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Transglutaminase Inhibition Reduces Fibrosis and Preserves Function in Experimental Chronic Kidney Disease

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ABSTRACT

Progressive tissue fibrosis is involved in debilitating diseases that affect organs including the lungs, liver, heart, skin, and kidneys. Recent evidence suggests that tissue transglutaminase, an enzyme that crosslinks proteins, may be involved in tissue fibrosis by crosslinking and stabilizing the extracellular matrix or by recruiting and activating the large latent transforming growth factor (TGF)-β1 complex. We treated rats that had undergone 5/6-nephrectomy with two different irreversible inhibitors of transglutaminase and found that both prevented a decline in kidney function and reduced the development of glomerulosclerosis and tubulointerstitial fibrosis by up to 77% and 92%, respectively. Treatment reduced the accumulation of collagen I and collagen III, with the primary mechanism of action being direct interference with the crosslinking of extracellular matrix rather than altered regulation of TGFβ1. We conclude that inhibition of transglutaminase offers a potential therapeutic option for chronic kidney disease and other conditions that result from tissue fibrosis.

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Tissue remodeling leading to scarring and fibrosis is associated with a number of debilitating diseases affecting major organs. Pathologic changes that are associated with scarring are often highly conserved between organs despite varying initial stimuli. The proliferation of fibroblasts and overproduction/accumulation of extracellular matrix (ECM) proteins is well characterized, with an array of growth factors and cytokines implicated in driving the disease; however, despite an extensive knowledge of the cellular and biochemical changes, there remains no effective therapy.

One enzyme that increasingly is associated with ECM is the protein–cross-linking enzyme tissue transglutaminase (TG2). TG2 is a member of the transglutaminase family of enzymes that have the capacity to cross-link proteins irreversibly through $\varepsilon(\gamma$-glutamyl) lysine iso-peptide bonds.[1] Although changes in TG2 have been described in scarring of the lung,[2] liver,[3] and heart[4] as well as in atherosclerosis,[5] the enzyme has been most extensively characterized in the kidney, where a persistent upregulation and cellular release of TG2 occurs in animal models and human biopsies.[6,7] In human kidney disease, there is strong correlation between levels of TG2 ($R^2 = 0.92$), $\varepsilon(\gamma$-glutamyl) lysine iso-peptide

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crosslinking ($R^2 = 0.86$) and the development of tissue scarring in all diseases investigated.[6]

Chronic kidney disease (CKD) affects up to 10% of the population[8] and contributes considerably to their morbidity and mortality. The progressive nature of CKD is associated with a relentless loss of renal tissue and its replacement by ECM, culminating in organ fibrosis and failure. Evidence suggests that TG2 has a multifunctional role in the progression of disease.[7,9] TG2 is trafficked to the extracellular environment, where the high Ca^{2+} levels result in activation of TG2,[10] leading to ECM cross-linking. This cross-linking can facilitate inappropriate deposition of ECM proteins[11,12] and confer resistance to the action of matrix metalloproteinases.[9] High intracellular levels of TG2 have also been associated with cell death by the cross-linking of cytoplasmic proteins after cell trauma and loss of Ca^{2+} homeostasis, possibly acting as an alternative to apoptosis in limiting necrosis.[13,14] This role of TG2 is thought to be independent of its proposed roles in apoptosis.[15] Cell deletion and tubular atrophy are major components of kidney scarring and contribute to progressive kidney insufficiency.[16] TG2 also has a key role in the matrix storage and activation of TGF-β1, through the cross-linking of the large latent TGF-β1–binding protein to the ECM.[17,18] It is interesting that TGF-β1 has also been shown to upregulate TG2 transcription.[19,20] Matrix-associated TG2 may also have a cell adhesion/migration role independent of its transamidating activity that is important in the wound response.[14,21] Despite this accumulating evidence to implicate TG2 as a key enzyme in the scarring process, a causative role for the enzyme still has not been established.

Here we applied two specific site-directed, irreversible inhibitors of TG by direct intrarenal delivery to the five sixths subtotal nephrectomy (SNx) model of progressive kidney scarring and fibrosis with the aim of determining whether TG inhibition can reduce scarring and preserve kidney architecture and function. For ascertainment of whether total or predominantly extracellular inhibition of the enzyme leads to comparable outcomes, compounds with different characteristics were chosen. 1,Dimethyl-2[(oxopropyl)thio]imidazolium (NTU283), originally synthesized as a factor XIIIa inhibitor,[22,23] is highly cell soluble and readily enters the intracellular compartment. The other compound, N-benzyloxycarbonyl-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine (NTU281), has poor transfer across the cell membrane, thereby limiting its action to the extracellular compartment.[24] We demonstrate that TG inhibition when applied from the onset of disease is effective at preventing tissue scarring and maintaining kidney function with beneficial effects through direct ECM modification.

RESULTS

General Observations

Rats that were treated with TG inhibitors showed no obvious phenotypic changes. Measurement of heart and liver enzymes demonstrated no deleterious effect of TG inhibition during the experimental period. Blood clot stability as measured by solubility in 5 M urea indicated that fibrin cross-linking was not affected. Rats that underwent SNx and were treated with TG inhibitors gained body weight (Table 1) at a slightly greater rate than untreated rats, but this achieved significance only for NTU281 at day 84 and was less than that in normal controls. Compensatory renal growth after SNx was not significantly altered by either inhibitor, although the mean values were less with both inhibitors (Table 1).

TG activity increased in SNx kidneys during the 84 d (Figure 1A). Inhibitor NTU283 decreased activity levels to below that of control rats ($P \leq 0.05$) at 7 d after SNx and to control levels by 28 d. At 84 d, activity in NTU283-treated rats rose to just under half that of untreated SNx rats (Figure 1A).

NTU281 has limited cell membrane permeability; therefore, its inhibitory activity is more accurately measured using a TG *in situ* activity assay optimized for measuring extracellular TG activity.[9] Extracellular TG activity was four-fold greater in remnant kidneys of SNx rats when compared with controls (Figure 1B). NTU281 prevented any increase in extracellular TG activity until 28 d and reduced the elevated level in SNx rats at 84 d by >60%. Both inhibitors were highly effective in preventing the seven-fold increase in the TG-mediated $\varepsilon(\gamma$-glutamyl) lysine cross-link associated with kidney scarring (Figure 1C). Importantly $\varepsilon(\gamma$-glutamyl) lysine cross-link levels were not significantly higher than those in control rats with either inhibitor at 84 d.

Kidney Scarring and Function

By 84 d after surgery, all rats that had undergone SNx had developed significant glomerulosclerosis (Figures 2 and 3A) associated with advanced tubulointerstitial scarring (Figures 2 and 3B) as assessed by Masson's trichrome staining. There was considerable flattening of the tubular epithelium, leading to significant tubular atrophy (Figure 2). Rats receiving TG in-

Table 1. Terminal body and kidney weight in TG inhibitor–treated SNx rats[a]

Parameter	Terminal Body Weight/g			Terminal Kidney Weight/g		
	7 D	28 D	84 D	7 D	28 D	84 D
Control	320 ± 14	349 ± 17	531 ± 7	1.07 ± 0.07	1.08 ± 0.01	1.37 ± 0.03
SNx	313 ± 11	354 ± 18	433 ± 25[b,c]	1.51 ± 0.16[b]	1.61 ± 0.09[b]	3.22 ± 0.22[b]
SNx + 281	290 ± 20	337 ± 6	488 ± 9[c]	1.54 ± 0.03[b]	1.88 ± 0.20[b]	2.94 ± 0.13[b]
SNx + 283	287 ± 17	320 ± 24	449 ± 20[b]	1.47 ± 0.09[b]	1.79 ± 0.08[b]	3.07 ± 0.29[b]

[a]Data are mean body and left kidney weights (g) ± SEM at 7, 28, and 84 d after SNx; $n = 5$ to 6 rats for each experimental group per time point. $P < 0.05$, [b]sham and [c]SNx.

