Combination therapy with tranilast and angiotensin-converting enzyme inhibition provides additional renoprotection in the remnant kidney model

DJ Kelly[1], Y Zhang[1], AJ Cox[1] and RE Gilbert[1,2]

[1]*Department of Medicine, University of Melbourne, St. Vincent's Hospital, Victoria, Australia and* [2]*Department of Medicine, University of Toronto, St. Michael's Hospital, Ontario, Canada*

Despite current therapy with agents that block the renin–angiotensin system, renal dysfunction continues to progress in a significant proportion of patients with kidney disease. Several pre-clinical studies have reported beneficial effects of tranilast, an inhibitor of transforming growth factor (TGF)-β's actions in a range of diseases that are characterized by fibrosis. However, whether such therapy provides additional benefits in renal disease, when added to angiotensin-converting enzyme (ACE) inhibition, has not been explored. We randomized subtotally (5/6) nephrectomized rats to receive vehicle, the ACE inhibitor, perindopril (6 mg/l), tranilast (400 mg/kg/day), or their combination for 12 weeks. When compared with sham-nephrectomized animals, subtotally nephrectomized animals had reduced creatinine clearance, proteinuria, glomerulosclerosis, interstitial fibrosis, tubular atrophy, and evidence of TGF-β activity, as indicated by the abundant nuclear staining of phosphorylated Smad2. These manifestations of injury and TGF-β activation were all attenuated by treatment with either tranilast or perindopril, with the latter also attenuating the animals' hypertension. When compared with single-agent treatment, the combination of tranilast and perindopril provided additional, incremental improvements in creatinine clearance, proteinuria, and glomerulosclerosis, and a reduction in nuclear phsopho-Smad2 beyond single-agent treatment. These findings indicate that the combination of tranilast and perindopril was superior to single-agent treatment on kidney structure and function in the remnant kidney model, and suggests the potential for such dual therapy in kidney disease that continues to progress despite blockade of the renin–angiotensin system.

Correspondence: *RE Gilbert, Department of Medicine, University of Toronto, Division of Endocrinology, St Michael's Hospital, 30 Bond Street, Toronto, Ontario, Canada M5B 1W8. E-mail: gilbert@medstv.unimelb.edu.au*

Received 5 May 2005; revised 8 December 2005; accepted 12 January 2006; published online 22 March 2006

Kidney International (2006) **69,** 1954–1960. doi:10.1038/sj.ki.5000376; published online 22 March 2006

KEYWORDS: transforming growth factor-β; tranilast; glomerulosclerosis; tubulointerstitium; tubular atrophy

Chronic kidney disease is a common and increasing public health problem in the US, Europe, and in most countries where reliable epidemiological data are available. For instance, in the US, approximately eight million people have impaired renal function (calculated glomerular filtration rate <60 ml/min/1.73 m^2, with a further 300 000 individuals in end-stage renal failure.[1]

Almost regardless of the insult, an initial critical nephron loss is frequently followed by progressive proteinuria and declining glomerular filtration rate, with an inexorable progression to end-stage renal disease. Current renoprotective therapy, aimed at slowing this progression, centers on blood pressure control and blockade of the renin–angiotensin system, for which data from controlled clinical trials provide a strong evidence base for treatment.[2] However, despite these important therapies, a considerable proportion of patients with chronic kidney disease continue to progress and new therapies are needed.

The pathological accumulation of extracellular matrix[3] and tubular atrophy[4] are classical histopathological features of progressive kidney disease that each correlate with declining renal function. Consistent with these findings, experimental data accumulated over more than a decade have repeatedly implicated the profibrotic and proapoptotic growth factor, transforming growth factor-β (TGF-β), in the pathogenesis of kidney injury,[5] making it a key target for renoprotective therapies.

To date, a number of strategies to reduce the expression or antagonize the effects of TGF-β have been evolved, including neutralizing TGF-β antibodies,[6,7] the glycosaminoglycan, decorin,[8] and small-molecule inhibitors of TGF-β action.[9] Among the latter is tranilast (*n*-[3,4-dimethoxycinnamoyl] anthranilic acid), an antifibrotic agent used in Japan for the

treatment of hypertrophic scars,[10] which has been shown to inhibit TGF-β-induced extracellular matrix production synthesis in cultured kidney cells[11] and to reduce renal injury in a number of disease models.[12–14]

Given the effectiveness, albeit incomplete, of angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs), any new therapy in clinical practice would need to be examined as an 'add-on' to these agents. We, therefore, sought to examine the renoprotective effects of combining tranilast with ACE inhibition, using the 5/6 nephrectomy model, a well-characterized model of non-inflammatory proteinuric renal disease in which critical nephron loss leads to progressive renal dysfunction.[15] In this model, TGF-β expression is increased but unaffected by tranilast.[12] Accordingly, in the present study, we also sought to determine the effects of tranilast on TGF-β-dependent collagen production.

RESULTS

Animal characteristics

All rats that underwent subtotal nephrectomy were hypertensive and developed substantial proteinuria and reduced creatinine clearance. Treatment of subtotal (5/6) nephrectomy (STNx) rats with perindopril attenuated all of these changes (Table 1). Tranilast also reduced proteinuria and improved creatinine clearance, albeit to a lesser extent than perindopril, but without affecting blood pressure. Without additional blood pressure lowering, the addition of tranilast to perindopril led to a further reduction in proteinuria and incremental improvement in creatinine clearance when compared with either drug as monotherapy (Table 1).

