NADPH oxidase subunits (NOX-1, p22phox, Rac-1) and tacrolimus-induced nephrotoxicity in a rat renal transplant model

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Abstract

Background. TGF-β and oxidative stress are known mediators of renal injury. However, the precise mechanisms by which TGF-β and oxidative stress may be involved in the development of nephrotoxicity are not known. We examined whether anti-TGF-β antibody limits nephrotoxicity produced by tacrolimus (TAC) and whether this altered genes that regulate oxidative stress.

Methods. Renal transplants were performed in Wistar-Furth and Lewis rat strains. Groups included: isograft controls; untreated allografts; allografts treated with 0.25 mg/kg TAC till 90 days with or without 1.0 mg/kg anti-TGF-β antibody or control antibody. Serum creatinine and BUN levels and renal histology were determined. Real time PCR and western analysis were used to quantify mRNA and protein expression.

Results. BUN and creatinine were elevated in TAC-treated rats. TAC increased expression of TGF-β (37-fold) and NADPH oxidase subunits, NOX-1 (18-fold), p22phox (31-fold) and Rac-1 mRNA (20-fold), respectively. Contrariwise, expression of antioxidant genes, superoxide dismutase (SOD) and thioredoxin (TRX) was decreased. Anti-TGF-β antibody but not control antibody reversed the TAC-induced changes in gene expression, renal histology and function.

Conclusions. Our findings suggest a potential for anti-TGF-β antibody as a novel adjunct therapeutic tool to prevent TAC-induced nephrotoxicity in transplant recipients. The mechanism of protection involves suppression of TGF-β and the expression of genes that regulate oxidative stress. Moreover, the specific up-regulation of NOX-1, a non-phagocytic NADPH oxidase subunit and its reversal by anti-TGF-β antibody strongly implicates for the first time the up-regulation of renal parenchymal cell NADPH oxidase in the aetiology of immunosuppression-induced nephrotoxicity.

Keywords: anti-TGF-β antibody; NADPH oxidase; nephrotoxicity; rat renal transplant model; tacrolimus; TGF-β

Introduction

The mechanism of immunosuppression-associated nephrotoxicity is not completely understood. Transforming growth factor-β (TGF-β) is one of the leading candidates for mediating nephrotoxicity since cyclosporine (CsA), tacrolimus (TAC) and sirolimus (SRL) induce TGF-β expression [1–7]. Oxidative stress can also stimulate TGF-β production. Oxidative stress has been considered to be an important mediator of immunosuppression-induced renal injury [8,9]. In fact, there is sufficient evidence showing that TGF-β alone can increase levels of reactive oxygen in cultured renal cells [10–13]. However, the precise mechanism by which TGF-β and immunosuppressive drugs increase reactive oxygen species has not been determined. The increased levels of reactive oxygen species might be due to a decrease in antioxidant genes such as the depletion of SOD as has been shown by exposure of rat hepatocytes to TGF-β [10]. Alternatively, increased levels of reactive oxygen might be increased due to the increased production of reactive oxygen species by certain oxidases.

NADPH oxidases are prime candidates as sources of reactive oxygen production. Phagocytic NADPH oxidase consists of a catalytic core of two membrane-associated subunits, gp91phox and p22phox. The protein gp91phox is considered to be the subunit responsible for phagocytic O₂⁻ production [14]. NADPH oxidase in the resting state becomes activated to produce O₂⁻ upon interaction with cytoplasmic components, (p47phox and p67phox), and Rac, a small GTPase. Activation of NADPH oxidase in the aetiology of immunosuppression-induced nephrotoxicity.
oxidase requires translocation of cytoplasmic components to the membrane fraction. Genetic ablation of NADPH oxidase subunits has been shown to inhibit $O_2^-$ production in inflammatory states [15,16].

In addition to phagocytic NADPH oxidases, there is emerging evidence of the presence of novel protein analogues of these NADPH oxidases that are non-phagocytic in origin. In kidney, NOX-1 is a membrane-bound NADPH oxidase subunit and part of the catalytic core which is a non-phagocytic analogue of the phagocytic subunit gp91phox [17]. The importance of non-phagocytic NADPH oxidase in renal pathology was demonstrated in studies showing that NOX-1 is increased in rat kidney stressed by infusion with angiotensin II [18].