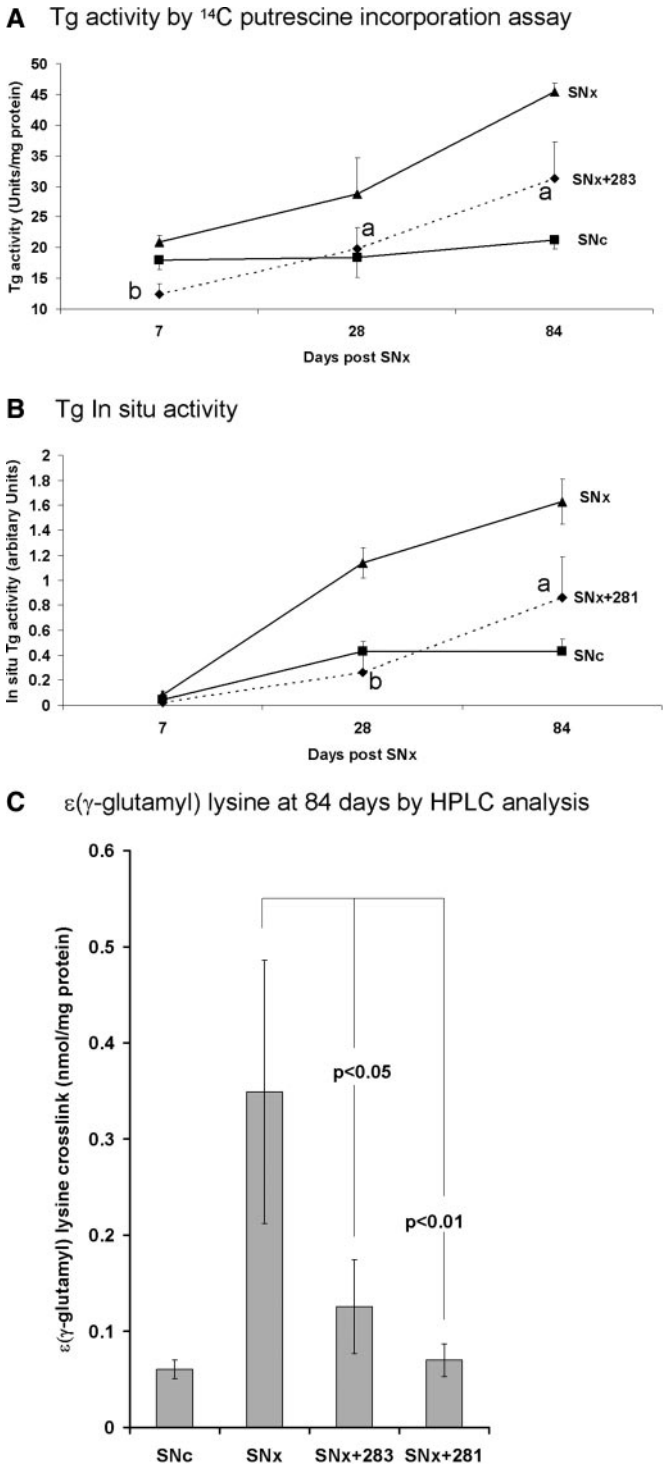

Figure 1. Effective TG inhibition. TG activity after SNx was assessed in a renal homogenate using the ^{14}C Putrescine incorporation assay for NTU283 (A) and an *in situ* activity assay on cryostat section for NTU281 (B). This was confirmed by measurement of TG catalyzed ε(γ-glutamyl) lysine cross-link levels at 84 d after SNx (C). Data are means ± SEM; $n = 5$ to 6 rats for each experimental group per time point, with assays performed in duplicate on three separate occasions. $^{a}P < 0.05$; $^{b}P < 0.01$.

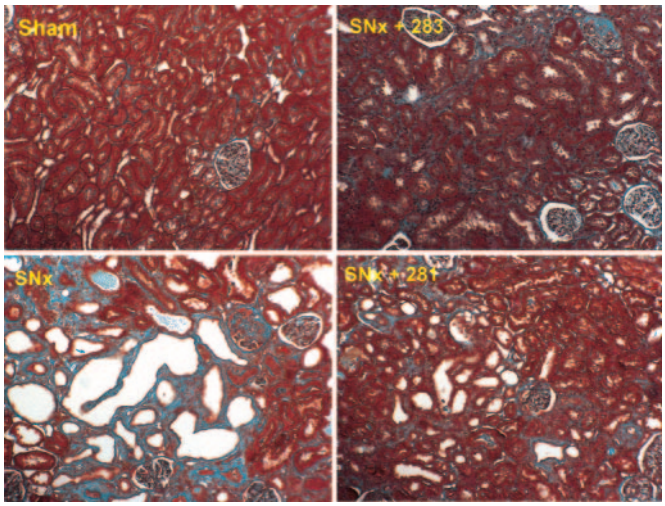

Figure 2. Effect of TG inhibition on kidney scarring at 84 d after SNx. Representative Masson's trichrome–stained kidney sections from sham rats, SNx rats, SNx rats that were treated with NTU281, and SNx rats that were treated with NTU283 at 84 d after SNx. Magnification, ×100.

hibitors had far less advanced disease throughout the remnant kidney, with the exception of one rat that received NTU281 and had an area of heavily scarred and fibrosed tissue. Although improved, the renal histology of treated rats did not seem completely normal. Most had areas of slightly thickened tubular basement membrane, and some had an enlarged tubular lumen, although minimal tubular atrophy was seen. There was a visible reduction in interstitial cell numbers (inflammatory and noninflammatory) in both treated groups. Masson's trichrome staining quantified by multiphase image analysis for assessment of collagen deposition demonstrated a significant improvement in the level of both glomerulosclerosis (Figure 3A) and tubulointerstitial scarring (Figure 3B) in both TG inhibitor–treated groups.

Measurement of kidney function by creatinine clearance showed a 50% reduction in the SNx group 7 d after SNx (Figure 4A). This increased slightly during the first month in response to compensatory renal growth but fell sharply between 28 and 84 d as the remnant kidney scarred. Both treated groups showed comparable reductions in creatinine clearance to the untreated rats until 28 d as a result of the reduction in renal mass associated with this model. After this, rats receiving inhibitors maintained renal function at approximately 50% of normal, whereas in untreated kidneys renal function fell below 20% of controls. Normalizing creatinine clearance for the degree of resection (*i.e.*, correcting for the surviving nephron number [Figure 4B]) indicated marked hyperfiltration in both treated and untreated remnant kidneys up to 28 d. After this, treated groups maintained this level of hyperfiltration, whereas the untreated rats had a dramatic reduction in the creatinine clearance. All clearance values were consistent, with TG inhibition improving serum creatinine levels (Figure 4C). Albu-