Histopathology

Subtotal nephrectomy led to significant glomerulosclerosis and tubulointerstitial fibrosis (Figures 1–3), with the proportional area of kidney cortex occupied by collagenous matrix increased more than 10-fold ($P<0.01$; Figure 1) compared with shams. Considerable inter-nephron heterogeneity in the extent of fibrosis and atrophy was also noted. When used as monotherapy, perindopril, and tranilast each reduced the extent of glomerulosclerosis in STNx rats, although the magnitude of this reduction was greater with perindopril than tranilast. The combination of the two agents resulted in a still greater reduction in glomerulosclerosis when compared with either single-agent treatment.

Tubular atrophy, absent in sham kidneys, was also a prominent feature of STNx kidneys (Figures 3, 4) that was reduced by perindopril and to a lesser extent by tranilast also. However, when used together, the perindopril and tranilast combination led to a greater diminution in the extent of tubular atrophy when compared with either agent as monotherapy.

All treatments substantially reduced the degree of interstitial fibrosis to a similar extent (Figures 1–4).

Phosphorylated Smad2

In contrast to its minimal immunostaining in sham animals, abundant phosphorylated Smad2 was noted in STNx kidneys (Figures 5, 6), where it was confined to the nuclei and noted

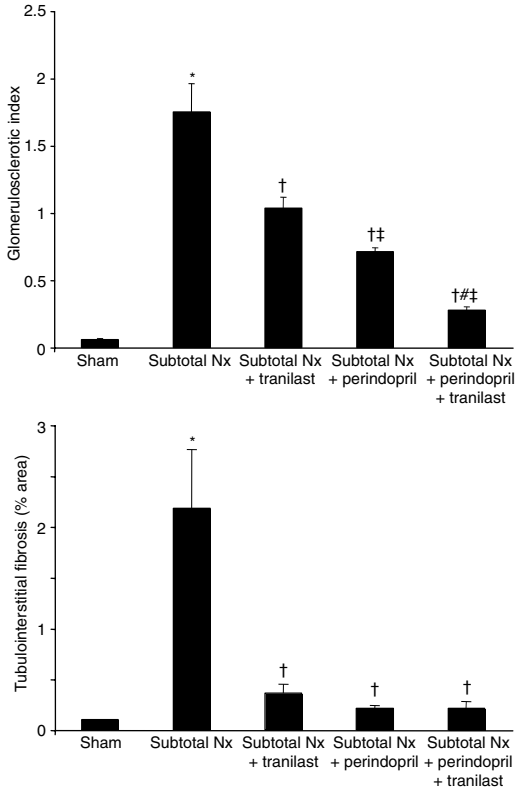

Figure 1 | Glomerulosclerotic Index (upper panel) and tubulointerstitial fibrosis (lower panel) in sham and STNx rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. Data are presented as mean ± s.e.m. *$P<0.01$ compared with shams, †$P<0.01$ versus untreated STNx rat kidneys. ‡$P<0.05$ versus STNx + tranilast. #$P<0.05$ versus monotherapy.

Table 1 | Animal characteristics

	Sham	STNx	STNx+tranilast	STNx+perindopril	STNx+tranilast+perindopril
N	10	10	15	15	17
Body weight (g)	560 ± 18	516 ± 9	475 ± 16	512 ± 13	514 ± 12
SBP (mm Hg)	135 ± 10	193 ± 11†	199 ± 9†	131 ± 8†,¶	124 ± 9†,¶
Proteinuria (mg/day)	1.3 ± 0.12	6.24 ± 0.94†	4.5 ± 0.71‡	2.5 ± 0.43‡	0.71 ± 0.24¶,§
Creatinine clearance (ml/min)	2.7 ± 0.14	0.60 ± 0.11†	0.86 ± 0.10†	1.23 ± 0.16‡	1.36 ± 0.11‡,§

SBP, systolic blood pressure; STNx, subtotal (5/6) nephrectomy. Data are expressed as mean ± s.e.m. *$P<0.05$, †$P<0.01$ versus sham, ‡$P<0.05$, ¶$P<0.01$ versus STNx, §$P<0.05$ versus STNx+perindopril.

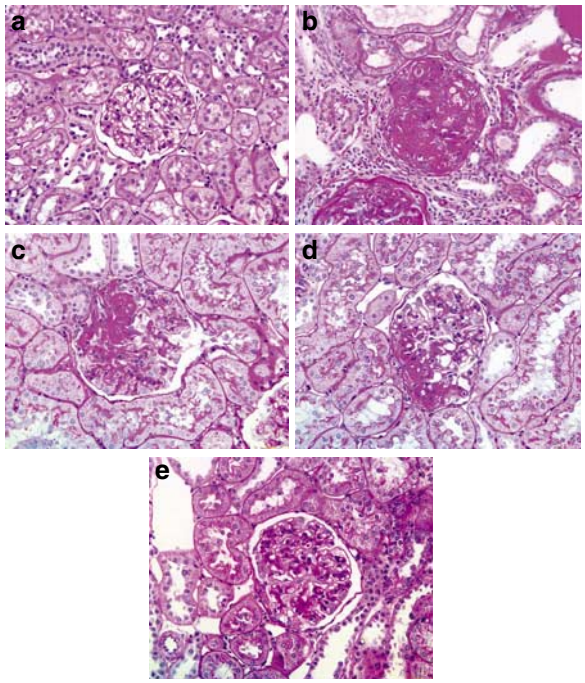

Figure 2 | Representative photomicrograph of periodic acid Schiff-stained sections from Sham, STNX rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. In (**a**) sham rats there is no glomerulosclerosis, while (**b**) STNx is associated with a dramatic increase in glomerulosclerosis. Treatment of STNx rats with (**c**) tranilast and (**d**) perindopril was associated with a reduction in extent of glomerulosclerosis. (**e**) Combination therapy with tranilast and perindopril was associated with less glomerulosclerosis than monotherapy. Original magnification $\times 360$.

