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Renal Extracellular Matrix Accumulation in Acute Puromycin Aminonucleoside Nephrosis in Rats

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Progressive renal fibrosis is considered to be the final common pathway leading to chronic renal insufficiency. In this study, the authors examined some of the cellular and molecular mechanisms regulating the renal accumulation of extracellular matrix (ECM) proteins using rats with puromycin aminonucleoside (PAN) nephrosis as an acute model system. Puromycin aminonucleoside rats developed reversible nephrotic syndrome accompanied by an interstitial infiltrate of monocytes. The number of interstitial fibroblasts expressing ST4 antigen did not increase. During the first 4 days, steady-state mRNA levels for all genes examined remained at or below control levels. At 1 week, nephrotic syndrome and interstitial inflammation were established, and a period of renal cell proliferation occurred, identified by increased histone mRNA levels and localized by tritiated thymine autoradiography to tubular epithelial cells and occasional interstitial cells. Transforming growth factor-beta (TGF-β) steady-state mRNA levels were increased eightfold, but returned to control levels by 3 weeks. At week 1, there was a 10- to 20-fold increase in kidney steady-state mRNA levels for genes encoding interstitial matrix proteins collagen I and fibronectin and basement membrane collagen IV. By in situ hybridization, α1(I) procollagen mRNA was localized to interstitial cells. Immunofluorescence microscopy demonstrated focal accumulation of ECM proteins in the tubulointerstitial compartment at 2 and 3 weeks, but by 6 weeks, kidney immunohistology was normal again. Steady-state mRNA levels for the matrix degrading metalloproteinase stromelysin remained at control values, whereas the levels for interstitial collagenase were normal at week 1 and increased twofold to threefold at 2 and 3 weeks. Steady-state mRNA levels for the tissue inhibitor of metalloproteinases (TIMP) increased fivefold at 1 week and returned to baseline values over the next 2 weeks. The results of this study suggest that tubulointerstitial ECM accumulation occurs in rats with acute PAN nephrosis because of the activation of genes encoding several matrix proteins and inhibition of matrix degradation mediated by TIMP. These events are reversed during the phase of recovery from nephrotic syndrome. Increased mRNA levels for TGF-β, possibly originating from inflammatory interstitial monocytes, are likely to be one of the mediators of the molecular events observed. (Am J Pathol 1992, 141:1381–1396)

Extracellular matrix (ECM) accumulation, recognized as fibrosis on histologic examination, is the pathologic indication of progressive renal injury.[1–3] Extracellular matrix accumulation occurs in both glomeruli and the interstitium in nearly all cases of chronic renal injury.[1–3] Renal fibrosis may result from relative parenchymal cell loss, so that the increase in ECM is apparent rather than real, or it may be due to the biosynthesis of excessive ECM relative to the parenchymal cell mass. González-Avila et al[4] found that the former process occurred after unilateral renal vein ligation and the latter occurred after ureteral obstruction in rat models. At a molecular level, ECM accumulation in any tissue is the net result of the balance between the synthesis of matrix proteins, proteoglycans, and other components of the matrix, and the degradation of these molecules, primarily by the metalloproteinase groups of enzymes.[5] The activity of this group of enzymes is inhibited by the tissue inhibitor of metalloproteinases (TIMP). The turnover of ECM proteins is regulated

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in part by various growth factors, particularly transforming growth factor-beta (TGF-β1), but others as well.[6–11]

Diamond et al[12] reported that rats given a single dose of puromycin aminonucleoside (PAN) developed reversible nephrotic syndrome that was followed by the appearance of diffuse interstitial fibrosis and focal segmental glomerulosclerosis 6 months after the injection. An interstitial cell infiltrate consisting predominantly of macrophages, originating from extrarenal sites, has been noted in the acute phase of this disease.[12–15] Feeding the rats an essential fatty-acid–deficient diet before and for 1 month after administration of PAN results in amelioration of the chronic injury.[12] Essentially fatty acid deficiency specifically inhibits macrophage infiltration in this disease[15]; thus it appears that the macrophage is an important, perhaps critical, cell involved in the production of ECM in this model. Little, however, is known about the mechanism of fibrosis in this model and its relationship to infiltrating leukocytes. An understanding of the pathophysiology of renal fibrosis should provide a scientific rationale for the exploration of therapeutic approaches aimed at ameliorating the progressive loss of renal function caused by renal fibrogenesis.

This study reports on a series of molecular and cellular changes that occur in rat kidneys during the first weeks after an injection of PAN, which may be relevant to the development of chronic renal injury. Using Northern blot analysis to evaluate changes in total kidney steady-state mRNA levels, we observed a striking increase for genes encoding interstitial ECM proteins collagen I and fibronectin and basement membrane collagen IV beginning at 1 week. At this same time, increased TIMP mRNA levels and constant mRNA levels for the metalloproteinases suggest that ECM degradation should be diminished. The onset of these changes is coordinated with the initiation of nephrotic syndrome accompanied by an interstitial infiltrate of mononuclear cells, tubular cell proliferation, and increased steady-state mRNA levels for TGF-β1. Accumulation of ECM proteins within the tubulointerstitium was confirmed by immunofluorescence microscopy.

Methods

Experimental Design

Acute PAN nephrosis was produced in female Lewis rats weighing 130 to 150 g in four separate experiments. The rats were fed normal rat chow and water *ad libitum*. In the first experiment, 30 rats were divided into five groups of six animals. On day 0, three of each group (PAN rats) were given an intraperitoneal injection of PAN (Sigma Chemical Co, St. Louis, MO), 15 mg/100 g body weight dissolved in 0.9% saline (15 mg/ml), and the other three control rats were given an intraperitoneal injection of the same volume of 0.9% saline (1 ml/100 g body weight). A right nephrectomy was performed through an incision in the flank under enflurane inhalation anesthesia at 0.5, 1, 2, 24, and 96 hours after the intraperitoneal injection. The kidney was flash-frozen in liquid nitrogen for extraction of total RNA.

In a second experiment, rats were divided into groups of six animals (three PAN rats and three controls) and given PAN or saline at time 0 as described above. One rat group was killed at 6 hours, 1, 2, and 3 weeks and the kidneys were harvested for RNA extraction. A third experiment was performed to evaluate renal matrix protein accumulation. One group of rats (four PAN rats and two controls) was killed at 1, 2, 3, 5, and 6 weeks, and sections of the renal cortex were flash-frozen in isopentane (precooled in liquid nitrogen) and stored at −70°C for immunofluorescence studies. Urinary albumin excretion rates (see below) were measured at weeks 1, 2, 3, and 6 for individual animals in experiments 2 and 3.

A fourth study was performed to validate the results of the initial Northern blot analysis by using total RNA from individual animals instead of RNA pooled from animals at each study point (see below). A group of PAN-injected ($n = 5$) and saline-injected ($n = 5$) rats were killed on day 10, and the kidneys were processed in the usual way.

An additional group of rats (three PAN and three control rats) was used for tritiated thymidine incorporation experiments (see below). One PAN rat and one control rat were killed at each of 5, 7, and 9 days.

Renal Function Parameters

Animals were housed individually in metabolic cages to obtain 24-hour collections of urine. The urinary albumin concentration was quantitated by radial immunodiffusion according to a modification of the technique of Mancini, as previously described.[13,16] The urinary albumin excretion was calculated for individual animals and expressed as total albumin/100 g body weight normalized to a 24-hour collection period.

The level of creatinine of each animal killed at 1, 2, 3, 5, and 6 weeks was determined from the plasma sample collected at the time of death by the Kodak Ektachem 700 method.

Renal Immunofluorescence Studies

Lymphohemopoietic cells in the renal interstitium of tissue obtained at 1, 2, 3, 5, and 6 weeks were characterized

and quantitated with the dual-fluorochrome labeling method and enumeration technique, as previously described.[13,14] The primary monoclonal antibodies used and their reactivities were OX19 for all T lymphocytes, excluding natural killer cells, OX8 for cytotoxic T lymphocytes and natural killer cells (Sera Lab, distributed by Dimensions Laboratory, Mississauga, Ontario, Canada), ED 1 for rat macrophages and monocytes (Serotec, Oxford, England), Mar 1 for rat macrophages and monocytes[17] (gift of Dr. A. Yamashita, Hamamatsu University School of Medicine, Hamamatsu, Japan), and ST4 for rat peripheral tissue fibroblasts[18] (gift from Dr. A. Sullivan, McGill University, Montreal, Canada). The results were expressed as the mean number of positive cells per 1000 tubulointerstitial cells. The results were compared with Bonferroni's *t*-test for independent means. A *P* value of less than 0.05 was considered significant.

Renal ECM proteins were examined by indirect immunofluorescence microscopy. The primary anti-sera used were sheep anti-human collagen I, goat anti-human collagen III and goat anti-human collagen IV (Southern Biotechnology Associates, Birmingham, AL), and rabbit anti-murine laminin and goat anti-human fibronectin (Dimension Laboratories Inc., Mississauga, Ontario, Canada). The secondary anti-sera were fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep IgG, FITC-conjugated rabbit anti-goat IgG, or FITC-conjugated goat anti-rabbit IgG antiserum (Organon Teknika Corp., West Chester, PA). The FITC-conjugated anti-sera were preabsorbed with rat plasma and shown to be nonreactive with control kidney sections.