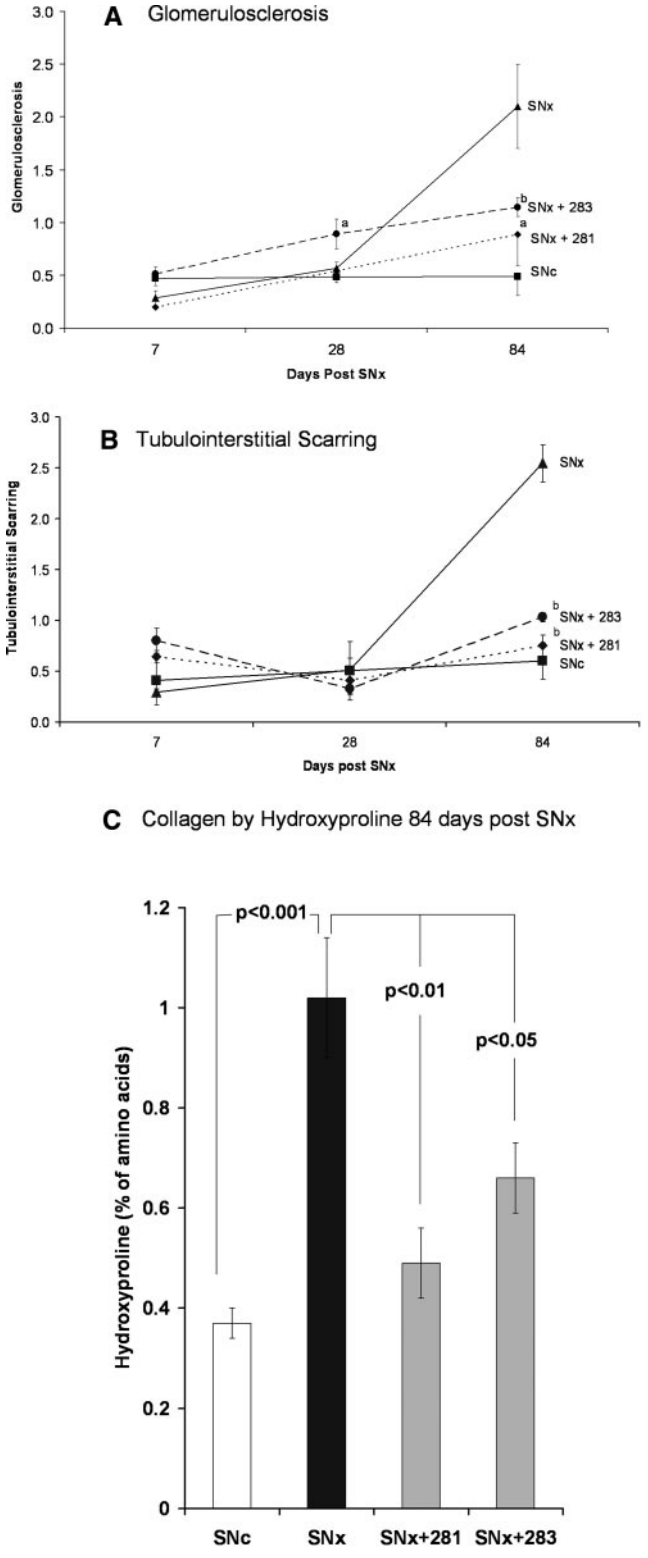

Figure 3. Quantification of renal scarring. The level of glomerulosclerosis (A) and tubulointerstitial scarring (B) was assessed by multiphase image analysis of Masson's trichrome–stained sections. Ten glomeruli or 10 fields of ×100 magnification were analyzed, respectively, for each rat on at least three sections. This was confirmed biochemically by measurement of total renal hydroxyproline (total collagen) levels day 84 (C). Data are means ± SEM; $n = 5$ to 6 rats for each experimental group per time point. $^{a}P < 0.05$; $^{b}P < 0.01$.

minuria in untreated SNx kidneys was 85-fold that of control rats by day 84 (Figure 4D), but treatment with NTU281 and NTU283 reduced this increase by 61 and 72%, respectively.

Changes in ECM Levels

Measurement of whole kidney collagen using hydroxyproline showed a 275% increase in untreated SNx kidneys by day 84 compared with controls (Figure 3C). In comparison, the hydroxyproline levels in remnant kidneys that were treated with TG inhibitors increased by only 33% with NTU281 (not significantly different from controls) and by just 70% with NTU283 (Figure 3C).

Immunohistochemical analysis (Figure 5) quantified by multiphase image analysis indicated significant increases in collagens I, III, and IV in the untreated remnant kidneys at day 84 (Figure 6A, i through iii). Collagen 1 changes were seen extensively in the glomeruli (Figure 5, row 1) but also in the tubulointerstitium (Figure 5, row 2). Collagen III changes were mainly confined to the interstitial space (Figure 5, row 3), whereas collagen IV was elevated both periglomerular and in the tubular basement membrane (Figure 5, row 4). Both inhibitors prevented or significantly reduced levels of immunoreactive collagens I and III. Only compound NTU281 was able to lower collagen IV. Fibronectin was also reduced by both inhibitors (Figure 6Aiv).

Measurement of mRNA levels of major ECM components and processing enzymes by Northern blot analysis (Figure 6B) indicated that SNx caused a significant induction in collagens I, III, and IV; fibronectin; matrix metalloproteinase-1; and tissue inhibitor of metalloproteinase-1 mRNA levels. Application of TG inhibitors, despite some variation in the mean values, caused no significant change in mRNA levels for any collagen measured compared with the untreated SNx. However, NTU281 significantly increased fibronectin and reduced tissue inhibitor of Matrix metalloproteinase-1 mRNA levels (Figure 6B, x and xii).

Interstitial Cells

Examination of Masson's trichrome–stained sections suggested that fewer interstitial cells were present in TG inhibitor–treated kidneys; however, staining for myofibroblasts using α-smooth muscle actin (α-SMA) showed that levels in the inhibitor-treated groups were still elevated and comparable to untreated remnant kidneys (Figure 6Av). Staining with ED1, a monocytic cell marker in rat, also showed no reduction with NTU283 but was reduced with NTU281 at 84 d (Figure 6Avi). At 28 d, both TG inhibitors elevated ED1-positive staining to levels greater than SNx alone. A five-fold increase over controls was observed in cells showing ED1 staining in the SNx, which was increased to 21- and 13-fold normal levels when treated with compounds NTU283 and NTU281, respectively ($P < 0001$).