Since oxidative stress can also stimulate TGF-β production, oxidative stress is also believed to mediate TGF-β-induced expression of extracellular matrix proteins. While the latter promotes fibrogenesis, the exact cascade of events initiated by immunosuppressant treatment such as activation of TGF-β and its downstream signals mediating events leading to the development of nephrotoxicity are not known. The potential role of TGF-β in mediating CsA-induced nephrotoxicity was demonstrated in one of our recent studies in which treatment with low-dose anti-TGF-β antibody prolonged graft survival and decreased nephrotoxicity in a rat cardiac transplant model [7].

Despite these new findings, it has not yet been determined if antagonizing the actions of TGF-β by antibody intervention can uniformly be applied to limit nephrotoxicity mediated by immunosuppressant drugs other than CsA. Contrary to prior dogma, the nephrotoxic effects of TAC have been determined to be similar to CsA [19–23]. Like CsA, we have determined that TAC also induces the expression of TGF-β [4].

The present study was planned to examine (i) if NADPH oxidase components and TGF-β mediate the nephrotoxic effects of TAC in a rat model of renal transplantation, (ii) if anti-TGF-β antibody neutralizes the effects of TGF-β but also inhibits expression of genes related to oxidative stress and the development of nephrotoxicity. We studied the effect of long-term treatment with TAC on renal function, histology, intragraft mRNA and protein expression of TGF-β.

Since NADPH oxidase subunits components (NOX-1, p22phox and Rac-1) are sources of $O_2^-$ production whereas SOD decreases $O_2^-$ and thioredoxin (TRX) decreases redox signalling, we also examined expression of these key genes in modulating oxidative stress. The role of TGF-β in mediating these changes was determined by a strategy involving treatment with anti-TGF-β antibody versus control antibody concomitant with TAC on nephrotoxicity. The results demonstrate that the nephrotoxic effects of TAC are mitigated by anti-TGF-β antibody. Furthermore, our findings show that the mechanism also involves preventing changes in gene expression that would otherwise promote oxidative stress.

### Methods

#### Rat renal transplantation

We used Lewis (LEW, RT1<sup>1</sup>) and Wistar-Furth (WF RT1<sup>1</sup>) rats, which represent complete genetic disparity at both major and minor histocompatibility loci. LEW or WF donor kidneys were transplanted into LEW recipient rats to represent isogeneic and allogeneic transplantation, respectively. In renal donors, following induction of general anesthesia with 50mg/kg pentobarbital i.p., a midline laparotomy was performed. The left kidney was mobilized on its vascular pedicle and the ureter was divided. Following heparinization (1000 U, i.p.), the left kidney was removed with a generous cuff of aorta and vena cava. The kidney was then flushed with iced saline, stored on ice and then transplanted. Through a midline laparotomy in the recipient (anaesthetized as above), the infrarenal aorta and vena cava were exposed. Using standard, established microvascular surgical techniques, the left donor kidney was anastomosed in an end-to-side fashion to the recipient vessels and the blood flow restored. The ureter was inserted through the bladder wall and stented with a short segment of PE50 tubing. Both kidneys of the recipients were removed and the only functional kidney in these transplant recipients was the transplanted kidney.

#### Animal monitoring, sacrifice, tissue harvesting

Rats housed in metabolic cages were monitored daily for urine output for evidence of graft rejection, which was established by a significant increase in weight gain and a decrease in urine output. Renal function was determined by changes in serum creatinine and BUN levels. At completion of the study period, animals were anaesthetized with sodium pentobarbital (50 mg/kg i.p). Following exsanguination via cardiac puncture, isografts and allografts were obtained for routine histology and immunohistochemistry, gene and protein expression (western analysis, real time RT–PCR).

