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Interstitial fibrosis in hypercholesterolemic rats: Role of oxidation, matrix synthesis, and proteolytic cascades

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Interstitial fibrosis in hypercholesterolemic rats: Role of oxidation, matrix synthesis and proteolytic cascades. Uninephrectomized rats with diet-induced hypercholesterolemia develop interstitial inflammation and fibrosis after 8 to 12 weeks. Fibrosis has been associated with the accumulation of lipid peroxidation products within the tubulointerstitium, along with increased renal mRNA levels for transforming growth factor beta-1 (TGF-β1), some matrix proteins, and the tissue inhibitor of metalloproteinases (TIMP-1). However, mRNA levels for urokinase-type plasminogen activator (uPA) have been found to be decreased. The purpose of the present study was to determine whether antioxidant therapy could attenuate interstitial fibrosis in hypercholesterolemic rats and to determine changes in the pattern of renal gene expression induced by antioxidant therapy. Three groups of uninephrectomized rats were studied after 12 weeks of feeding standard rat chow, an atherogenic diet (standard chow plus 4% cholesterol/1% cholic acid), or an atherogenic diet supplemented with high doses of the antioxidants probucol and vitamin E. Rats fed the atherogenic diet developed hypercholesterolemia and a 56% increase in total kidney collagen compared with rats fed standard chow. In comparison, the hypercholesterolemic rats treated with antioxidants had normal levels of renal lipid peroxidation products and a normal kidney collagen content. In contrast, there were no significant differences in urinary albumin excretion rates or the number of interstitial macrophages between the two hypercholesterolemic groups. Compared with the untreated hypercholesterolemic group, antioxidant therapy induced significant reductions in renal mRNA levels for procollagen III (to 60% of untreated levels), collagen IV (60%), and TIMP-1 (20%), while uPA levels were significantly increased (to 210%). Paradoxically, antioxidant therapy was associated with a significant increase in renal TGF-β1 mRNA levels (to 150%), although TGF-β1 protein expression shifted from interstitial to tubular epithelial cells in predominance. The results of the present study demonstrate the efficiency of antioxidant therapy in preventing renal interstitial fibrosis in hypercholesterolemic rats with a single kidney. Based on changes in renal gene expression at the mRNA level, impaired matrix protein synthesis and increased intrarenal activity of the metalloproteinases and uPA/plasmin may play a role in the attenuation of fibrosis.

Progressive renal failure is characterized histologically by the accumulation of extracellular matrix proteins within the renal interstitium and progressive tubular atrophy. This final common pathway of progressive tubulointerstitial damage predicts the degree of renal functional impairment and long-term prognosis. The factors that have been implicated in the initiation of this cascade are numerous and include cytokines, growth factors, angiotensin II, endothelin, complement proteins, proteolytic enzymes, iron, reactive oxygen metabolites, phosphate, and mechanical factors related to tubular obstruction [reviewed in 1]. Abnormalities in lipid metabolism may also play a role in progressive renal disease, particularly at the level of the glomerulus [2]. However, there is increasing evidence that lipoprotein abnormalities associated with renal disease may also play an active role in progressive tubulointerstitial disease. This hypothesis first gained popularity in 1982 when Moorhead and colleagues [3] suggested that lipoproteins produced by the liver in response to proteinuria might contribute to progressive renal disease.

In studies of animal models of hypercholesterolemia caused by genetic factors (such as in obese Zucker rats) or induced by high dietary cholesterol, histologic evidence of tubulointerstitial disease has occasionally been reported [4–8]. Hyperlipidemia worsens tubulointerstitial damage in rats with chronic anti-Thy-1 glomerulonephritis [9]. We recently reported that uninephrectomized rats fed a high cholesterol diet develop tubulointerstitial disease, but that in the absence of pre-existing renal disease the process appears to be indolent [10]. It took eight weeks before a significant interstitial infiltrate of macrophages could be detected; significant interstitial fibrosis did not develop until 12 weeks. In that study we speculated that oxidized low-density lipoprotein (LDL) may play an active role in mediating the tubulointerstitial disease, as the level of renal lipid peroxidation products was significantly increased and oxidized LDL was detected within tubular and interstitial cells.

Once oxidized, LDL is not eliminated by the hepatic native LDL receptor system. Instead, these modified lipoproteins bind to a family of scavenger receptors that includes macrophage type-I and type-II class A scavenger receptors, CD36, scavenger receptor BI, macrosialin/CD68, MARCO and FcγRII [11–18]. Macrophages are an important source of scavenger receptors and are thought to be the precursor of the lipid-laden "foam cell" that characterizes the early atherosclerotic lesion. Renal tubular cells are able to internalize oxidized LDL, although the specific receptor pathways involved are not yet known. Uptake of oxidized LDL by scavenger receptors may trigger a cascade of responses leading to fibrogenesis. The exact mechanisms whereby LDL is oxidized in the kidney remain to be explained and are likely

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multifactorial. One possibility is a superoxide-dependent mechanism, a pathway of LDL oxidation that has been identified using cultured human renal tubular cells [19].