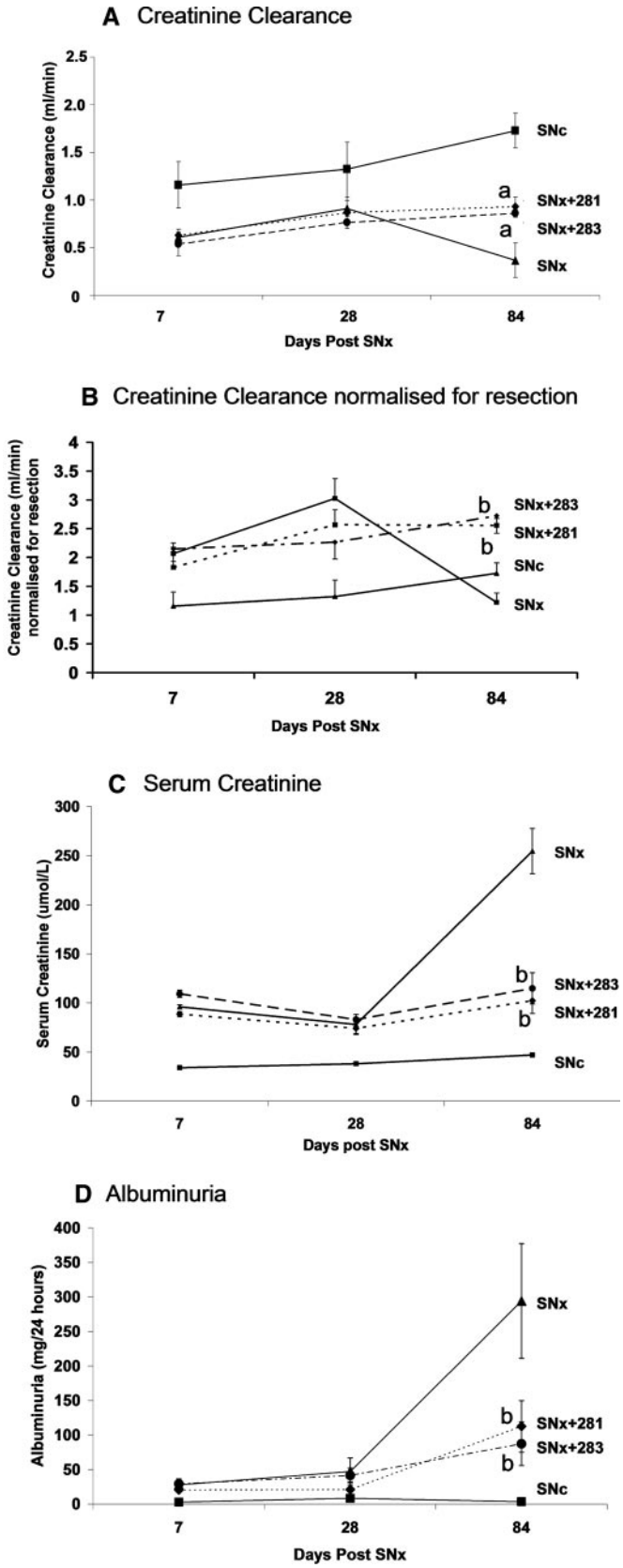

Figure 4. Clinical markers of kidney function and damage. Kidney function was assessed by measurement of creatinine clearance (A), which was normalized for nephron number after resection (B) and serum creatinine (C). Albuminuria (D) was used to assess glomerular leakage. Data are means ± SEM; $n = 5$ to 6 rats for each experimental group per time point. [a]$P < 0.05$; [b]$P < 0.01$.

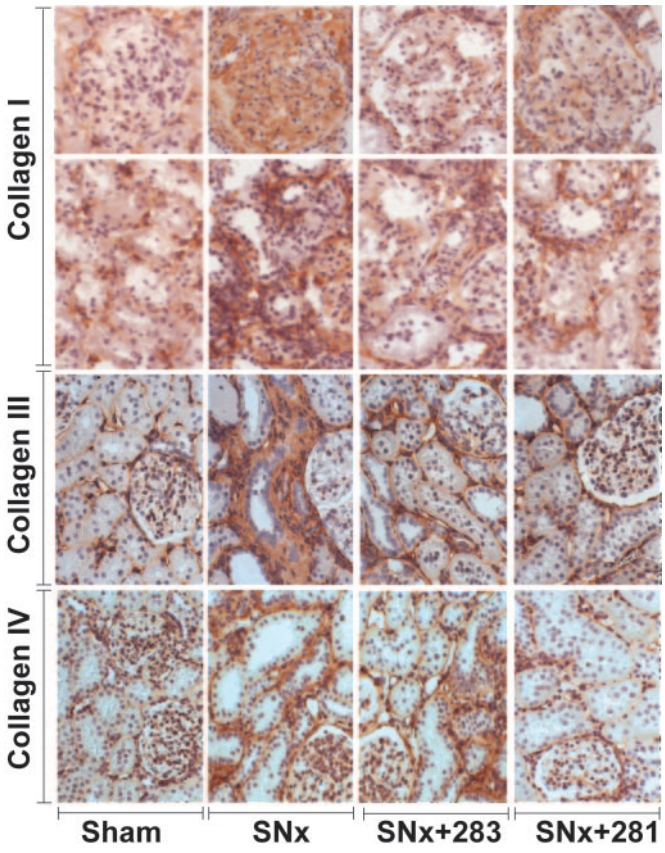

Figure 5. Collagen immunohistochemistry. Representative immunohistochemical stained sections for collagen I (rows 1 and 2, cryostat sections), collagen III (row 3, paraffin embedded sections) and collagen IV (row 4, paraffin-embedded sections) at 84 d after SNx. Magnification, ×200.

TGF-β Activation

Remnant kidney scarring was associated with a significant 30% increase in biologically active renal TGF-β from day 28 d onward (Figure 7A). This increase in active TGF-β was associated with a 2.5-fold increase in total TGF-β protein (Figure 7B) that was mRNA dependent (data not shown), although the percentage of the available latent TGF-β actually activated was one third lower in the untreated SNx (Figure 7C) compared with control rats. Treatment with TG inhibitors did not reduce the levels of active TGF-β1, with levels on the whole being slightly raised throughout the time course and significantly elevated with NTU283 from 28 d onward. TG inhibition did not consistently alter total TGF-β levels compared with untreated SNx rats. After 84 d of treatment, TG inhibition led to a higher conversion rate of latent TGF-β than in the untreated SNx rats (Figure 6C).

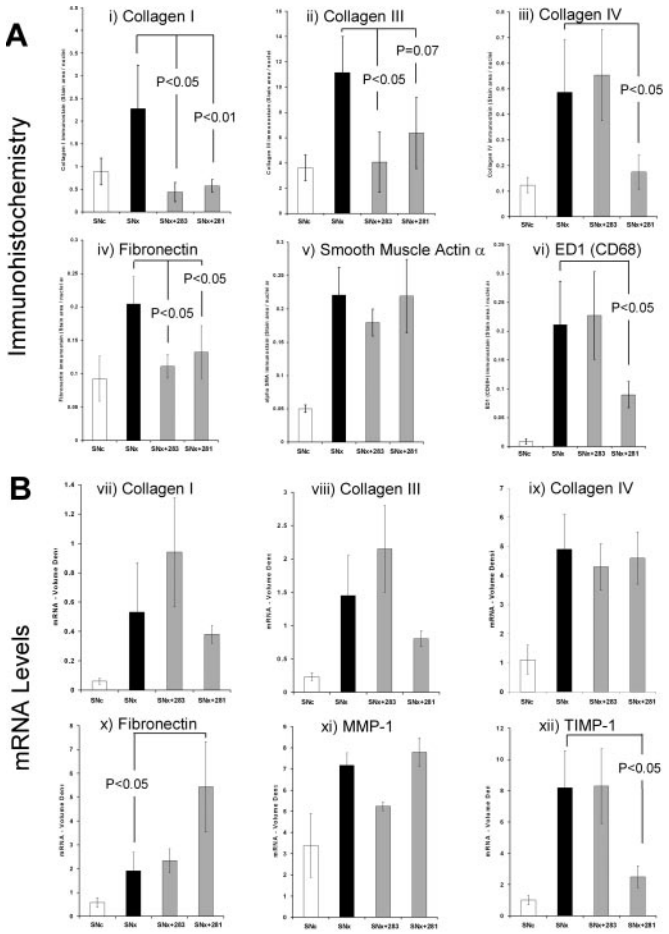

Figure 6. Changes in protein and mRNA levels of molecules implicated in the scarring process at 84 d after SNx. (A) Kidney levels of collagen I (i), collagen III (ii), collagen IV (iii), and fibronectin (iv) protein were assessed by computerized multiphase analysis of 10 immunohistochemistry stained fields per animal on a minimum of three sections. Levels of myofibroblasts (v) and monocytic cells (vi) were assessed similarly using α-SMA and ED1 as markers respectively. (B) mRNA levels of collagen I (vii), collagen III (viii), collagen iv (ix), fibronectin (x), matrix metalloproteinase-1 (xi), and tissue inhibitor of metalloproteinase-1 (xii) were assessed by Northern blot analysis using volume densitometry measurements corrected for loading using cyclophilin. Data are means ± SEM; $n = 5$ to 6 rats for each experimental group per time point. Each hybridization was performed in twice on duplicate blots.