The accumulation of ECM proteins within the tubulointerstitium (along the tubular basement membranes [TBM] and in the interstitial space) was assessed semiquantitatively by fluorescence microscopy. At least 50 random fields (contained within a 10-mm × 10-mm eyepiece grid) were each assigned an arbitrary intensity score from 1 to 3, where 1 represented a normal pattern of tubulointerstitial distribution determined separately for each individual ECM protein; 2, a slightly increased ECM staining; and 3, a markedly increased staining. The deposition of ECM proteins within glomeruli (glomerular basement membranes [GBM] and mesangium) was also assessed in 50 random glomerular cross sections with a similar technique and an arbitrary scale of 1 to 3, with reference to a control pattern equal to 1. A total of two control and four PAN rat kidneys were evaluated at 1, 2, 3, 5, and 6 weeks. Because the pattern of staining was similar in all control animals (weeks 1 to 6), the results for the control animals were pooled and compared with each experimental group with Bonferroni's *t*-test for independent means. A *P* value of less than 0.05 was considered significant. The results were expressed as the percentage of fields with a score greater than 1.0. All scoring was done by one of the investigators, who was blinded to the animal group at the time of evaluation.

RNA Extraction and Northern Blotting

Total cellular RNA was isolated from each individual rat kidney according to the method of Chirgwin et al.[19] We confirmed the RNA integrity by fractionation on 1.2% agarose-formaldehyde gels containing ethidium bromide and observing the ribosomal bands.[20] The concentration of the RNA was determined by spectroscopy at 260 nm. An equal amount of RNA from each of the animals in a particular experimental or control group was pooled for the preparation of Northern blots for studies 1 and 2. In study 4, RNA from individual animals was processed separately. Northern blotting was performed according to standard methods.[20,21] After the electrophoretic separation of RNA on agarose-formaldehyde gels containing ethidium bromide, the gel was photographed (Polaroid MP-4 LAND camera with 665 professional instant pack film, Polaroid, Cambridge, MA) with ultraviolet transillumination (LKB 2011 Macrovue Transilluminator, Pharmacia LKB Biotechnology, Uppsala, Sweden). We then transferred the RNA in the gel to a nylon membrane and fixed the RNA by baking it at 80°C for 2 hours.

cDNA Probes and Hybridization Conditions

The cDNA probes used were for rat α1(I) procollagen[22] (supplied by Dr. S. Thorgeirsson, National Cancer Institute, Bethesda, MD), rat TGF-β1[23] (supplied by Dr. S. W. Qian, National Cancer Institute Bethesda, MD), rat fibronectin lambda-rlf-1[24] (supplied by Dr. R. Hynes, Center for Cancer Research, Massachusetts, Institute of Technology, Cambridge, MA), murine probes for α1(IV) procollagen pPE 123 and α2(IV) procollagen pPE18[25] (supplied by Dr. M. Kurkinen, University of New Jersey-Rutgers Medical School, Piscaway, NJ), rabbit stromelysin $pS1_2$ and collagenase $pC1_1$[26] (supplied by Dr. Z. Werb, University of California, San Francisco, CA), murine TIMP[27] (supplied by Dr. D. T. Denhardt, Rutgers University, Piscataway, New Jersey), and human histone (H3)[28] (supplied by Dr. J. Stein, University of Florida, Gainesville, FL). Complementary DNA probes were radiolabeled by random priming with the Klenow fragment of *Escherichia coli* DNA polymerase I in the presence of ^{32}P dCTP (3000 Ci/mmol) with a commercial kit (Multiprime DNA Labelling System, Amersham International, United Kingdom). ^{32}P dCTP-labeled cDNA probes were separated from unincorporated nucleotides by gel filtration

with Sephadex G-50 (Nick Columns Pharmacia LKB Biotechnology).

The membrane was prehybridized in 50% deionized formamide, 6.25× SSPE (750 mmol/l NaCl, 50 mmol/l NaH_2PO_4, 5 mmol/l disodium ethylenediamine tetraacetic acid, pH 7.4), 10% dextran sulfate, 0.8% sodium dodecyl sulfate (SDS), 5× Denhardt's solution (0.1% of each of bovine serum albumin, polyvinyl pyrrolidone, and Ficoll 400), and 100 μg/ml denatured salmon sperm DNA for 12 hours at 42°C. Hybridization was carried out with freshly prepared prehybridization solution containing 10^6 cpm labeled cDNA probe per milliliter hybridization solution for 24 hours at 42°C.

After hybridization, the membranes were washed sequentially in 5× SSPE and 0.2% SDS for 20 minutes at room temperature and then in conditions of increasing stringency in which the SSPE concentration was changed to 2× SSPE, 0.2× SSPE, and 0.1× SSPE at 42°C. The radioactivity emitted by the filters was monitored during the washing with a hand-held beta-emission counter, and when nonspecific emissions were not detected, the washing was stopped and the filters were autoradiographed with two intensifying screens (Dupont Cronex, Wilmington, DE) for 24 to 72 hours. Some of the Northern blots were stripped with a wash of 0.1× SCC (3 mol/l NaCl, 0.3 mol/l trisodium citrate, pH 7.0) containing 0.1% SDS at 85°C for 15 minutes and reprobed once with a different cDNA probe.

Quantitation of mRNA Signals

Messenger RNA signals on autoradiographs were quantitated by laser densitometry (Ultroscan XL Enhanced Laser Densitometer, Pharmacia LKB Biotechnology). Similarly, the negative images of the photographs of the formaldehyde gels stained with ethidium bromide were scanned for the density of the ribosomal band (ie, 28S or 18S) that was most closely associated with the mRNA signal of interest to quantitate the amount of RNA loaded onto the gels. Two different durations of exposure were used for the autoradiographs to ensure that the density readings were in the linear range. If there was a difference in the RNA loading of the gel wells of more than 20%, the gel was not used. The densitometry reading of each band on the autoradiograph was adjusted for any RNA loading inequality by division of the densitometry result for the Northern blot band by the density of the closest ribosomal band. This technique of correction for RNA loading discrepancies has been used by others[29] and was used in preference to quantitation of single-copy genes such as actin or glyceraldehyde-3-phosphate dehydrogenase, because our preliminary studies suggested that the steady-state mRNA levels for these genes were variable during the early time points after PAN administration.

Tritiated Thymidine Incorporation and Autoradiography

Experimental and control rats were given 1 μCi/g body weight methyl-^{3}H thymidine (Amersham International,

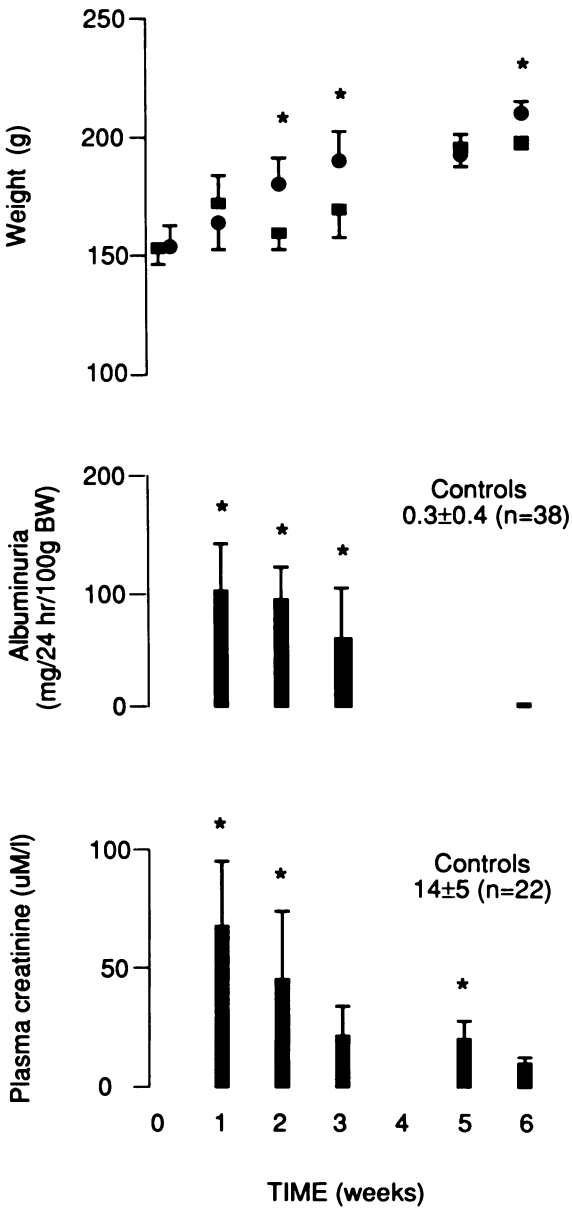


Figure 1. *Mean rat weights, albuminuria, and plasma creatinine values plotted against time. The results for the weight of PAN rats are indicated by square symbols and those for control rat groups by circles. The data for all experiments have been combined. The bars represent 1 SD. The albuminuria and creatinine results for the control rats have been combined from all time points because no difference was found between results for each study period.* *P *value less than 0.05.*

Amersham, United Kingdom) intravenously and killed 2 hours later. The kidneys were flash frozen in isopentane and stored at −70°C. Four-micron–thick cryostat sections were placed on poly-L-lysine–subbed slides, fixed in 4% paraformaldehyde for 1.5 minutes, and processed for autoradiography according to the method of Culling et al.[30]

In Situ *Hybridization*

Procollagen α1 (I) was selected as representative of interstitial matrix proteins for *in situ* hybridization studies. *In situ* hybridization was performed on frozen renal tissue from the rat groups killed at 2 weeks. Single-stranded sense and anti-sense RNA probes of procollagen α1(I) were synthesized, labeled with ^{35}S, and partially hydrolyzed to yield approximately 0.15 to 0.3 kb fragments, according to the method of Nakatsukasa et al.[22] *In situ* hybridization was performed with 9-μ-thick frozen-tissue sections placed on poly-L-lysine–subbed slides. The slides were fixed for 1 minute in 4% paraformaldehyde. The protocol of Simmons et al[31] then was followed exactly.

