test.

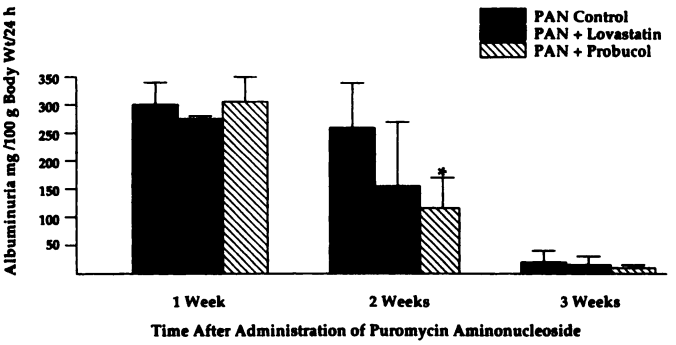

Figure 3. Urinary albumin excretion rates in rats after a single dose of PA. Results are mean ± 1 SD. *$P < 0.05$ by Bonferroni's t test.

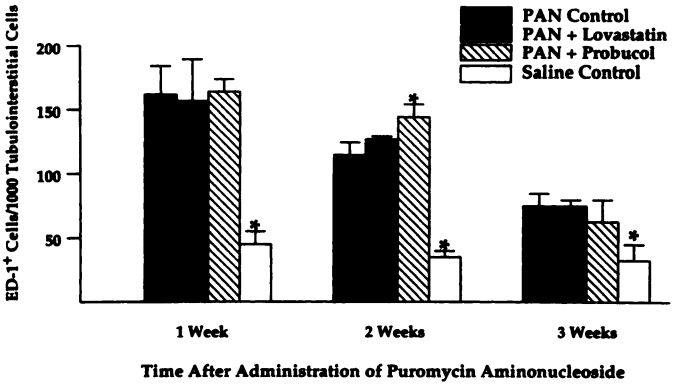

Figure 4. Interstitial macrophages in rats after a single dose of PA. Results are mean ± 1 SD. *$P < 0.05$ by Bonferroni's t test.

immunostaining for collagen III and fibronectin. Expressed as the percentage of interstitial fields with increased staining, the results were as follows: 0 ± 0, 37 ± 7, 54 ± 5, and 37 ± 7% fibronectin; and 0 ± 0, 28 ± 17, 30 ± 17, and 37 ± 18% collagen III for saline controls, PAN controls, PAN plus lovastatin, and PAN plus probucol, respectively.

Study Two: Multidose PA-Induced Nephrosis

At the termination of the study on day 35, the animals had significant proteinuria, but the degree of proteinuria (day 21)

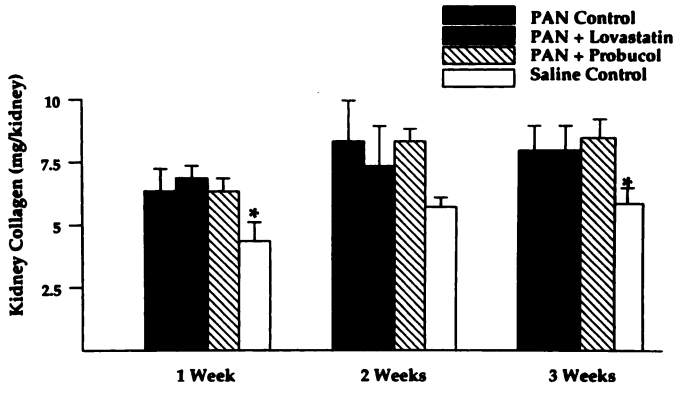

Figure 5. Total kidney collagen levels in rats after a single dose of PA. Results are mean ± 1 SD. *$P < 0.05$ by Bonferroni's t test.

was considerably less than during the nephrotic phase (day 8) (Table 2). At this time, serum cholesterol levels in the proteinuric animals were slightly increased; this difference was not statistically significant (Table 2). As in the first study, renal lipid peroxidation products were not increased at 2 wk in the PA-treated groups. Hepatic lipid peroxidation products were increased in all of the proteinuric rats, except in the group that was treated with probucol. All three groups of PA-treated rats had similar degrees of proteinuria on day 8 (Table 2). On day 21, all PA-treated animals still had proteinuria, although quantitatively less (11 to 36%) than the day 8 levels. The probucol-treated rats actually had significantly more albuminuria.