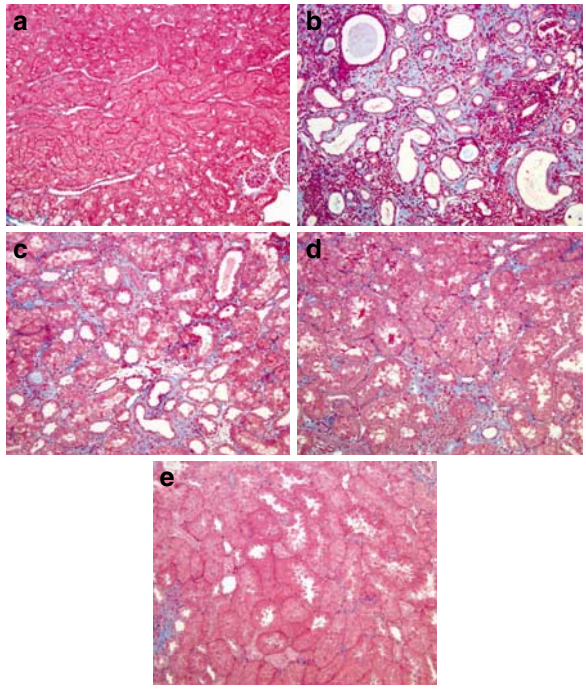

Figure 3 | Representative trichrome-stained sections showing tubulointerstitial fibrosis and atrophy in Sham, STNx rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. In (**a**) sham, there is no cortical tubular fibrosis or atrophy, while (**b**) STNx is associated with an increase in interstitial fibrosis (blue) and atrophic tubules (arrows). Treatment of STNx rats with (**c**) tranilast, (**d**) perindopril, and (**e**) combination therapy was associated with a reduction in both tubular fibrosis and atrophy. Original magnification $\times 380$.

to be particularly prominent in areas of fibrosis and atrophy (Figure 6). In contrast, phosphorylated Smad2 immunostaining was substantially lower in STNx rats treated with perindopril and with tranilast. However, animals treated with the combination of perindopril and tranilast showed less phosphorylated Smad2 immunostaining than did rats treated with either agent as monotherapy. Glomerular and tubulointerstitial regions showed similar patterns of phosphorylated Smad2 immunostaining.

In vitro *studies*. In cultured rat mesangial cells, TGF-β led to a significant increase in extracellular matrix production, as measured by ^{3}H-proline incorporation. This was attenuated by tranilast at 100 μM, although ^{3}H-proline incorporation remained higher than in controls. However, at 300 μM tranilast, ^{3}H-proline incorporation was similar to that in control cells (Figure 7).

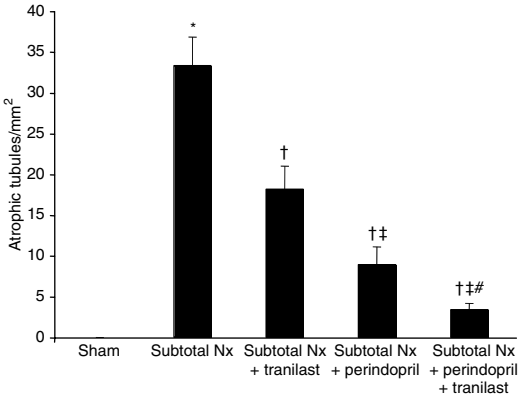

Figure 4 | Quantitation of atrophic tubules in rat kidneys from sham, STNx rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. Data are presented as mean $\pm$ s.e.m. $^*P<0.01$ compared with shams, $^\dagger P<0.01$ versus untreated STNx rat kidneys. $^\ddagger P<0.05$ versus STNx + tranilast. $^\# P<0.05$ versus monotherapy.

DISCUSSION

Pivotal experimental studies[16,17] and small clinical trials,[18,19] performed more than two decades ago, first demonstrated the beneficial effects of blood pressure lowering and later ACE inhibitor therapy on the progression of kidney disease, laying the foundations for major clinical trials in both diabetic[20–22] and non-diabetic kidney disease.[23,24] However, despite the widespread use of ACE inhibitors and ARBs in kidney disease, renal dysfunction continues to progress in the majority of patients, eventually leading to end-stage renal disease, in those who do not succumb to its co-morbidities. The present study demonstrates that therapy designed to

antagonize the effects of TGF-β, another pathogenetic factor in renal disease, resulted in an incremental renoprotective effect on the structural and functional manifestations of renal injury in the subtotally nephrectomized rat, when added to background ACE inhibitor therapy.

TGF-β is a multi-functional cytokine that has long been associated with the pathogenesis of progressive kidney disease, implicated in particular, with the development of fibrosis and atrophy.[25,26] It is synthesized as a 391 amino-acid precursor molecule with little biological activity, requiring cleavage of its N-terminal latency-associated peptide to give rise to its active form. In addition, the biological effects of TGF-β may also be modified by the presence of the proteoglycan decorin[27] and the scavenging protein alpha 2-macroglobulin.[28] Given these factors that may modulate TGF-β activity, we therefore assessed its biological effects by examining one of its specific intracellular actions, the phosphorylation and nuclear translocation of the TGF-β receptor-activated protein, Smad2.[29] Although only minimal phospho-Smad2 immunostaining was detected in the kidney of sham rats, those from subtotally nephrectomized animals showed prominent nuclear staining of phosphorylated Smad2. In contrast to untreated animals, those receiving either tranilast or perindopril as single agents showed substantial reductions in nuclear phospho-Smad2. Moreover, the combination of the two agents resulted in further incremental reductions in nuclear phospho-Smad2 that were greater than seen when either agent was used on its own.

