Research Article

EXPRESSION OF BMP-7 AND TGF- β IN THALIDOMIDE TREATED RENAL FIBROSIS MICE

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ABSTRACT

Experimental unilateral ureteral obstruction (UUO) produces tubule interstitial inflammation and fibrosis, reproducing obstructive nephropathy in humans. The present study evaluated the effects of thalidomide in an established model of UUO-induced renal fibrosis. C57BL/6 mice were divided into three groups that were treated or not treated with 5 mg/kg thalidomide beginning on day 1. Unilateral ureteral obstruction was performed on day 0. Blood was collected from the abdominal aorta and then centrifuged. Serum was separated to determine creatinine levels. Urinary protein and creatinine were determined, and kidney tissues were harvested and used to quantify bone morphogenic protein 7 (BMP-7) and transforming growth factor β (TGF-β) mRNA expression using real-time polymerase chain reaction 1, 4, 7, and 14 days after surgery. Thalidomide significantly reduced serum creatinine levels on all days of evaluation. Thalidomide treatment also reduced TGF-β expression and increased BMP-7 expression, thus reversing the TGF-β/BMP-7 mRNA expression ratio and demonstrating a protective effect. Thalidomide ameliorated renal dysfunction by decreasing the TGF-β/BMP-7 mRNA expression ratio.

Keywords: Renal fibrosis, Unilateral ureteral obstruction, Thalidomide.

1. INTRODUCTION

Urinary tract obstruction is a common pathological condition in the adult and pediatric populations. Autopsy studies have identified hydronephrosis in 2-3% of adults and 2% of infants[1, 2]. Tissue fibrosis is a leading cause of morbidity and mortality. An estimated 45% of deaths in the United States can be attributed to fibrotic disorders[3].

Renal tubular interstitial fibrosis is a chronic and progressive process that is characterized by the loss of renal tubules and deposition of extracellular matrix components. It follows chronic tissue inflammation and leads to organ scarring and a subsequent loss of function[4]. Experimental unilateral ureteral obstruction (UUO) produces tubule interstitial inflammation and fibrosis, reproducing obstructive nephropathy in humans[5]. In progressive obstructive nephropathy, the renal tubules dilate, and tubular epithelial cells undergo apoptosis, leading to tubular atrophy[6].

Profibrotic transforming growth factor β (TGF-β) mediates both clinical and experimental renal fibrosis[7]. Previous studies found that the suppression of TGF-β signaling prevented renal fibrosis[8]. The TGF-β-induced epithelial-to-mesenchymal transition can be blocked by upregulating bone morphogenic protein 7 (BMP-7) [9]. BMP-7 has protective effects on the kidney by decreasing apoptosis, exerting anti-fibrotic activity, and stabilizing or even

reversing renal fibrosis, thus improving renal function[10].

Due to its potent antiinflammatory and immunomodulatory properties, thalidomide was selected for this study. This compound was synthesized and introduced in the 1950s as an over-the-counter medication[11, 12]. Initially it was used as a sedative and anti-emetic agent, but it was promptly banned because of its teratogenic effects[13, 14]. Recent studies showed that thalidomide has a wide range of biological effects by modulating the production of cytokines, and pointed out that their therapeutic application may represent an alternative strategy in some diseases[15-18]. Particularly in animals with UUO-induced fibrosis, thalidomide treatment improved renal function by modulating proinflammatory cytokines[19].

Whereas other mechanisms may be involved in the beneficial effect of thalidomide in renal fibrosis, this study evaluated their effects on the TGF-β/BMP-7 mRNA expression ratio in mice with UUO.

2. MATERIALS AND METHODS

2.1 Animals

Isogenic male C57BL/6 mice (H2-A^b; 5-6 weeks of age, 20-25 g) were provided by the Central Animal House of the State University of Maringá (Paraná, Brazil). The animals were housed at 22°C ± 2°C under a 12 h/12 h light/dark cycle. All of the animals were housed in individual standard cages and had free access to water and food. The experimental protocols were approved by the Ethical Committee on Animal Experimentation of the State University of Maringá (CEAE/UEM 041/2008).

2.2 Experimental model of unilateral ureteral obstruction

The mice were briefly anesthetized with ketamine (100 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.; Agribans, São Paulo, Brazil). A left lateral incision was made, and UUO was achieved by occluding the left ureter with 3-0 silk thread and then sectioning it. The abdominal cavity was reopened, and the viscera were placed back into the abdominal cavity. The abdomen was then closed with sutures. The animals were then returned to single cages with warm indirect light until they completely recovered from anesthesia. The animals were assigned to the following groups: sham animals, fibrotic animals that were not treated with thalidomide (n = 8), and fibrotic animals that were treated with thalidomide (n = 8). Thalidomide was prepared by dissolving 5 mg/kg in 0.1 ml of 0.5% carboxymethylcellulose (CMC; Sigma-Aldrich, St. Louis, MO, USA), followed by vortexing with sterile glass for 5 min. The drug was administered by gavage 1 day before surgery and daily thereafter until animal sacrifice.