The earlier increase in the number of interstitial macrophages had reversed in the PAN control rats by day 35. In the nephrotic rats treated with either antioxidant, there was a significant persistent macrophage infiltrate on day 35 (Table 3). Proteinuric rats had significantly increased total kidney collagen and kidney weight compared with those of the saline-treated control group (Table 3). Antioxidant therapy failed to attenuate the increase in either the renal collagen content or the kidney weight. Immunostaining also confirmed interstitial fibrosis. Increased interstitial staining for collagen III was found in 0 ± 0% interstitial fields for saline controls, 18 ± 5% for PAN controls, 17 ± 10% for PAN plus probucol, and 15 ± 8% for PAN plus vitamin E.

The renal cortical mRNA levels for TGF-β1 were increased in the nephrotic rats on day 35. However, as with the day 21 urinary albumin excretion rates, levels for individual animals were quite variable within each PAN group, and, consequently, the mean TGF-β1 mRNA values were not significantly different from those of the saline control group (Table 4, Figure 6). There was, in fact, a significant positive correlation between albuminuria on day 21 and renal cortical TGF-β1 mRNA levels on day 35 ($r = 0.70$). It is noteworthy that the renal cortical mRNA levels for TGF-β1 were significantly increased in the nephrotic kidneys that were removed on day 12, *i.e.*, 14.5 ± 5.7 *versus* 1.0 ± 0.4 arbitrary densitometric units (PAN *versus* saline control groups). Neither probucol nor vitamin E treatment induced a significant change in the day 12 renal

Table 2. Multidose PA-induced nephrosis: biochemical profile[a]

Group	Serum Cholesterol (mmol/L)	Lipid Peroxidation Products		Albuminuria (mg/100 g per 24 h)	
		Kidney (nmol MDA/ g wet wt)	Liver (nmol MDA/ μg protein)		
	on Day 35	on Day 12	on Day 35	On Day 8	On Day 21
Saline controls	1.7 ± 0.4	113 ± 10	867 ± 122[b]	1 ± 1[b]	1 ± 1[b]
PAN controls	2.5 ± 0.5	111 ± 6	1024 ± 152	350 ± 92	39 ± 15
PAN + probucol	1.9 ± 0.4	108 ± 22	868 ± 25[b]	209 ± 76	75 ± 26[b]
PAN + vitamin E	3.1 ± 1.5	105 ± 18	1043 ± 102	221 ± 87	60 ± 39

[a] PA, puromycin aminonucleoside; MDA, malondialdehyde; PAN, puromycin aminonucleoside nephrosis.
[b] $P < 0.05$ (Bonferroni's t test).

Table 3. Multidose PA-induced nephrosis: interstitial inflammation and fibrosis

Group	ED-1$^+$ Interstitial Macrophages (number per 1000 tubulointersti-tial cells)	Total Kidney Collagen (mg/kidney) on Day 35	Kidney Weight (mg) on Day 35
Saline controls	28 ± 8	5.4 ± 0.5[a]	1060 ± 124[a]
PAN controls	25 ± 5	7.4 ± 0.7	1388 ± 116
PAN + probucol	54 ± 21[a]	10.1 ± 2.6	1873 ± 465
PAN + vitamin E	67 ± 41	9.3 ± 1.8	1774 ± 370

[a] $P < 0.05$ (Bonferroni's t test). Abbreviations as in Table 2.

cortical TGF-β1 mRNA levels (10.8 ± 2.5 and 11.3 ± 3.3 arbitrary densitometric units for the probucol and vitamin E groups, respectively).

Compared with the saline control group, the PAN control group had a significant increase in renal cortical mRNA levels for the interstitial matrix proteins procollagen α1(I), procollagen α1(III), and fibronectin on day 35 (Table 4, Figure 7). Treatment with either probucol or vitamin E did not significantly change the matrix protein mRNA levels relative to those of the PAN control group. Antioxidant treatment also failed to induce a significant difference in renal cortical mRNA levels for the protease inhibitors TIMP-1 and PAI-1 (Table 4).

Discussion

Rats with acute and reversible PA-induced nephrosis develop an interstitial infiltrate of macrophages followed by focal interstitial fibrosis. We have shown previously that in the absence of persistent proteinuria and tubulointerstitial nephritis, the foci of interstitial fibrosis actually disappear again between weeks 3 and 6 (12). At the peak of the nephrotic syndrome, these rats develop severe hypercholesterolemia, and an increase in the level of renal lipid peroxidation products ensues. The goal of the present studies was to determine whether relatively short-term but severe nephrotic hypercholesterolemia might have damaging effects on the tubulointerstitial compartment of the kidney. Our results suggest that hypercholesterolemia does not play a significant role in the pathogenesis of the interstitial fibrosis that develops in the course of acute PA-induced nephrosis. In contrast, previous studies have demonstrated a significant beneficial effect of antioxidants in chronic models of PA nephrosis of 12 to 32 wk duration (8–10).