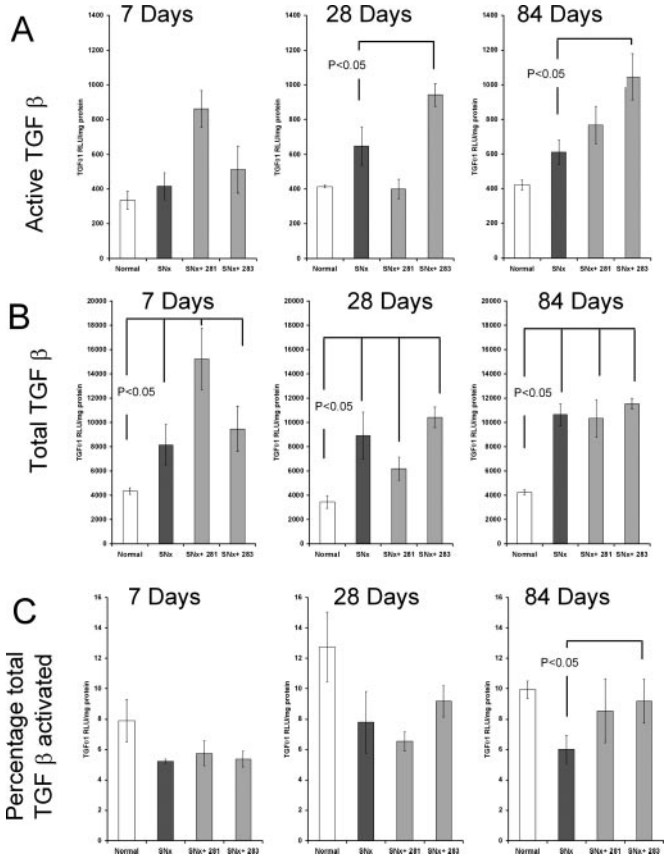

Figure 7. Effect of TG inhibition on TGF-β1 activation. Active TGF-β was assessed in kidney homogenate supernatants using the plasminogen activator inhibitor-1 luciferase reporter construct in mink lung cells (A). Total TGF-β was determined by acid activating the homogenate (B) and the level of conversion of total to active calculated (C). Data are means ± SEM; $n = 5$ to 6 rats for each experimental group per time point. Each kidney was assayed in triplicate on three separate occasions.

Blood Pressure

For assessment of whether NTU283 and NTU281 could affect BP, a separate group of SNx rats were left for 60 d to develop hypertension (Table 2). After application of both TG inhibitors at day 60, after 7 d of infusion, there was no significant difference in BP between treated and untreated SNx rats (Table 2) within time-matched groups; however, after 30 d of treatment, there was a tendency for rats that were treated with NTU283 to have a higher BP, although this was significant only with diastolic BP (Table 2).

Correlation Analysis

Across all time points, the TG product, ε(γ-glutamyl) lysine cross-link, correlated well with levels of tubulointerstitial scarring (Masson's trichrome staining; $r = 0.72$, $P < 0.001$). Cross-link levels also related well ($P < 0.05$) with collagen I ($r = 0.53$), collagen IV ($r = 0.51$), and collagen III ($r = 0.34$) when measured individually. Measurements of collagen by hydroxyproline revealed a better association with immunoreactive collagen I ($r = 0.49$, $P < 0.01$) than either collagen III ($r = 0.34$, $P < 0.05$) or collagen IV ($r = 0.33$, $P < 0.05$), suggesting collagen 1 to be the predominant collagen. Creatinine clearance correlated inversely with scarring ($r = -0.6$, $P < 0.01$) and particularly collagen III ($r = -0.73$, $P < 0.001$), with a weaker link to cross-link ($r = -0.35$, $P < 0.05$). ED1 staining showed no correlation to cross-link levels even when compound NTU283 data were excluded ($r = 1.1$, NS), although there was good association with scarring ($r = 0.62$, $P < 0.01$)

Table 2. BP in TG inhibitor–treated SNx rats[a]

BP	Before TG Inhibitor		After TG Inhibitor	
	Day 0 (before SNx)	Day 60 (after SNx)	Day 67 (7 d after TG Inhibitor)	Day 90 (30 d after TG Inhibitor)
Systolic				
control	140 ± 4	147 ± 12	147 ± 12	150 ± 7
SNx	139 ± 4	182 ± 2[b]	177 ± 5[b]	188 ± 5[b]
SNx + NTU281	144 ± 1	168 ± 3[b,c]	171 ± 3[b]	184 ± 9[b]
SNx + NTU283	150 ± 11	182 ± 24[b]	191 ± 21[b]	208 ± 17[b]
Mean				
control	115 ± 3	120 ± 7	119 ± 8	108 ± 3
SNx	106 ± 3[b]	151 ± 4[b]	145 ± 7[b]	144 ± 3[b]
SNx + NTU281	115 ± 4	140 ± 2[b]	137 ± 3[b]	140 ± 7[b]
SNx + NTU283	122 ± 13	154 ± 23[b]	151 ± 25[b]	171 ± 24[b]
Diastolic				
control	104 ± 5	105 ± 5	105 ± 7	87 ± 5
SNx	90 ± 4[b]	134 ± 5[b]	109 ± 12[b]	123 ± 2[b]
SNx + NTU281	100 ± 6	126 ± 3[b]	119 ± 4[b]	117 ± 9[b]
SNx + NTU283	108 ± 14	139 ± 23[b]	130 ± 27[b]	151 ± 27[b,c]

[a]Rats underwent SNx. After 60 d, kidneys were infused with NTU281 and NTU283. Both systolic and diastolic BP (five readings per rat per measurement) was assessed by computerized tail-cuff plethysmography before SNx, 60 days after SNx (before TG inhibitors), and then 7 and 30 d after application of TG inhibitors. Data are mean BP ± SEM; $n = 3$ rats for each experimental group per time point.
$P < 0.05$, [b]sham and [c]untreated SNx.

DISCUSSION

TG2-mediated protein cross-linking has been associated with the progression of kidney disease and insufficiency in experimental models and in humans.[6,7] Here, using the SNx model of chronic renal fibrosis, we demonstrate that two structurally distinct irreversible TG inhibitors directed toward the cysteine residue in the enzyme active site both are able to reduce intrarenal TG activity. Importantly, this reduction in TG activity is paralleled by a reduction in the product of the enzyme, the $\varepsilon(\gamma$-glutamyl) lysine cross-link. Consequently, the progressive fibrotic phase that takes place between 4 and 12 wk is significantly blunted, resulting in reduced glomerular and interstitial scarring in the treated groups. Neither inhibitor caused a reduction in BP. Importantly, inhibition of protein cross-linking by TG maintains renal function at a level that is associated with the reduction in renal mass in this model and does not progressively decline, whereas the reduction in glomerulosclerosis (59% [NTU283] and 75% [NTU281]) is in line with improved albuminuria.

Transcription of the major ECM proteins as well as matrix metalloproteinase-1 are not significantly different from untreated levels using either inhibitor. This suggests that the major driving fibrotic stimuli remain and that TG inhibition is likely to be interfering with ECM deposition and expansion in the diseased kidneys. This is consistent with cell culture studies[25] using NTU283 in an *in vitro* model of hyperglycemia; using proximal tubular cells, glucose-induced ECM accumulation was reduced by 75%. Importantly, these reductions occurred without altering ECM transcription levels or the amount of available active TGF-β1. Mechanistically, we[26,27] previously demonstrated that incubation of TG2 with both collagens 1 and III leads to an increased rate of fibril formation and that both isolated collagen or a conditioned cell matrix once cross-linked by TG2 becomes more resistant to proteolytic decay.[9] In cell matrix turnover experiments, we[26,27] also demonstrated that the increased presence of matrix-associated TG2 leads to a reduced rate of matrix turnover, facilitating deposition and accumulation.