Results

Clinical and Renal Functional Parameters

Animal weights, urinary albumin excretion rates, and plasma creatinine values for all experiments are summarized in Figure 1. In the first experiment, two of the PAN rats did not develop proteinuria, presumably because of the injection of PAN into the intestine, and one of the control rats had intermittent proteinuria. One rat in the second experiment died. These rats have been excluded from the analysis. The PAN rats developed gross ascites between the first and third week of the experiments. The PAN rats had little weight gain for the 3 weeks after the administration of PAN. The transient increase in their weight at 1 week was probably due to edema because they all had nephrotic range proteinuria. The mean plasma creatinine was increased in the PAN rats at 1, 2, and 5 weeks compared with that in the control rats ($P < 0.05$).

Identification and Quantitation of Interstitial Cells

The PAN-treated rats developed significant interstitial nephritis characterized as an influx of monocytes with lesser but significant numbers of T lymphocytes (Figure 2). The intensity of the interstitial inflammation reached a peak at 1 and 2 weeks and had completely disappeared by 6 weeks. Interstitial fibroblasts expressing the ST4 antigen decreased in number at 2 and 3 weeks.

Cell Proliferation Studies

The mRNA for histone, a gene that is tightly coupled to DNA synthesis, was markedly increased at 1 week in the PAN rat group but not at other times (Figure 3). Tritiated thymidine incorporation autoradiography in the control rats disclosed an occasional positive cell in the tubules and glomeruli. An impressive increase in the number of labeled cells was found in PAN rats at 7 and 9 days. Most of the dividing cells were tubular epithelial cells (Figure 4),

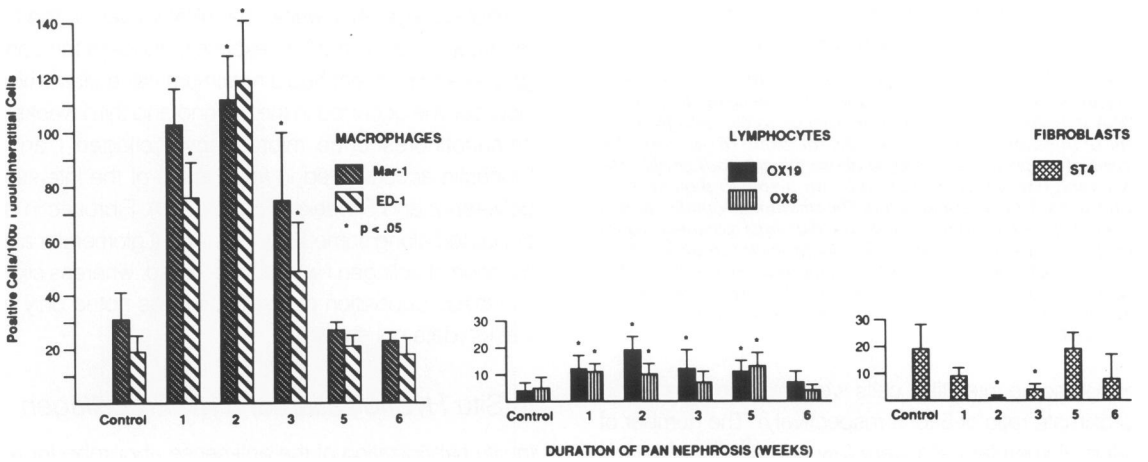


Figure 2. *The mean number of interstitial cells bearing ED-1 and Mar-1 antigens (macrophages), OX19 (all T lymphocytes), OX8 (cytotoxic T lymphocytes), and ST4 (rat peripheral fibroblasts) per thousand tubulointerstitial cells is plotted against time. The bars represent 1 SD. The controls have been pooled because all values at different time points were similar.*

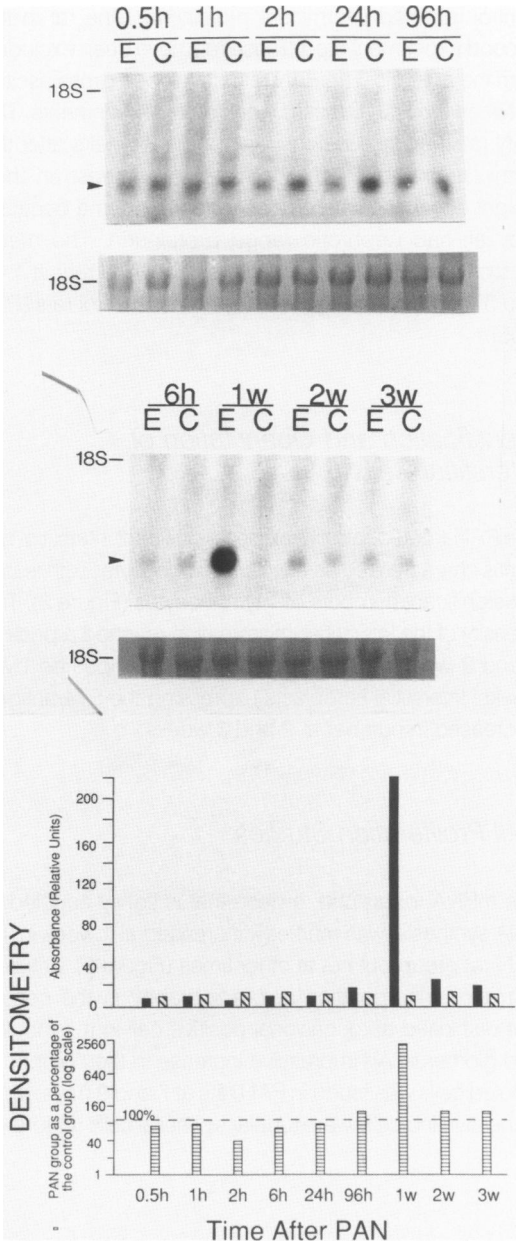


Figure 3. *Northern blot and densitometry analysis of whole kidney steady-state mRNA levels for histone H3, a gene tightly coupled to DNA synthesis. The time in hours (hr) or weeks (wk) from the initial IP injection of PAN for the PAN-rat group (E) or saline for the control-rat group (C) is shown above the autoradiograph. The mRNA band (0.8 kb) is highlighted by an arrow to show its relationship to the 18S ribosomal band. The ethidium-bromide–stained gel below the autoradiograph shows that approximately equal amounts of RNA were loaded. The densitometry analysis of the autoradiograph corrected for any RNA loading errors is plotted in the upper bar graph. The absorbance of the PAN group mRNA band is expressed as a percentage of that for the control group in the lower graph.*

although some interstitial cells were also positive (at an approximate ratio of 5 to 1, respectively). The number of labeled glomerular cells were few and similar in control and PAN rats.

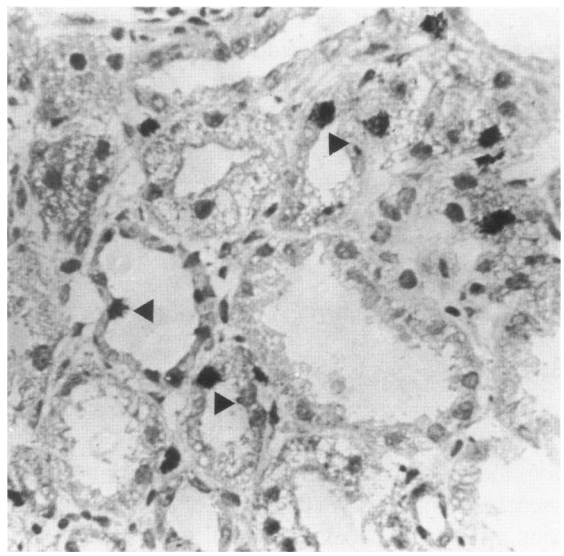


Figure 4. *Autoradiograph of a kidney tissue section from a rat with aminonucleoside nephrosis (day 7) injected with tritiated thymidine. Arrowheads highlight some of the tubular epithelial cell nuclei demonstrating ^3H thymidine uptake (×400).*

TGF-β1 Gene Expression

The steady mRNA levels for TGF-β1 remained at or below control levels during the first 4 days after PAN administration (Figure 5). At 1 week, the mRNA levels were increased 7.5-fold. This increase was sustained, but to a lesser degree, at weeks 2 and 3.