It is quite clear from studies in humans and mice with genetic forms of hypercholesterolemia that the renal vessels, but not the renal parenchyma itself, are the primary targets for lipid-induced damage (32–34). However, within the milieu of an injured kidney, the renal parenchyma may become more susceptible to the damaging effects of hypercholesterolemia (35). A delicate balance normally exists between the level of intrarenal oxidants and antioxidants (36). In the face of an abundance of reactive oxygen metabolites, as often occurs in acute renal diseases including PAN (37–39), it is likely that there is an increased rate of intrarenal LDL oxidation. These changes provide at least a theoretical framework for the increased susceptibility of the injured kidney to lipid-mediated damage. Although hypercholesterolemia is thought to mediate renal injury after LDL is oxidized, it should be emphasized that intrarenal LDL oxidation likely generates a heterogeneous mixture of products and the specific molecular species that mediates renal injury is still unknown. In the present study, the thiobarbituric acid assay was used as a surrogate marker of the extent of lipid peroxidation within the kidney. This assay measures levels of malondialdehyde, a secondary product of lipid peroxidation. Malondialdehyde-modified LDL is one form of oxidized LDL that is associated with cholesterol-mediated vascular disease. Yet, until the specific lipid peroxidation products that mediate renal interstitial disease are identified, it remains unclear whether this assay truly reflects LDL oxidation products in the present study.

Oxidized LDL has been implicated as a mediator of macrophage recruitment. With the use of *in vitro* systems, it has been shown that oxidized LDL itself has chemotactic properties (13), as well as indirect effects mediated by the upregulation of chemokines such as MCP-1 (14). In addition, oxidized LDL may stimulate the expression of adhesion molecules such as the β2 integrins (40), intercellular adhesion molecule-1 (41), and

Table 4. Multidose PAN-induced nephrosis: day 35 renal cortical mRNA levels[a]

Group	Renal Cortical mRNA Levels[b]					
	TGF-β1	Procollagen α1(I)	Procollagen α1(III)	Fibronectin	TIMP-1	PAI-1
Saline controls	1.0 ± 0.4	1.0 ± 1.1[c]	1.0 ± 0.6[c]	1.0 ± 0.5[c]	1.0 ± 0.2	1.0 ± 0.4
PAN controls	3.6 ± 2.8	4.3 ± 1.8	4.3 ± 2.1	2.7 ± 0.9	1.5 ± 0.5	1.5 ± 0.8
PAN + probucol	5.0 ± 1.7	2.5 ± 1.1	4.9 ± 2.4	3.3 ± 0.3	1.8 ± 0.5	1.7 ± 0.7
PAN + vitamin E	3.8 ± 1.6	6.5 ± 4.8	3.8 ± 1.2	3.3 ± 1.4	2.7 ± 2.1	2.7 ± 2.4

[a] TIMP-1, tissue inhibitor of metalloproteinases-1; PAI-1, plasminogen-activator inhibitor-1. Other abbreviations as in Tables 1 and 2.
[b] Results are expressed in arbitrary densitometric units as mean ± 1 SD.
[c] $P < 0.05$ (Bonferroni's t test).

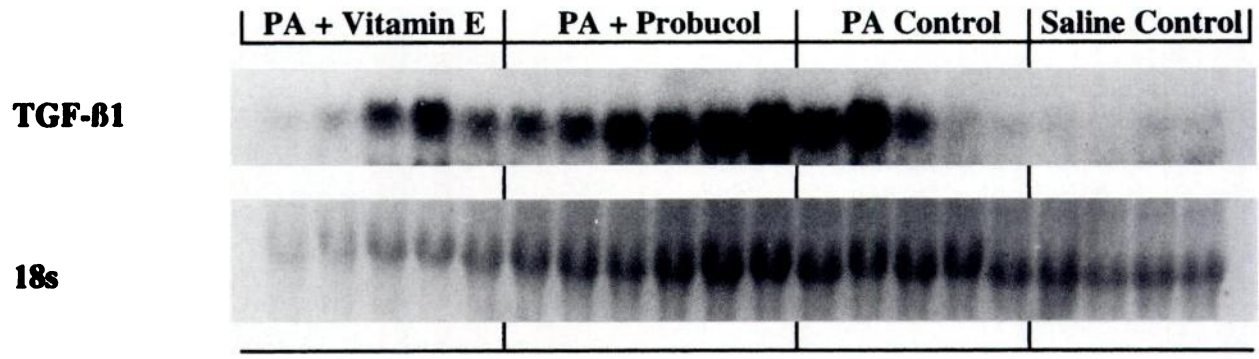

Figure 6. Northern blot illustrating renal cortical mRNA levels for transforming growth factor-β1 on day 35 in rats given three doses of PA. The mean densitometric scores in arbitrary units after correction for RNA loading inequality were 1.0 ± 0.4 for saline controls, 3.6 ± 2.8 for PA controls, 5.0 ± 1.7 for PA and probucol, and 3.8 ± 1.6 PA and vitamin E.