Whereas both inhibitors prevent the accumulation of collagens I and III, only NTU281 is able to reduce collagen IV. The *in vitro* use of NTU283[25] and the data generated so far *in vivo* allow only speculation as to the reason behind the discrepant action of the inhibitors on collagen IV. It may be simply a matter of the inhibitor's intrarenal concentration and distribution. Both compounds have comparable IC_{50} (50 μM) when measured against TG2,[28] but NTU281 has a greater molecular mass than NTU283 and, unlike NTU283, has poor cell solubility.[24] With the direct intrarenal delivery system used, it is likely that the extracellular levels of NTU281 are higher than NTU283 and NTU281 is therefore more able to target the tubular epithelial basement membrane. This is supported by the fact that when inhibition is measured by *in situ* TG activity assay in cryostat sections, NTU283 is approximately 30% less effective at the dosages used (data not shown).

An important question with TG inhibitors is whether their effects on matrix accumulation are through inhibition of TG2's cross-linking of matrix proteins, and/or whether it is an indirect effect *via* inhibition of TG2's role in the activation of matrix-bound TGF-β1. Rifkin *et al.*[17] strongly suggested a role for TG2 in the activation of the ECM-associated large latent TGF-β1 complex. Hence any action would be through reduction in either active or total (recruited) TGF-β, from the elevated levels after SNx.[29] Our data failed to show supportive

evidence for such a hypothesis in this model. The possibility that increased TGF-β1 synthesis overcame any inhibition of activation by TG2 was excluded, because no increased expression of TGF-β1 mRNA in treated groups was observed. It is therefore reasonable to exclude changes in TGF-β1 activation as a mechanism for TG2 action in this model. The inability of TG inhibitors to influence TGF-β1 activation is in accordance with our *in vitro* studies.[25]

A lower interstitial cell number in the two inhibitor-treated groups by the end of the time course was very evident; however, using α-SMA as a myofibroblast marker indicated a similar level of staining in the treated and untreated groups. Likewise, ED1 staining for monocytic cells remained high when cells were treated with NTU283, suggesting the presence of an alternative interstitial cell type that is lower in the treated groups. With NTU281, the number of ED1-positive cells was reduced at 84 d, which would in part account for the lower interstitial cell number. One possible explanation for the reduction in monocytic inflammatory cells by the ECM-targeted NTU281 inhibitor is the inhibition of TG2 cross-linking in the activation of the secretory form of phospholipase A2.[30] Inhibition of the secretory form of phospholipase A2 would reduce the release of arachidonic acid from the cell membrane during inflammation, which is the rate-limiting step in the biosynthesis of eicosanoids by cyclo-oxygenase (reviewed by Miele[31]), thereby modulating the inflammatory response.

The specificity of the two inhibitors when used in an *in vivo* model given the similar structures of the eight TG so far characterized (reviewed by Lorand and Graham[32]) needs addressing. It is likely that both inhibitors will target all TG, although with varying potencies; however, only two known TG are likely to be associated with the ECM: TG2 and factor XIII. In the initial stages of wound repair, we cannot rule out any effects on factor XIIIa, although this enzyme is likely to be less important in the later stages of ECM deposition (reviewed by Verderio *et al.*[33]). NTU281 is predominantly found in the extracellular environment,[23] which is supported by our own studies using tissue slices and tissue homogenates, in which the efficacy of NTU281 is evident only when assaying TG activity in cryostat sections, which predominantly measures extracellular activity. Because NTU281 is equally as effective as NTU283, this confirms that the predominant beneficial effect of both inhibitors is extracellular. Importantly, low cell internalization of NTU281 limits nonspecific effects on other TG or other potential thiol-containing enzymes in the intracellular space, and the presence of other thiol-containing enzyme targets in the extracellular space is limited. NTU283 (and its analogues) was originally targeted toward factor XIIIa[22,23] but is equally effective against TG2,[25] TG1, and TG3 and has undergone extensive *in vitro* trials to ascertain its specificity against the active site thiol of TG. The testing of the NTU283 family of compounds against a range of both thiol and serine proteases has also indicated no efficacy against these enzymes when tested up to 1-mM concentration.[22] We cannot rule out that other intracellular targets may be affected by NTU283; however, the testing of both NTU283 and NTU281 against another key thiol target, the caspases, has shown no effect on caspase 3 when tested at concentrations up to 1 mM (data not shown). Even though both compounds have renal activity when given subcutaneously, we chose to deliver both compounds directly to the kidney *via* an intrarenal cannula fed from an osmotic minipump.[34] This gave us three major advantages. First, this approach requires much less compound, which is an important consideration because large-scale synthesis is not economical on a laboratory scale. Second, the direct delivery prevents complication of the data by secondary or systemic effects after long-term delivery (should they occur). Third, it provides an easy and accurate dosing regimen without daily application. This delivery system has been extensively tested.[34] Insertion of the cannula does cause some minor remodeling immediately adjacent to it, which has to be excluded from any analysis, but using radiolabeled markers and lissamine green dyes showed that distribution throughout the kidney is excellent and devoid of any major gradient effect.[34]

The data presented show a significant improvement in both kidney function and prevention of renal remodeling in kidneys that were treated with site-directed, irreversible inhibitors of TG activity, thereby confirming the importance of TG2 in disease progression and as a potential therapeutic target for the prevention of tissue scarring.[2–5] Although we recognize that key questions still need to be answered, such as the effectiveness of TG inhibition on well-established disease, the comparison of TG inhibition against existing regimens, and the effects of long-term administration of this class of compound, this study for the first time demonstrates a targeted and effective intervention strategy that offers the potential to reduce tissue scarring in a wide range of fibrotic disorders.

CONCISE METHODS

SNx Model of Renal Scarring

Male Wistar rats (250 to 300 g) were subjected to SNx by ligation and removal of the left kidney using a flank incision. Upper and lower poles of the remaining kidney were resected to cause a loss of five sixths of the renal mass. Control rats were subjected to a sham operation.

Synthesis of TG Inhibitors

NTU283[23] and NTU281[28] were synthesized according to published methods.[28] Compound purity was determined by nuclear magnetic resonance and mass spectrometry. Inhibitor efficacy was verified against renal TG by application of inhibitors at 100 and 500 μm to a 20% kidney homogenate with activity measured using the [^{14}C]putrescine incorporation assay.[7]

Application of TG Inhibitors

A 0.58-mm bore polythene cannula was heat-sealed at 1 mm and a 9-mm section fenestrated 2 mm from the seal. The open end was attached to a 2ml4 Azlet osmotic minipump (Charles River, Margate, UK). At resection, the cannula was inserted longitudinally through

one cut pole of the remnant such that the fenestrated area was within the renal parenchyma. The cannula was secured and then run through the muscle wall to the pump located on the scruff. This approach has been shown to give continual, low-gradient delivery throughout the remnant kidney by both radiolabeled markers and lissamine green delivery.[34] Pumps were loaded with either PBS (vehicle) or 50-mM solutions of NTU281 or NTU283. Pumps were replaced every 4 wk as they became exhausted. TG inhibitor dosage was based on 28-d pilot studies, using a concentration that would completely inhibit the increase in TG activity after SNx.

Protocol

Rats were maintained at 20°C and 45% humidity on a 12-h light/dark cycle and allowed free access to standard rat food and tap water. All procedures were carried out under license according to regulations laid down by Her Majesty's Government, United Kingdom (Animals Scientific Procedures Act, 1986).

Experimental groups ($n = 5$ to 6 per group per time point) of control, SNx, SNx+PBS, SNx+NTU281 and SNx+NTU283 rats were killed at 7, 28, and 84 d after SNx. A terminal blood sample and 24-h urine collection were obtained. The recovered remnant kidney was quartered. One quarter was fixed in neutral-buffered formalin and paraffin embedded, and the remainder was snap-frozen in liquid nitrogen.

