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Endostatin, an antiangiogenic protein, is expressed in the unilateral ureteral obstruction mice model

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ABSTRACT

***Background*: Extracellular matrix accumulation, epithelial-to-mesenchymal transition, tubular atrophy and loss of peritubular capillary network are hallmarks of tubulointerstitial injury in progressive renal diseases. In this study, we analyzed endostatin expression in kidneys subjected to unilateral ureteral obstruction (UUO).**

***Methods*: Collagen XVIII mRNA expression was evaluated by real-time polymerase chain reaction (PCR). Endostatin and CD31 protein levels were analyzed by Western blot and immunohistochemistry. In vitro quantification of collagen XVIII and fibrosis-related genes in HK2 cells was performed by real-time PCR.**

***Results*: UUO significantly increased collagen XVIII mRNA expression and released a 30-kDa endostatin fragment. Immunohistochemistry revealed endostatin expression increased in injured tissue, mainly on tubular cells. Of interest, expression of CD31 was significantly reduced by UUO. Endostatin administration in vitro did not modify the expression of genes related to fibrosis development. However, in vitro TGF-β1 administration induced expression of collagen XVIII/endostatin mRNA in human tubular cells.**

***Conclusion*: Endostatin is expressed during the progression of renal fibrosis in vitro and in vivo, suggesting a role for endostatin in development of tubulointerstitial injury.**

Key words: *Endostatin, Proximal tubular cells, Tubulointerstitial fibrosis, Unilateral ureteral obstruction*

INTRODUCTION

Tubulointerstitial fibrosis is a common feature of progressive renal disease (1) and strongly correlates with deterioration of renal function. Fibrosis of the tubulointerstitial compartment is characterized by excessive matrix protein production, epithelial-to-mesenchymal transition, monocyte infiltration and tubular atrophy (2, 3). Unilateral ureteral obstruction (UUO) is a representative experimental model of tubulointerstitial fibrosis that involves several cellular and molecular events (4).

Accumulating evidence suggests that tubular damage results in loss of peritubular capillaries, impairing blood flow delivery. Interstitial fibrosis also impairs oxygen diffusion and supply to tubular cells, inducing chronic hypoxia in that compartment (5). Loss of peritubular capillaries and tubulointerstitial hypoxia are noted even in early stage of progressive renal disease, when interstitial fibrosis is mild (6). In vitro studies suggest that hypoxia induces fibrosis in proximal tubular epithelial cells (7, 8), consistent with in vivo data (9).

Endostatin, a fragment cleaved from the C terminus of collagen XVIII, is a specific inhibitor of endothelial cell proliferation, migration, invasion and tube formation (10). Mice treated with nitric oxide synthase inhibitors exhibited up-regulation of collagen XVIII/endostatin and rarefaction of capillary profiles. This was accompanied by increased expression of transforming growth factor β1 (TGF-β1) and connective tissue growth factor (CTGF) in the kidney (11).

In this study, we demonstrated that interstitial fibrosis induced by UUO increases the expression of collagen XVIII mRNA and endostatin protein. In vitro, endostatin was not able to modify the expression of fibrosis-related genes.

However, in vitro administration of TGF-β1 stimulated collagen XVIII mRNA expression, corroborating the data found in vivo. We also detected down-regulation of CD31 expression after UUO, indicating loss of capillaries concomitant with increased expression of endostatin.

Subjects and methods

Unilateral ureteral obstruction

A UUO model was induced in adult male C57BL/6 mice (3-5 months of age, 20-25 g) under pentobarbital-induced anesthesia. The right ureter was ligated with 4-0 silk at 2 locations and cut between ligatures to prevent urinary tract infection (obstructed kidney). The animals were sacrificed 1, 3 or 5 days after the procedure. Contralateral and obstructed kidney were removed and processed for analysis. All procedures were approved by the ethics committee on research of the Federal University of São Paulo.