The molecular pathways that lead to interstitial fibrosis once LDL is oxidized within the kidney are not yet clear. Our previous study of uninephrectomized hypercholesterolemic rats identified several candidates. Modest but significant increases in renal mRNA levels for procollagen α1(I) and procollagen α1(III) suggested that matrix protein synthesis rates were increased. Matrix protein synthesis could be a direct consequence of LDL oxidation or an indirect one regulated by the production of fibrogenic cytokines such as TGF-β1. In our initial study, renal TGF-β1 mRNA levels were significantly elevated and bioactive TGF-β1 protein was identified in both tubular and interstitial cells. Our previous study suggested that in addition to increased matrix protein synthesis, impaired matrix protein turnover also contributed to interstitial fibrosis. Two proteolytic pathways were implicated. The first is the metalloproteinase family [20]. The metalloproteinases are a group of enzymes characterized in part by zinc-dependent enzyme activity. Together these enzymes are able to degrade virtually all matrix proteins. Members of this family include gelatinases, collagenases, and stromelysins. Enzyme activity can be blocked by the non-covalent interaction of latent and/or active enzymes with tissue inhibitors of metalloproteinases (TIMP). The renal disease that developed in the hypercholesterolemic rats was characterized by up-regulated expression of TIMP-1. The second proteolytic pathway is the plasminogen activator/plasmin pathway [21]. Renal tubules constitutively produce significant quantities of urokinase-type plasminogen activator (uPA). During the three-month study period, renal uPA mRNA levels were down-regulated, while expression of plasminogen activator inhibitor-1 (PAI-1) was unchanged, which are changes that would predict decreased intrarenal generation of plasmin. Plasmin has important effects on matrix turnover by virtue of its ability to degrade selected matrix proteins, but more importantly to activate latent metalloproteinases.

The present study was designed to to determine whether antioxidant therapy could attenuate interstitial fibrosis in hypercholesterolemic rats and to determine changes in the pattern of renal gene expression induced by antioxidant therapy.

METHODS

Experimental design

Thirty-nine female Sprague-Dawley rats weighing 100 to 120 g were purchased from Charles River Breeding Laboratories (Wilmington, MA, USA). We performed a left nephrectomy on each animal five days before the study began.

To examine the effects of antioxidant therapy on renal interstitial fibrosis, three groups of seven animals were studied. The control group was fed standard rat chow containing 270 mg cholesterol per kilogram (Purina Mills Inc., Richmond, IN, USA). The high-cholesterol group was fed standard rat chow supplemented with 4% cholesterol and 1% cholic acid (Purina Test Diets). The high-cholesterol/antioxidant group was fed the same high-cholesterol diet supplement, with the addition of vitamin E (alpha-tocopherol acetate, 150 IU per 30 mg) and 2% probucol powder (kindly provided by Marion Merrell Dow Inc., Cincinnati, OH, USA). Vitamin E and probucol were added to the high-cholesterol diet and prepared as pellets of chow by Purina Mills Inc. A combination of two high-dose antioxidants was used because we had just completed a study in rats with acute nephrotic syndrome and found that neither supplement alone had a significant anti-fibrotic effect [22]. The rats were housed individually in metabolic cages at 8 and 12 weeks to collect spontaneously voided urine over a 24 hour period. At 12 weeks all animals were killed by exsanguination under general anesthesia by inhalation of nitrous oxide, oxygen, and enflurane. The right kidney was carefully decapsulated and weighed (wet weight). The kidney was divided in half longitudinally. One half was carefully weighed, frozen in liquid nitrogen, and stored at $-70°$ for total collagen measurement. The other half of the kidney was divided into two pieces, one of which was snap-frozen in isopentane pre-cooled in liquid nitrogen for subsequent immunostaining. The final piece was snap-frozen in liquid nitrogen and stored at -70°C until RNA extraction.

To measure renal lipid peroxidation products, a second study was performed. Eighteen uninephrectomized female Sprague-Dawley rats were divided into three groups of six animals each, fed the control diet, the high-cholesterol diet, and the high-cholesterol/antioxidant diet, respectively. The animals were killed as described above at the end of five weeks. This earlier time point was used as our previous study showed little difference in the degree of elevation of renal peroxidation products at 4, 8 and 12 weeks [10]. The right kidney was removed, decapsulated, frozen in liquid nitrogen, and stored at -70°C for measurement of lipid peroxidation products.

Biochemical studies

Urinary albumin concentrations were measured by radial immunodiffusion according to our previously described methods [23]. Plasma creatinine, total cholesterol and triglyceride levels were measured on individual samples of heparinized blood obtained when the rats were exsanguinated. Plasma vitamin E levels were measured on three randomly selected animals from each of the three dietary groups by a liquid chromatographic method modified from that of Catignani and Bieri [24].