2.3 Renal function outcome

Blood was collected from the abdominal aorta under anesthesia and then centrifuged. The serum was separated to determine creatinine (Cr) levels. Urinary protein/creatinine was measured in samples that were collected from the bladder in all three groups of animals.

2.4 Gene profiles

Kidney samples were quickly stored in liquid nitrogen. Total RNA was isolated from the kidney using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the RNA concentration was determined by spectrophotometry at an absorbance wavelength of 260 nm. First-strand cDNAs were synthesized using MML-V reverse transcriptase (Promega, Madison, WI, USA). Reverse-transcription polymerase chain reaction (PCR) was performed using the SYBR Green real-time PCR assay (Applied Biosystem, Foster City, CA, USA). The primer sequences were the following: BMP-7 (sense, 5'-AGA ATT CTT CCA CCC TGC ATA CC-3'; antisense, 5'-TCC TTA TAG ATC CTG AAT TGG GCT-3'), TGF-β (sense, 5'-AAC TAT TGC TTC AGC TCC AGA GAG A-3'; antisense, 5'-AGT TGG ATG GTA GCC CTT G-3'). The cycling conditions were the following: 10 min at 95°C, followed by 45 cycles of 20 s at 58°C and 20 s at 72°C. The analyses were performed using Sequence Detection Software 1.9. The mRNA expression was normalized to hypoxanthine guanine phosphoribosyltransferase (HPRT) as the endogenous control (sense, 5'-CTC ATG CAC TGA TTA TGG AGA GGA C-3'; antisense, 5'-GCA GGT CAG CAA AGA ACT TAT AGC C-3'). The data were expressed relative to the expression of the matched normal control. The TGF-β/BMP-7 mRNA expression ratio was determined to evaluate improvements in renal fibrosis.

2.5 Statistical analysis

The data are expressed as mean ± SEM. The results were statistically analyzed using two-way analysis of variance (ANOVA) or one-way ANOVA followed by the Tukey test. Values of $p < 0.05$ were considered statistically significant.

3. RESULTS

Thalidomide attenuated impairments in renal function, reflected by serum creatinine levels, on all days of observation compared with untreated UUO mice. Sham animals had significantly lower serum creatinine levels (0.47 ± 0.03 mg/dL) compared with UUO animals on post-operative day 1 (0.82 ± 0.04 mg/dL, $p < 0.0001$), day 4 (0.64 ± 0.03 mg/dL, $p = 0.0047$), day 7 (0.65 ± 0.02 mg/dL, $p = 0.0001$), and day 14 (0.65 ± 0.02 mg/dL, $p = 0.0001$). Thalidomide treatment restored renal function, reflected by creatinine levels, in the fibrotic groups on post-operative day 1 (0.50 ± 0.02 mg/dL, $p = 0.287$), day 4 (0.50 ± 0.02 mg/dL, $p = 0.3900$), day 7 (0.48 ± 0.01 mg/dL, $p = 0.7757$), and day 14 (0.48 ± 0.02 mg/dL, $p = 0.6857$). A significant reduction of creatine levels was observed in thalidomide-treated UUO animals compared with untreated UUO animals on post-operative day 1 (0.51 ± 0.02 mg/dL *vs*. 0.82 ± 0.04 mg/dL, respectively, $p < 0.0001$), day 4 (0.49 ± 0.02 mg/dL *vs*. 0.64 ± 0.03 mg/dL, respectively, $p = 0.0075$), day 7 (0.48 ± 0.02 mg/dL *vs*. 0.65 ± 0.02 mg/dL, $p < 0.0001$), and day 14 (0.48 ± 0.02 mg/dL *vs*. 0.74 ± 0.04 mg/dL, $p = 0.0003$; Figue 1).

The urinary protein/creatinine ratio was evaluated in all groups on post-operative days 1, 4, 7, and 14. No significant differences in the urinary protein/creatinine ratio were found between the sham and UUO groups on post-operative day 1 (sham: 0.46 ± 0.04; untreated UUO: 0.56 ± 0.08, $p = 0.3622$; thalidomide-treated UUO: 0.38 ± 0.04, $p = 0.2598$), day 4 (sham: 0.46 ± 0.04; untreated UUO: 0.38 ± 0.03, $p = 0.2210$; thalidomide-treated UUO: 0.57 ± 0.09, $p = 0.3128$), day 7 (sham: 0.46 ± 0.04; untreated UUO: 0.33 ± 0.06, $p = 0.1273$; thalidomide-treated UUO: 0.46 ± 0.09, $p = 0.9932$), and day 14 (sham: 0.46 ± 0.04; untreated UUO: 0.54 ± 0.06, $p = 0.3653$; thalidomide-treated UUO: 0.63 ± 0.04, $p = 0.0516$; Figure 2).