Cell line and culture conditions

HK2 cells (American Type Culture Collection number CRL-2190) were cultured in a humidified atmosphere containing 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium and Ham's F12 (DMEM/F12; Invitrogen, Carlsbad, CA, USA) and supplemented with 100 U/mL penicillin, 50 mg/mL streptomycin and 10% fetal bovine serum (FBS; Invitrogen) until 60% confluence, when the medium was replaced by DMEM/F12 for 24 hours. After that, cells were incubated with TGF-β1 (2 ng/mL, Sigma-Aldrich, St Louis, IL, USA) or endostatin (200 ng/mL, Alpha Diagnostic International, San Antonio, TX, USA) for 6 or 24 hours.

Quantitative real-time PCR

Total RNA from kidney or cell pellets was extracted using an RNeasy kit (Qiagen, Hilden, Germany). Samples were submitted to cDNA synthesis (Superscript-II; Invitrogen), and quantitative real-time polymerase chain reaction (qPCR) was carried out using the ABI 7700 thermocycler (Applied Biosystems, Foster City, CA, USA) and Quantitect SYBR Green I kit (Qiagen), according to the manufacturers' recommendations. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. Expression of target gene was normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels. Specificity of each reaction was confirmed by melting curve analysis and agarose gel electrophoresis.

Protein extraction and Western blot analysis

Kidneys were homogenized in lysis buffer (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, 1 mM sodium orthovanadate, 1 mM sodium fluoride, protease inhibitors, pH 8.0) and analyzed by Western blot. Membranes were incubated with a mouse anti-endostatin antibody (Res.16; kindly donated by Dr Ritva Heljasvaara, University of Oulu, Finland), anti-GAPDH (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-CD31 (1:2,000; BD Biosciences Pharmingen, San Jose, CA, USA) and developed through chemiluminescence in x-ray films (GE HealthCare, Piscataway, NJ, USA). Images were acquired on a GS-710 densitometer and analyzed by the software QuantityOne (Bio-Rad, Hercules, CA, USA).