Total kidney collagen was calculated as previously described [25] based on measurements of the hydroxyproline concentration in kidney homogenates according to the technique of Kivirikko, Laitinen and Prockop [26]. Collagen was assumed to contain 12.7% hydroxyproline by weight. Final results, based on the wet weight of the kidney, were expressed as milligrams of collagen per kidney. Renal peroxidation products were assessed using thiobarbituric acid assay to measure the renal malondialdehyde content according to the methods of Ohkawa, Ohishi and Yagi [27].

Renal immunofluorescence studies

The number of interstitial macrophages expressing the ED-1 cytoplasmic marker (Serotec, Oxford, UK) was determined on acetone-fixed kidney cryosections 3 μm thick using the dual fluorochrome labeling and enumeration technique previously described [10].

The accumulation of interstitial matrix proteins in the renal interstitium was assessed semiquantitatively, as previously described [28]. In brief, at least 50 random interstitial fields of the cortical interstitium were evaluated by fluorescence microscopy for evidence of increased staining for interstitial matrix proteins. Each field was defined as the area contained within a 10 mm $\times$ 10 mm eyepiece grid (magnification $\times$400). Each field was evaluated

Table 1. Renal function

Group	Urinary albumin *mg/100 g body wt/day* Week 8	Urinary albumin *mg/100 g body wt/day* Week 12	Body weight *g*	Serum creatinine *μmol/liter*	Kidney weight *g*
Control group	0.3 ± 0.4[a]	0.5 ± 0.7[a]	285 ± 22	49 ± 4	1.4 ± 0.1[a]
High-cholesterol group	15 ± 15	23 ± 16	301 ± 24	52 ± 4	2.0 ± 0.4
High-cholesterol/antioxidant group	10 ± 11	18 ± 23	296 ± 20	44 ± 3[a]	1.5 ± 0.2[a]

Results are means ± 1 SD. The body weight, serum creatinine and kidney weight are mean values at the time of sacrifice.
[a] $P < 0.05$ compared with the high-cholesterol group, by Mann-Whitney U test

by the investigator (A.E.) who was blinded to the animal group at the time of the evaluation. The range of normal staining for the matrix protein of interest was first determined for three control animals. Each of the 50 fields was then determined to have either a normal or an increased area of interstitial staining. Equivocal fields were assigned to the normal group. For each animal the results were expressed as the percent of interstitial fields with a definite increase in the area stained for each specific interstitial matrix protein. The primary antibodies used were: sheep anti-human collagen I; goat anti-human collagen III (Southern Biotechnology Associates, Birmingham, AL, USA); rabbit anti-human tenascin (a gift from Dr. H. Erickson, Duke University, NC, USA); and a murine monoclonal antibody to human extracellular domain A (EDA+) fibronectin, an alternatively spliced form of fibronectin (a gift from Dr. L. Zardi, Institute Nazionale perla Rererca sul Concro, Italy).

Tissue sections from each animal group were evaluated by indirect immunofluorescence staining for the pattern of expression of TIMP-1 (rabbit anti-bovine TIMP-1, a gift from Dr. Y.A. DeClerk, Children's Hospital of Los Angeles, Los Angeles, CA, USA), uPA (rabbit anti-human uPA from American Diagnostica Inc., Greenwich, CT, USA), and TGF-β1 [rabbit anti-TGF-β1 (anti-LC) antibody, a gift from Dr. K. Flanders, National Institutes of Health, Bethesda, MD, USA]. The secondary antisera for detecting polyclonal antibodies were fluorescein isothiocyanate (FITC)–conjugated goat anti-rabbit IgG and FITC-conjugated rabbit anti-goat IgG antisera (Chemicon International Inc., Temecula, CA, USA). The secondary antiserum for detecting murine monoclonal antibodies was FITC-conjugated goat anti-mouse IgG (Zymed Laboratories Inc., San Francisco, CA, USA). The FITC-conjugated antisera were absorbed with normal rat plasma; lack of staining showed them to be nonreactive with control kidney sections. Code numbers were used to blind the investigator to the animal group in each case.

We isolated total renal cortical RNA according to the guanidinium isothiocyanate–cesium chloride method of Chirgwin and co-workers [29], separated it by electrophoresis in a 1% formaldehyde agarose gel, transferred it to a nylon-membrane Gene Screen (NEN Research Products, Boston, MA, USA), and probed it with ^{32}P-labeled dCTP. For these studies, RNA samples of 10 μg from each animal in the high-cholesterol and the high-cholesterol/antioxidant groups were loaded onto separate wells on the same gel to permit direct comparison of results. 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 previously described [10]. To simplify the presentation of the data, final results were expressed as the ratios of mean densitometric scores.

