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## Research Article

Tissue transglutaminase is a calcium-dependent enzyme that catalyzes the cross-linking of polypeptide chains, including those of extracellular matrix (ECM) proteins, through the formation of epsilon-(gamma-glutamyl) lysine bonds. This crosslinking leads to the formation of protein polymers that are highly resistant to degradation. As a consequence, the enzyme has been implicated in the deposition of ECM protein in fibrotic diseases such as pulmonary fibrosis and atherosclerosis. In this study, we have investigated the involvement of tissue transglutaminase in the development of kidney fibrosis in adult male Wistar rats submitted to subtotal nephrectomy (SNx). Groups of six rats were killed on days 7, 30, 90, and 120 after SNx. As previously described, these rats developed progressive glomerulosclerosis and tubulo-interstitial fibrosis. The tissue level of epsilon-(gamma-glutamyl) lysine cross-link (as determined by exhaustive proteolytic digestion followed by cation exchange chromatography) increased from 3.47 $\pm$ 0.94 (mean $\pm$ SEM) in controls to 13.24 $\pm$ 1.43 nmol/g protein 90 d after SNx,  $P \leq 0.01$ . Levels of epsilon-(gamma-glutamyl) lysine cross-link correlated well with the renal fibrosis score throughout the 120 observation days ( $r = 0.78$ ,  $P \leq 0.01$ ). Tissue homogenates showed no significant change in overall transglutaminase activity ( $^{14}\text{C}$  putrescine incorporation assay) unless adjusted for the loss of viable tubule cells, when an increase from 5.77 $\pm$ 0.35 to 13.93 $\pm$ 4.21 U/mg DNA in cytosolic tissue transglutaminase activity was seen. This increase was supported [...]

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# The Role of Transglutaminase in the Rat Subtotal Nephrectomy Model of Renal Fibrosis

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## Abstract

Tissue transglutaminase is a calcium-dependent enzyme that catalyzes the cross-linking of polypeptide chains, including those of extracellular matrix (ECM) proteins, through the formation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds. This cross-linking leads to the formation of protein polymers that are highly resistant to degradation. As a consequence, the enzyme has been implicated in the deposition of ECM protein in fibrotic diseases such as pulmonary fibrosis and atherosclerosis.

In this study, we have investigated the involvement of tissue transglutaminase in the development of kidney fibrosis in adult male Wistar rats submitted to subtotal nephrectomy (SNx). Groups of six rats were killed on days 7, 30, 90, and 120 after SNx. As previously described, these rats developed progressive glomerulosclerosis and tubulo-interstitial fibrosis. The tissue level of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link (as determined by exhaustive proteolytic digestion followed by cation exchange chromatography) increased from  $3.47 \pm 0.94$  (mean  $\pm$  SEM) in controls to  $13.24 \pm 1.43$  nmol/g protein 90 d after SNx,  $P \leq 0.01$ . Levels of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link correlated well with the renal fibrosis score throughout the 120 observation days ( $r = 0.78$ ,  $P \leq 0.01$ ). Tissue homogenates showed no significant change in overall transglutaminase activity ( $^{14}\text{C}$  putrescine incorporation assay) unless adjusted for the loss of viable tubule cells, when an increase from  $5.77 \pm 0.35$  to  $13.93 \pm 4.21$  U/mg DNA in cytosolic tissue transglutaminase activity was seen. This increase was supported by Western blot analysis, showing a parallel increase in renal tissue transglutaminase content. Immunohistochemistry demonstrated that this large increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link and tissue transglutaminase took place predominantly in the cytoplasm of tubular cells, while immunofluorescence also showed low levels of the  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link in the extracellular renal interstitial space.

Timothy S. Johnson and Martin Griffin contributed equally to this work.

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The number of cells showing increases in tissue transglutaminase and its cross-link product,  $\epsilon$ -( $\gamma$ -glutamyl) lysine appeared greater than those showing signs of typical apoptosis as determined by in situ end-labeling. This observed association between tissue transglutaminase,  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link, and renal tubulointerstitial scarring in rats submitted to SNx suggests that tissue transglutaminase may play an important role in the development of experimental renal fibrosis and the associated loss of tubule integrity. (*J. Clin. Invest.* 1997. 99:2950–2960.) Key words: kidney •  $\epsilon$ -( $\gamma$ -glutamyl) lysine • tubule • scarring • subtotal nephrectomy

## Introduction

The progression of chronic renal insufficiency is characterized by a relentless fibrosis of the kidney. Both increased synthesis and decreased breakdown of the renal extracellular matrix (ECM)<sup>1</sup> have been implicated (1, 2). The latter may be due to changes in the ECM-regulating enzymes, including a fall in renal metalloproteinases, or an increase in their inhibitors (tissue inhibitors of metalloproteinases and plasminogen activator inhibitors) (3, 4). Another factor in the pathogenesis of renal fibrosis may be the resistance of the deposited ECM to breakdown. This resistance has been put forward as a contributing factor in the development of other fibrogenic processes, including lung fibrosis and atherosclerosis (5–8). Tissue transglutaminase-induced cross-linking of the ECM may underlie its resistance to breakdown.

Transglutaminases (E.C.2.3.2.13) are calcium-dependent enzymes which catalyze the posttranslational modification of proteins through an acyl transfer reaction between the  $\gamma$  carboxamide group of a peptide-bound glutamyl residue and various primary amines (9). Covalent cross-links using  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds are stable and resistant to enzymatic, chemical, and mechanical disruption (9). Endopeptidases capable of hydrolyzing the  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links formed by transglutaminases have not been described in vertebrates, and even lysosomes do not contain enzymes capable of splitting the  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds (10–12). A number of transglutaminase enzymes have been characterized which have distinct genes, structures, and physiological functions. Examples include the plasma Factor XIIIa involved in cross-linking fibrin during wound healing (13), and the keratinocyte transglutaminase involved in the formation of the cornified envelope during terminal differentiation (14, 15). A further transglutaminase, the tissue transglutaminase, is the most widespread member of this family, and is present in many different cell types.

1. Abbreviations used in this paper: ECM, extracellular matrix; SNx, subtotal nephrectomy.

Recent evidence suggests that the tissue transglutaminase may have a number of key roles in cells. These include a role in programmed cell death (16–18), cell adhesion (19), and interactions between the cell and its ECM via the cross-linking of proteins such as fibronectin, laminin, nidogen, and collagens types I, III, and IV (7, 20, 21). In addition, tissue transglutaminase has been demonstrated recently to play a role in the covalent stabilization of collagen fibrillar formation through cross-linking of the core fibrils (22). Involvement in ECM diseases has also been noted (5), as raised tissue transglutaminase levels have been detected in paraquat-induced pulmonary fibrosis. Since then, transglutaminase has been ascribed roles in atherosclerosis (6–8), cataract formation (23), and neurofibrillary tangles in Alzheimer's disease (24) and tau-containing polymers (25).

In view of the possible role of tissue transglutaminase in diseases characterized by perturbations in cell death and ECM metabolism, we have designed this study to investigate the role of tissue transglutaminase in renal fibrosis induced in rats by subtotal nephrectomy. We aimed to determine whether the expression of the enzyme was raised in scarred kidneys, to localize its expression, and to detect changes in its cross-link product.