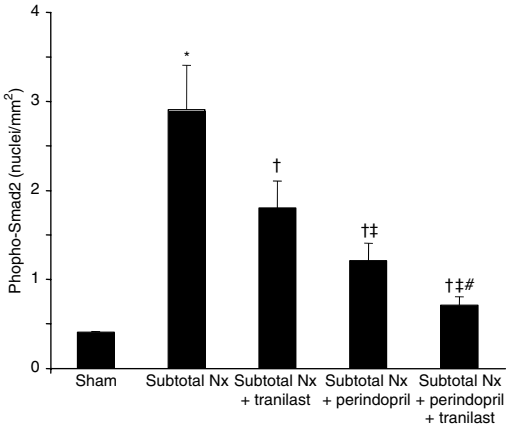

Figure 5 | Quantitation of tubulointerstitial (upper panel) and glomerular phospho-Smad 2 (lower panel) immunostaining in rat kidneys from sham, STNx rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. *$P<0.01$ compared with shams, $^{\dagger}P<0.01$ versus untreated STNx rat kidneys. $^{\ddagger}P<0.05$ versus STNx + Tran. $^{\#}P<0.05$ versus monotherapy.

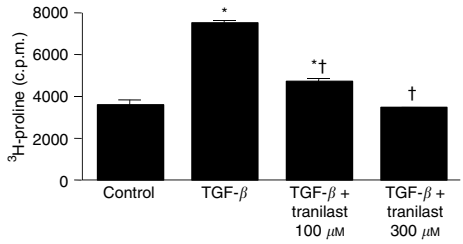

Figure 7 | *In vitro* effects of tranilast on TGF-β induced ^{3}H-proline incorporation in cultured rat mesangial cells. Values are expressed as mean ± s.e.m. *$P<0.01$ versus cells grown in control medium, $^{\dagger}P<0.01$ versus TGF-β-treated cells.

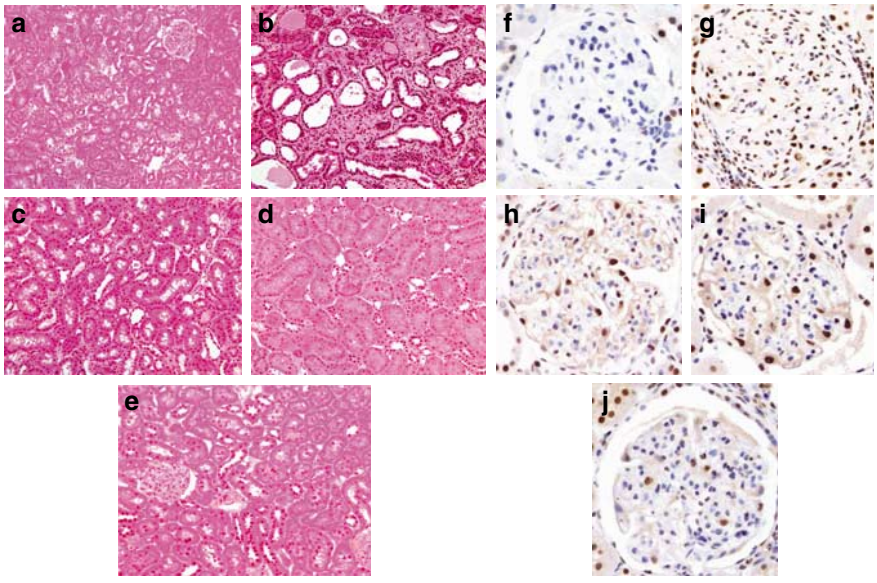

Figure 6 | Representative photomicrographs of (a–e) glomeruli and (f–j) tubulointerstitium immunostained for phospho-Smad 2 in Sham, STNx rats treated with tranilast, perindopril, or a combination of tranilast and perindopril. In (**a**, **f**) sham there is little evidence of phospho-Smad 2, while (**b**, **g**) STNx is associated with an increase immunostaining for phospho-Smad 2. (**c**, **h**) Treatment of STNx rats with tranilast, (**d**, **i**) perindopril, and (**e**, **j**) combination therapy was associated with an incremental reduction in phospho-Smad 2 immunostaining in the tubular epithelium. Original magnification × 380.

Blood pressure reduction and blockade of the renin–angiotensin system are key elements in attenuating the progression of chronic renal disease. We therefore used a dose of perindopril that in previous dose-finding studies by our group had been shown to effectively reduce blood pressure and provide renal protection,[30] with higher doses of the compound failing to provide further benefit.[31] As previously described in the 5/6 nephrectomy model,[12,16] treatment with either tranilast or ACE inhibitor, in the present report, led to improved glomerular filtration rate and diminution in proteinuria. However, in contrast to perindopril, tranilast exerted its renoprotective effects without affecting hypertension. Moreover, despite its lack of effect on systemic pressure, 'add-on' therapy with tranilast led to further incremental benefits on glomerular filtration rate and proteinuria beyond that with perindopril alone.