Interstitial Matrix Proteins Collagen I and Fibronectin

The variations in the steady-state mRNA levels for α1(I) procollagen (Figure 6) and fibronectin (Figure 7) during the course of acute PAN nephrosis were similar. During the first 4 days, the mRNA levels in PAN rat kidneys were decreased to a variable extent compared with those in control kidneys. At 1 week, the mRNA levels in the PAN rat groups were in marked excess of those of the control groups and had reached a maximum value after which a slow decline occurred in the second and third weeks. By immunofluorescence microscopy, collagen I and fibronectin accumulated in focal areas of the interstitium between 2 and 5 weeks (Figures 8, 9). Fibronectin also deposited along some TBM. Significant glomerular accumulation of collagen I was not observed, whereas slightly increased deposition of fibronectin was noted only at 2 weeks (data not shown).

In Situ *Hybridization of α1(I) Procollagen*

In situ hybridization of the anti-sense riboprobe for α1(I) procollagen was found in a scant but even distribution in

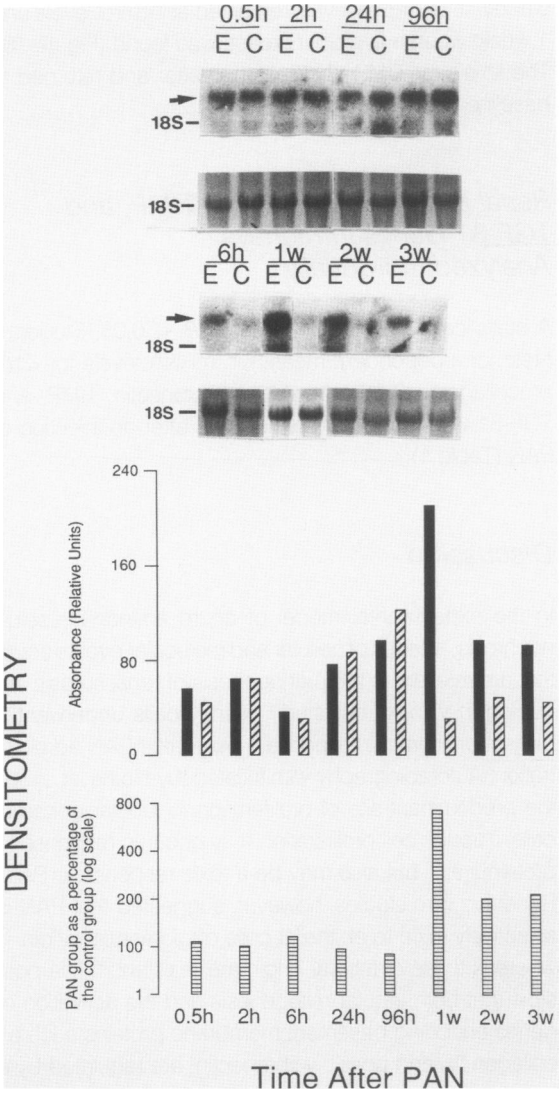


Figure 5. *Northern blot and densitometry analysis of mRNA for TGF-β1 (2.5 kb), arranged as described in Figure 3.*

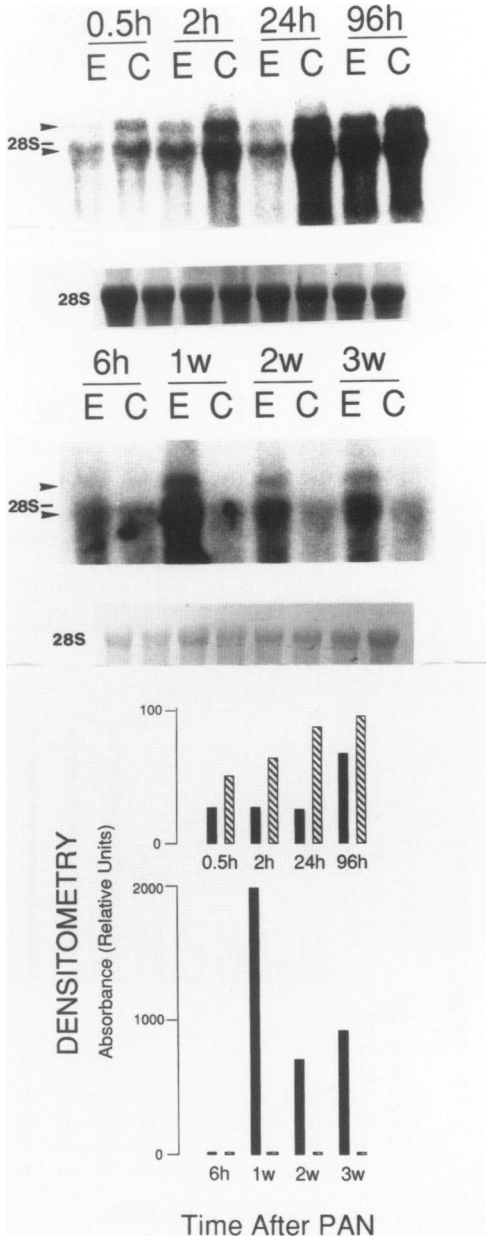


Figure 6. *Northern blot and densitometry analysis of whole kidney mRNA for α1(I) procollagen. The time in hours (hr) or weeks (wk) from the initial IP injection of PAN for the PAN-rat group (E) or saline for the control-rat group (C) is shown above the autoradiograph. The mRNA bands (4.7 and 5.7 kb) are highlighted by arrows to show their relationship to the 28S ribosomal band. The ethidium-bromide-stained gel below the autoradiograph shows that approximately equal amounts of RNA were loaded. The densitometry analysis of the autoradiograph corrected for any RNA loading errors is plotted in the graphs below.*

the interstitium of control rat kidneys at 2 weeks. In contrast, hybridization in the widened interstitium of PAN rats was dense (Figure 10). The antisense probe also hybridized to the adventitia of blood vessels, but failed to hybridize to glomerular or tubular cells in either control or PAN rats. The sense riboprobe did not bind to control or PAN renal tissue.

Basement Membrane Collagen IV

Total kidney steady-state mRNA levels for genes encoding α1(IV) procollagen (Figure 11) and α2(IV) procollagen (Figure 12) showed a trend that was similar to those of the interstitial ECM proteins; levels were at or below control values during the first 4 days, and a marked increase occurred at 1 week that persisted at 2 and 3 weeks. By immunofluorescence microscopy, collagen IV increased along some TBM and appeared in small amounts in interstitial spaces (Figures 9, 13) between 2 and 5 weeks. The glomerular pattern of staining for col-

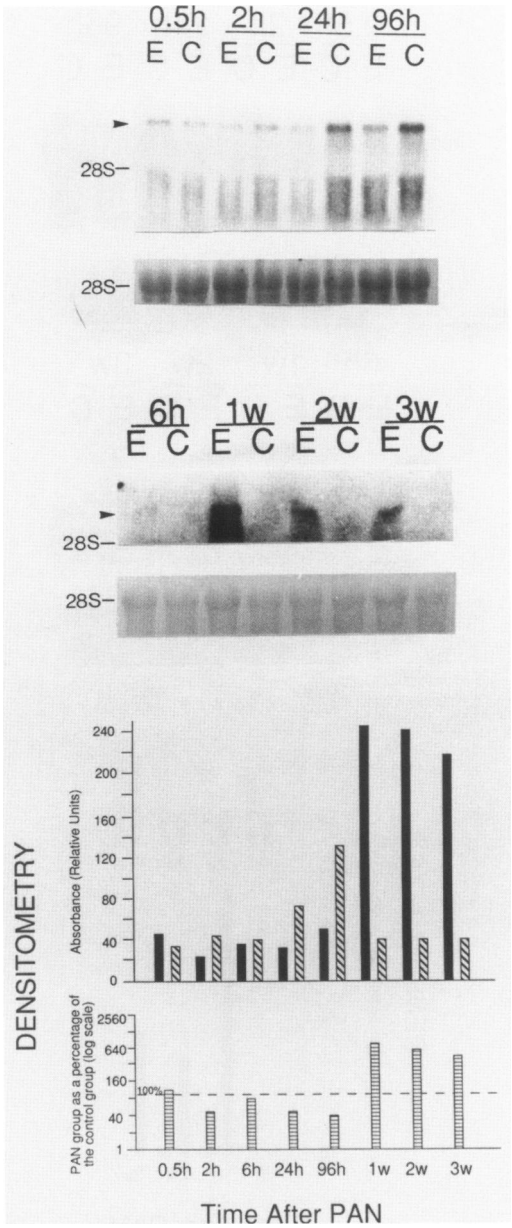


Figure 7. *Northern blot and densitometry analysis of mRNA for fibronectin (8.0 kb) arranged as described in Figure 3.*

lagen IV was similar to that for control animals at all experimental periods (data not shown).