Proteinuria and Albuminuria

Twenty-four-hour albuminuria was measured using the Bethyl Laboratories rat albumin ELISA kit (BioGnosis, Hailsham, UK) as per the manufacturer's instructions.

Renal Scarring

Four-micrometer paraffin sections were stained with Masson's trichrome (stains collagenous material green and cytoplasm purple). For determination of tubulointerstitial scarring, 10 fields (cortex) at ×100 magnification were acquired using a CC-12 digital camera (Soft Imaging Systems, Muenster, Germany). For glomerulosclerosis, 10 glomeruli (×400) were acquired. Scarring was assessed using three-phase analysis (green, purple, and white) using Analysis 3.2 software (Soft Imaging Systems) ensuring total phase coverage in excess of 95%. The tubulointerstitial scarring index was determined by dividing the area of green collagenous stain by the purple cellular stain, thus correcting for cell number and tubular dilation. Glomerulosclerosis was calculated by dividing green collagenous stain with the unstained white area to take account of changes in Bowman's space, hypercellularity, and intercapillary area.

Measurement of TG Activity in Kidney Tissues

Putrescine Incorporation Assay.

TG activity in kidney homogenates was measured using the [^{14}C]putrescine incorporation into N,N^{1}-dimethylcasein assay as described previously.[7] Results are corrected to U/mg protein (1 U equals 1 nmol putrescine incorporated per hour at 37°C).

In situ *Activity Assay.*

Unfixed cryostat sections were incubated with the TG primary amine substrate fluorescein (FITC) cadaverine (Molecular Probes, Leiden, Netherlands) as described previously.[9] Incorporated cadaverine was revealed using a mouse anti-FITC mAb and visualized with a goat anti-mouse Cy5 (indodicarbocyanine)-conjugated antibody (Stratech Scientific, Newmarket, UK). Sections were acquired using confocal microscopy (Leica DMRBE; Leica, Wetzlar, Germany) at 650 nm, and the level of cadaverine incorporation was determined using multiphase analysis as already described. This technique was used to measure the TG inhibitory effects of NTU281.

Measurements of Renal ε(γ-Glutamyl)-Lysine Iso-Dipeptide.

Samples were subjected to exhaustive proteolytic digestion with subtilisin, pronase, leucine amino peptidase, prolidase, and carboxypeptidase Y. ε(γ-Glutamyl)-lysine levels in the digests were measured by cation exchange chromatography using an Ultrapac 8 cation exchange resin (8 ± 0.5-μm particle size) on an LKB 4151 amino acid analyser (Pharmacia, Cambridge, UK) using a modification of a lithium citrate buffer method as described previously.[35]

Measurement of mRNA Levels.

Total RNA was extracted using Trizol (Life Technologies-BRL, Paisley, UK) from each rat in the study and subjected to Northern blot analysis as described previously using published random primed cDNA sequences.[36] Hybridized Northern blots were exposed to Biomax MS film, and autoradiographs were quantified by scanning densitometry using a Biorad GS-690 densitometer and Molecular Analyst version 4 software (Biorad, Hemel Hempstesd, UK). Densitometry values were corrected for loading using repeat probings with the housekeeping gene cyclophilin. Hybridization and analysis were performed in duplicate on separate blots for consistency, although data given originate from a single representative autoradiograph.

Immunohistochemical Measurements

Staining was carried out on 4-μm, neutral-buffered, formalin-fixed, paraffin-embedded sections using a biotinylated secondary antibody/extravidin-horseradish peroxidase approach. After antigen revealing, antibodies were applied as follows: Goat anti collagen III (1:10; Southern Biotechnology, Birmingham, AL), rabbit anti–collagen IV (1:35; ICN, High Wycombe, UK), monoclonal anti-fibronectin (1:50; Sigma, Poole, UK), monoclonal anti–α-SMA (1:100; Dako, Ely, UK), and monoclonal anti-ED1 (CD68; 1:50; Serotec, Kidlington, UK). Collagen 1 immunohistochemistry was performed on 10-μm cryostat sections using a goat polyclonal antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA). Multiphase image analysis was performed as already described, with staining normalized to nuclear area.

Hydroxyproline Analysis

Kidney homogenates were hydrolyzed in 6 M HCl at 100°C for 16 h, and amino acids were recovered using the EZ:Faast amino acid analysis kit (Phenomenex, Macclesfield, UK) according to the manufacturer's instructions. The amino acid and hydroxyproline content was measured against internal amino acid standards by gas chromatography using a 9890N Network GC system (Agilent Technologies, Wokingham, UK).

Active and Total TGF-β

TGF-β biologic activity was measured using the mink lung epithelial cell bioassay using the TGF-β1 response element on the plasminogen activator inhibitor-1 promoter hooked up to the luciferase reporter gene.[37] Kidney homogenates (10% wt/vol) were centrifuged at 30,000 $\times$ *g* (30 min, 4°C), and the supernatant was filter-sterilized through 4-mm Millex Filter Units (Millipore, Watford, UK). Samples were diluted 1:25 in serum-free DMEM containing 0.1% (wt/vol) BSA and placed onto 1.6×10^4 mink lung epithelial cells in a 96-well plate. After 22 h, cells were lysed with Glo Lysis buffer (Promega, Madison, WI). A total of 50 μl of cell lysate was mixed with 50 μl of Bright-Glo luciferase substrate (Promega) and loaded onto a Berthold (Redbourn, UK) Autolumat Plus LB 953 luminometer. The addition of NTU283 and NTU281 at concentrations up to 400 μM to assays of normal and SNx did not alter values obtained. Total TGF-β1 was measured after acid activation using hydrochloric acid for 1 h at 4°C.

Effect of Transglutaminase Inhibitors on BP

For assessment of whether TG inhibitors affected BP, nine rats were subjected to SNx and three to a sham operation. Sixty days after SNx, once significant hypertension had developed, all SNx rats had an intrarenal cannula fitted. Three were infused with PBS, three with NTU281, and three with NTU283 from implanted osmotic minipumps. Both systolic and diastolic BP were measured by computerized tail-cuff plethysmography (IITC Life Science, Woodland Hills, CA) with rats restrained in Perspex tubes before SNx, 60 d after SNx, and then 7 and 30 d after continual infusion of TG inhibitors. At each point, systolic, diastolic, and mean BP were attained from an average of five consecutive readings.

Statistical Analyses

Comparison between experimental groups was undertaken using either the *t* test with Bonferroni correction or one-way ANOVA as appropriate. A probability of 95% ($P \leq 0.05$) was taken as significant.

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DISCLOSURE

None.