The cDNA probes used were: rat α1(I) procollagen (supplied by Dr. S. Thorgeirsson, National Cancer Institute, Bethesda, MD, USA); murine α1(III) procollagen (supplied by Dr. B. deCrombrugghe, Anderson Cancer Center, The University of Texas, Houston, TX, USA) [30]; murine α2(IV) procollagen (supplied by Dr. M. Kurkinen, Rutgers Medical School, University of New Jersey, Piscataway, NJ, USA); fibronectin (supplied by Dr. R. Hynes, Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA, USA); rabbit stromelysin (supplied by Dr. Z. Werb, University of California, San Francisco, CA, USA); murine TIMP-1 (supplied by Dr. R. Khokha, London Regional Cancer Centre, London, ON, Canada); rat plasminogen-activator inhibitor-1 (rat PAI-1; supplied by Dr. Z.R. Gelehrter, University of Michigan, Ann Arbor, MI, USA); uPA (supplied by Dr. J. Degan, Children's Hospital Research Foundation, Cincinnati, OH, USA); and murine α2-antiplasmin (supplied by Dr. P.A. Menoud, University of Geneva, Medical School, Geneva, Switzerland) [31]. Unreferenced antibodies and cDNA probes are cited in our previous publication [10].

Statistics

Results were compared using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

RESULTS

Renal function

The antioxidant therapy had no significant effect on urinary albumin excretion rates in rats fed a high-cholesterol diet (Table 1). Serum creatinine and final kidney weight, however, were significantly lower in the hypercholesterolemic animals treated with antioxidants. Creatinine clearance rates calculated on 24-hour urine collections of spontaneously voided urine, expressed as ml per minute per 300 g body wt, were not significantly different in the three groups: 0.77 ± 0.16 for the control group; 0.73 ± 0.17 for the high-cholesterol group; and 0.91 ± 0.35 for the high-cholesterol/antioxidant group.

Lipid abnormalities

Rats fed a high-cholesterol diet developed significant hypercholesterolemia and elevated renal levels of lipid peroxidation products compared with rats fed standard chow. These changes were attenuated by antioxidant therapy (Table 2). Serum triglyceride levels were not significantly different: 0.9 ± 0.2 mmol/liter for the control group; 1.3 ± 0.6 mmol/liter for the high-cholesterol group; and 1.4 ± 0.4 mmol/liter for the high-cholesterol/antioxidant group. Serum vitamin E levels were elevated in the hypercholesterolemic animals that were treated with antioxidants: 22 ± 33

Table 2. Lipid abnormalities

Group	Serum cholesterol *mmol/liter*	Renal products *nmol/g*
Control group	2 ± 0.2[a]	122 ± 8[a]
High-cholesterol group	19 ± 5	144 ± 8
High-cholesterol/antioxidant group	10 ± 2[a]	125 ± 9

Results are means ± 1 SD. "Renal products" are lipid peroxidation products, in nanomoles of malondialdehyde per wet-weight gram of tissue, measured at week 5.

[a] $P < 0.05$ compared with the high-cholesterol group, by Mann-Whitney U test.

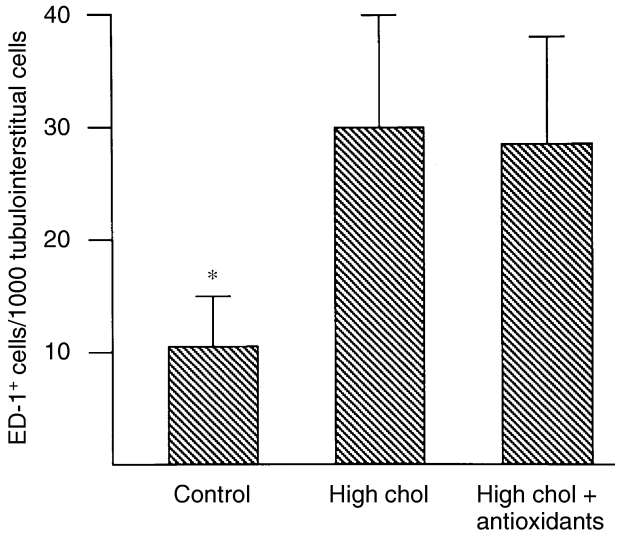


Fig. 1. Interstitial macrophages, identified as cells expressing the ED-1 cytoplasmic antigen, for the control, high-cholesterol, and high-cholesterol/antioxidant groups. Results are means ± 1 SD. *$P < 0.05$ compared to the high-cholesterol group.

μmol/liter for the control group; 82 ± 37 μmol/liter for the high-cholesterol group; and 342 ± 77 μmol/liter for the high-cholesterol/antioxidant group.

Renal interstitial inflammation

The number of interstitial macrophages increased in rats fed the high-cholesterol diet, a response that was not altered by the addition of antioxidant therapy (Fig. 1).

Renal fibrosis

The total kidney collagen content was increased in rats fed a high-cholesterol diet compared with control rats. The increase in renal collagen was prevented by treatment with antioxidant therapy (Fig. 2). Immunostaining confirmed the accumulation of several matrix proteins in the renal interstitium of untreated hypercholesterolemic rats. Compared with animals fed a normal diet, hypercholesterolemic rats showed increased interstitial staining for the following average percentages of interstitial fields: collagen I, 19%; collagen III, 12%; EDA^+ fibronectin, 11%; and tenascin, 16%. In antioxidant-treated hypercholesterolemic rats, these numbers were decreased to: collagen I, 14%; collagen III, 1%; EDA^+ fibronectin, 2%; and tenascin, 3%.