Metalloproteinases and TIMP

No difference was found in the kidney steady-state mRNA levels for stromelysin (Figure 14). Levels for interstitial collagenase were similar to those for control animals, except at weeks 2 and 3, when there was a twofold to threefold increase in interstitial collagenase mRNA levels (Figure 15). In PAN-treated rats, the renal TIMP steady-state mRNA levels remained at control levels until 1 week, when a fivefold increase was found (Figure 16). The levels for TIMP declined thereafter and returned to baseline values by 3 weeks.

Renal Expression of Matrix, TIMP, and TGF-β1 Genes in Kidneys Analyzed Individually

A statistically significant increase ($P < 0.05$, Student's *t*-test for independent means) in mRNA levels for α1(I) procollagen, α2(IV) procollagen, fibronectin, TIMP, and TGF-β1 was found for rats 10 days after an injection of PAN (Table 1).

Discussion

In the experimental model of acute aminonucleoside nephrosis, a series of cellular and molecular events occur that are relevant to the pathogenesis of renal fibrosis occurring *in vivo*. In our study, kidney cells underwent a distinct proliferative response 1 week after PAN administration. Autoradiography with tritiated thymidine localized the predominant site of proliferation to tubular epithelial cells. Tubular cell proliferation may occur in response to proteinuria,[32] but also may be a toxic response to PAN. Recent *in vitro* studies, however, suggested that PAN is selectively toxic to epithelial cells of glomerular origin,[33] whereas those of tubular origin are resistant.[34] It is possible that both tubular mitogenesis and the activation of genes encoding basement membrane proteins such as collagen IV, and possibly fibronectin, are regulated by a common activation pathway triggered by peptide growth factors such as platelet-derived growth factors[35–37] or basic fibroblast growth factor.[38–40]

Genes encoding the basement membrane collagens α1(IV) collagen and α2(IV) collagen in the kidney are both activated in aminonucleoside nephrosis. The steady-state mRNA levels for both α1(IV) and α2(IV) collagen remained at or below control values during the first 4 days. At 1 week, the total kidney mRNA level for α1(IV) collagen was increased 14-fold, whereas that for α2(IV) collagen was increased eightfold. Although in the current study the intrarenal site of collagen IV gene activation was not determined, it is likely that extraglomerular sources, particularly tubular cells, make a significant contribution, for the following reasons: 1) immunofluorescence microscopy showed an increase in tubulointerstitial collagen IV protein between 2 and 5 weeks, whereas a change in glomerular distribution of collagen IV was not detected; 2) Nakamura and colleagues,[41] using glomeruli isolated from rats with aminonucleoside nephrosis, ob-

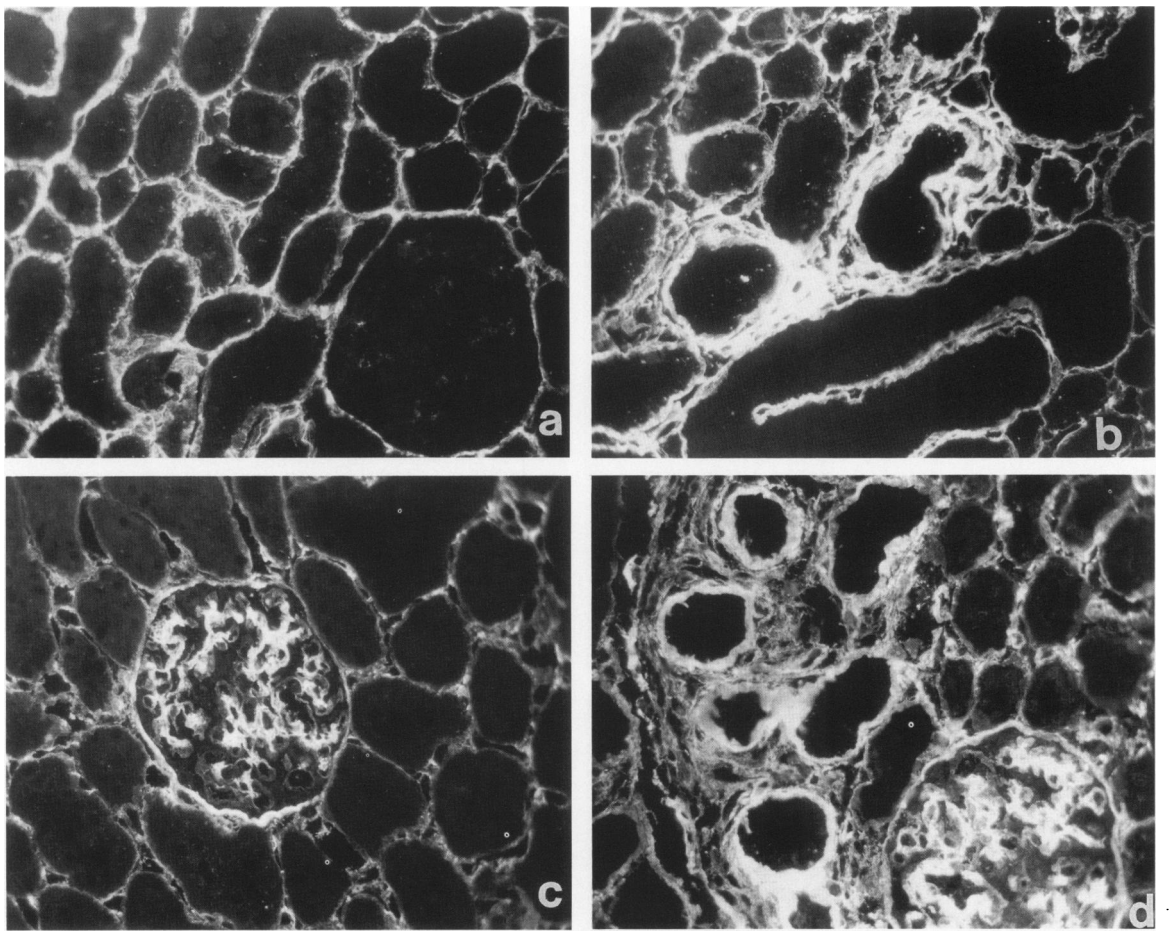
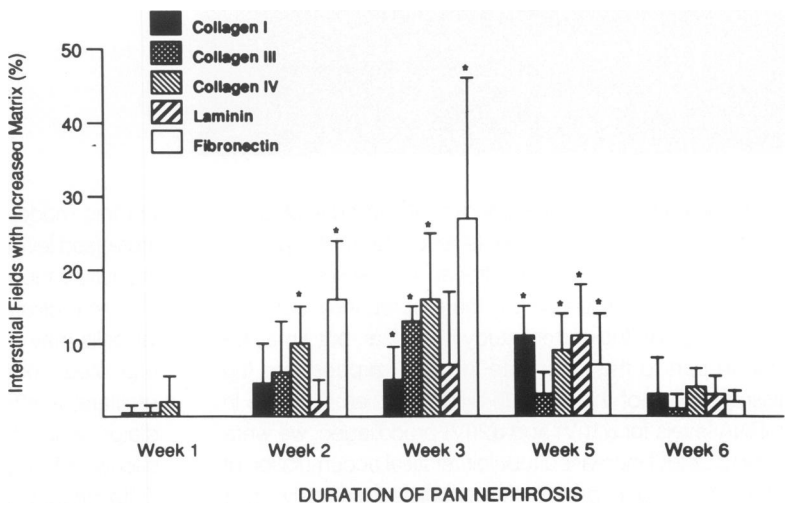


Figure 8. *Immunofluorescence photomicrographs illustrating the renal distribution of the interstitial extracellular matrix proteins collagen I* (a, b) *and fibronectin* (c, d). *Compared to the control animals* (a, c) *after 3 weeks of aminonucleoside nephrosis focal areas of the interstitium showed increased deposition of collagen I* (b) *and fibronectin* (d). *Fibronectin also accumulated along some tubular basement membranes (×260).*

served a small (1.5-fold) increase in glomerular α1(IV) collagen mRNA levels at 8 days, less than the increase observed in the current study; 3) glomeruli constitute approximately 3% of the total kidney volume,[42] making it likely that the current study focuses to a significant extent on steady-state mRNA levels from nonglomerular sites.

Figure 9. *The quantity of the ECM proteins, collagens I, III, and IV, fibronectin, and laminin in the tubulointerstitium of PAN rats estimated with fluorescence microscopy and expressed relative to an arbitrary control value of 1.0. The normal distribution of each extracellular matrix protein was established individually and assigned an arbitrary value of 1.0. The results are expressed as the percent of fields with an abnormal intensity score of 2.0 or 3.0.* *$P < 0.05$.