The temporal development of fibrosis significantly increased the TGF-β/BMP-7 mRNA expression ratio on post-operative day 1 (1.79 ± 0.03, $p = 0.0003$), day 4 (3.31 ± 0.56, $p = 0.0043$), day 7 (10.2 ± 0.44, $p = 0.0005$), and day 14 (13.6 ± 3.63, $p = 0.0169$) compared with the sham group (0.79 ± 0.03). Thalidomide treatment in fibrotic animals significantly decreased the TGF-β/BMP-7 mRNA expression ratio to levels that were similar to the sham group on post-operative day 7 (2.75 ± 0.13, $p < 0.0001$) and day 14 (1.83 ± 0.27, $p = 0.0092$). On post-operative day 1 (1.98 ± 0.30, $p = 0.0081$) and day 4 (3.52 ± 0.74, $p = 0.0110$) the TGF-β/BMP-7 mRNA expression ratio was similar to fibrotic animals on post-operative day 1 (1.79 ± 0.03, $p = 0.0003$) and day 4 (3.31 ± 0.56, $p = 0.0043$; Figure 3).

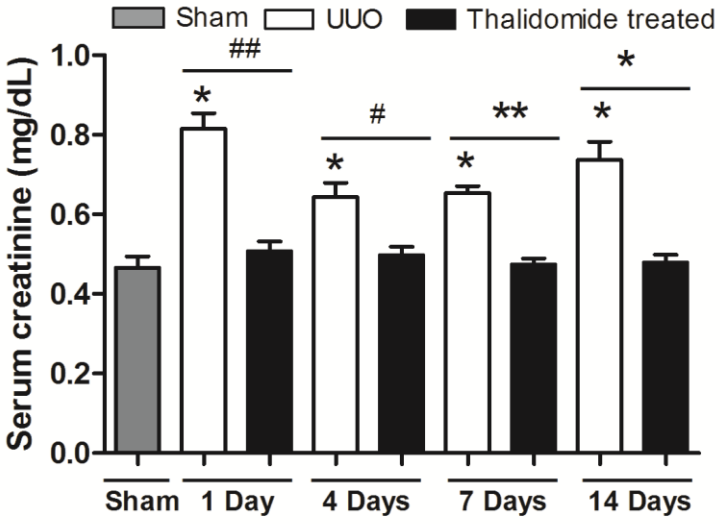

Fig. 1: Serum creatinine levels in sham animals, animals with unilateral ureteral obstruction (UUO) that were not treated with thalidomide, and animals with UUO that were treated with thalidomide (5 mg/kg; daily by gavage) 1, 4, 7, and 14 days after surgery ($n = 8$). The results are expressed as mean ± SEM. *$p < 0.05$, compared with sham group; $^{\#}p < 0.05$, compared with untreated UUO group.

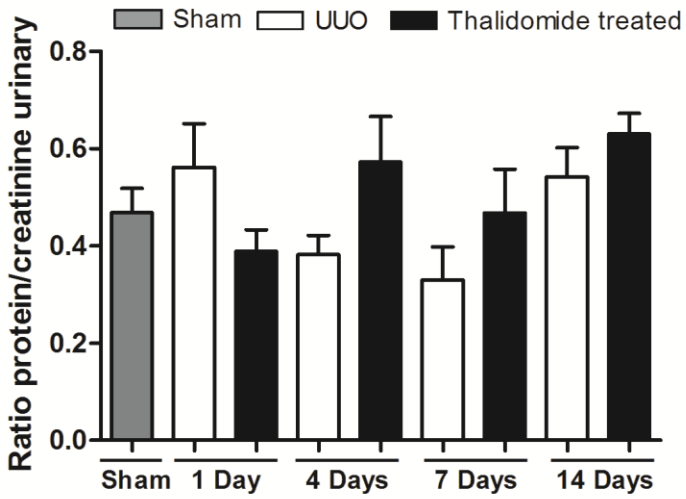

Fig. 2: Ratio of urinary protein/creatinine in sham animals, animals with unilateral ureteral obstruction (UUO) that were not treated with thalidomide, and animals with UUO that were treated with thalidomide (5 mg/kg; daily by gavage) 1, 4, 7, and 14 days after surgery (*n* = 8). The results are expressed as mean ± SEM. **p* < 0.05, compared with sham group; #*p* < 0.05, compared with untreated UUO group.