#### Experimental groups

The following groups of animals ($n = 3$ in each group) were used for this study. Group A: isografts kept for 90 days; Group B: 0.25 mg/kg i.m TAC for 90 days; Group C: TAC + anti-TGF-β antibody (1D11, 1 mg/kg/twice a week); Group D: TAC + control antibody (1 mg/kg/twice a week i.m). Anti-TGF-antibody designated as 1D11 is a murine monoclonal antibody (IgG1), which specifically neutralizes the biological activity of TGF-β 1, 2 and 3. The antibody recognizes active but not latent TGF-β. Control antibody (13C4) is also a murine monoclonal antibody (IgG1) that specifically binds shigella toxin. Both antibodies were produced and purified at Genzyme Corporation (Cambridge, MA, USA) and were determined to be free of detectable endotoxin [4]. Based on our experience [7], we designed this study using an antibody dose of 1.0 mg/kg. In contrast to high dose (2.5 mg/kg), this low antibody dose (1.0 mg/kg) limits immunosuppression-induced nephrotoxicity without altering graft survival in the rat [7].
BUN and creatinine levels

Assay of serum levels of BUN and creatinine were performed to assess renal function using specific kits (Wako Chemicals Inc., Richmond, VA and Oxford Biomedical Research, Oxford, MI, USA, respectively).

Allograft histology, immunohistochemistry and quantification of histopathological changes

A portion of transplanted kidneys from each animal was fixed in formalin and paraffin imbedded. Haematoxylin and eosin (H&E) and periodic acid schiff (PAS) staining were used to assess histological changes. Two different individuals graded the histopathological findings using numbered slides that did not give any indication from what group the sample was derived. We quantified renal histological changes by standard procedures. Using light microscopy, we evaluated the severity of the glomerular injury score according to established methods [1,4]. In each field, we counted at least 50–60 glomeruli for each specimen and score the lesions into five grades. In H&E stained renal tissue sections, histopathological grading was defined as: score 0, no proliferation, almost normal histology; score 1, about 25% segmental lesion; score 2, segmental lesion >25 but <50%; score 3, fibrotic lesions with diffuse proliferation; score 4, almost completely fibrotic changes. Using PAS staining, we determined the extent of extracellular matrix protein accumulation; the grading was not performed on matrix deposition. The interlobular arteries in H&E stained slides and the lesions were scored.

Plasma TGF-β protein and immunochemistry in renal tissues

Plasma levels of TGF-β protein were measured as previously described [5]. Intragraft protein expression of TGF- β was studied using immunohistochemistry [1,2,4]. Formalin-fixed tissues were paraffin-embedded, sliced into fine sections, deparaffinized in xylene and rehydrated in graded ethanol tissues were paraffin-embedded, sliced into fine sections, deparaffinized in xylene and rehydrated in graded ethanol.

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<th>Anti-sense sequence</th>
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Detection of mRNA by real time PCR

We performed real time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA). RNAs were isolated from renal tissues using a kit from Promega (Madison, USA) and reverse-transcribed into cDNAs by using a cDNA synthesis kit from invitrogen (Carlsbad, CA). The specificity of primers was tested by running a regular PCR for 40 cycles at 95°C for 20 s and 60°C for 1 min, and followed separating in ethidium bromide containing agarose gels. The primer sequences are given in Table 1. The real time PCR was performed using a SYBR supermix kit (Bio-RAD), and running for 40 cycles at 95°C for 20 s and 60°C for 1 min. The PCR efficiency was also examined by serially diluting the template cDNA and the melting curve data was collected to check the PCR specificity and proper negative controls were included in each assay. The mRNA level for each gene for each sample was normalized to β-actin mRNA and was presented as 2[(Ct/β-actin)]^+ Ct/gene of interest] as described [24].

Western analysis

Frozen tissues were homogenized in ice-cold PBS with 1% Triton X-100, 1 mM phenylmethysulfonyl fluoride, 35 ng/ml pepstatin A and 10 ng/ml leupeptin. After centrifugation, 50g of protein was electrophoresed by SDS PAGE as described in our laboratory [24]. Blots were probed with appropriate dilutions of a polyclonal anti-p22phox antibody.
NADPH oxidase and transplant nephrotoxicity

creatinine levels (but not control antibody significantly inhibited TAC-induced recipients compared to isografts (significant increase in serum creatinine (0.83 ± 0.06 vs 0.52 ± 0.09 mg/dl; P < 0.04) was observed in TAC-treated recipients compared to time-matched isograft transplant controls. Similar to BUN levels, anti-TGF-β antibody (0.48 ± 0.08 mg/dl; P < 0.02) but not control antibody (0.78 ± 0.09) inhibited TAC-induced creatinine levels (Figure 1B).