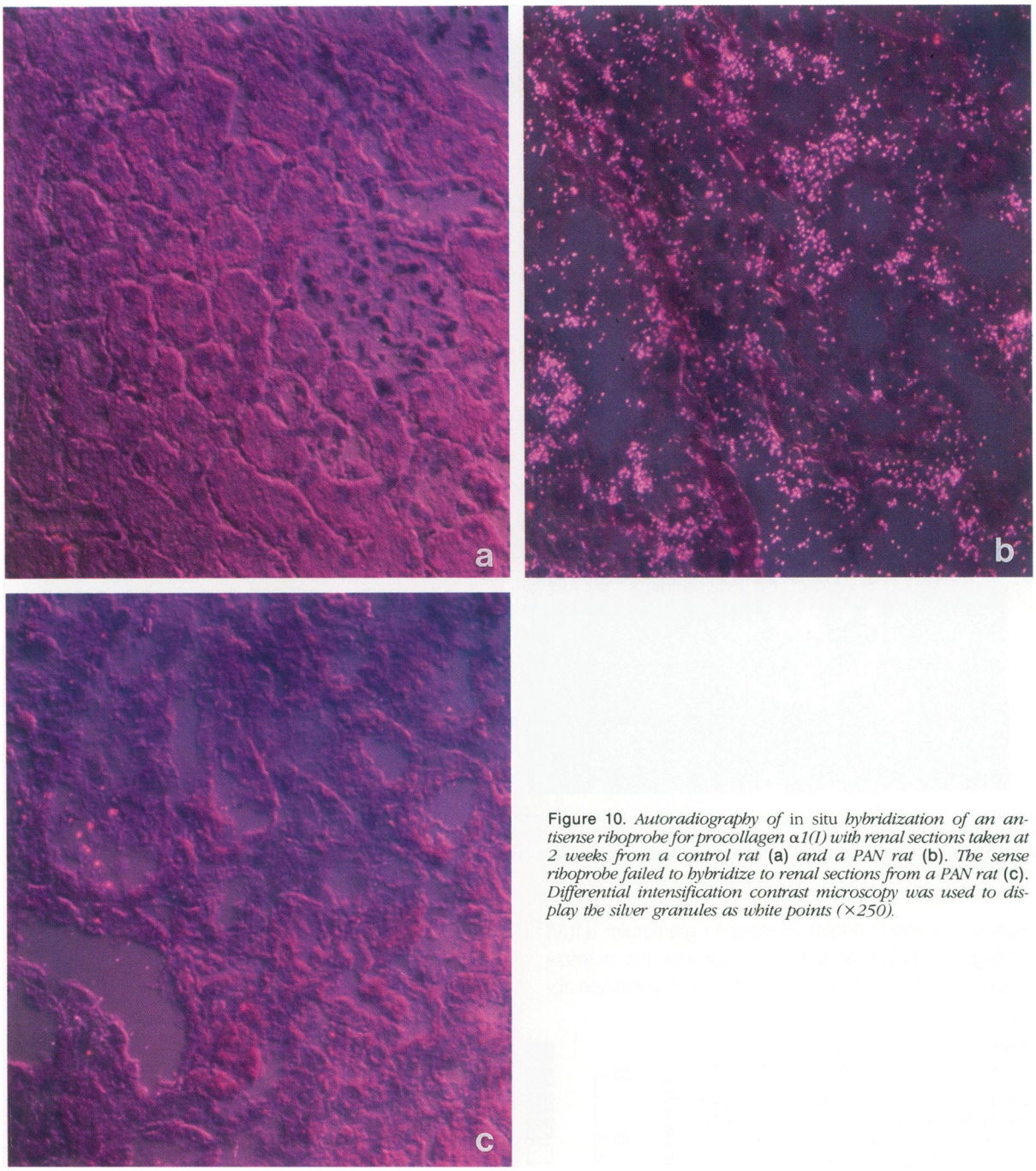


Figure 10. *Autoradiography of* in situ *hybridization of an antisense riboprobe for procollagen α1(I) with renal sections taken at 2 weeks from a control rat* (a) *and a PAN rat* (b). *The sense riboprobe failed to hybridize to renal sections from a PAN rat* (c). *Differential intensification contrast microscopy was used to display the silver granules as white points (×250).*

It is interesting that Nakamura et al[43] failed to observe an increase in medullary mRNA levels for α1(IV) procollagen 2, 8, 14, and 20 days after an intraperitoneal injection of PAN. The reason for the discrepancy between their study and the current study is unclear, but it may be due in part to the difference in cDNA probes and the tissue source of the RNA. In addition to an increase in mRNA levels for α1(IV) and α2(IV) procollagen, we were able to detect increased tubulointerstitial accumulation of type IV collagen protein immunohistochemically. In a chronic model of PAN-induced nephrosis, we reported increased levels of matrix proteins in both the cortical and medullary tubulointerstitial compartments.[44]

The proliferative and biosynthetic responses of tubular cells may be independently rather than coordinately regulated. For example, TGF-β, which often has an antiproliferative effect on cultured cells,[45–49] is a potent mediator of fibrosis both *in vitro* and *in vivo*.[50,51] Biologic effects of TGF-β include increased production of several ECM proteins and TIMP, whereas the synthesis of the

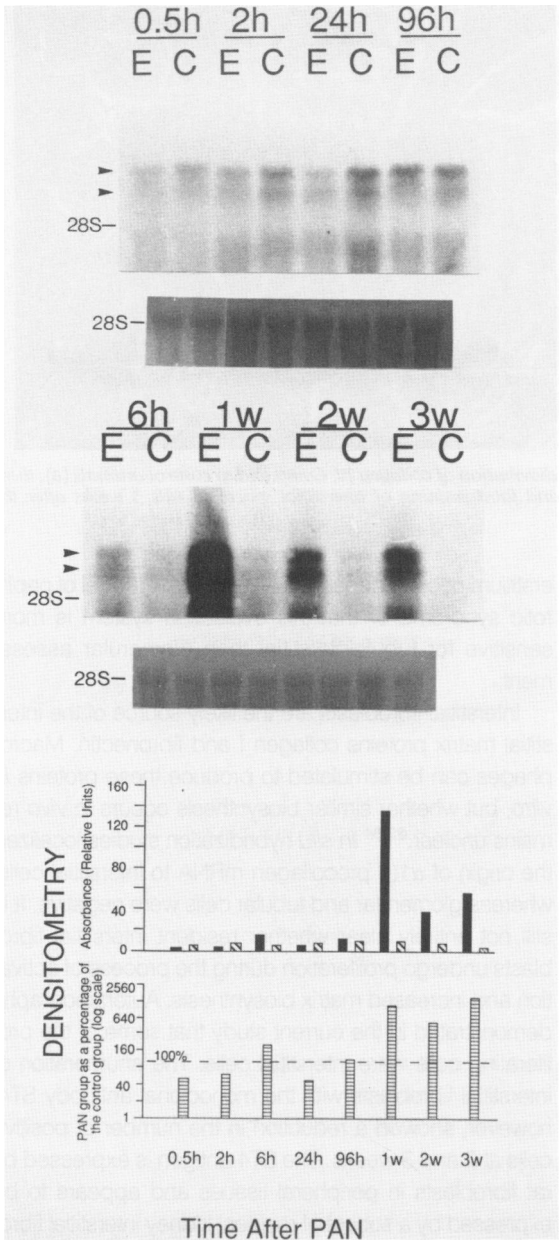


Figure 11. *Northern blot and densitometry analysis of mRNA for α1(IV) procollagen (6.2 and 6.8 kb), arranged as described in Figure 3.*

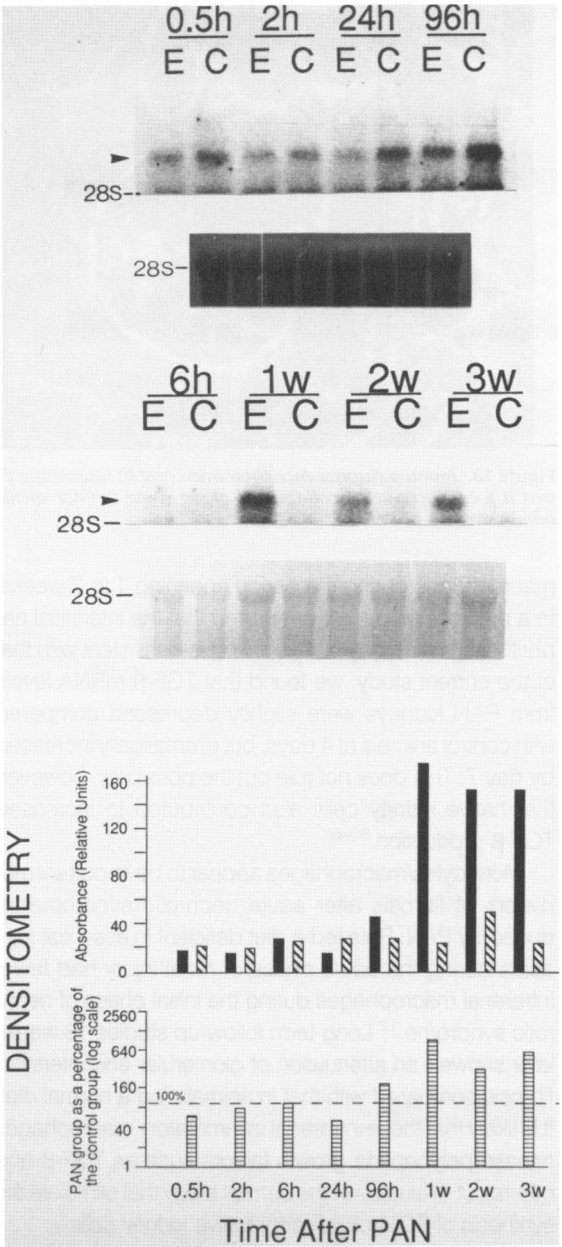


Figure 12. *Northern blot and densitometry analysis of mRNA for α2(IV) procollagen (6.4 kb), arranged as described in Figure 3.*