## Methods

### *Experimental animals and protocol*

Male Wistar rats (Sheffield University strain) of approximately similar weight (350–400 g) and age (8–10 wk) were subjected to subtotal (5/6th) nephrectomy (SNx) by complete removal of the left kidney and upper and lower pole resections of the right kidney. Rats were housed 2–4 to a cage and maintained at 20°C and 45% humidity on a 12-h light/dark cycle. They were allowed free access to standard rat chow (Labsure Ltd., Cambridge, United Kingdom) and tap water. At days 7, 30, 90, and 120 after SNx, experimental groups of six rats were killed, and the remnant kidneys were removed. At each time point, four control animals were also killed that had been subjected to surgical manipulation of the kidneys at the time of the nephrectomy. Age- and weight-matched spontaneously hypertensive rats ( $n = 8$ ) were also studied to control for systemic hypertension. Before death, all rats had their renal function (serum creatinine and creatinine clearance), proteinuria, and systolic blood pressure measured. Creatinine was measured by standard autoanalyzer technique, proteinuria by the Biuret method, and blood pressure by tail-cuff plethysmography. All procedures were carried out under license according to regulations laid down by Her Majesty's Government, United Kingdom (Animals Scientific Procedures Act, 1986).

### *Assessment of renal scarring*

Analysis of renal scarring was determined on formol calcium-fixed, paraffin-embedded sections (4  $\mu\text{m}$ ) stained by hematoxylin and eosin (H & E) or Masson's trichrome. One of the authors (A. Meguid El Nahas) scored the severity of glomerulosclerosis and tubulo-interstitial scarring, blinded to their code, according to a previously described arbitrary score (from 1 to 4) (26). Scores were attributed as follows: glomerulosclerosis score (1–4): 1 = normal glomeruli; 2 = presence of mild segmental glomerulosclerosis affecting < 25% of the glomerular tuft; 3 = moderate segmental sclerosis affecting 25–50% of the tuft, 4 = diffuse severe glomerulosclerosis affecting > 50% of the tuft including glomeruli with total tuft obliteration, fibrosis, and obsolescence (see Fig. 1, *bottom*). A minimum of 25 glomeruli per kidney/remnant kidney was examined, and the mean of the glomerular scores was taken to represent the severity of glomerulosclerosis for a given rat.

Tubulointerstitial scarring was scored as follows: (1–4): 1 = normal tubules and interstitium; 2 = mild tubular atrophy and interstitial

fibrosis; 3 = moderate tubular atrophy and dilatation with marked interstitial fibrosis; 4 = end-stage kidney with extensive interstitial fibrosis and few remaining atrophic tubules (see Fig. 1, *top*). A score was given to each microscopic field viewed at a magnification of 200. A minimum of 10 fields was scored per kidney/remnant kidney, and the mean value was taken to represent the tubulointerstitial score for a given rat.

To express biochemical measurements in relation to the amount of residual renal tissue within remnant kidneys, the amount of tubular tissue was determined using both volume and numerical density methods (27). Determination of tubular cell volume was made using a 25-point lattice projected onto an H & E-stained section. The percent volume of tubule cells was calculated by dividing the number of points overlying tubule cells by the total number of points. Estimation of tubule cell number was made by numerical density calculations. Tubule nuclei were counted on H & E-stained sections in 10 light microscopic fields ( $\times 200$ ) of predetermined area. Calculation of tubule cell number per kidney was made assuming a nuclei diameter of 6  $\mu\text{m}$  and a density of 1  $\text{g}/\text{cm}^3$  in control and diseased tissue.

When determining the amount of tubular tissue remaining (when calculated as a percentage of the control at each time point after nephrectomy), there was no statistical difference between the volume density and numerical density methods. Tubular tissue correction calculations were made using the numerical density values.

### *Measurement of tissue transglutaminase*

*Antigen.* Levels of transglutaminase antigen were determined by immunoprobings of Western blots. Tissue homogenates were separated on a 10% (wt/vol) polyacrylamide denaturing gel using the method of Laemmli (28), and then electroblotted (Immun-Lite PVDF membranes; Biorad Laboratories, Herfordshire, United Kingdom). The Western blot was then probed with a polyclonal goat anti-guinea pig liver transglutaminase (TCS Biologicals, United Kingdom). Primary antibody binding was revealed using an anti-goat alkaline phosphatase (Vector Laboratories Ltd., Peterborough, United Kingdom) and the Immun-Lite chemiluminescent detection system (Biorad) at 25°C.

*Activity.* Transglutaminase activity was measured by the  $\text{Ca}^{2+}$ -dependent incorporation of [1,4- $^{14}\text{C}$ ]putrescine into  $N',N'$ -dimethylcasein as described previously (29). Briefly, tissue homogenates were incubated for 20 min at 37°C with [1,4- $^{14}\text{C}$ ]putrescine (2.5 mM, 3.97 mCi/mmol; Amersham International, Little Chalfont, United Kingdom), dithiothreitol (3.8 mM), calcium chloride (2.5 mM), and dimethylcasein (5 mg/ml). A sample of the homogenate was then spotted onto 3-mm filter paper and precipitated with ice-cold 10% (wt/vol) TCA. After washing, the amount of putrescine incorporation into precipitated protein was determined by scintillation counting. Values were then corrected for DNA content. 1 U of activity is equivalent to 1 nmol of putrescine incorporated per hour at 37°C.

### *Distribution of tissue transglutaminase and $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link*

The distribution of immunoreactive transglutaminase and its cross-link,  $\epsilon$ -( $\gamma$ -glutamyl) lysine present in proteins, was determined by immunohistochemistry and immunofluorescence.

For immunohistochemistry, 4- $\mu\text{m}$  sections were cut from formol calcium-fixed paraffin-embedded tissue. These sections were deparaffinized using xylene, rehydrated, and endogenous peroxidase activity was quenched with 3% (vol/vol) hydrogen peroxide in phosphate-buffered saline (PBS). Antigen was further revealed by proteinase K digestion for 7.5 min (20  $\mu\text{g}/\text{ml}$ ) at room temperature. Sections were then blocked with appropriate blocking serum (filtered 20% [vol/vol] nonimmune serum in PBS for 20 min at room temperature), and the primary antibody (monoclonal anti-guinea pig liver transglutaminase [Cub7402] (30) at 1:200 dilution, polyclonal rabbit anti-rat liver transglutaminase at 1:100 dilution, monoclonal anti- $\epsilon$ -( $\gamma$ -glutamyl) lysine (31) at 1:20 dilution (kind gift of G. Quash and S. El-Alaoui, Laboratoire d'Immunochimie, Faculte de Medecine Lyon-Sud, France)