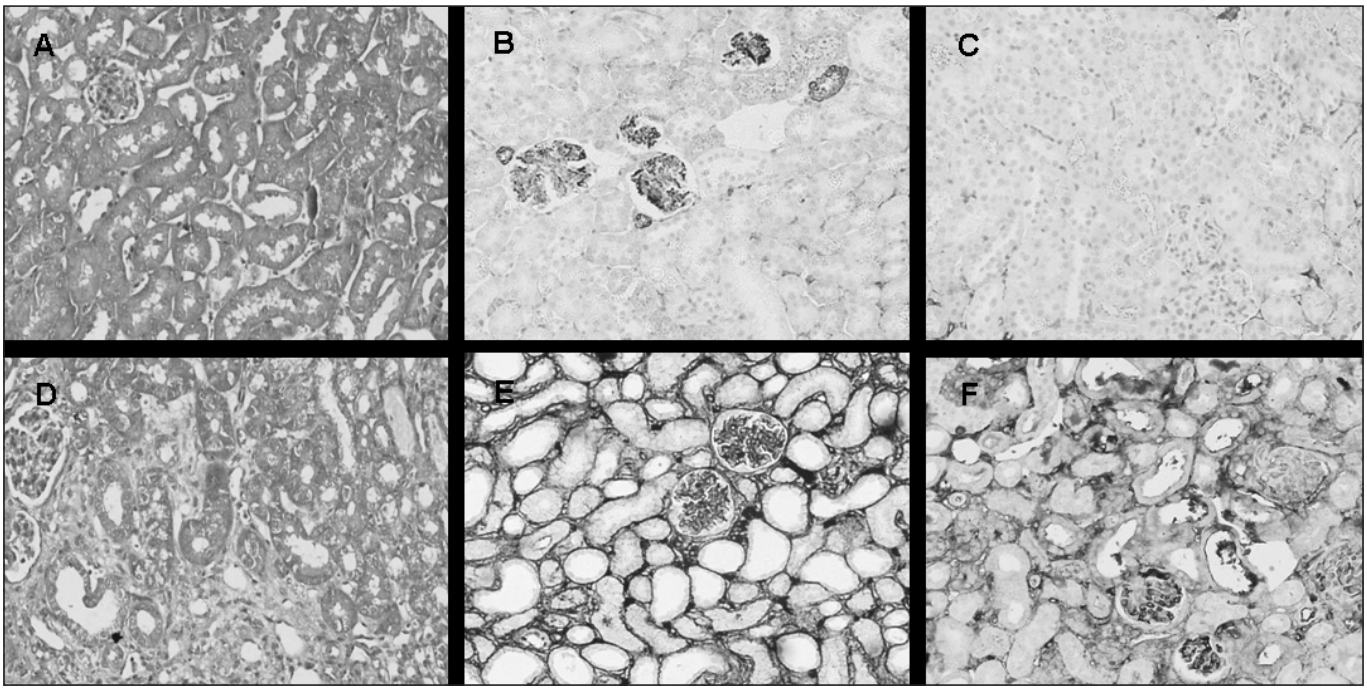
Immunohistochemistry

Kidneys were removed, fixed in formalin, embedded in paraffin and sectioned (4 μm thick), and immunohistochemistry was performed or Masson's trichrome stain (Sigma-Aldrich) applied, with heat-induced epitope retrieval. The slides were processed for identification of macrophage infiltration (anti-CD68, clone KP1, 1:200; Dako Cytomation, Carpinteria, CA, USA), myofibroblast differentiation (anti-α-smooth muscle actin antibody, clone HHF35, 1:200; Dako Cytomation) and endostatin (anti-endostatin antibody, 1:100; Chemicon, Temecula, CA, USA), followed by diaminobenzidine detection system (Sigma-Aldrich, USA). Images were collected (digital camera DXM1200F, Nikon, Japan) at ×200 magnification.

Results

UUO procedure promoted renal fibrosis

UUO promoted renal interstitial fibrosis, characterized by tubular atrophy and interstitial matrix deposition, whereas no histological change was found in the contralateral kidney. Analysis of Masson's trichrome staining revealed that ureteral obstruction elicited intense interstitial collagen deposition when compared with control kidneys (Fig. 1A,



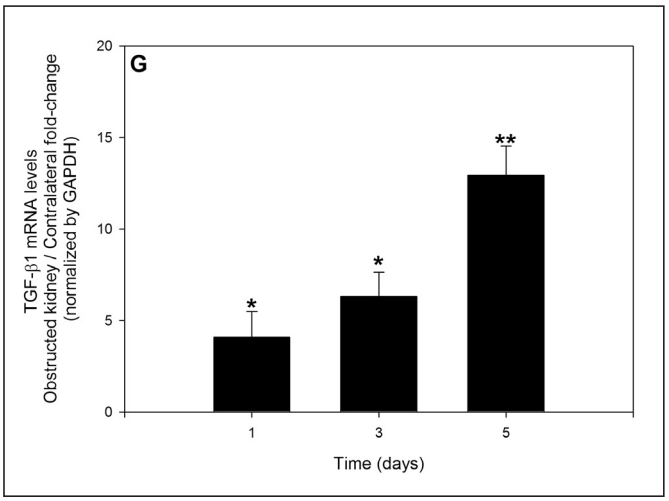
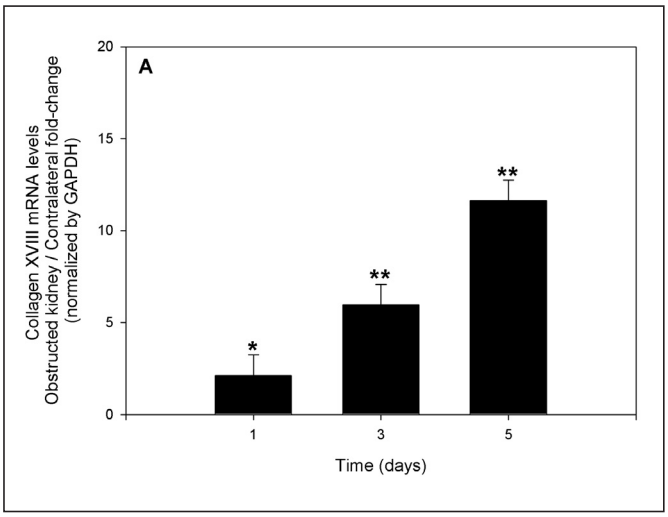