matrix-degrading metalloproteinases is decreased. In the current study, kidney steady-state mRNA levels for TGF-β were increased 7.5-fold at 1 week and persisted at two times control levels at 2 and 3 weeks. In an experimental model of anti-GBM disease in rabbits, increased renal cortical mRNA levels for collagens I and IV have been associated with increased production of TGF-β by diseased renal cortical tissue.[52,53] In an experimental model of glomerular injury targeted to mesangial cells, Border and his colleagues[54] recently reported that the *in vivo* administration of TGF-β–neutralizing antiserum prevented glomerular fibrosis. Transforming growth factor-beta also has been implicated as an important mediator of ECM accumulation leading to fibrosis in several other organs.[55–58]

The cellular origin of TGF-β mRNA in acute aminonucleoside nephrosis requires further investigation. Cells of the monocyte/macrophage lineage are one potential source. Once released, TGF-β also may function as a monocyte-chemotactic factor to amplify the renal inflammatory response.[59] In the current study, there is a striking association between TGF-β mRNA levels and the presence of an interstitial infiltrate of monocytes/

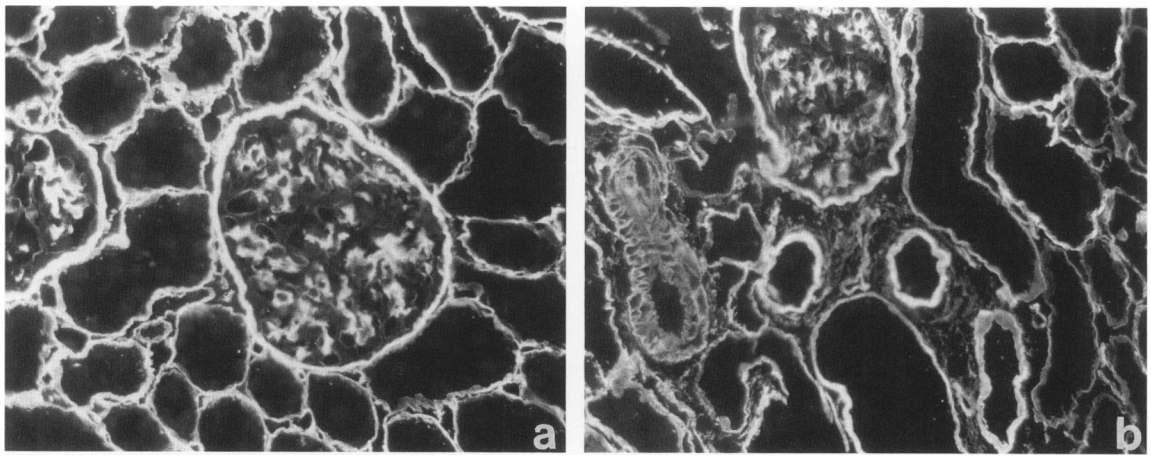


Figure 13. *Immunofluorescence photomicrographs illustrating the renal distribution of collagen IV. Compared to control animals* **(a)**, *there was a focal increase of collagen IV along some tubular membranes and faint staining of interstitial spaces in rats 3 weeks after the administration of PAN* **(b)** *(×270).*

macrophages during the period spanning 1 to 3 weeks. In a previous study,[13] we reported that the interstitial nephritis begins on day 5. This timing is consistent with that of the current study: we found that TGF-β mRNA levels from PAN kidneys were slightly depressed compared with control animals at 4 days, but dramatically increased by day 7. This does not rule out the possibility, however, that native kidney cells also contributed to increased TGF-β production.[60–62]

Monocytes/macrophages appear to be important mediators of fibrosis after acute nephrotic syndrome induced by PAN. Rats fed a diet deficient in essential fatty acids during the acute phase of renal injury had fewer intrarenal macrophages during the initial phase of nephrotic syndrome.[12] Long-term follow-up studies 18 weeks later showed an attenuation of glomerular and interstitial fibrosis compared with that in animals fed a normal diet. It is likely that these intrarenal inflammatory macrophages release polypeptide growth factors such as TGF-β and others not evaluated in the current study that stimulate the synthesis of ECM proteins by native kidney cells.

In addition to increased renal mRNA and protein levels for the basement membrane protein collagen IV, we observed an increase in interstitial matrix proteins in this study. In the experimental group, steady-state mRNA levels for procollagen α1(I) and fibronectin rose from levels below control values at 4 days to maximum levels at 7 days, and increased 20-fold and 10-fold, respectively. Elevated levels persisted at weeks 2 and 3. Accumulation of fibronectin and collagen I could be detected in the tubulointerstitium at 2 and 3 weeks by immunofluorescence microscopy. Glomerular deposition of collagen I did not occur, whereas accumulation of fibronectin was modest and only evident at the 2-week observation period. The results of immunohistochemical analysis suggest that events occurring in the glomeruli and tubulointerstitium occur independently in the PAN model of nephrotic syndrome or that this evaluation system is more sensitive for tubulointerstitial than glomerular assessment.

Interstitial fibroblasts are the likely source of the interstitial matrix proteins collagen I and fibronectin. Macrophages can be stimulated to produce these proteins *in vitro*, but whether similar biosynthesis occurs *in vivo* remains unclear.[63,64] *In situ* hybridization studies localized the origin of α1(I) procollagen mRNA to interstitial cells, whereas glomerular and tubular cells were negative. It is still not entirely clear whether resident interstitial fibroblasts undergo proliferation during the process of activation and increased matrix biosynthesis. Autoradiography demonstrated in the current study that some of the proliferating cells were interstitial cells. The enumeration of interstitial fibroblasts with the monoclonal antibody ST4, however, showed a reduction in the number of positive cells at 2 and 3 weeks. The ST4 antigen is expressed by rat fibroblasts in peripheral tissues and appears to be expressed by a subset of resident kidney interstitial fibroblasts.[18] It is unknown whether the reduction in $ST4^+$ interstitial cells represents a decrease in fibroblast numbers, or more likely, an alteration in cell phenotype associated with their activation. Further investigations are necessary to delineate the full cascade of molecular mediators that trigger renal interstitial fibroblasts to synthesize new ECM proteins *in vivo*.[65] The elevated levels of TGF-β mRNA suggest that this growth factor is one likely candidate.

Our immunohistochemical studies demonstrate that early accumulation of several ECM proteins occurs within the tubulointerstitial compartment of the kidney during the nephrotic state. As the proteinuria reverses and interstitial inflammation subsides during weeks 5 and 6, accumulated ECM proteins actually seem to disappear from the

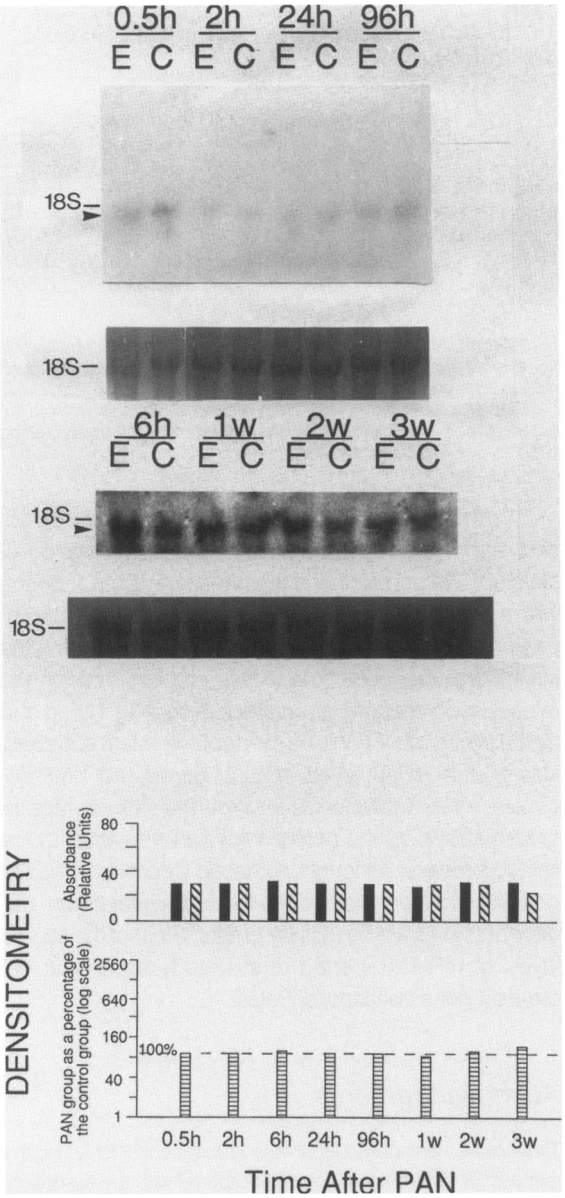


Figure 14. *Northern blot and densitometry analysis for stromelysin (1.9 kb), arranged as described in Figure 3.*