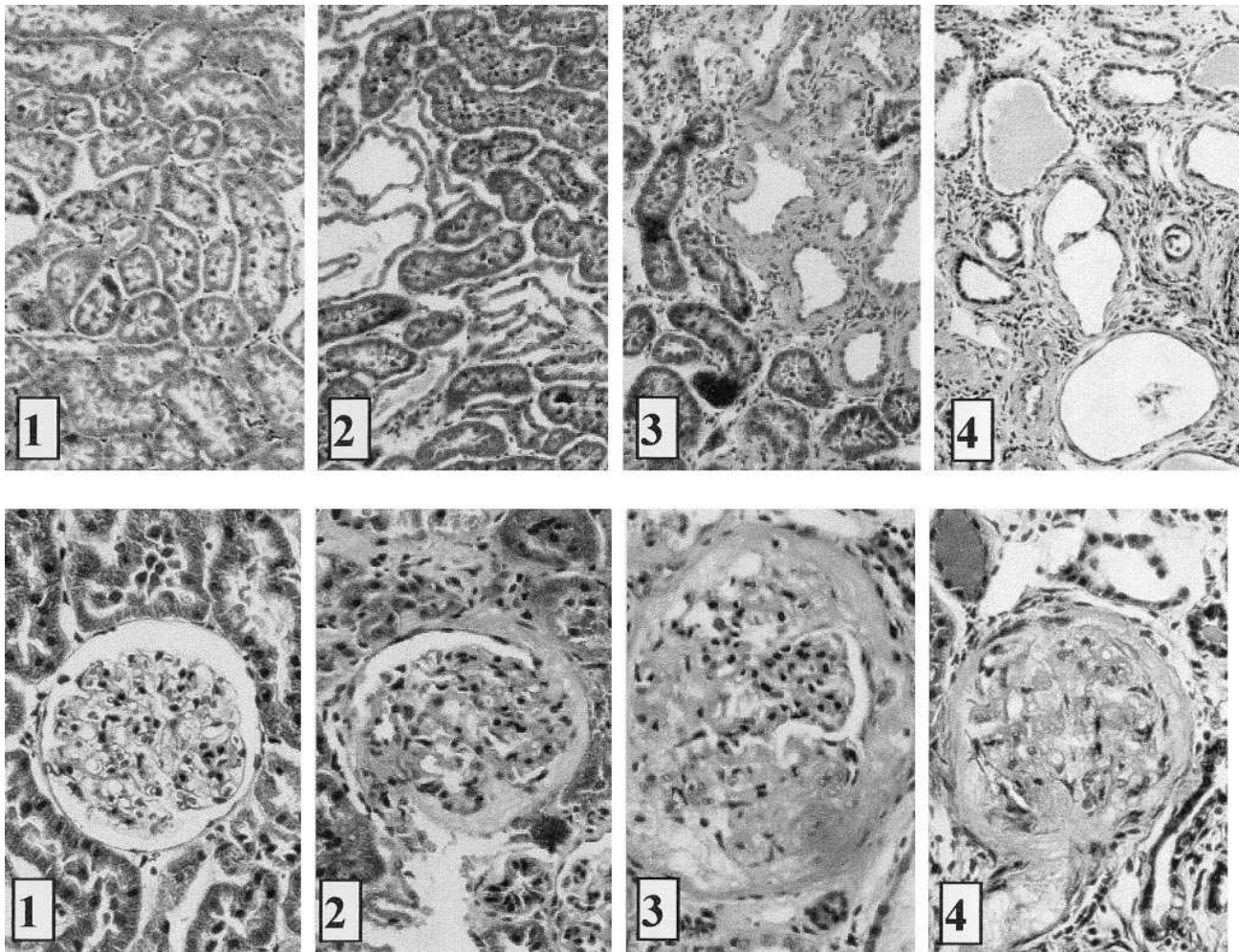


Figure 1. Masson's Trichrome staining of kidney sections demonstrating the scarring index measurements scale from 1 to 4. (Top), Tubule scarring; (bottom), glomerular scarring.

applied overnight at 4°C. The monoclonal anti- $\epsilon$ -( $\gamma$ -glutamyl) lysine antibody (No. 15D<sub>4</sub>H<sub>5</sub>) showed no cross-reactivity to conjugated polyamines, acetylated polyamines, or to acetylated lysine. Primary antibody was revealed using the relevant peroxidase-conjugated secondary antibody (Dako, United Kingdom) at 1:500 dilution and staining for peroxidase activity using 3-amino-9-ethylcarbazole or 3,3'-diaminobenzidine tetrachloride (Sigma Chemical Co., Poole, United Kingdom). Sections were then counterstained with Mayers hematoxylin for 5 min before mounting.

For immunofluorescence studies, 4  $\mu$ m formol calcium-fixed, paraffin-embedded sections were treated as described above (but omitting the quenching step for endogenous peroxidase) using the monoclonal anti-guinea pig liver antibody (Cub 7402) or the anti- $\epsilon$ -( $\gamma$ -glutamyl) lysine antibody (15D<sub>4</sub>H<sub>5</sub>). A fluorescein conjugated anti-mouse secondary antibody (Dako) was then used at a dilution of 1:100. Sections were counterstained in propidium iodide (Sigma) and mounted in Vectorshield (Vector Laboratories).

#### Quantitation of $\epsilon$ -( $\gamma$ -glutamyl) lysine

Transverse sections of kidney of  $\sim$  100 mg wet wt (taken from either above or below the papilla region) were used for the measurement of  $N$ - $\epsilon$ -( $\gamma$ -glutamyl) lysine isopeptide content. After removal of lipids, cell proteins were subjected to sequential exhaustive proteolytic digestion over several days using subtilisin, pronase E, leucine aminopeptidase, prolidase, and carboxypeptidase Y (Sigma). Preparifica-

tion of dipeptide from interfering amino acids was undertaken by cation exchange chromatography (100  $\times$  10 mm Dowex-50  $\times$  4-400) column as previously described (17). Analysis of the  $\epsilon$ -( $\gamma$ -glutamyl) lysine dipeptide was undertaken by cation exchange chromatography using an LKB 4151-amino acid analyzer by a modification of that previously described using lithium citrate buffers (32). Separation of the dipeptide was achieved on a stainless steel 5  $\times$  250 mm column containing Ultrapac 8 cation exchange resin ( $8 \pm 0.5$   $\mu$ m-particle size; Pharmacia LKB Biotechnology, Hertfordshire, United Kingdom) using a constant temperature of 25°C and a stepwise gradient of increasing molarity and changing pH of lithium citrate buffers as described previously (32). The program of elution involved 9 min of 0.2 M lithium citrate buffer, pH 2.8, 23 min of 0.3 M lithium citrate buffer, pH 3.0, and 63.5 min of 0.6 M lithium citrate buffer, pH 2.6, at a flow rate of 25 ml/h. Detection of the dipeptide which eluted around 88 min was achieved using postcolumn derivatization with  $\sigma$ -phthalaldehyde/2, $\beta$ -mercaptoethanol at a flow rate of 20 ml/h which was analyzed in a fluorescent detector (LS1, Perkin-Elmer Corp., Beaconsfield, UK) (340 nm excitation, 450 nm emission). Data acquisition was by means of an interface box (900 series; Nelson Analytical, Cupertino, CA). Chromatograms were stored, compared, and analyzed using an IBM compatible computer and software (model 2600, version 5; Nelson Analytical). The amount of  $\epsilon$ -( $\gamma$ -glutamyl) lysine in each sample was quantified by standard addition of  $\epsilon$ -( $\gamma$ -glutamyl) lysine dipeptide as previously described (32).  $\epsilon$ -( $\gamma$ -glutamyl) lysine is ex-

Table I. Serum Creatinine, Proteinuria, Systolic Blood Pressure, Body Weight, Scarring Index, and Percentage of Control Tubule Mass at Death (Mean±SEM) in Control, Hypertensive, and SNx Animals (Time Course)