Histopathologically, kidney disease is characterized by glomerulosclerosis, tubulointerstitial fibrosis, and atrophy. When used as monotherapy, tranilast and perindopril each reduced the extent of glomerulosclerosis and tubulointerstitial fibrosis in subtotally nephrectomized rats. Moreover, when both drugs were used in combination, a further reduction in glomerulosclerosis, beyond that seen with single-agent treatment, was also noted. In addition to fibrosis, tubular atrophy has also been long recognized as an indicator of renal disease severity and progression,[4,32] where its development is thought to reflect apoptosis, rather than necrosis of epithelial cells.[33,34] Consistent with previous reports, we also found that both ACE inhibition and tranilast treatment reduced tubular atrophy.[12,35] However, the combination of these two agents, as used in the present study, showed a further beneficial effect on the extent of tubular atrophy, beyond that found with either single-agent treatment.

Overexpression of TGF-β is a characteristic feature of many human kidney diseases and of the 5/6 nephrectomy model in rats.[35] Although ACE inhibitors and ARBs attenuate this overexpression, tranilast does not.[12] However, as shown in the present study, tranilast effectively reduces TGF-β-induced collagen production. These separate, but related mechanisms of RAS blockade and tranilast in inhibiting TGF-β are consistent with their additive effects of these agents when used together in the setting of progressive renal disease.

In clinical studies, additional renal protection can be achieved with increasing the dose of ARB[36] or by combining ARBs and ACE inhibitor together.[37] In our own studies, we have previously explored the dose–response relationship between renal protection and perindopril dose, demonstrating a maximal perindopril effect at 2 mg/kg/day and also showing that higher doses of perindopril such as the 6 mg/kg/day, used in the present study, have similar efficacy to combining perindopril with the ARB, valsartan.[30,31] Accordingly, in the current study, we used the 6 mg/kg/day dose to determine whether an alternative non-RAS-dependent treatment may provide additional efficacy.

Agents used in experimental studies to antagonize the effects of TGF-β in kidney disease have included neutralizing antibodies[7] and the proteoglycan, decorin.[27] In the present study, we explored an alternative approach, using an orally active compound that is currently in clinical use, and that has previously been shown to block TGF-β-induced fibrosis *in vitro*[11,38,39] and in a number of *in vivo* settings.[11,12,14,40] The findings of the present study thus complement those in other disease models that have examined the effects of adding TGF-β inhibitory strategies to background therapy with ACE inhibitor or ARB. For instance, in studies of experimental diabetic nephropathy, Benigni *et al*[6] found that the addition of the TGF-β neutralizing monoclonal antibody, 1D11, to lisinopril led to an incremental attenuation of renal injury. Similarly, in studies of experimental mesangial proliferative glomerulonephritis, using the same antibody, Yu *et al*[41] also reported additional benefits by combining this agent with enalapril. In the present study, we examined animals that had undergone STNx. This well-characterized model of non-inflammatory proteinuric renal disease features many of the structural and functional attributes of chronic progressive kidney disease in humans, leading to its frequent use in exploring the pathogenetic pathways that contribute to the relentless progressive nature of kidney disease.[42]

In summary, treatment with tranilast in addition to ACE inhibitor therapy resulted in an incremental attenuation in the structural and functional manifestations of injury in association with evidence of reduced activation of the TGF-β-signalling pathway. In the context of a recent pilot study in humans combining tranilast with RAS blockade in diabetic nephropathy,[43] the findings of the present study suggest that tranilast may also provide a novel strategy for the treatment of non-diabetic kidney disease characterized by fibrotic scarring.

MATERIALS AND METHODS

Animals

A total of 80 male Sprague–Dawley rats weighing 200–250 g were randomized to eight groups of 10 animals each. Anesthesia was achieved by the intraperitoneal administration of pentobarbital (6 mg/100 g body weight, Boehringer Ingelheim, Artarmon, NSW, Australia). A total of 60 rats underwent STNx performed by right subcapsular nephrectomy and infarction of approximately two-thirds of the left kidney by selective ligation of two of 3–4 extrarenal branches of the left renal artery. Animals were then randomly assigned to the following groups: STNx and vehicle or STNx with either tranilast (400 mg/kg/day by twice daily gavage, Pharm Chemical, Shanghai Lansheng Corporation, China), perindopril (6 mg/kg/day, drinking water) or a combination of tranilast and perindopril. The control group underwent sham surgery consisting of laparotomy and manipulation of both kidneys before wound closure. Rats were housed in a temperature (22°C)-controlled room with *ad libitum* access to commercial standard rat chow (Norco Co-Operative Ltd, Lismore, NSW, Australia) and water during the entire study. Rats from each group were killed at 12 weeks postsurgery. On killing, the remnant (left) kidney was excised, decapsulated, then sliced sagitally with one-half immersion fixed in 10% neutral-buffered formalin and embedded in paraffin for histology, and the other half frozen in liquid nitrogen. All experiments adhered to the guidelines of the Animal Welfare and Ethics Committee of the St Vincent's Hospital and the National Health and Medical Research Foundation of Australia.

Renal function

Body weight was measured weekly. Plasma creatinine was measured by autoanalyzer (Beckman Instrumentals, Palo Alto, CA, USA) at the beginning and end of the study. Systolic blood pressure was measured in conscious rats using an occlusive tail-cuff plethysmograph attached to a pneumatic pulse transducer (Narco Bio-system Inc., Houston, TX, USA). Before killing, rats were housed in metabolic cages for 24 h for subsequent measurement of urinary creatinine and albumin excretion using a rat-specific radioimmune assay.[44]

Tissue preparation

Rats were anesthetized (Nembutal 60 mg/kg body weight intraperitoneally, Boehringer-Ingelheim, Australia) and the abdominal aorta cannulated with an 18-G needle. Perfusion–exsanguination commenced at SBP (180–220 mm Hg) via the abdominal aorta with 0.1 M phosphate-buffered saline, pH 7.4 (20–50 ml) to remove circulating blood, and the inferior vena cava adjacent to the renal vein was simultaneously severed, allowing free flow of the perfusate. After clearance of circulating blood, 10% buffered formalin was perfused for a further 5 min (100–200 ml) to fix the tissues.