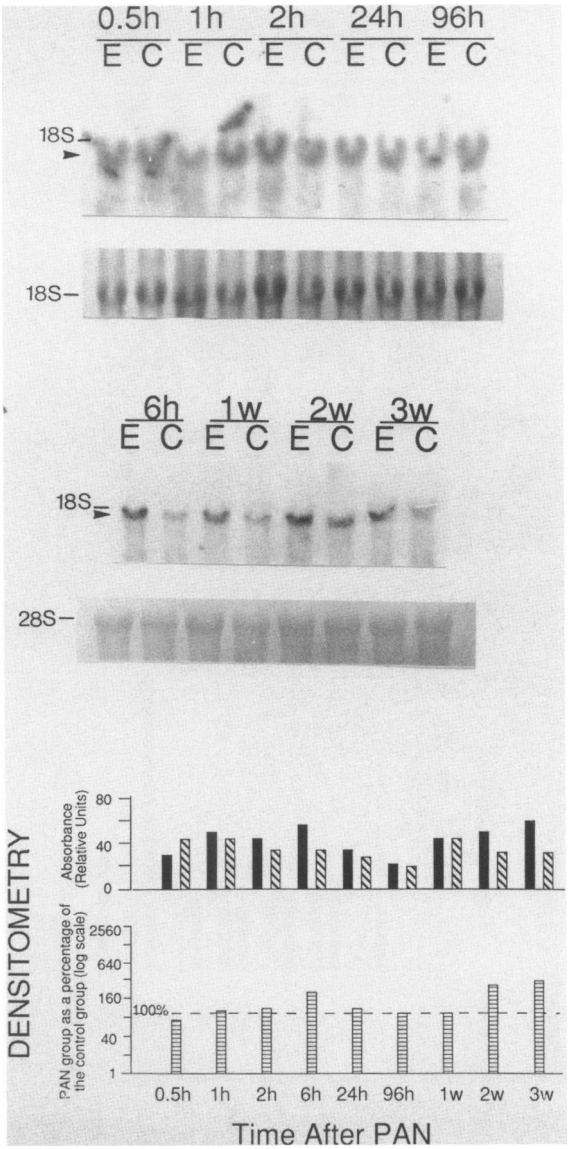


Figure 15. *Northern blot and densitometry analysis for interstitial collagenase (1.9 kb), arranged as described in Figure 3.*

tubulointerstitium. We must be cautious, however, in the interpretation of these immunohistochemical studies of matrix protein accumulation because they were performed semiquantitatively; this technique also may fail to detect hidden antigenic determinants. Molecular studies of steady-state mRNA levels for genes encoding proteins of the matrix degradation pathway provide a possible explanation for this observation. If the changes in steady-state mRNA levels observed were associated with increased synthesis and activation of these regulatory proteins, an increased rate of matrix degradation would occur during the phase of declining proteinuria. Interstitial collagenase degrades collagens I, II, III, VIII, and X, whereas stromelysin has wider activity: it degrades several ECM proteins, including collagens IV, V, VII, fibronectin, laminin, and denatured collagen I.[66,67] Both enzymes are inhibited by TIMP. In the current study, mRNA levels for interstitial collagenase and stromelysin appear to be independently regulated. In nephrotic rats, the levels for both proteases were similar to those for control animals at week 1, but only interstitial collagenase levels were increased (twofold to threefold) at 2 and 3 weeks. Conversely, the mRNA level for the enzyme inhibitor TIMP is increased fivefold at 1 week and returns to baseline values by 3 weeks.

Additional studies are necessary to identify the cellular origin of these metalloproteinases and their inhibitor. The timing of the molecular events that occur in the kidneys of rats with PAN-induced acute nephrotic syndrome

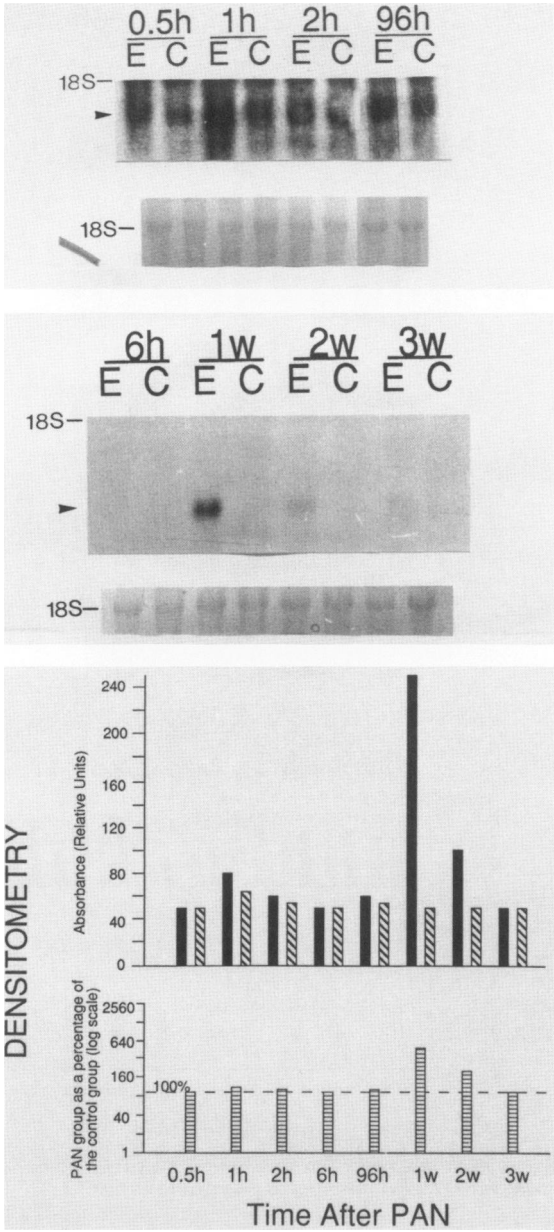


Figure 16. *Northern blot and densitometry analysis of mRNA for TIMP (1.0 kb), arranged as described in Figure 3.*

may be unique to this experimental model—especially during the first few days. Although the mechanism of action of puromycin aminonucleoside is not fully understood, earlier studies[68,69] reported that this drug interferes with RNA synthesis. A specific drug-related effect might account for the low mRNA levels found during the first 4 days of the current study. Maintenance of the nephrotic state and interstitial inflammation by multiple PAN injections is associated with continued ECM accumulation and persistently elevated renal mRNA levels for TGF-β, several ECM genes, and TIMP, whereas the levels for interstitial collagenase and stromelysin remained unchanged.[44]

Table 1. *Densitometric Analysis of Matrix, TIMP, and TGF-β1 Genes in the Kidneys of Rats with PAN-induced Nephrosis (Day 10)*

	Day 10 PAN rats	Day 10 control rats	*P* value*
α1(I) procollagen	2.4 ± 0.6**	1.0 ± 0.2	0.001
α2(IV) procollagen	3.0 ± 1.4	1.0 ± 0.2	0.015
Fibronectin	3.2 ± 0.7	1.0 ± 0.2	<0.001
TIMP	10.8 ± 2.4	1.0 ± 0.6	0.001
TGF-β1	3.0 ± 1.2	1.0 ± 0.2	0.007

* Student's *t*-test for independent means.

** Results are mean ± 1 SD expressed as arbitrary densitometric units, corrected for any inequality in RNA loading and standardized to a mean control value of 1.0 unit.

Kidney total RNA (20 μg) from individual animals, n = 5 for experimental, n = 5 for control, was analyzed by Northern blotting with the cDNA probes indicated.

In summary, the current study of acute aminonucleoside nephrosis reports simultaneous up-regulation of several ECM genes during the proteinuric phase, during which time matrix degradation may be inhibited by increased production of TIMP. Macrophages infiltrating the interstitium may contribute to this process through the release of polypeptide growth factors such as TGF-β and by the synthesis of TIMP. As the nephrotic state subsides, steady-state mRNA levels for ECM genes and TIMP decrease, whereas the levels for interstitial collagenase increase, providing the potential for partial reversal of the fibrotic process. Although increased deposition of ECM proteins was demonstrated in the tubulointerstitium, additional studies are necessary to establish that the altered levels of mRNA reported in this study are due to increased gene transcription rates.

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