Days after SNx	Body weight	Serum creatinine	Systolic blood pressure	Proteinuria	Scarring index	% Normal tubule mass remaining
	g	μmol/liter	mmHg	mg/24 h		
7 d						
SNx (n = 6)	294.18±6.12	74.2±5.67 <sup>‡</sup>	150.4±7.31*	49.8±3.36*	1.53±0.08 (T) 1.55±0.08 (G)	86
Sham (n = 4)	(343.1±14.85)	(32.67±2.44)	(139.2±0.74)	(26.5±1.05)	—	—
30 d						
SNx (n = 6)	332±13.06	79.35±6.77 <sup>‡</sup>	164.6±4.43*	93.9±5.73 <sup>‡</sup>	2.63±0.13 (T) 2.48±0.12 (G)	52
Sham (n = 4)	—	—	—	—	—	—
90 d						
SNx (n = 6)	404.5±26.25	89.71±5.51 <sup>‡</sup>	176.17±2.91*	256.2±6.45 <sup>‡</sup>	3.22±0.36 (T) 3.29±0.25 (G)	31
Sham (n = 4)	(440±16.60)	(32.11±1.18)	(142.7±1.43)	(36.7±4.60)	—	—
SHR (n = 4)	404.5±7.60	33.8±2.1	189±5.9	49.8±3.35	—	96
120 d						
SNx (n = 6)	390.7±21.55	308.7±20.21 <sup>‡</sup>	175±2.88*	194±7.48 <sup>‡</sup>	3.07±0.38 (T) 3.09±0.37 (G)	42
Sham (n = 4)	(500±28.40)	(33.4±3.1)	(150.4±8.96)	(27.84±2.15)	—	—
SHR (n = 4)	464±9.17	31.75±1.70	195±7.07 <sup>‡</sup>	56.4±6.15	—	94

The numbers in parentheses refer to sham-operated animals. T, tubular; G, glomerular. \* $P \leq 0.05$  compared to respective sham; <sup>‡</sup> $P \leq 0.01$  compared to respective sham.

pressed as nanomoles per gram of protein. Sample protein was determined both before and after the subtilisin digestion using the DC protein assay system (Biorad) as per manufacturer's instructions.

#### Detection of apoptosis

This relied on in situ end-labeling using a modification of the ApopTag™ system (Oncor Inc., Gaithersburg, MD). Tissue was fixed in formal calcium overnight, and was embedded in paraffin. 5-μm sections were deparaffinized using xylene, rehydrated, and the tissue was digested with 20 μg/ml proteinase K for 15 min at room temperature. Endogenous peroxidase activity was quenched with 2% (vol/vol) hydrogen peroxide for 5 min. Application of terminal transferase enzyme in equilibration buffer containing dUTP labeled with digoxigenin, and binding of the anti-digoxigenin peroxidase-conjugated antibody was performed as per manufacturer's instructions. 3-amino-9-ethylcarbazole substrate was applied to reveal end-labeling sites, and was incubated at room temperature for up to 30 min. The section was then counterstained with Mayers hematoxylin for 5 min at room temperature.

This technique provides a method of identifying cells undergoing apoptosis by the ability of the terminal transferase enzyme to attach digoxigenin-labeled nucleotides to chromatin fragmented in the early stages of the apoptotic program.

#### Statistical analysis

Results are given as mean±standard error of the mean (M±SEM). Differences between groups were determined by one-way analysis of variance (ANOVA). Correlations between variables were determined by linear regression analysis. Probability of < 5% was taken as significant.

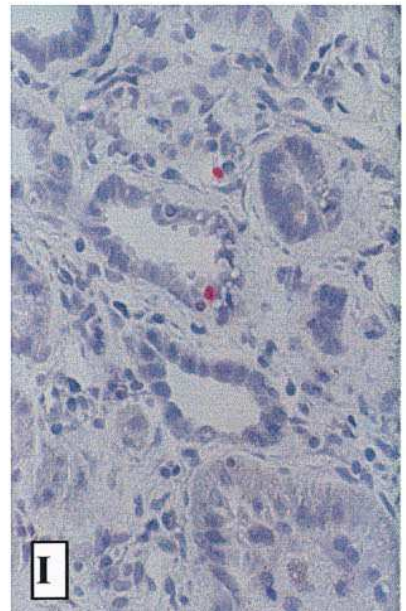
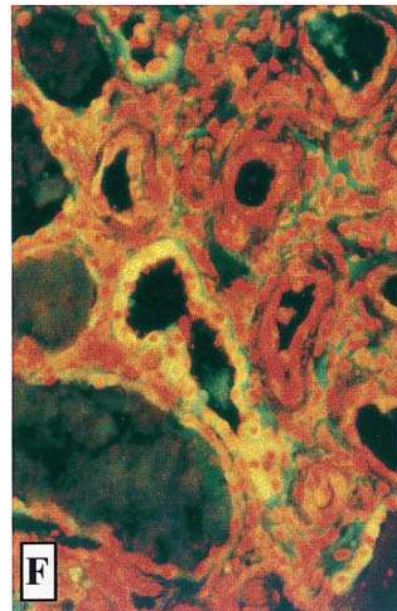
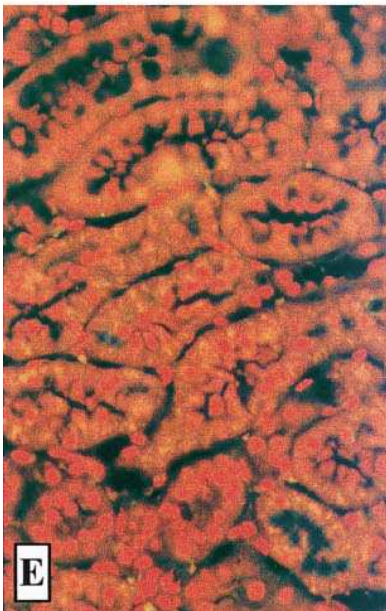
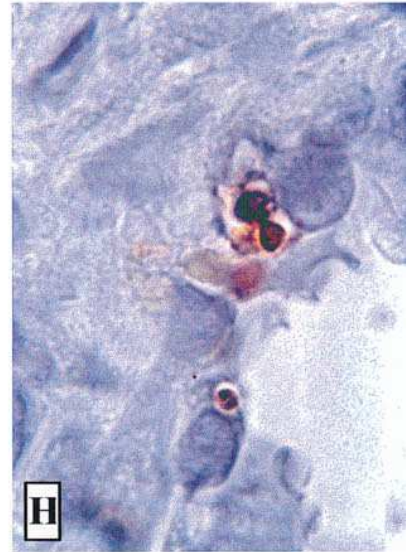
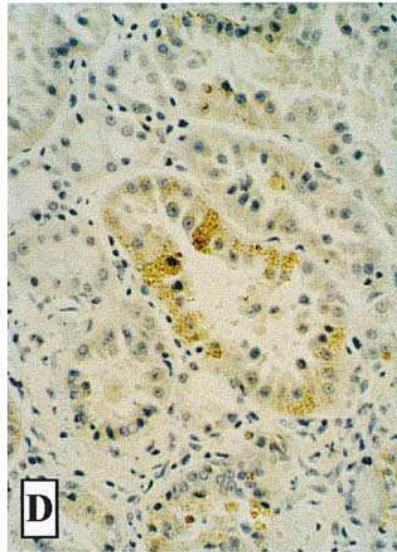
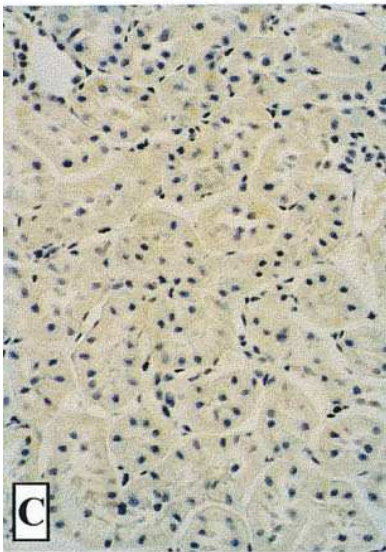
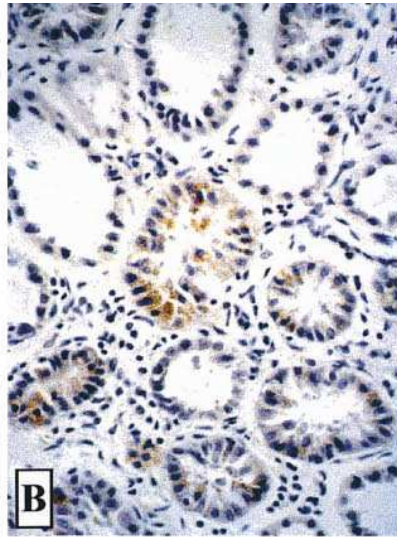
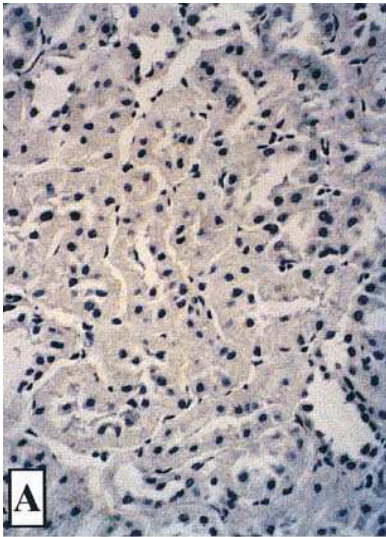
## Results