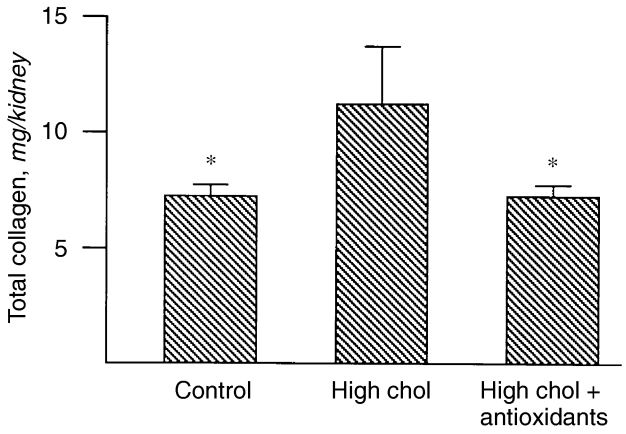


Fig. 2. Total kidney collagen content for the control, high-cholesterol, and high-cholesterol/antioxidant rats. Results are means ± 1 SD. *$P < 0.05$ compared to the high-cholesterol group.

Table 3. Renal gene expression

Gene family and gene	Densitometric ratio	P value[a]
1. Extracellular matrix proteins		
Procollagen α1(I)	0.9	NS
Procollagen α1(III)	0.6	0.035
Procollagen α2(IV)	0.6	0.015
Fibronectin	0.5	0.05
2. Metalloproteinases/inhibitors		
Stromelysin	0.8	NS
TIMP-1	0.2	0.007
3. Plasminogen activators/inhibitors		
uPA	2.1	0.018
PAI-1	1.5	NS
α2-antiplasmin	0.8	NS
4. Growth factors		
TGF-β1	1.5	0.003

The densitometric ratio compares the results of the high-cholesterol/antioxidants group to those of the high-cholesterol group. Each ratio is calculated from the mean densitometric scores after correction for any RNA loading inequality. In other words, the corresponding ratio for the closest ribosomal band (18s or 28s) is 1.0 for each gene evaluated. Abbreviations are: TIMP-1, the tissue inhibitor of metalloproteinases; uPA, urokinase-type plasminogen activator; PAI-1, plasminogen activator inhibitor-1; TGF-β1, transforming growth factor β-1.

[a] Mann-Whitney U test analysis of individual densitometric results, expressed as arbitrary units for each of the three animals in each group. $P < 0.05$ was considered statistically significant.

Renal gene expression in hypercholesterolemic rats

The renal expression of four extracellular matrix genes was evaluated. Only procollagen α1(III) and α2(IV) levels were significantly decreased by antioxidant therapy (Table 3). Antioxidant therapy significantly decreased renal TIMP-1 mRNA levels to an average of 20% relative to the untreated animals (Table 3 and Fig. 3), while stromelysin levels were similar in both hypercholesterolemic groups (Table 3). Immunostaining showed TIMP-1 protein appearing *de novo* in the interstitium of the hypercholesterolemic rats (Fig. 4). The overall intensity of interstitial staining for TIMP-1 staining was decreased in the antioxidant-treated group.

Antioxidant therapy significantly increased renal urokinase

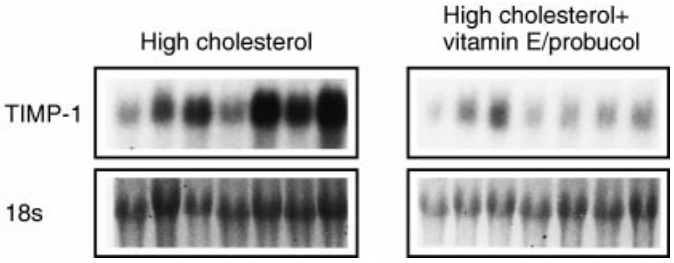
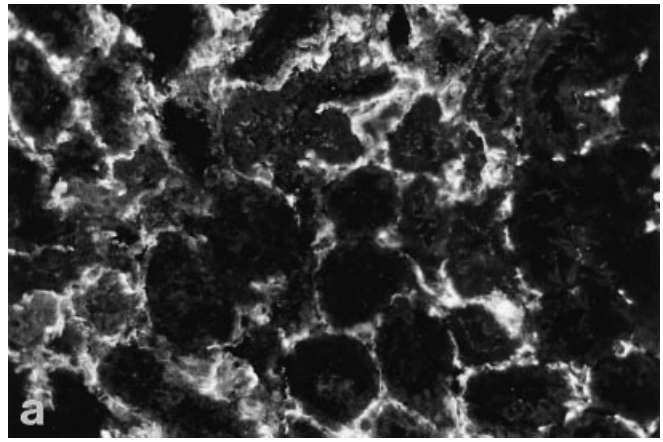


Fig. 3. Renal mRNA levels of tissue inhibitor of metalloproteinases (TIMP-1). By Northern blot analysis on the same autoradiograph, the mean densitometric score for the high-cholesterol group was 1.0 ± 0.7 arbitrary units, versus 0.2 ± 0.1 arbitrary units for the high-cholesterol/antioxidant group. This difference was statistically significant.