Anti-TGF-β antibody and TAC-induced expression of intragraft TGF-β, NOX-1, p22phox and Rac-1 mRNA

We performed a real time PCR analysis for intragraft expression of TGF-β, NOX-1 and p22phox mRNA in renal tissues from isografts, TAC, TAC + anti-TGF-β antibody and TAC + control antibody-treated animals at 90 days post-transplantation. The results are presented with respect to isograft transplant controls. Treatment with TAC increased mRNA expression of TGF-β (by 37-fold), NOX-1 (by 18-fold), and p22phox (by 31-fold) and Rac-1 (by 20-fold). Increases in gene expression were inhibited by anti-TGF-β (P < 0.01; P < 0.04, 0.008, P < 0.04 and P < 0.03, respectively) but were not inhibited by control antibody (Figure 2A). The mRNA expression of gp91phox was undetectable in all the groups and the levels of β-actin mRNA (in terms of Ct value) were similar in each group.

Differential intragraft mRNA expression of TGF-β, SOD and TRX

Since we observed that treatment of renal transplant recipients with TAC resulted in increased intragraft expression of TGF-β and NADPH oxidase subunit components, the next step was to determine the effect of prolonged treatment with TAC on antioxidant genes. We examined intragraft mRNA expression of SOD and TRX and compared it with TGF-β mRNA expression. The data demonstrate that SOD and TRX mRNA decreased whereas TGF-β increased in TAC-treated rats compared to isograft transplant controls. SOD mRNA was partially reversed whereas TRX mRNA was completely reversed in TAC + anti-TGF-β antibody-treated rats. We calculated the fold-increase in expression of TGF-β, SOD and TRX mRNA. Treatment with TAC resulted in a 37-fold increase in TGF-β whereas mRNA expression of SOD and TRX decreased 27- and 80-fold, respectively. These changes were reversed by anti-TGF-β antibody (Figure 2B).

Intragraft expression of p22phox and NOX-1 protein

We studied the expression of p22phox and NOX-1 protein in kidney tissues from isografts, and
treated with TAC. The protein lysates (10 μg) were electrophoresed, transferred to nitrocellulose paper and probed with anti-p22phox and anti NOX-1 antibodies. The results (Figure 2C) show that treatment with TAC resulted in increased intragraft p22phox and NOX-1 protein. More significantly, anti-TGF-β treatment decreased TAC induced increased intragraft p22phox and NOX-1 protein expression.

**Treatment with TAC results in the transplant-nephrotoxicity specific renal histological changes**

We examined the effect of TAC, TAC + a control antibody and TAC + anti-TGF-β antibody treatment on morphological changes by histopathological examination in H&E- and PAS-stained thin kidney sections. We scored the morphological changes. The light microscopic findings of kidneys of transplant control rats from isografts (panels 5 and 6) showed normal glomeruli, afferent arterioles and tubule cells. In sharp contrast, the renal tissues of rats treated with TAC showed marked histological changes including, severe to moderate epical blebbing, hyalinization, glomerular basement thickening, a pattern of tubulo-interstitial fibrosis including arteriopathy of afferent arteriole (panel 1) and terminal portions of the interlobular arteries. These changes were not observed in co-administration of TAC and anti-TGF-β antibody (panels 3 and 4). However, similar changes can be seen in animals treated with TAC + a control antibody.

Histology in TAC + control antibody-treated animals was not different than TAC alone (not shown). Renal toxicity specific to proximal tubular epithelial cells specific changes specific to renal toxicity can be seen in TAC and TAC + control antibody-treated rat transplant recipients and not in isografts or TAC + anti-TGF-β antibody-treated rats (Figure 3B). TAC-induced tubular changes (tubular atrophy/vacuolization, hyalinization are also shown in Figure 3B. These changes were seen only in TAC and TAC + control antibody-treated renal transplant recipients but not in isografts and TAC + anti-TGF-β antibody-treated transplant recipients. The quantitative analysis (Figure 3C) demonstrated a statistically significant (P < 0.036) score in TAC-treated recipients compared to the control isografts which were not different than TAC + anti-TGF-β antibody-treated allografts.