Histopathology

Changes in kidney structure were assessed in a masked protocol in at least 25 randomly selected tissue sections from each group studied. Sections were stained with Mayer's hematoxylin and eosin, periodic acid Schiff's stain, or Masson's modified trichrome to demonstrate collagen matrix.

Glomerulosclerosis

The extent of glomerulosclerosis was determined in 3 μm kidney sections stained with periodic acid Schiff's stain, as described previously.[35] In brief, 50–80 glomeruli from each rat were examined in a masked protocol. The degree of sclerosis in each glomerulus was graded on a scale of 0–4 as described previously with Grade 0, normal; Grade 1, sclerotic area upto 25% (minimal); Grade 2, sclerotic area 25–50% (moderate); Grade 3, sclerotic area 50–75% (moderate to severe), and Grade 4, sclerotic area 75–100% (severe). A glomerulosclerotic index (GSI) was then calculated using the formula:

$$\mathrm{GSI} = \sum_{i=0}^{4} \mathrm{Fi(i)}$$

where Fi is the fraction of glomeruli in the rat with a given score (i).

Tubular atrophy

Tubular atrophy was assessed in kidney sections stained with Masson's trichrome. At $\times 40$ magnification (Olympus BX-50 light microscope, Olympus, Tokyo, Japan), six random and non-overlapping fields from each slide (two slides analyzed for $N = 6$ rats/group) were selected. Atrophic tubules were identified as described previously,[26] with results expressed as the number of atrophied tubules per field of kidney cortex.

TGF-β receptor activation

Activation of the TGF-β receptor was assessed by quantifying the nuclear expression of phosphorylated Smad2 using a rabbit anti-phospho-Smad2 antibody (Cell Signalling Technology, Boston, MA, USA) that detects endogenous Smad2 only when dually phosphorylated at Ser463 and Ser465. Sections were immunostained, as described previously,[45] according to the manufacturer's instructions. Sections incubated with 1:10 NGS, instead of the primary antiserum, served as the negative control.

Quantitation of matrix deposition and phospho-Smad2 expression

The extent of phospho-Smad2 immunostaining was also quantified using computer-assisted image analysis, as reported previously.[12] For glomerular phospho-Smad2, 10 glomeruli were examined from each rat and the number of glomerular nuclei that showed phospho-Smad2 immunolabelling were counted and expressed as positive nuclei/per glomerulus. For the tubulointerstitium, five random non-overlapping fields from six rats per group were captured and digitized using a BX50 microscope attached to a Fujix HC5000 digital camera. Digital images were then loaded onto a Pentium III IBM computer. The accumulation of matrix within the tubulointerstitium was similarly assessed on Masson's trichrome-stained sections using computer-assisted image analysis, as reported previously.[46,47] An area of blue on a trichrome-stained sections (for matrix) or brown on immunostained sections (for phospho-Smad2) were selected for their color ranges and the proportional area of tissue with their respective ranges of color was then quantified. Calculation of the proportional area stained blue (matrix) or brown (phospho-Smad2) was then determined using image analysis (AIS, Analytical imaging Station Version 6.0, Ontario, Canada).

Cell culture studies

To determine the effects of tranilast on glomerular collagen production *in vitro*, we studied a well-characterized cloned rat mesangial cell line[48] (gift of David Nikolic-Patterson), which was cultured in Dulbecco's modified Eagle's medium (Gibco™; Invitrogen, Grand Island, NY, USA) with fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in humidified 5% CO_2 atmosphere at 37°C. Cells were plated into 24-well dishes at low density and allowed to attach overnight. The subconfluent cells were then serum starved overnight in 0.1% fetal bovine serum before treatment with or without tranilast. Tranilast, 100 and 300 μM, was then added to the wells, followed 4 h later by ^{3}H-proline (1 μCi/well) and TGF-β 5 ng/ml (R&D systems, Minneapolis, MN, USA). Cells were harvested 48 h poststimulation, washed twice with ice-cold phosphate-buffered saline, and twice with 10% trichloroacetic acid, 500 μl 1 M NaOH, and then neutralized with 500 μl 1 M HCl. Incorporation of exogenous ^{3}H-proline (L-[2,3,4,5-^{3}H]-proline; Amersham Biosciences, Piscataway, NJ, USA) was then measured using a liquid scintillation counter (Wallac 1410; Amersham Biosciences). Cell viability was assessed by trypan blue exclusion.

Statistics

Data are expressed as means $\pm$ s.e.m. unless otherwise stated. Statistical significance was determined by a two-way analysis of variance with a Fishers *post-hoc* comparison. Owing to its skew distribution, data on albuminuria were log transformed prior to analysis and expressed as geometric mean x/tolerance factor. All analyses were performed using Statview II + Graphics package (Abacus Concepts, Berkeley, CA, USA) on an Apple Macintosh G4 computer (Apple Computer Inc., Cupertino, CA, USA). A P-value < 0.05 was regarded as statistically significant.