**General observations.** SNx rats demonstrated a steady increase in proteinuria and serum creatinine with time (Table I), indi-

cating progressive renal insufficiency. Systolic blood pressure was significantly raised in SNx rats from day 7 onward, and was comparable on days 90 and 120 to that of SHRs (Table I). All rats survived the subtotal nephrectomy and the experimental time course. The level of kidney fibrosis rose steadily throughout the experimental time course, showing severe scarring (Fig. 1, 3 and 4) by day 90 after SNx. Tubular and glomerular scarring indices were comparable at each time point ( $r = 0.97$ ,  $P \leq 0.01$ ). While proteinuria showed a strong correlation with overall renal scarring ( $r = 0.79$ ,  $P \leq 0.01$ ), the reciprocal of serum creatinine demonstrated a weak correlation with scarring ( $r = 0.49$ ,  $P \leq 0.05$ ).

**Immunohistochemistry.** In control kidneys, the immunohistochemistry revealed a very low background staining for both transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link that was predominantly intracellular, and in the main restricted to tubule cells. In SNx kidneys, there was a visible rise in both transglutaminase and its product throughout the time course. This rise was almost exclusively intracellular, and was confined to the tubule cells. The staining for both of these reached a maximum between days 90 and 120 (Fig. 2, A–D). In most cases, cells that appeared to give intense staining for  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link still demonstrated intact nuclei. A quantitative analysis of these changes over the time course of the experiment is described in the biochemical assessment of transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link.

Although the immunohistochemistry demonstrated a primarily intracellular location for both transglutaminase and the  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link, a low level of stain for cross-link was occasionally observed in the interstitial space surrounding the tubules in late-stage SNx animals (data not shown) that was not found in controls. The more sensitive immunofluores-



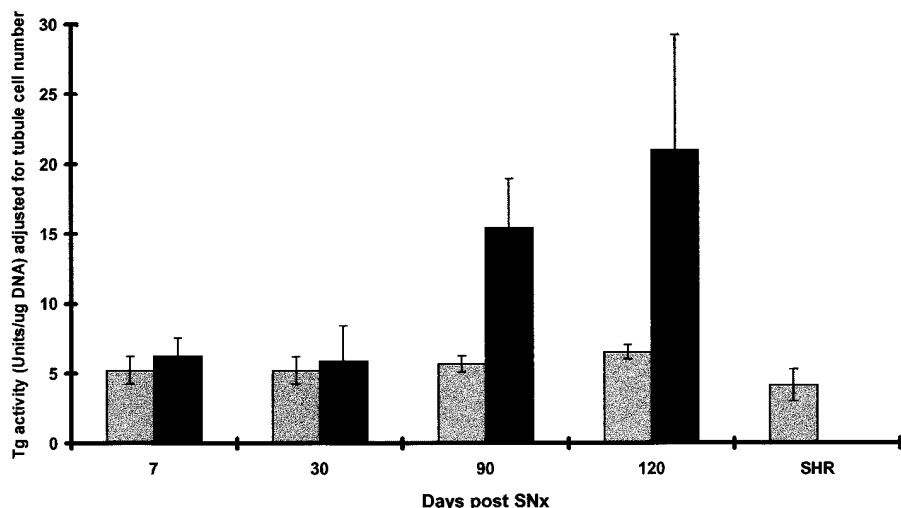


Figure 3. Transglutaminase activity measurement from control/hypertensive (SHR) (shaded bars), and SNx (black bars) animals as determined by a [<sup>14</sup>C]putrescine incorporation into N',N'-dimethyl casein assay. Data represent mean transglutaminase activity (U/ $\mu$ g DNA) adjusted for tubule cell number  $\pm$  SEM from four or more animals.

cence, while still demonstrating the majority of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link in SNx tissue to be intracellular, clearly shows  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link associated with the ECM in the peritubular fibrotic lesions (Fig. 2, E and F).

There was also a strong staining for both transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link in what appeared to be cellular fragments found in a number of tubule lumen in heavily scarred kidneys. These detached fragments displayed a morphology that could be described as similar to an apoptotic body (Fig. 2, B and G).

**Transglutaminase levels.** Tissue levels of transglutaminase as measured by activity (data not shown) initially showed a slight, but not significant, decrease in transglutaminase levels when normalized for either protein or DNA in the nephrectomized animals compared to controls. When this decrease was corrected for the number of surviving tubule cells (that appeared to be the prime transglutaminase-expressing cells as shown by immunohistochemistry) (Table I), then a threefold increase was seen at 90 d ( $P \leq 0.05$ ), rising to fivefold by day 120 ( $P \leq 0.01$ ) (Fig. 3). Transglutaminase antigen levels determined by Western blot analysis of tissue homogenates (Fig. 4) also showed an increase when adjusted for tubule cell number. This increase demonstrated a fivefold increase by day 90 after nephrectomy ( $P \leq 0.01$ ), rising to eightfold by day 120 ( $P \leq 0.01$ ) (Fig. 5). A weak positive correlation was noted between tubule-adjusted tissue transglutaminase levels and the severity of tubulointerstitial fibrosis ( $r = 0.46$ ,  $P = 0.08$ ).