**Tacrolimus increases intragraft TGF-β protein expression**

We have also examined intragraft expression of TGF-β protein in kidney tissues from recipients of renal transplants treated with TAC, TAC + anti-TGF-β antibody, TAC + control antibody-treated or untreated allografts. Renal sections were stained with anti-TGF-β antibody as earlier described by our group [7]. Beside hyalinization and arteriopathy tubulo-interstitial fibrosis and glomerular basement membrane thickening, a positive staining for TGF-β protein can be seen in TAC and TAC + control antibody-treated recipients but not in TAC + anti-TGF-β antibody-treated recipients (Figure 4A). The intragraft expression of TGF-β protein in TAC + anti-TGF-β antibody-treated recipients was slightly higher compared to the isografts, based on our experience; this suggests that the anti-TGF-β antibody only neutralized the induced but not the constitutive TGF-β protein expression.

**Effect of treatment with TAC on circulating levels of TGF-β protein**

These results (Figure 4B) demonstrate that compared to isografts, long-term treatment of TAC resulted in a
significant increase in circulating levels of TGF-β protein (9.8 ± 1.7 vs 57 ± 6 ng/mL; *p < 0.01). There was no difference in TGF-β levels among isografts and TAC + control antibody-treated recipients (57 ± 6 vs 52 ± 6 ng/ml); however, a statistically significant difference (9 ± 1.3 vs 57 ± 6 ng/ml; *P < 0.01) was observed between TAC and TAC + anti-TGF-β antibody-treated recipients (Figure 4B).

**Discussion**

Immunosuppression-induced nephrotoxicity remains an unavoidable and significant problem in organ transplantation. Furthermore, there is a lack of sufficient understanding of its pathogenesis and mechanism(s) involved. The results of the present study for the first time demonstrate that besides TGF-β, oxidative stress also contributes significantly to this process. This action occurs through two separate mechanisms. The first mechanism is by down-regulation of antioxidant defense genes which detoxify reactive oxygen such as SOD and TRX, which detoxify reactive oxygen. The second mechanism is by up-regulation of NADPH oxidase subunit genes which increase reactive oxygen production.

We used a rat renal transplantation model with long-term treatment with TAC that results in renal dysfunction as determined by increases in BUN and creatinine and by changes in renal histopathology. Treatment with TAC also increased intragraft expression of TGF-β mRNA and protein expression as well as the circulating levels of TGF-β protein. This is similar to our previous findings in a rat cardiac transplant model treated with CsA [7]. In that model using anti-TGF-β antibody treatment, we were able to document the important role of CsA-induced up-regulation of TGF-β in nephrotoxicity using an anti-TGF-β antibody strategy. In the present study, we found that treatment with anti-TGF-β antibody inhibited TAC-associated renal dysfunction. This conclusion is based upon the documented by inhibition of changes in BUN, creatinine levels and renal histopathology. These results suggest that anti-TGF-β antibody treatment may protect against nephrotoxicity in renal transplant recipients by potentially neutralizing the induced TGF-β protein and gene expression.

Immunosuppressive agents such as CsA and TAC have been shown to promote generation of reactive oxygen species in a number of cell types [25–29]. Despite this, the exact mechanism of how reactive oxygen levels are up-regulated by these agents remains unresolved. One theory is that reactive oxygen species are by-products of the metabolism of immunosuppressive drugs [27]. According to this, the normal $O_2^-$ due to CsA induced inhibition of NADPH cytochrome P-450 reductase might have resulted in $O_2^-$, increased NADPH activity and lipid peroxidation [30–32]. Currently, there is no evidence if a similar mechanism of drug metabolism also plays a role in the TAC-induced increased $O_2^-$. Reactive oxygen species as well as the end-products of lipid peroxidation like 4-hydroxy-2, 3-nonenal induce expression of TGF-β in renal tissue 1 h after TAC administration in rats [33]. These data suggest that TAC-induced nephrotoxicity may be related to increased oxidative stress and lipid peroxidation.