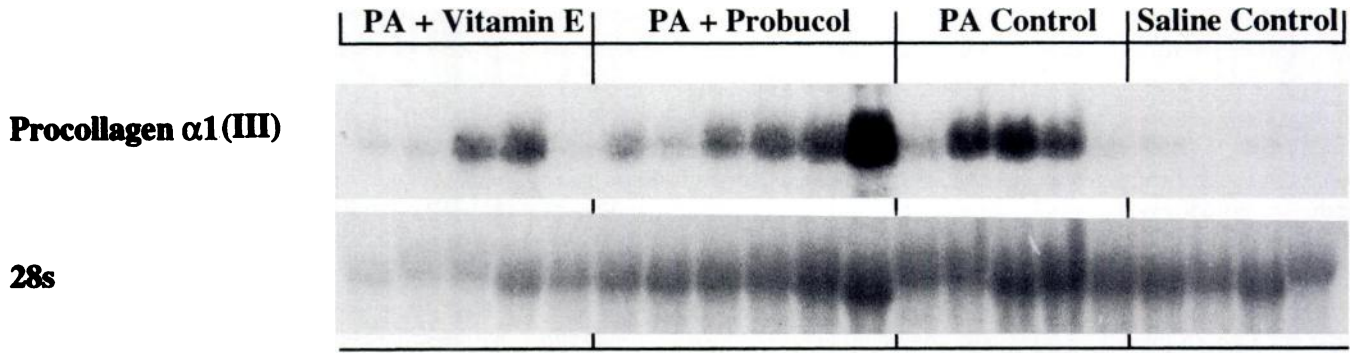

Figure 7. Northern blot illustrating renal cortical mRNA levels for procollagen α1(III) on day 35 in rats given three doses of PA. The mean densitometric scores in arbitrary units after correction for RNA loading inequality were 1.0 ± 0.6 for saline controls, 4.3 ± 2.1 for PA controls, 4.9 ± 2.4 for PA and probucol, and 3.8 ± 1.2 for PA and vitamin E.

vascular cell adhesion molecule-1 (42). In a previous work, we observed an increase in the renal expression of MCP-1, vascular cell adhesion molecule-1, and intercellular adhesion molecule-1 in rats with PAN (43) (A. Eddy, unpublished observations). Yet, despite these theoretical possibilities, in the present study, antioxidant therapy failed to prevent the interstitial infiltrate of macrophages. Elucidation of the molecular basis of interstitial monocyte recruitment is of great interest, because the persistence of these cells within the renal interstitium correlates with progressive tubulointerstitial damage (15). Until now, however, it has been impossible to show that the selective inhibition of a single molecular pathway of monocyte recruitment will prevent interstitial nephritis in PA-induced nephrosis (44,45). We have suggested that there may be such redundancy in the monocyte recruitment mechanisms that a cocktail approach may be necessary, *i.e.*, blocking several pathways simultaneously to achieve a significant inhibitory effect. Thus, as in previous studies, the present experiments

also show that inhibitory therapy targeted to a single molecule—in this case oxidized LDL—did not attenuate interstitial fibrosis.

Our previous studies (12,19,20) of PA-induced nephrosis in rats suggested an important role for TGF-β1 in the mediation of interstitial fibrosis. Although the mechanism of TGF-β1 upregulation in interstitial fibrosis remains unknown, one possibility based on *in vitro* studies is that oxidized LDL may stimulate TGF-β1 production. This is true at least for cultured human glomerular mesangial (17) and epithelial (16) cells. Previous studies (46) suggested that interstitial cells (macrophages and myofibroblasts), along with tubular epithelial cells, are the major sites of TGF-β1 production in experimental models of interstitial fibrosis. At the mRNA level, the usual pattern of gene expression in PAN rat kidneys is one that would be predicted after TGF-β1 activation: increased expression of the genes encoding matrix proteins and of the protease inhibitors TIMP-1 and PAI-1. The present study found that in PAN rats, despite normalization of renal lipid peroxidation products by probucol therapy, renal cortical mRNA levels for TGF-β1 were not decreased, suggesting that the intrarenal oxidation of LDL over a relatively short period of time does not play a key role in the stimulation of TGF-β1 gene transcription. Oxidized LDL also did not increase TGF-β1 production by cultured endothelial cells (47). Therefore, it is possible that within the *in vivo* milieu of the kidney, oxidized LDL is not a major acute stimulus for TGF-β1 synthesis by tubulointerstitial cells.