ACKNOWLEDGMENTS

This project was supported by a program grant from the National Health and Medical research Council of Australia and the Juvenile

Diabetes Research Foundation. We thank Mariana Pacheco and Jemma Court for excellent animal husbandry and Laura Di Rago for technical assistance. Darren Kelly is the recipient of a Career Development Award from the Juvenile Diabetes Research Foundation.

REFERENCES

1. Coresh J, Astor BC, Greene T *et al.* Prevalence of chronic kidney disease and decreased kidney function in the adult US population: Third National Health and Nutrition Examination Survey. *Am J Kidney Dis* 2003; **41**: 1–12.
2. Ruggenenti P, Schieppati A, Remuzzi G. Progression, remission, regression of chronic renal diseases. *Lancet* 2001; **357**: 1601–1608.
3. Bohle A, Wehrmann M, Bogenschutz O *et al.* The pathogenesis of chronic renal failure in diabetic nephropathy: investigation of 488 cases of diabetic glomerulosclerosis. *Pathol Res Pract* 1991; **187**: 251–259.
4. Risdon RA, Sloper JC, de Vardener HE. Relationship between renal function and histological changes found in renal biopsy specimens from patients with persistent glomerulonephritis. *Lancet* 1968; **ii**: 363–366.
5. Border WA, Brees D, Noble NA. Transforming growth factor-beta and extracellular matrix deposition in the kidney. *Contrib Nephrol* 1994; **107**: 140–145.
6. Benigni A, Zoja C, Corna D *et al.* Add-on anti-TGF-beta antibody to ACE inhibitor arrests progressive diabetic nephropathy in the rat. *J Am Soc Nephrol* 2003; **14**: 1816–1824.
7. Ziyadeh FN, Hoffman BB, Han DC *et al.* Long-term prevention of renal insufficiency, excess matrix gene expression, and glomerular mesangial matrix expansion by treatment with monoclonal antitransforming growth factor-beta antibody in db/db diabetic mice (see comments). *Proc Natl Acad Sci USA* 2000; **97**: 8015–8020.
8. Border WA, Noble NA, Yamamoto T *et al.* Natural inhibitor of transforming growth factor-beta protects against scarring in experimental kidney disease. *Nature* 1992; **360**: 361–364.
9. McGowan TA, Zhu Y, Sharma K. Transforming growth factor-beta: a clinical target for the treatment of diabetic nephropathy. *Curr Diab Rep* 2004; **4**: 447–454.
10. Shigeki S, Murakami T, Yata N *et al.* Treatment of keloid and hypertrophic scars by iontophoretic transdermal delivery of tranilast. *Scand J Plast Reconstr Surg Hand Surg* 1997; **31**: 151–158.
11. Mifsud S, Kelly DJ, Qi W *et al.* Intervention with tranilast attenuates renal pathology and albuminuria in advanced experimental diabetic nephropathy. *Nephron* 2003; **95**: 83–91.
12. Kelly DJ, Zhang Y, Gow R *et al.* Tranilast attenuates structural and functional aspects of renal injury in the remnant kidney model. *J Am Soc Nephrol* 2004; **15**: 2619–2629.
13. Mifsud S, Kelly DJ, Qi W *et al.* Intervention with tranilast attenuates renal pathology and albuminuria in advanced experimental diabetic nephropathy. *Nephron Physiol* 2003; **95**: p83–p91.
14. Miyajima A, Asano T, Yoshimura I *et al.* Tranilast ameliorates renal tubular damage in unilateral ureteral obstruction. *J Urol* 2001; **165**: 1714–1718.
15. Brenner BM, Meyer TW, Hostetter TH. Dietary protein intake and the progressive nature of kidney disease: the role of hemodynamically mediated glomerular injury in the pathogenesis of progressive glomerular sclerosis in aging, renal ablation, and intrinsic renal disease. *N Engl J Med* 1982; **307**: 652–659.
16. Anderson S, Rennke H, Brenner B. Therapeutic advantages of converting enzyme inhibitors in arresting progressive renal disease associated with systemic hypertension in the rat. *J Clin Invest* 1986; **77**: 1993–2000.
17. Zatz R, Dunn BR, Meyer TW *et al.* Prevention of diabetic glomerulopathy by pharmacological amelioration of glomerular capillary hypertension. *J Clin Invest* 1986; **77**: 1925–1930.
18. Parving HH, Andersen AR, Smidt UM *et al.* Early aggressive antihypertensive treatment reduces rate of decline in kidney function in diabetic nephropathy. *Lancet* 1983; **1**: 1175–1179.
19. Bjorck S, Nyberg G, Mulec H *et al.* Beneficial effects of angiotensin converting enzyme inhibition on renal function in patients with diabetic nephropathy. *Br Med J (Clin Res Ed)* 1986; **293**: 471–474.
20. Lewis EJ, Hunsicker LG, Bain RP *et al.* The effect of angiotensin-converting-enzyme inhibition on diabetic nephropathy. *N Engl J Med* 1993; **329**: 1456–1462.
21. Lewis EJ, Hunsicker LG, Clarke WR *et al.* Renoprotective effect of the angiotensin-receptor antagonist irbesartan in patients with nephropathy due to type 2 diabetes. *N Engl J Med* 2001; **345**: 851–860.
22. Brenner BM, Cooper ME, de Zeeuw D *et al.* Effects of losartan on renal and cardiovascular outcomes in patients with type 2 diabetes and nephropathy. *N Engl J Med* 2001; **345**: 861–869.