It has also been determined that reactive oxygen species enhance TGF-β release in vitro and in vivo [34]. Thus, increased reactive oxygen may be both a promoter of TGF-β expression as well as a potential mediator of downstream actions of TGF-β. Regarding the latter, we have previously shown that TGF-β increased albumin permeability in isolated glomeruli and that this injury was antagonized by SOD or dimethylthiourea [35]. This finding suggests a potential role of $O_2$ and hydroxyl radicals as downstream mediators of the nephrotoxic actions of TGF-β. The results of the present study demonstrate that not only intragraft mRNA and protein expression of TGF-β are significantly increased in rat renal transplant recipients treated with TAC. In addition, our study shows for the first time that the marked up-regulation of NADPH oxidase components ($p22^{phox}$, NOX-1, Rac-1) are significant sources for increased $O_2^-$ production in TAC-induced nephrotoxicity. This suggests that interventions to limit actions of TGF-β such as in this study using anti-TGF-β antibody might also have additional benefits to limit downstream actions of oxidative stress and nephrotoxicity associated with long-term treatment of transplant recipients with TAC.

In our study, we were able to show for the first time that expression of major antioxidant genes, SOD and TRX, are down-regulated in renal transplants treated with TAC. These proteins are primary enzymes important in detoxifying reactive oxygen species in cells. We found that co-administration with anti-TGF-β antibody inhibited the down-regulation in SOD and TRX gene expression. While TGF-β has previously been shown to decrease MnSOD in cultured hepatocytes [10], it has not, however, been determined that this action may occur in other cell types or tissues including renal cells/tissues.

In our renal transplant model, the antagonizing of the down-regulation of SOD and TRX by anti-TGF-β gives evidence of a novel potential pathway by which TAC may induce nephrotoxicity in transplant recipients. Our study describes this novel finding that TAC also alters the redox state of cells by down-regulating TRX expression. To the best of our knowledge, TRX expression has not been previously determined for any immunosuppressant agent. Furthermore, our findings suggest a new source contributing to enhanced oxidative stress in this condition.

This conclusion arises from our other novel findings that anti-TGF-β antibody but not control antibody prevents TAC-induced up-regulation of NADPH oxidase gene and protein expression. Taken together with our findings with SOD and TRX, we find that TAC has potentially two mechanisms of increasing oxidative stress; one by
Fig. 3. Effect of TGF-β antibody on renal histology. (A) The light microscopic findings of kidneys of control rats (isografts) showed normal glomeruli, afferent arterioles and tubule cells (panels 5 and 6). In sharp contrast, the renal tissues of rats, which received TAC showed marked histological changes including, severe to moderate epical blebbing, hyalinization, glomerular basement thickening, a pattern of tubulo-interstitial fibrosis and arteriolopathy of afferent arteriole (panel 1, black arrow) and terminal portions of the interlobular arteries. These changes were not observed in co-administration of TAC and anti-TGF-β antibody (panels 3 and 4). Tubular thickening, remodelling and arteriolopathy tubulo-interstitial fibrosis and glomerular basement membrane thickening can be seen in TAC-treated recipients (panels 1 and 2) but not in TAC + anti-TGF-β antibody-treated recipients (panels 3 and 4). B: Tubular changes such as dilation, swelling, thickening, remodelling and hyalinization are seen in TAC and TAC + control antibody-treated recipients but not in the isografts or TAC + anti-TGF-β antibody-treated recipients, magnification 400x. (C) Statistically significant semi-quantitative changes (*P < 0.036) in kidneys from TAC-treated rat renal transplant recipients compared to TAC + anti-TGF-β antibody are shown. There were no differences in TAC alone vs TAC + control antibody and isografts vs TAC + anti-TGF-β antibody-treated animals.
up-regulating genes that increase $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$ production and a second by down-regulating genes that remove or scavenge $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$. Our findings on the collective role of TGF-β and $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$ are also supported by studies on other models of renal injury such as salt-sensitive Dahl rats [36,37] and an angiotensin-induced hypertension model [38]. In these studies, the improvement in the renal injury was associated with a simultaneous inhibition of TGF-β and oxidative stress.