An alternative explanation for the failure of antioxidant therapy to attenuate interstitial fibrosis is the possibility that prolonged exposure to oxidized LDL is required before a significant fibrogenic response ensues, likely involving TGF-β1 along with other fibrogenic cytokines. In rats with a solitary kidney, it is clear that the development of interstitial fibrosis after exposure to significant hypercholesterolemia is an indolent process that develops over months rather than days or weeks (44). It was with this possibility in mind that the second study was designed to examine both early outcomes at day 12 and late outcomes after a longer period of proteinuria (*i.e.*, at day 35). Although uninephrectomy and additional small doses of PA in this second group of experiments caused persistent and increased albuminuria (range, 40 to 75 mg/24 h on day 21 compared with 10 to 23 mg/24 h in rats given the single dose), when the animals were killed on day 35 their serum cholesterol levels were only modestly increased compared with those of the saline control animals.

It is conceivable that hypercholesterolemia of a relatively short duration does not generate specific profibrotic LDL oxidation products within the tubulointerstitial compartment, despite the fact that renal levels of thiobarbituric acid-reactive substances were significantly increased in nephrotic animals and normalized by probucol therapy. Little is known about either the molecular basis of LDL oxidation within the kidney or the nature of the oxidized products that promote fibrosis. Two distinct processes of lipid peroxidation have been proposed (48,49). First, intrinsic renal cells or inflammatory cells may release reactive oxygen metabolites, such as superoxide anion, that subsequently peroxidize LDL. Within the kidney, reactive oxygen metabolites are detoxified by antioxidant enzymes, including superoxide dismutases, catalases, and glutathione peroxidases (50). A relative antioxidant deficiency within the kidney might also facilitate lipid peroxidation. Superoxide and peroxynitrites have been implicated in lipid peroxidation at sites of atherosclerosis (51). Cultured human renal tubular cells have been shown to oxidize native LDL by a superoxide-dependent mechanism (52). A body of evidence exists to support a role for reactive oxygen metabolites in the acute glomerular injury that develops in rats treated with PA (37–39). Whether these reactive species are the same as those that oxidize LDL within the kidney, however, is not known. The second possible mechanism of intrarenal LDL oxidation is through the intracellular synthesis of lipoperoxides that are subsequently transferred to LDL. Possible enzymes that may generate intracellular lipoperoxides include lipoprotein lipase, cholesterol esterase, lipoxygenases, and phospholipases (49).

In contrast to the renal effects of hypercholesterolemia that are sustained over several months, hypercholesterolemia of a few weeks' duration, as in the present study, does not appear to damage the interstitium. This also may be due in part to the paucity of oxidized LDL receptors on the target tubular and interstitial cells at the onset of nephrosis. Although interstitial macrophages likely express adequate scavenger receptors (53,54), interstitial foam cells representing cholesterol-laden macrophages (55,56) are not a feature of this acute disease. Tubular epithelial cells have been shown to internalize oxidized LDL *in vitro* (52,57), but the specific receptor pathways have not yet been elucidated. Candidate receptors include macrophage type I and type II scavenger receptors (58), scavenger receptor BI (59), macrosialin/CD68 (60), MARCO (61), CD36 (62), and Fcγ RII (63).

Finally, we want to propose an explanation for the failure of antioxidant therapy to attenuate tubulointerstitial disease in rats with acute PA-induced nephrosis. We speculate that this is due to the coexistence of another factor (or factors) that dominates the potential mediators of tubulointerstitial injury during acute and reversible nephrotic syndrome. Proteinuria itself is a likely candidate, particularly if it is of nonselective composition. Persistent significant proteinuria has been proposed as one of the mediators of progressive tubulointerstitial damage in several human and experimental models of renal disease (20,64–67).

In summary, rats with acute PA-induced nephrosis develop tubulointerstitial disease characterized by an increase in the number of interstitial macrophages and fibrosis. Treatment of nephrotic rats with probucol, but not with vitamin E or lovastatin, was efficacious in reducing serum cholesterol levels, as well as in promoting intrarenal antioxidant effects. Yet, despite these effects, the interstitial infiltrate of macrophages and interstitial fibrosis were not attenuated in the probucol-treated group.