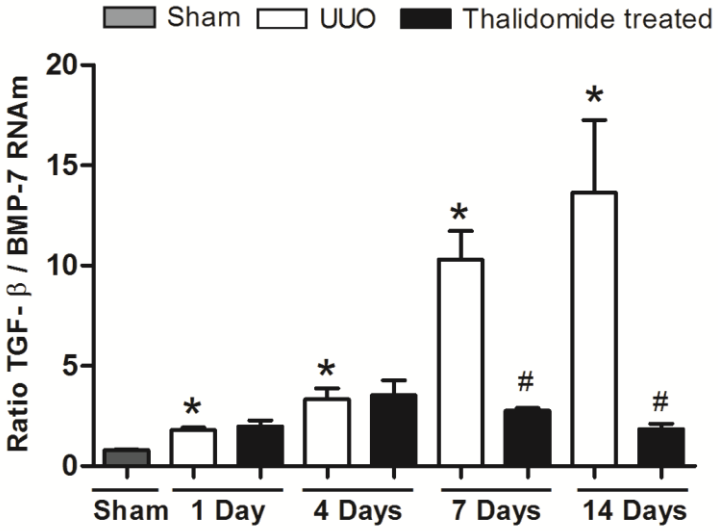

Fig. 3: Ratio of TGF-β/BMP-7 mRNA expression in sham animals, animals with unilateral ureteral obstruction (UUO) that were not treated with thalidomide, and animals with UUO that were treated with thalidomide (5 mg/kg; daily by gavage) 1, 4, 7, and 14 days after surgery (*n* = 8). The results are expressed as mean ± SEM. **p* < 0.05, compared with sham group; #*p* < 0.05, compared with untreated UUO group.

4. DISCUSSION

Unilateral ureteral obstruction in rats and mice produces tubule interstitial inflammation and fibrosis, which appears to reproduce the human condition that is produced by obstructive nephropathy[20]. Previous studies showed that the inflammatory response occurs throughout the development of chronic renal disease and interstitial fibrosis, in which infiltrated inflammatory cells play an important role in chronic inflammation in renal fibrosis[19,21].

Proinflammatory mediators and growth factors that are produced by inflammatory cells promote the proliferation of renal fibroblasts and *trans* differentiation of renal tubular epithelial cells and stimulate local cells to produce TGF-β and other fibrogenic factors[22]. Activated renal tubular epithelial cells also produce inflammatory factors, including

interleukin-1β (IL-1β) and tumor necrosis factor-α[23]. Collagen types I and III are also the major chronic kidney disease components that accumulate in the kidney during renal tubulointerstitial fibrosis[24].

One important pathway for renal fibrosis is the activation of poly(ADP-ribose) polymerase 1 (PARP1), a nuclear proinflammatory protein that can regulate gene expression as a transcriptional coactivator and can regulate protein function that is related to renal fibrosis via poly(ADP-ribosyl)ation[25]. PARP1 stimulation appears to be involved in the regulation of cellular inflammatory processes that contribute to renal fibrosis and are functionally associated with neutrophil recruitment and TNF-α production, among other factors[26-28]. The loss of PARP1 is related to TNF-α production by renal tubular cells in response to UUO, but it does not specifically reflect the downregulation of TGF-β expression[29].

A recent study by our group investigated the effects of thalidomide treatment in kidney disease. The data suggested the involvement of antiinflammatory and immunomodulatory mechanisms that can modify the fibrosis framework by reducing the serum levels of proinflammatory factors, such TNF-α, IL-1β, IL-6, IL-10, and IL- 17, in addition to a decrease in the accumulation of interstitial collagen types I and III[19].

The present study observed improvements in UUO-induced renal fibrosis that occurred through changes in the levels of TGF-β and BMP-7 mRNA that resulted in a decrease in the TFG-β/BMP-7 mRNA expression ratio. The activation of fibrogenic genes is triggered by TGF-β signals that act on intracellular proteins that counteract the activity of BMP-7 (i.e., an antifibrinogenic factor). TGF-β enhances the phosphorylation of mitogen-activated protein kinases and Smad2/3 in fibrosis, which was found to be inhibited by thalidomide through inhibition of the p38 and Smad3 signaling pathways[30]. Thalidomide treatment may also reduce TGF-β expression in renal injury, consistent with previous studies that reported the beneficial effects of thalidomide in other chronic diseases[31-34].

5. CONCLUSION

In conclusion, the present results provide evidence that thalidomide improves renal function in mice with UUO by reducing the TGF-β/BMP7 mRNA expression ratio without causing overt renal complications.

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