REFERENCES

1. Lorand L, Conrad SM: Transglutaminases. *Mol Cell Biochem* 58: 9–35, 1984
2. Griffin M, Smith LL, Wynne J: Changes in transglutaminase activity in an experimental model of pulmonary fibrosis induced by paraquat. *Br J Exp Pathol* 60: 653–661, 1979
3. Mirza A, Liu SL, Frizell E, Zhu J, Maddukuri S, Martinez J, Davies P, Schwarting R, Norton P, Zern MA: A role for tissue transglutaminase in hepatic injury and fibrogenesis, and its regulation by NF-kappaB. *Am J Physiol* 272: G281–G288, 1997
4. Small K, Feng JF, Lorenz J, Donnelly ET, Yu A, Im MJ, Dorn GW, 2nd, Liggett SB: Cardiac specific overexpression of transglutaminase II (G(h)) results in a unique hypertrophy phenotype independent of phospholipase C activation. *J Biol Chem* 274: 21291–21296, 1999
5. Bowness JM, Venditti M, Tarr AH, Taylor JR: Increase in epsilon-(gamma-glutamyl)lysine crosslinks in atherosclerotic aortas. *Atherosclerosis* 111: 247–253, 1994
6. Johnson TS, El-Koraie AF, Skill NJ, Baddour NM, El Nahas AM, Njloma M, Adam AG, Griffin M: Tissue transglutaminase and the progression of human renal scarring. *J Am Soc Nephrol* 14: 2052–2062, 2003
7. Johnson TS, Griffin M, Thomas GL, Skill J, Cox A, Yang B, Nicholas B, Birckbichler PJ, Muchaneta-Kubara C, Meguid El Nahas A: The role of transglutaminase in the rat subtotal nephrectomy model of renal fibrosis. *J Clin Invest* 99: 2950–2960, 1997
8. Coresh J, Byrd-Holt D, Astor BC, Briggs JP, Eggers PW, Lacher DA, Hostetter TH: Chronic kidney disease awareness, prevalence, and trends among U.S. adults, 1999 to 2000. *J Am Soc Nephrol* 16: 180–188, 2005
9. Johnson TS, Skill NJ, El Nahas AM, Oldroyd SD, Thomas GL, Douthwaite JA, Haylor JL, Griffin M: Transglutaminase transcription and antigen translocation in experimental renal scarring. *J Am Soc Nephrol* 10: 2146–2157, 1999
10. Smethurst PA, Griffin M: Measurement of tissue transglutaminase activity in a permeabilized cell system: Its regulation by Ca2+ and nucleotides. *Biochem J* 313: 803–808, 1996
11. Kleman JP, Aeschlimann D, Paulsson M, van der Rest M: Transglutaminase-catalyzed cross-linking of fibrils of collagen V/XI in A204 rhabdomyosarcoma cells. *Biochemistry* 34: 13768–13775, 1995
12. Aeschlimann D, Paulsson M: Cross-linking of laminin-nidogen complexes by tissue transglutaminase: A novel mechanism for basement membrane stabilization. *J Biol Chem,* 266: 15308–15317, 1991
13. Johnson TS, Scholfield CI, Parry J, Griffin M: Induction of tissue transglutaminase by dexamethasone: Its correlation to receptor number and transglutaminase-mediated cell death in a series of malignant hamster fibrosarcomas. *Biochem J* 331: 105–112, 1998
14. Verderio E, Nicholas B, Gross S, Griffin M: Regulated expression of tissue transglutaminase in Swiss 3T3 fibroblasts: Effects on the processing of fibronectin, cell attachment, and cell death. *Exp Cell Res,* 239: 119–138, 1998
15. Knight RL, Hand D, Piacentini M, Griffin M: Characterization of the transglutaminase-mediated large molecular weight polymer from rat liver: Its relationship to apoptosis. *Eur J Cell Biol* 60: 210–216, 1993
16. Thomas GL, Yang B, Wagner BE, Savill J, El Nahas AM: Cellular apoptosis and proliferation in experimental renal fibrosis. *Nephrol Dial Transplant* 13: 2216–2226, 1998
17. Nunes I, Gleizes PE, Metz CN, Rifkin DB: Latent transforming growth factor-beta binding protein domains involved in activation and transglutaminase-dependent cross-linking of latent transforming growth factor-beta. *J Cell Biol* 136: 1151–1163, 1997
18. Verderio E, Gaudry C, Gross S, Smith C, Downes S, Griffin M: Regulation of cell surface tissue transglutaminase: Effects on matrix storage of latent transforming growth factor-beta binding protein-1. *J Histochem Cytochem* 47: 1417–1432, 1999
19. Douthwaite JA, Johnson TS, Haylor JL, Watson P, El Nahas AM: Effects of transforming growth factor-beta1 on renal extracellular matrix components and their regulating proteins. *J Am Soc Nephrol* 10: 2109–2119, 1999
20. George MD, Vollberg TM, Floyd EE, Stein JP, Jetten AM: Regulation of transglutaminase type II by transforming growth factor-beta 1 in normal and transformed human epidermal keratinocytes. *J Biol Chem* 265: 11098–11104, 1990

21. Akimov SS, Belkin AM: Cell-surface transglutaminase promotes fibronectin assembly via interaction with the gelatin-binding domain of fibronectin: A role in TGFbeta-dependent matrix deposition. *J Cell Sci* 114: 2989–3000, 2001
22. Baldwin JJ, Remy DC, Claremon DA, United States Patent 5098707, filed 29 March 1991, published 24 March 1992
23. Hound KF, Doshi K, Gaul S: Transglutaminase inhibition by 2-[(2-oxopropyl) 10] imidazolium derivatives: Mechanism of factor IIIa inactivation. *Biochemistry* 33: 10109–10119, 1994
24. Baumgartner W, Golenhofen N, Weth A, Hiiragi T, Saint R, Griffin M, Drenckhahn D: Role of transglutaminase 1 in stabilisation of intercellular junctions of the vascular endothelium. *Histochem Cell Biol* 122: 17–25, 2004
25. Skill NJ, Johnson TS, Coutts IG, Saint RE, Fisher M, Huang L, El Nahas AM, Collighan RJ, Griffin M: Inhibition of transglutaminase activity reduces extracellular matrix accumulation induced by high glucose levels in proximal tubular epithelial cells. *J Biol Chem* 279: 47754–47762, 2004
26. Jones RA, Kotsakis P, Johnson TS, Chau D, Ali S, Melino G, Griffin M: Matrix changes induced by transglutaminase 2 lead to inhibition of angiogenesis and tumor growth. *Cell Death Differ* 13: 1442–1453, 2006
27. Gross SR, Balklava Z, Griffin M: Importance of tissue transglutaminase in repair of extracellular matrices and cell death of dermal fibroblasts after exposure to a solarium ultraviolet A source. *J Invest Dermatol* 121: 412–423, 2003
28. Griffin M, Coutts IG, Saint R: Novel Compounds and Methods of Using the Same. International Publication Number WO 2004/113363. GB patent PCT/GB2004/002569, 2004
29. Border WA, Noble NA: TGF-beta in kidney fibrosis: A target for gene therapy. *Kidney Int* 51: 1388–1396, 1997
30. Cordella-Miele E, Miele L, Mukherjee AB: A novel transglutaminase-mediated post-translational modification of phospholipase A2 dramatically increases its catalytic activity. *J Biol Chem* 265: 17180–17188, 1990
31. Miele L: New weapons against inflammation: Dual inhibitors of phospholipase A2 and transglutaminase. *J Clin Invest* 111: 19–21, 2003
32. Lorand L, Graham RM: Transglutaminases: Crosslinking enzymes with pleiotropic functions. *Nat Rev Mol Cell Biol* 4: 140–156, 2003
33. Verderio EA, Johnson TS, Griffin M: Transglutaminases in wound healing and inflammation. *Prog Exp Tumor Res* 38: 89–114, 2005
34. Oldroyd SD, Thomas GL, Gabbiani G, El Nahas AM: Interferon-gamma inhibits experimental renal fibrosis. *Kidney Int* 56: 2116–2127, 1999
35. Griffin M, Wilson J: Detection of epsilon(gamma-glutamyl) lysine. *Mol Cell Biochem* 58: 37–49, 1984
36. Johnson TS, Haylor JL, Thomas GL, Fisher M, El Nahas AM: Matrix metalloproteinases and their inhibitions in experimental renal scarring. *Exp Nephrol* 10: 182–195, 2002
37. Abe M, Harpel JG, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB: An assay for transforming growth factor-beta using cells transfected with a plasminogen activator inhibitor-1 promoter-luciferase construct. *Anal Biochem* 216: 276–284, 1994