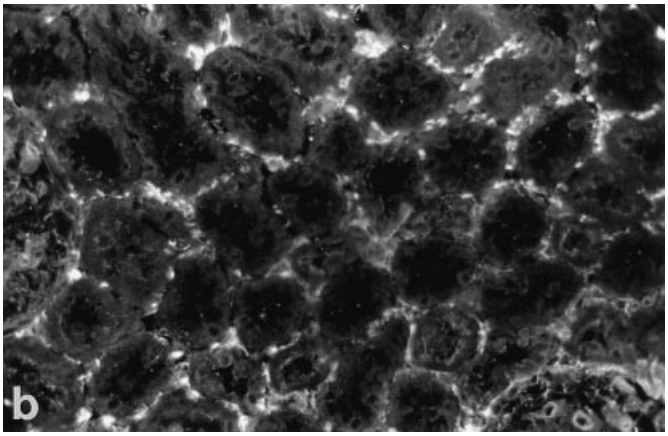


Fig. 4. Immunofluorescence photomicrograph illustrating (*a*) the *de novo* expression of tissue inhibitor of metalloproteinases (TIMP-1) protein in the kidney of a rat with diet-induced hypercholesterolemia, and (*b*) the decrease in overall intensity of interstitial TIMP-1 staining in the hypercholesterolemic animals treated with antioxidants. Magnification ×240.

mRNA levels relative to the untreated hypercholesterolemic group (Table 3 and Fig. 5). On immunostained slides, all tubules appeared weakly positive for urokinase, and it was difficult to discern a clear difference between the two groups. Renal mRNA levels for the protease inhibitors PAI-1 and α2-antiplasmin were similar in both hypercholesterolemic groups (Table 3).

Renal TGF-β1 mRNA levels were not decreased but rather

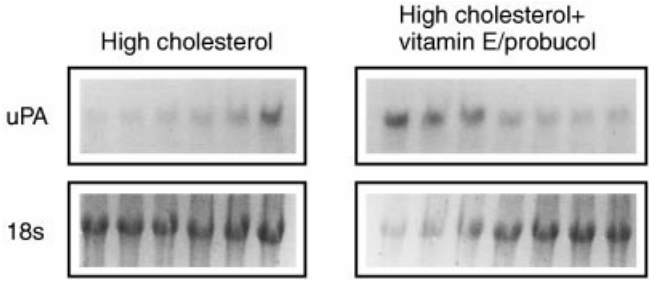


Fig. 5. Renal mRNA levels of urokinase-type plasminogen activator (uPA). The mean densitometric score for the high-cholesterol group by Northern blot analysis on the same autoradiograph, 1.0 ± 0.3 arbitrary units, versus 2.1 ± 1.2 arbitrary units for the high-cholesterol/antioxidant group. This difference was statistically significant.

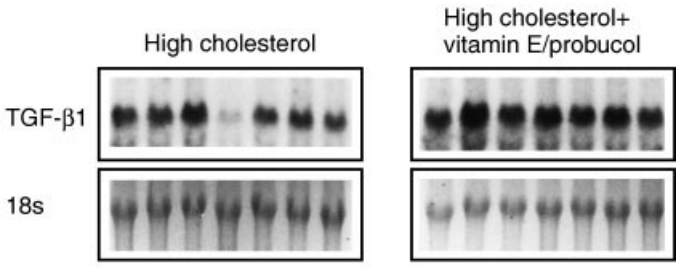


Fig. 6. Renal mRNA levels of transforming growth factor β-1 (TGF-β1). By Northern blot analysis on the same autoradiograph, the mean densitometric score for the high-cholesterol group was 1.0 ± 0.3 arbitrary units, versus 1.5 ± 0.2 arbitrary units for the high-cholesterol/antioxidant group. This difference was statistically significant.

were significantly increased, to 50% above the levels observed in the untreated group (Fig. 6). This observation was reproducible on several different RNA blots. In the high-cholesterol group, staining for bioactive TGF-β1 identified several positive interstitial cells as well as some positive tubules (Fig. 7a). Immunostaining for bioactive TGF-β1 shifted in the antioxidant-treated group to more striking tubular staining, both a diffuse and a granular cytoplasmic pattern (Fig. 7b).