23. The GISEN Group (Gruppo Italiano di Studi Epidemiologici in Nefrologia). Randomised placebo-controlled trial of effect of ramipril on decline in glomerular filtration rate and risk of terminal renal failure in proteinuric, non-diabetic nephropathy. *Lancet* 1997; **349**: 1857–1863.
24. Ruggenenti P, Perna A, Gherardi G *et al.* Renoprotective properties of ACE-inhibition in non-diabetic nephropathies with non-nephrotic proteinuria. *Lancet* 1999; **354**: 359–364.
25. Border WA, Noble NA. Transforming growth factor-β in tissue fibrosis. *New Engl J Med* 1994; **331**: 1286–1392.
26. Kelly DJ, Cox AJ, Tolcos M *et al.* Attenuation of tubular apoptosis by blockade of the renin–angiotensin system in diabetic Ren-2 rats. *Kidney Int* 2002; **61**: 31–39.
27. Yamaguchi Y, Mann DM, Ruoslahti E. Negative regulation of transforming growth factor-β by the proteoglycan decorin. *Nature* 1990; **346**: 281–284.
28. O'Connor-McCourt MD, Wakefield LM. Latent transforming growth factor β in serum. *J Biol Chem* 1987; **262**: 14090–14099.
29. Massague J, Chen YG. Controlling TGF-beta signaling. *Genes Dev* 2000; **14**: 627–644.
30. Mifsud SA, Skinner SL, Cooper ME *et al.* Effects of low-dose and early versus late perindopril treatment on the progression of severe diabetic nephropathy in (mREN-2)27 rats. *J Am Soc Nephrol* 2002; **13**: 684–692.
31. Wilkinson-Berka JL, Gibbs NJ, Cooper ME *et al.* Renoprotective and anti-hypertensive effects of combined valsartan and perindopril in progressive diabetic nephropathy in the transgenic (mRen-2)27 rat. *Nephrol Dial Transplant* 2001; **16**: 1343–1349.
32. Schainuck LI, Striker GE, Cutler RE *et al.* Structural-functional correlations in renla disease. Part II. The correlations. *Hum Pathol* 1970; **1**: 631–641.
33. Schelling JR, Cleveland RP. Involvement of Fas-dependent apoptosis in renal tubular epithelial cell deletion in chronic renal failure. *Kidney Int* 1999; **56**: 1313–1316.
34. Schelling JR, Nkemere N, Kopp JB *et al.* Fas-dependent fratricidal apoptosis is a mechanism of tubular epithelial cell deletion in chronic renal failure. *Lab Invest* 1998; **78**: 813–824.
35. Wu L, Cox A, Roe C *et al.* Transforming growth factor β1 and renal injury following subtotal nephrectomy in the rat: role of the renin–angiotensin system. *Kidney Int* 1997; **51**: 1553–1567.
36. Rossing K, Schjoedt KJ, Jensen BR *et al.* Enhanced renoprotective effects of ultrahigh doses of irbesartan in patients with type 2 diabetes and microalbuminuria. *Kidney Int* 2005; **68**: 1190–1198.
37. Nakao N, Yoshimura A, Morita H *et al.* Combination treatment of angiotensin-II receptor blocker and angiotensin-converting-enzyme inhibitor in non-diabetic renal disease (COOPERATE): a randomised controlled trial. *Lancet* 2003; **361**: 117–124.
38. Yamada H, Tajima S, Nishikawa T *et al.* Tranilast, a selective inhibitor of collagen synthesis in human skin fibroblasts. *J Biochem (Tokyo)* 1994; **116**: 892–897.
39. Miyazawa K, Kikuchi S, Fukuyama J *et al.* Inhibition of PDGF- and TGF-beta 1-induced collagen synthesis, migration and proliferation by tranilast in vascular smooth muscle cells from spontaneously hypertensive rats. *Atherosclerosis* 1995; **118**: 213–221.
40. Pinto YM, Pinto-Sietsma SJ, Philipp T *et al.* Reduction in left ventricular messenger RNA for transforming growth factor beta(1) attenuates left ventricular fibrosis and improves survival without lowering blood pressure in the hypertensive TGR(mRen2)27 rat. *Hypertension* 2000; **36**: 747–754.
41. Yu L, Border WA, Anderson I *et al.* Combining TGF-beta inhibition and angiotensin II blockade results in enhanced antifibrotic effect. *Kidney Int* 2004; **66**: 1774–1784.
42. Brenner BM. Hemodynamically mediated glomerular injury and the progressive nature of kidney disease. *Kidney Int* 1983; **23**: 647–655.
43. Soma J, Sugawara T, Huang YD *et al.* Tranilast slows the progression of advanced diabetic nephropathy. *Nephron* 2002; **92**: 693–698.
44. Kelly DJ, Hepper C, Zhang Y *et al.* Protein kinase C β inhibition attenuates the progression of experimental diabetic nephropathy in the presence of continued hypertension. *Diabetes* 2003; **52**: 512–518.
45. Kelly DJ, Cox AJ, Gow RM *et al.* Platelet-derived growth factor receptor transactivation mediates the trophic effects of angiotensin II *in vivo*. *Hypertension* 2004; **44**: 195–202.
46. Lehr HA, Mankoff DA, Corwin D *et al.* Application of photoshop-based image analysis to quantification of hormone receptor expression in breast cancer. *J Histochem Cytochem* 1997; **45**: 1559–1565.
47. Lehr HA, van der Loos CM, Teeling P *et al.* Complete chromogen separation and analysis in double immunohistochemical stains using Photoshop-based image analysis. *J Histochem Cytochem* 1999; **47**: 119–126.
48. Kakizaki Y, Kraft N, Atkins RC. Differential control of mesangial cell proliferation by interferon-gamma. *Clin Exp Immunol* 1991; **85**: 157–163.