**Estimation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine.** The level of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link in control animals (as assessed by cation exchange chromatography of proteolytically digested samples) remained constant throughout the experimental period, whereas a fivefold increase in tissue levels was detected by day 90 in the SNx animals ( $3.47 \pm 0.94$  to  $13.24 \pm 1.43$  nmol/g protein,  $P \leq 0.01$ ). When these figures are corrected for the num-

ber of tubule cells within the tissue, then a 29-fold increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link is observed in the SNx animals compared to controls at 120 d ( $P \leq 0.01$ ) (Fig. 6). This increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine is in agreement with the findings from immunohistochemistry, demonstrating a good correlation between transglutaminase antigen levels and its  $\epsilon$ -( $\gamma$ -glutamyl) lysine product ( $r = 0.76$ ,  $P \leq 0.025$ ). Furthermore, a good correlation was observed between tubulointerstitial scarring and levels of tubule cell number adjusted  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links at each time point in the experimental series (Fig. 7), with a strong correlation between individual animal levels of tubule cell number-adjusted  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links and the severity of tubulointerstitial scarring (Fig. 8,  $r = 0.86$ ,  $P \leq 0.01$ ). In addition, the glomerular scarring index also showed a high correlation with levels of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link ( $r = 0.89$ ,  $P \leq 0.01$ ).

**In situ end-labeling as a measure of cellular apoptosis.** In situ end-labeling (ApopTag<sup>TM</sup>) was used as an assessment of the degree of apoptosis in this study. Fig. 2 H shows a typical ApopTag<sup>TM</sup>-staining of a cell in apoptosis. This cell demonstrates condensed chromatin that is being pinched off into an apoptotic body, while a neighboring cell is phagocytosing an apoptotic body. Visual comparison of a severely scarred kidney stained for either transglutaminase or  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link (Fig. 2, B and D) with in situ end-labeling (Fig. 2 I) demonstrates that cells showing DNA cleavage tend to be discrete cells within a few tubules, whereas increased transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links typically affect the entire tubule, with the majority of tubular cells within a damaged area showing staining. Fig. 2 clearly demonstrates the large differences between cells expressing DNA cleavage and transglutaminase in typical fields from scarred kidney. It cannot, however, be ruled out that cells showing increased levels of intracellular  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links have under-

Figure 2. Representative sections from normal and scarred kidneys at day 90 after SNx. A, B, and G are stained for tissue transglutaminase, C and D are stained for  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link using immunohistochemistry. E and F are stained for  $\epsilon$ -( $\gamma$ -glutamyl) lysine using an immunofluorescence stain. A, C, and E are sections from control kidneys, while B, D, and F are from scarred kidneys. H and I are sections from a scarred kidney that has been in situ end-labeled with a digoxigenin-labeled nucleotide (apoptosis).

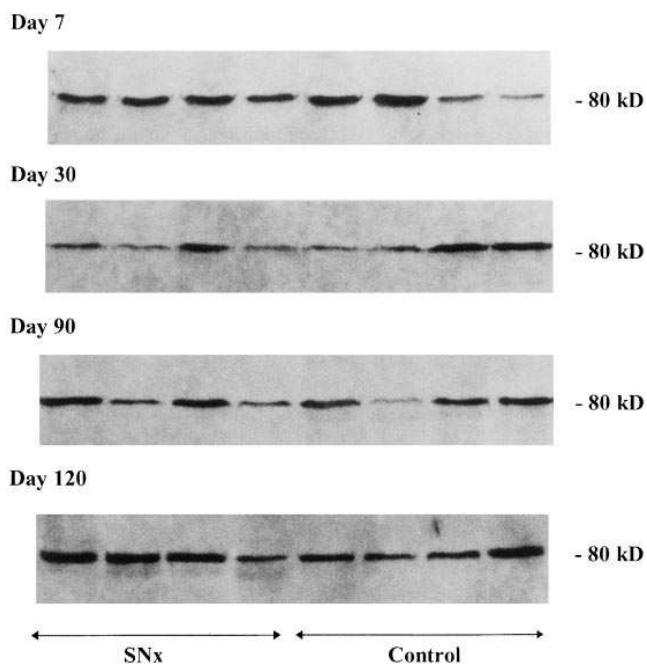


Figure 4. Western blot of kidney homogenates from control and SNx animals immunoprobed with a polyclonal anti-guinea pig liver transglutaminase as described in Methods.

gone cleavage of their DNA into large fragments (33) which would not be detected by the techniques used in this study.

## Discussion

In this study, we have investigated tissue transglutaminase protein levels in experimental renal fibrosis by both activity and antigen measurements, and have determined the enzyme product, the  $\epsilon$ -( $\gamma$ -glutamyl) lysine dipeptide cross-link. We have also localized both transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine by immunohistochemistry and immunofluorescence. In addition, as transglutaminase is one of the few known effector elements of apoptosis, or programmed cell death, we have in-

vestigated if changes in transglutaminase expression are a consequence of variations in apoptosis as detected by in situ end-labeling techniques.

The data presented clearly show an increase in transglutaminase-mediated protein cross-linking with renal disease progression. This increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link detected both immunohistochemically and by cation exchange chromatography of digested samples, indicates that the tissue transglutaminase observed predominantly in renal tubules is functionally active. The in situ end-labeling data coupled with light microscopy morphology demonstrate that the increase in both the enzyme and its product is not a consequence of the induction of apoptosis. Cleavage of DNA into larger fragments, (33) however, or activation of interleukin-converting enzymes (ICE) like serine proteases (34) cannot be ruled out since immunohistochemical methods to detect this type of damage are still unavailable. Immunohistochemical investigations revealed that the increases in transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine were seen exclusively in tubule cells, and that this increase was predominantly intracellular. Immunofluorescence, however, showed that detectable levels of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links were also present in the peritubular extracellular matrix in remnant kidneys, although these levels were usually in the late stages of the disease process. Although the increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links is most likely to result from the increases in tissue transglutaminase, it cannot be ruled out that such an increase in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links may also be because of the activation of Factor XIIIa during the influx of plasma into the damaged areas.

We noted a strong correlation between tubulointerstitial scarring and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link levels suggesting a causal association. The higher correlation shown between renal scarring and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link levels when compared to the correlation of renal scarring with tissue transglutaminase levels is not surprising, as a small transient rise in tissue transglutaminase expression or activation of the residual transglutaminase present in tubular cells would be expected to lead to large and cumulative increases in  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link products over several months.