The findings of the increased mRNA expression of NADPH oxidase components ($\text{p}22\text{phox}$, NOX-1 and Rac-1) by TAC distinguishes it from studies showing renal dysfunction due to salt loading in the Dahl S rat in which $\text{p}22\text{phox}$ and Rac-1 mRNA were not induced but rather $\text{gp}91\text{phox}$ and $\text{p}47\text{phox}$ [39]. This suggests that up-regulation of specific NADPH oxidases may mediate renal pathology under different conditions.

In addition to phagocytic NADPH oxidases, there is emerging evidence that non-phagocytic NADPH

Fig. 4. Intragraft expression of TGF-β protein. Representative slides of immuno-histochemical staining for TGF-β in renal tissues from isografts, TAC, TAC + anti-TGF-β and TAC + control antibody-treated animals are shown. The results demonstrate a significantly higher staining of intragraft TGF-β protein expression in TAC-treated recipients which was not different in control antibody + TAC-treated recipients. TGF-β protein staining in animals treated with TAC + anti-TGF-β antibody (1.0 mg/kg) was abolished, however, was slightly higher than isografts (A). (B) Circulating levels of TGF-β protein. Circulating levels of TGF-β protein in plasma samples were quantified by ELISA. A statistically significant increased expression in TAC-treated recipients can be seen compared to isograft transplant controls. A statistically significant decrease of TGF-β levels can be seen in TAC + anti-TGF-β antibody compared to TAC-treated animals; whereas, no difference with TAC + control antibody-treated recipients can be seen. *$P < 0.01$; **$P < 0.002$. 

up-regulating genes that increase $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$ production and a second by down-regulating genes that remove or scavenge $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$. Our findings on the collective role of TGF-β and $\text{O}_2^-\text{C}\text{C}_1\text{C}_5\text{C}_0$ are also supported by studies on other models of renal injury such as salt-sensitive Dahl rats [36,37] and an angiotensin-induced hypertension model [38]. In these studies, the improvement in the renal injury was associated with a simultaneous inhibition of TGF-β and oxidative stress.

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In addition to phagocytic NADPH oxidases, there is emerging evidence that non-phagocytic NADPH oxidases
oxidases are present in many cells and could be important sources for $O_2^-$ production in inflammatory states. In kidney, NOX-1 is the non-phagocytic membrane analogue of the phagocytic subunit gp91-phox. Despite this knowledge, the detailed analysis of these regulatory components in the events leading to increased $O_2^-$ production and initiation of events leading to nephrotoxicity have not been explored.

Our findings with gp91-phox do not support a significant role of phagocytic NADPH oxidase from infiltrating inflammatory cells in this model of TAC-induced nephrotoxicity. Thus, this distinguishes it from the role of phagocytic NADPH oxidases in both salt loading-induced and angiotensin-induced nephropathy. In contrast, our additional findings of up-regulation in NOX-1 and its reversal by TGF-$\beta$ antibody for the first time implicate non-phagocytic NADPH oxidase as a major role in TAC-induced nephrotoxicity as well. We suggest that this may be a direct action on the non-phagocytic NADPH oxidase unit within renal cells per se. Indeed, supporting this concept are new data from our laboratory showing that TGF-$\beta$ has the direct capacity to increase NADPH oxidase gene expression in proximal epithelial cells in culture and that this is blocked by TGF-$\beta$ siRNA (unpublished observations). In summary, the results from this study demonstrated that the expression of TGF-$\beta$ and NADPH oxidase components is increased while that of antioxidant genes SOD and TRX is decreased in TAC-treated rat renal transplant recipients. These effects were reversed in recipients treated with TAC + anti-TGF-$\beta$ antibody but not the control antibody. This suggests that TGF-$\beta$ may be a direct mediator of both the oxidative stress and nephrotoxicity in TAC-treated transplant recipients.

Collectively, our studies demonstrate that TGF-$\beta$ and $O_2^-$ participate in the events leading to renal damage. Therefore, a strategy to inhibit TGF-$\beta$ and/or $O_2^-$ would assist in the prolongation of graft survival without unwanted side effects of TAC for transplant recipients.

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Conflict of interest statement. None declared.

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