DISCUSSION

In this study, combined antioxidant treatment with probucol and vitamin E essentially prevented the early interstitial fibrosis that developed in the untreated uninephrectomized rats with severe hypercholesterolemia. These findings are consistent with the reported efficacy of the antioxidants vitamin E or probucol in attenuating tubulointerstitial disease in rats with chronic puromycin aminonucleoside nephrosis [32–34]. Probucol therapy has been reported to decrease serum cholesterol levels and the severity of tubulointerstitial disease that develops in rats following subtotal nephrectomy [35]. We chose combined high-dose therapy with vitamin E and probucol to achieve maximal antioxidant effect. In a recent study [22], we found that short-term treatment of nephrotic rats with either vitamin E or probucol alone failed to alter the degree of interstitial fibrosis that developed between three and six weeks. We speculated that factors other than hyperlipidemia were the predominant fibrogenic forces; however, considering the alternative explanation, that the nephrotic rats received suboptimal antioxidant treatment, we selected combined

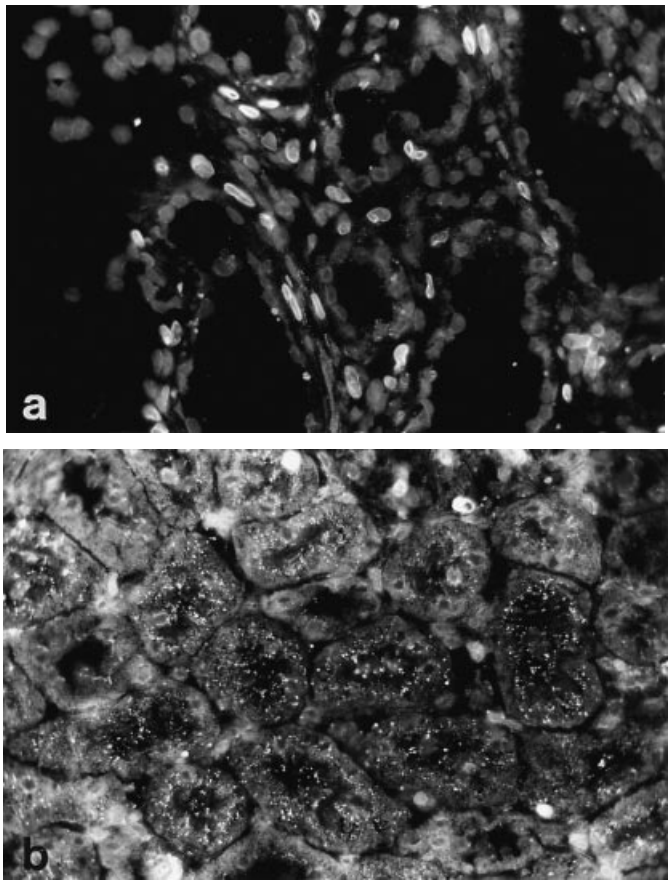


Fig. 7. Immunofluorescence photomicrographs illustrating expression of transforming growth factor β-1 (TGF-β1) in the tubules and interstitium of rats with hypercholesterolemia. (*a*) In rats with diet-induced hypercholesterolemia, several TGF-β1–positive cells can be observed in the interstitium. (*b*) In hypercholesterolemic rats treated with antioxidants, protein expression of TGF-β1 shifted toward a predominantly tubular pattern. Magnification ×260.

therapy for the present study. Whether single-drug therapy would have been equally efficacious remains unknown. It should be emphasized that the present study was terminated after three months of hypercholesterolemia, when interstitial fibrosis is evident histologically but when it is not yet severe enough to have a major effect on nephron function.

Why did antioxidant therapy prevent tubulointerstitial damage? There are three possibilities. (*1*) The drugs themselves may have had direct antifibrotic effects. Unfortunately, few data are currently available to support or refute this possibility. (*2*) The drugs decreased the intrarenal generation of oxidized lipoproteins, with subsequent fibrosis-preventing effects. In addition to their antioxidant effects, the combination of vitamin E and probucol significantly reduced serum cholesterol levels, an effect likely due to the modest lipid-lowering effects of probucol [36]. (*3*) The antioxidant therapy did cause a significant reduction in serum cholesterol levels. It is conceivable the the lowered cholesterol levels had a beneficial effect that was independent of the level of lipid peroxidation products within the kidney.

Renal lipid peroxidation products were normalized by antioxidant therapy. Although oxidized LDL has been reported to have monocyte chemotactic activity *in vitro*, the number of interstitial macrophages was similar in both hypercholesterolemic groups, which suggests that oxidized LDL is not a significant interstitial monocyte chemoattractant in this model. Proteinuria has been implicated in the pathogenesis of tubulointerstitial disease [37], and hypercholesterolemia has been shown to aggravate glomerular damage [38, 39]. Nonetheless, in the present study antioxidant therapy did not significantly reduce the severity of albuminuria in the hypercholesterolemic animals. It seems reasonable to hypothesize that the intrarenal oxidation of LDL generated bioactive products that participated in the fibrogenic cascade. Binding of oxidized LDL to receptors on tubular and/or interstitial cells could be important. Although the evaluation of renal mRNA levels was limited to the 12-week timepoint and may not reflect earlier events, nonetheless, the results provide some insight into possible fibrogenic pathways. Oxidized low-density lipoproteins themselves may stimulate production of matrix proteins [40]. The antioxidant-treated animals had significantly lower mRNA levels for procollagens III and IV at 12 weeks. The reason for the lack of significant effect by therapy on procollagen I levels is unclear, but is supported by the observation that interstitial collagen I immunostaining was similar in the untreated and treated hypercholesterolemic animals (19% vs. 14% of interstitial fields, respectively, showing increased interstitial deposits).