Increased  $\epsilon$ -( $\gamma$ -glutamyl) lysine levels correlate well with both tubulo-interstitial and glomerular scarring, yet we can only detect raised transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine

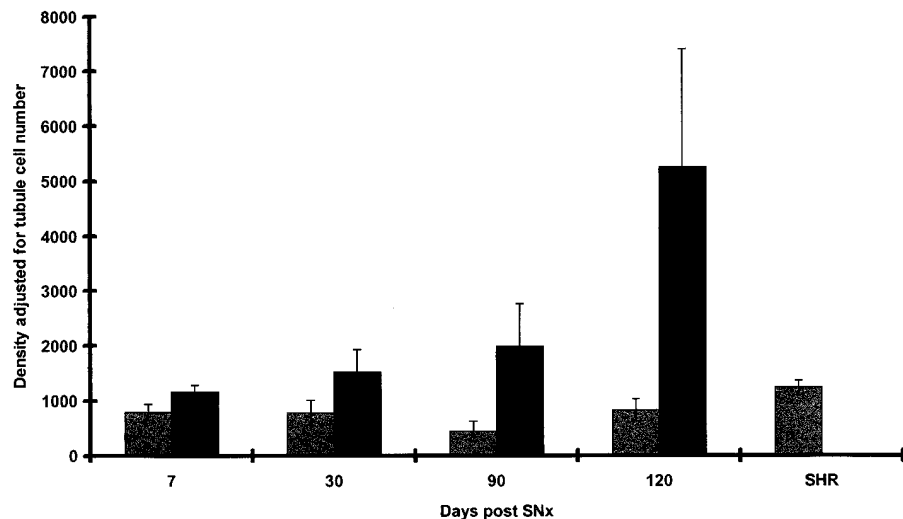


Figure 5. Densitometric analysis of the Western blot in Fig. 3 showing control/hypertensive (SHR) animals (shaded bars) and SNx animals (black bars). Data represent mean density (arbitrary units) adjusted for tubule cell number  $\pm$  SEM from four animals.



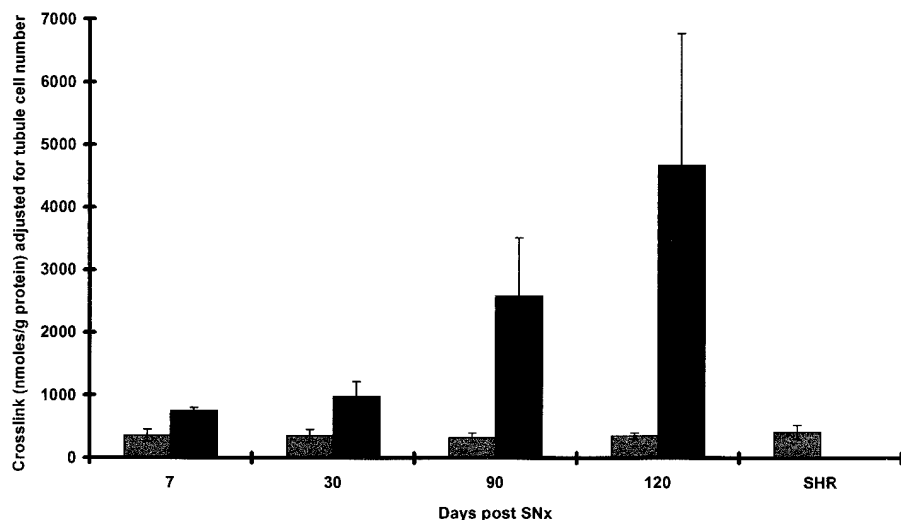


Figure 6.  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link measurements adjusted for surviving tubule cell number for control/hypertensive (SHR) (shaded bars) and SNx animals (black bars) as determined by exhaustive proteolytic digestion and cation exchange chromatography as described in Methods. Data represent mean cross-link values (nmol/g protein)  $\pm$  SEM from four or more animals.

in tubule cells. This observation lends itself to the inference that tissue transglutaminase may not play a role in glomerular scarring, which is not unusual since the mechanisms of scarring of the two structures are diverse, and tubular cells (unlike those of the glomeruli) are rich sources of enzymes. The close correlation between glomerular and tubular scarring, however, does not preclude causal interactions whereby the transglutaminase-mediated cross-linking of tubular cells, and the associated tubulointerstitial fibrosis, may contribute to the sclerosis of the glomeruli (35). Finally, it cannot be excluded that there is a failure to attach or inaccessibility of antibodies to the insoluble material of the glomerular basement membrane. For instance, tissue transglutaminase's tight association with fibronectin (36), and the fact that tissue transglutaminase is also a substrate for its own catalytic action (which results in the enzyme being cross-linked into the matrix), makes immunochemical antigen detection difficult.

Our data indicate a good degree of correlation between in-

creased tissue transglutaminase expression and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link. Although we could not demonstrate that the increased enzyme levels precede the accumulation of its cross-link product, the specificity of the transglutaminase enzymatic reaction suggests a causal association between the enzyme activity and its product. The lack of a strong correlation between renal transglutaminase content and scarring is to be expected, as small effective enzyme changes over prolonged periods can lead to disproportionate increases in the nondegradable  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link product. Thus, correlation between the product and not enzyme levels provides a more accurate determination of the enzyme's effects.

Most hypotheses relating to the pathogenesis of renal fibrosis have focused on the increased synthesis of the ECM (1, 2) or its decreased breakdown (3, 4). Little attention has been paid to qualitative changes in renal ECM preventing its breakdown. If the ECM was deposited in such a way as to reduce the ability of proteases to degrade it, possibly by covalent stabi-

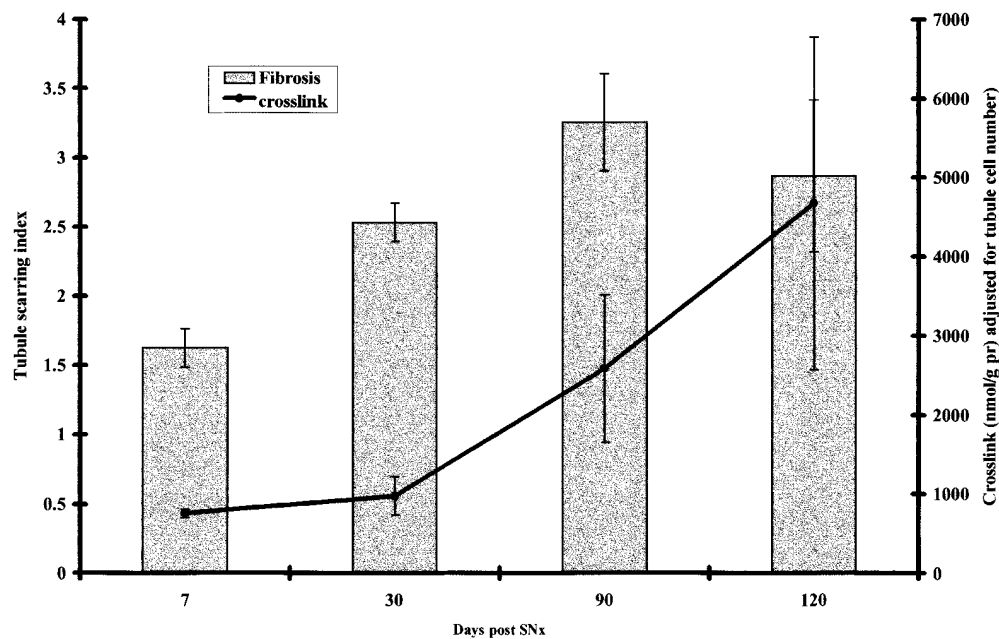


Figure 7. Level of renal scarring in SNx animals as determined by pathological morphology assessment compared with SNx  $\epsilon$ -( $\gamma$ -glutamyl) lysine levels. Data represent mean values  $\pm$  SEM from four or more animals.