Acknowledgments

This work was supported by grants from Baxter Extramural Grant Program and The Medical Research Council of Canada (MT-11553).

We acknowledge Marion Merrell Dow (Cincinnati, OH) for providing the probucol powder; Merck, Sharp, and Dohme for providing the lovastatin; and the numerous investigators mentioned who so generously shared their cDNA and antibody probes with us. We also acknowledge the work of Dana Debernyj in typing this manuscript. This article was prepared with the assistance of Editorial Services, The Hospital for Sick Children, Toronto, Ontario, Canada.

References

1. Keane WF: Lipids and the kidney. *Kidney Int* 46: 910–920, 1994
2. Diamond JR: Effect of lipid abnormalities on the progression of renal damage: Analogous pathobiologic mechanisms in glomerulosclerosis and atherosclerosis. *Kidney Int* 39[Suppl 31]: S29–S34, 1991
3. Kasiske BL, O'Donnell MP, Cleary MP, Keane WF, Phillips F: Effects of reduced renal mass on tissue lipids and renal injury in hyperlipidemic rats. *Kidney Int* 35: 40–47, 1989
4. Kasiske BL, O'Donnell MP, Schmitz PG, Kim Y, Keane WF: Renal injury of diet-induced hypercholesterolemia in rats. *Kidney Int* 37: 880–891, 1990
5. Magil AB: Tubulointerstitial lesions in young Zucker rats. *Am J Kidney Dis* 25: 478–485, 1995
6. Peric-Golia L, Peric-Golia M: Aortic and renal lesions in hypercholesterolemic adult, male, virgin Sprague-Dawley rats. *Atherosclerosis* 46: 57–65, 1983
7. Wellmann KF, Volk BW: Renal changes in experimental hypercholesterolemia in normal and in subdiabetic rabbits. II. Long-term studies. *Lab Invest* 24: 144–155, 1971
8. Trachtman H, Schwob N, Maesaka J, Valderrama E: Dietary vitamin E supplementation ameliorates renal injury in chronic puromycin aminonucleoside nephropathy. *J Am Soc Nephrol* 5: 1811–1819, 1995
9. Lee HS, Jeong JY, Kim BC, Kim YS, Zhang YZ, Chung HK: Dietary antioxidant inhibits lipoprotein oxidation and renal injury in experimental focal segmental glomerulosclerosis. *Kidney Int* 51: 1151–1159, 1997
10. Magil A: Inhibition of progression of chronic puromycin aminonucleoside nephrosis by probucol, an antioxidant. *J Am Soc Nephrol* 7: 2340–2347, 1996
11. Eddy AA, Michael AF: Acute tubulointerstitial nephritis associated with aminonucleoside nephrosis. *Kidney Int* 33: 14–23, 1988
12. Jones CL, Buch S, Post M, McCulloch L, Liu E, Eddy AA: Renal extracellular matrix accumulation in acute puromycin aminonucleoside nephrosis in rats. *Am J Pathol* 141: 1381–1396, 1992
13. Quinn MT, Parthasarathy S, Fong LG, Steinberg D: Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc Natl Acad Sci USA* 84: 2995–2998, 1987
14. Ylä-Herttuala S, Lipton BA, Rosenfeld ME, Sarkioja T, Yoshimura T, Leonard EJ, Witztum JL, Steinberg D: Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc Natl Acad Sci USA* 88: 5252–5256, 1991
15. Eddy AA: Interstitial macrophages as mediators of renal fibrosis. *Exp Nephrol* 3: 76–79, 1995
16. Ding G, van Goor H, Ricardo SD, Orlowski JM, Diamond JR: Oxidized LDL stimulates the expression of TGF-β and fibronectin in human glomerular epithelial cells. *Kidney Int* 51: 147–154, 1997