The results of the present study suggest that alterations in matrix turnover also play an important role in the pathogenesis of the interstitial fibrosis associated with severe hypercholesterolemia. In our previous study [10], TIMP-1 mRNA levels in rats fed a high-cholesterol diet were found to be significantly increased to 140%, 270%, and 270% of control levels at 4, 8, and 12 weeks. The present study observed a reversal of this change with antioxidant supplementation, with renal TIMP-1 mRNA levels reduced to 20% of untreated levels and a similiar reduction in immunodetectable TIMP-1 protein. To the best of our knowledge, the ability of oxidized LDL to directly stimulate TIMP-1 production has not been investigated. It is perhaps relevant that overexpression of TIMP-1 has been reported in atherosclerotic plaques, although metalloproteinase expression was also up-regulated in the same areas, and a net increase in gelatinolytic activity was reported [41].

Our study also suggests that impaired intrarenal uPA and plasmin activity is a feature of tubulointerstitial disease and that this imbalance can be reversed by antioxidant therapy. Urokinase-type plasminogen activator is produced constitutively by tubular cells [42]. In our original study in uninephrectomized hypercholesterolemic rats, down-regulation of renal uPA mRNA levels was a persistent feature, with levels sustained at 40% relative to control levels throughout weeks 4, 8, and 12. The most abundant intrarenal plasminogen activator inhibitor, PAI-1 [43], was not down-regulated to offset this change. Taken together, these findings predict a significant reduction in intrarenal urokinase activity and plasmin generation during the phase of active interstitial fibrosis. Treatment of hypercholesterolemic animals with antioxidant therapy attenuated this decrease in uPA expression. Renal uPA mRNA levels in the high-cholesterol/antioxidant group were twice those in the untreated group. Both PAI-1 and α2-antiplasmin levels were similar in both hypercholesterolemic groups; we measured α2-antiplasmin levels in response to the recent report of high intrarenal levels of this uPA inhibitor [31]. The ability of oxidized LDL to inhibit uPA production has not been investigated *in vitro*. The plasmin activator/plasmin pathway

has also been implicated in atherogenesis, although overexpression of both plasminogen activators and inhibitors has been reported [44–46].

The surprising result of the present study is the pattern of TGF-β1 expression. In our initial study, we had speculated that oxidized LDL–stimulated TGF-β1 production could be a unifying hypothesis to explain all of the molecular features of tubulointerstitial disease associated with severe hypercholesterolemia, such as increased matrix gene and TIMP expression and decreased uPA expression [40]. *In vitro* studies, however, are conflicting. In one study [47], oxidized LDL failed to stimulate TGF-β1 expression in cultured endothelial cells. However, Ding and colleagues [48] have recently reported enhanced expression of TGF-β1 by cultured human glomerular epithelial cells in the presence of oxidized LDL. It remains difficult to explain why antioxidant therapy should increase renal TGF-β1 expression. Vitamin E has been reported to induce a TGF-β1–dependent antiproliferative effect *in vitro*, but this appears to be at post-transcriptional levels [49, 50].

Before concluding that TGF-β1 does not have fibrosis-promoting effects in rats with hypercholesterolemia, a few additional points need to be raised. (*1*) The pattern of expression of bioactive TGF-β1 protein was changed by antioxidant therapy. Fewer positive interstitial cells were observed, and most of the protein was localized to tubular cells in the antioxidant-treated group. Perhaps TGF-β1 produced by interstitial myofibroblasts and/or macrophages is most relevant to interstitial fibrosis, as previously suggested [51]. (*2*) The ability of TGF-β1 to initiate fibrogenic signals depends on the absence of its inhibitors and the availability of dimerizing type I and II receptors [52], factors that were not investigated in the present study.

Is there another molecule whose expression is stimulated by oxidized LDL that might account for the observed changes in the expression of TIMP-1 and uPA in the kidneys of the hypercholesterolemic rats? Several factors in addition to TGF-β1 have been identified that may stimulate TIMP-1 expression, including: epidermal growth factor; transforming growth factor-α; tumor necrosis factor; interleukins-1, -6, and -10; oncostatin M; thrombin; platelet-derived growth factors; and phorbol esters [53–55]. Very few factors, except drugs such as colchicine and vinblastine, have been reported to decrease uPA expression [21, 54, 56]. From the literature, we were only able to identify two factors that increase TIMP-1 and decrease uPA expression—lipopolysaccharide and glucocorticoids—and neither of these can be readily implicated in the current study.

In summary, the present study demonstrates that the renal interstitial fibrosis that develops insidiously in uninephrectomized rats with severe hypercholesterolemia can be reversed with antioxidant therapy combining vitamin E and probucol. Studies of renal gene expression demonstrate that antioxidant therapy is associated with lower mRNA levels for genes encoding extracellular matrix proteins and TIMP-1, and higher uPA mRNA levels. We speculate that intrarenal oxidation of LDL may elicit a fibrogenic response in the renal interstitium, leading to both an increase matrix synthesis and a decrease in matrix degradation.

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