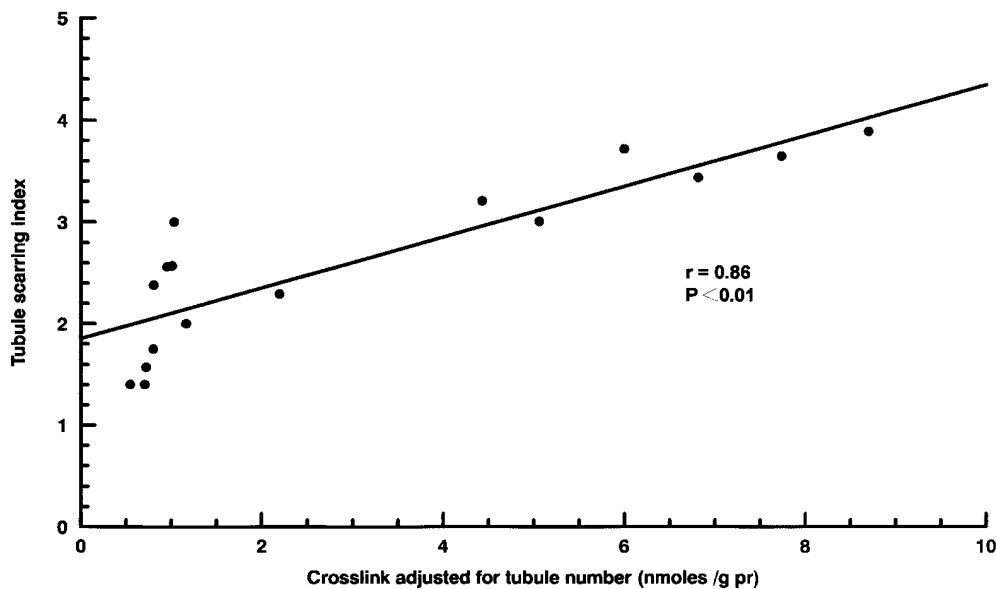


Figure 8. Linear regression correlation of cross-link data with tubule scarring index.  $r = 0.86$ ,  $P \leq 0.01$ .

zation of the fibrils by incorporation of  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-links (22) that could potentially inhibit the action of specific collagenases required before general endopeptidase degradation (37), then this stabilization would move the deposition-degradation balance towards accumulation and fibrosis. This mechanism may be the case if ECM polymers were formed with a high proportion of bonds resistant to proteolytic degradation, such as  $\epsilon$ -( $\gamma$ -glutamyl) lysine bonds formed by transglutaminase (9). Such a mechanism has been postulated to explain the fibrotic process taking place within the walls of atherosclerotic vessels where the cross-linking of matrix proteins is increased (6–8). The process also is similar to the cross-linking of fibrin by Factor XIII, which makes the cross-linked fibrin much more resistant to plasmin. The presence of transglutaminase and its product in scarred renal tubules would therefore support this hypothesis. To explain the predominantly intracellular localization of the tissue transglutaminase enzyme to the contrasting dual location (intracellular/extracellular) of its cross-linked product, it is tempting to speculate that these enzymes cross-link the ECM intracellularly, as it is produced by tubular cells before its extracellular deposition. It cannot be excluded, however, that small amounts of transglutaminase, undetectable by immunohistochemistry, are released from the basolateral aspect of tubular cells in response to tissue injury, and contribute to the extracellular cross-linking of ECM proteins. Although tissue transglutaminase is essentially an intracellular enzyme (it has no leader sequence or evidence for protein glycosylation), there is good evidence to indicate transglutaminase activity in the extracellular environment (38) where its involvement in matrix assembly and basement membrane stabilization is well described (7, 21, 22, 39, 40). The mechanism of its release from cells into the extracellular environment, however, is still to be elucidated.

The association between tubular tissue transglutaminase and renal fibrosis may also involve transforming growth factor- $\beta$  (TGF- $\beta$ ). Transglutaminase has been shown to play a key role in the activation of latent TGF- $\beta$ 1 (38), while TGF- $\beta$ 1 can in turn lead to de novo synthesis of tissue transglutaminase (41).

TGF- $\beta$ 1 is one of the major fibrogenic growth factors within the kidney, and has been implicated in its fibrosis (42). In that respect, it is interesting to observe that the tubular distribution of transglutaminase corresponds to that reported for TGF- $\beta$  within scarred kidneys (43, 44). Another factor which may facilitate transglutaminase-induced cross-linking in scarred kidneys is the known increase in its putrescine substrate (used in formation of  $N',N'$ -bis[ $\gamma$ -glutamyl]polyamine linkage) within these kidneys from exogenous sources (45). Thus, the scarred kidney, and in particular its tubular cells, would provide the optimal environment for the action of tissue transglutaminase and the generation of its cross-linked products.

Apoptosis is known to take place within remnant kidneys, and seems to predominate within tubular cells (46). Tissue transglutaminase is a known effector enzyme of the cellular apoptotic process (6, 11). The activation of cellular transglutaminase is thought to lead to the assembly of highly cross-linked protein shells (apoptotic envelopes) within the dying cell, which temporarily stabilizes the cell's integrity before clearance by phagocytosis, and shedding into body cavities (17, 18). It was therefore important to determine whether the tubular transglutaminase is associated with apoptotic cells. Our initial data indicate that there were significantly more tubular cells expressing tissue transglutaminase and increased  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link levels when compared to those showing signs of classical apoptosis as determined by in situ end-labeling, although it is important to stress the limitations of detecting apoptosis by this technique. It should be noted, however, that many cells expressing transglutaminase seem to have normal-looking nuclei. What we are observing seems to be a situation where there is a high intracellular tubular tissue transglutaminase and  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link content in the absence of the associated DNA cleavage (detectable by in situ end-labeling) characteristic of apoptosis or well advanced necrosis.

This tubular response to injury, involving transglutaminase-mediated cytoplasmic peptide cross-linking, may be an attempt to stabilize the injured tubules and maintain structural integrity (47–51) before ECM deposition. Such a function

would also be comparable to the Factor XIIIa (the plasma transglutaminase) during wound healing (13, 52). Furthermore, excessive levels of protein cross-linking within a cell are likely to influence cell function, and consequently viability. Therefore, it would appear from our data that we are potentially observing a transglutaminase-induced/mediated cell death in these tubule cells, which may be similar to that taking place in the formation of terminally differentiated keratinocytes by keratinocyte transglutaminase (15). Furthermore, tubular structural and morphological changes similar to the ones we observed in cross-linked tubular cells have been associated with the loss of renal function (53). It may be that this mechanism of tubular cell cross-linking is another contributor to the loss of tubular cell mass in renal scarring along with necrosis and apoptosis.

At the present stage, we can only speculate as to the underlying biochemistry of the progression of renal scarring and the role of transglutaminase in the fibrotic event, although it is clear that there are perturbations of transglutaminase and its major product  $\epsilon$ -( $\gamma$ -glutamyl) lysine cross-link in this disease. The observation that these perturbations occur in other fibrotic lesions (5–8) would suggest an important role for this enzyme in this disease. Further studies are necessary, however, using specific transglutaminase inhibitors, to gain a clear insight as to how transglutaminase is involved in the fibrotic process.

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