17. Lee HS, Kim BC, Kim YS, Choi KH, Chung HK: Involvement of oxidation in LDL-induced collagen gene regulation in mesangial cells. *Kidney Int* 50: 1582–1590, 1996
18. Glasser RJ, Velosa JA, Michael AF: Experimental model of focal sclerosis. I. Relationship to protein excretion in aminonucleoside nephrosis. *Lab Invest* 36: 519–526, 1977
19. Jones CL, Buch S, Post M, McCulloch L, Liu E, Eddy AA: The pathogenesis of interstitial fibrosis in chronic purine aminonucleoside nephrosis. *Kidney Int* 40: 1020–1031, 1991
20. Eddy A: Protein restriction reduces transforming growth factor-β and interstitial fibrosis in nephrotic syndrome. *Am J Physiol* 266: F884–F893, 1994
21. Kivirikko KI, Laitinen O, Prockop DJ: Modifications of a specific assay for hydroxyproline in urine. *Anal Biochem* 19: 249–255, 1967
22. Ohkawa H, Ohishi N, Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 95: 351–358, 1979
23. Dijkstra CD, Döpp EA, Joling P, Kraal G: The heterogeneity of mononuclear phagocytes in lymphoid organs: Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 54: 589–599, 1985
24. Balza E, Borsi L, Allemanni G, Zardi L: Transforming growth factor β regulates the levels of different fibronectin isoforms in normal human cultured fibroblasts. *FEBS Lett* 228: 42–44, 1988
25. Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ: Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* 18: 5294–5299, 1979
26. Nakatsukasa H, Nagy P, Evarts RP, Hsia C-C, Marsden E, Thorgeirsson SS: Cellular distribution of transforming growth factor-β1 and procollagen types I, III, and IV transcripts in carbon tetrachloride-induced rat liver fibrosis. *J Clin Invest* 85: 1833–1843, 1990
27. Toman PD, de Crombrugghe B: The mouse type-III procollagen-encoding gene: Genomic cloning and complete DNA sequence. *Gene (Amst)* 147: 161–168, 1994
28. Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO: Three different fibronectin mRNAs arise by alternative splicing within the coding region. *Cell* 35: 421–431, 1983
29. Khokha R, Denhardt DT: On the use of anti-sense RNA: Down-regulation of mRNA encoding a metalloproteinase inhibitor. *Anticancer Res* 7: 653–660, 1987
30. Zehab R, Gelehrter TD: Cloning and sequencing of cDNA for the rat plasminogen activator inhibitor-1. *Gene (Amst)* 73: 459–468, 1988
31. Qian SW, Kondaiah P, Roberts AB, Sporn MB: cDNA cloning by PCR of rat transforming growth factor β-1. *Nucleic Acids Res* 18: 3059, 1990
32. Chien KR: Genes and physiology: Molecular physiology in genetically engineered animals. *J Clin Invest* 97: 901–909, 1996
33. Breslow JL: Transgenic mouse models of lipoprotein metabolism and atherosclerosis. *Proc Natl Acad Sci USA* 90: 8314–8318, 1993
34. Helmchen U, Wenzel UO: Benign and malignant nephrosclerosis and renovascular disease. In: *Renal Pathology with Clinical and Functional Correlations*, edited by Tisher CC, Brenner BM, Philadelphia, J. B. Lippincott, 1994, pp 1201–1238
35. Reichenberg Y, Pomeranz A, Schurr D, Levy E, Stankiewicz H, Elath U, Rosenmann E, Drukker A: Dietary-induced hyperlipidemia and renal function in the uremic rat. *Child Nephrol Urol* 11: 1–5, 1991
36. Yoshioka T, Bills T, Moore-Jarrett T, Greene HL, Burr IM,

Ichikawa I: Role of intrinsic antioxidant enzymes in renal oxidant injury. *Kidney Int* 38: 282–288, 1990
37. Diamond JR, Bonventre JV, Karnovsky MJ: A role for oxygen free radicals in aminonucleoside nephrosis. *Kidney Int* 29: 478–483, 1986
38. Thakur V, Walker PD, Shah SV: Evidence suggesting a role for hydroxyl radical in puromycin aminonucleoside-induced proteinuria. *Kidney Int* 34: 494–499, 1988
39. Ricardo SD, Bertram JF, Ryan GB: Antioxidants protect podocyte foot processes in puromycin aminonucleoside-treated rats. *J Am Soc Nephrol* 4: 1974–1986, 1994
40. Lehr H-A, Kröber M, Hübner C, Vajkoczy P, Menger MD, Nolte D, Kohlschütter A, Messmer K: Stimulation of leukocyte/endothelium interaction by oxidized low-density lipoprotein in hairless mice: Involvement of CD11b/CD18 adhesion receptor complex. *Lab Invest* 68: 388–395, 1993
41. Kume N, Cybulsky MI, Gimbrone MAJ: Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J Clin Invest* 90: 1138–1144, 1992
42. Khan BV, Parthasarathy SS, Alexander RW, Medford RM: Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J Clin Invest* 95: 1262–1270, 1995
43. Eddy AA, Warren JS: Expression and function of monocyte chemoattractant protein-1 in experimental nephrotic syndrome. *Clin Immunol Immunopathol* 78: 140–151, 1996
44. Eddy AA: Expression of genes that promote renal interstitial fibrosis in rats with proteinuria. *Kidney Int* 49[Suppl 54]: S49–S54, 1996
45. Eddy AA: Experimental insights into the tubulointerstitial disease accompanying primary glomerular lesions. *J Am Soc Nephrol* 5: 1273–1287, 1994
46. Eddy AA: Molecular insights into renal interstitial fibrosis. *J Am Soc Nephrol* 7: 2495–2508, 1996
47. Rajavashisth TB, Andalibi A, Territo MC, Berliner JA, Navab M, Fogelman AM, Lusis AJ: Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature (Lond)* 344: 254–257, 1990
48. Witztum JL, Steinberg D: Role of oxidized low density lipoprotein in atherogenesis. *J Clin Invest* 88: 1785–1792, 1991
49. Simionescu M, Simionescu N: Proatherosclerotic events: Pathobiochemical changes occurring in the arterial wall before monocyte migration. *FASEB J* 7: 1359–1366, 1993
50. Oberley TD, Sempf JM, Oberley LW: Immunohistochemical localization of antioxidant enzymes during hamster kidney development. *Histochem J* 27: 575–586, 1995
51. White CR, Brock TA, Chang L-Y, Crapo J, Briscoe P, Ku D, Bradley WA, Gianturco SH, Gore J, Freeman BA, Tarpey MM: Superoxide and peroxynitrite in atherosclerosis. *Proc Natl Acad Sci USA* 91: 1044–1048, 1994
52. Ong ACM, Moorhead JF: Tubular lipidosis: Epiphenomenon or pathogenetic lesion in human renal disease? *Kidney Int* 45: 753–762, 1994
53. Matsumoto A, Naito M, Itakura H, Ikemoto S, Asaoka H, Hayakawa I, Kanamori H, Aburatani H, Takaku F, Suzuki H, Kobari Y, Miyal T, Takahashi K, Cohen EH, Wydro R, Housman DE, Kodama T: Human macrophage scavenger receptors: Primary structure, expression, and localization in atherosclerotic lesions. *Proc Natl Acad Sci USA* 87: 9133–9137, 1990
54. Kodama T, Freeman M, Rohrer L, Zabrecky J, Matsudaira P, Krieger M: Type 1 macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature (Lond)* 343: 531–535, 1990
55. Schönholzer KW, Waldron M, Magil AB: Intraglomerular foam cells and human focal glomerulosclerosis. *Nephron* 62: 130–136, 1992
56. Regnström J, Nilsson J: Lipid oxidation and inflammation-induced intimal fibrosis in coronary heart disease. *J Lab Clin Med* 124: 162–168, 1994
57. Agarwal A, Balla J, Croatt A, Nath K: Oxidized LDL injures renal tubular epithelial cells: Implications for tubulointerstitial disease [Abstract]. *J Am Soc Nephrol* 5: 935, 1994
58. Freeman M, Ashkenas J, Rees DJG, Kingsley DM, Copeland NG, Jenkins NA, Krieger M: An ancient, highly conserved family of cysteine-rich protein domains revealed by cloning type I and type II murine macrophage scavenger receptors. *Proc Natl Acad Sci USA* 87: 8810–8814, 1990
59. Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH, Krieger M: Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* 271: 518–520, 1996
60. Holness CL, da Silva RP, Fawcell J, Gordon S, Simmons D: Macrosialin, a mouse macrophage-restricted glycoprotein, is a member of the lamp/lgp family. *J Biol Chem* 268: 9661–9666, 1993
61. Elomaa O, Kangas M, Sahlberg C, Tuukkanen J, Sormunen R, Liakka A, Thesleff I, Kraal G, Tryggvason K: Cloning of a novel bacteria-binding receptor structurally related to scavenger receptors and expressed in a subset of macrophages. *Cell* 80: 603–609, 1995
62. Endemann G, Stanton LW, Madden KS, Bryant CM, White RT, Protter AA: CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* 268: 11811–11816, 1993
63. Stanton LW, White RT, Bryant CM, Protter AA, Endemann G: A macrophage Fc receptor for IgG is also a receptor for oxidized low density lipoprotein. *J Biol Chem* 267: 22446–22451, 1992
64. Eddy AA: Interstitial nephritis induced by heterologous protein-overload proteinuria. *Am J Pathol* 135: 719–733, 1989
65. Remuzzi G, Ruggenenti P, Benigni A: Understanding the nature of renal disease progression. *Kidney Int* 51: 2–15, 1997
66. Burton C, Harris KPG: The role of proteinuria in the progression of chronic renal failure. *Am J Kidney Dis* 27: 765–775, 1996
67. Perna A, Remuzzi G: Abnormal permeability to proteins and glomerular lesions: A meta-analysis of experimental and human studies. *Am J Kidney Dis* 27: 34–41, 1996