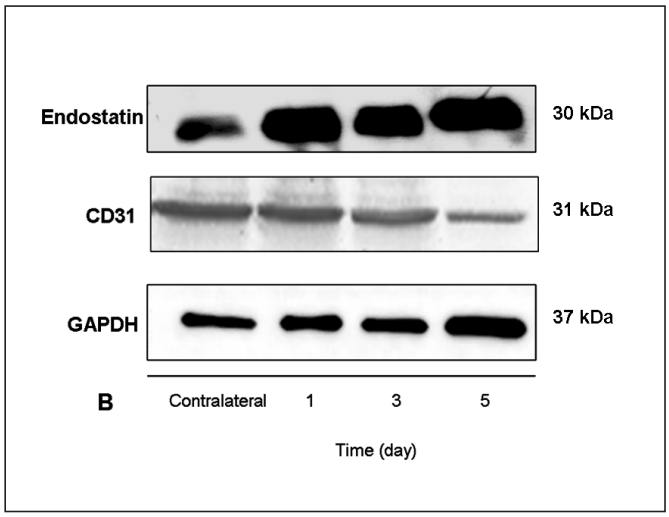
Fig. 1 - Tubulointerstitial fibrosis. Sections of contralateral (A-C) and obstructed kidneys (D-F), stained with Masson's trichrome for collagen deposition (A, D), representative interstitial α-smooth muscle actin expression (B, E) and macrophage infiltration (C, F), 5 days after the procedure. Each image was randomly acquired from the cortex area and is representative of 5 animals. Magnification ×200. G) qPCR analysis of TGF-β1 relative expression in obstructed/contralateral kidney, normalized by GAPDH. Each bar represents mean ± SEM, n=5; *p<0.05, **p<0.01, vs. contralateral.

D). UUO also increased the interstitial myofibroblast population, characterized by the expression of α-smooth muscle actin (α-SMA) surrounding the peritubular and periglomerular spaces (Fig. 1E). This contrasted with reduced α-SMA expression in the contralateral kidney (Fig. 1B). The infiltration of ED-1 positive macrophages in the contralateral kidneys was not significant (Fig. 1C). However, it was markedly present in obstructed kidneys (Fig. 1D). In addition, progression of renal fibrosis induced by ureteral obstruction involves TGF-β1 expression. As demonstrated in Figure 1G, obstructed kidneys significantly express high levels of TGF-β1 (12.9 ± 1.6 fold change at day 5, n=6). These results indicated that inflammation and epithelial-to-mesenchymal transition, classic components of tubulointerstitial fibrotic disease, took place in our model.

UUO is associated with increased endostatin expression

After UUO, injured kidneys showed a significant increase in tubulointerstitial fibrosis. We further examined the expression of collagen XVIII mRNA, the endostatin precursor. qPCR analysis revealed that collagen XVIII was upregulated after UUO (Fig. 2A). Increased expression of collagen XVIII mRNA was observed 24 hours after ureteral ligation (2.1 ± 0.3 fold change, n=5, p<0.05) and this effect was progressive (11.6 ± 1.3 fold change at day 5, n=5, p<0.01). Of interest, signals of collagen XVIII mRNA were not significantly changed in contralateral kidneys (data not





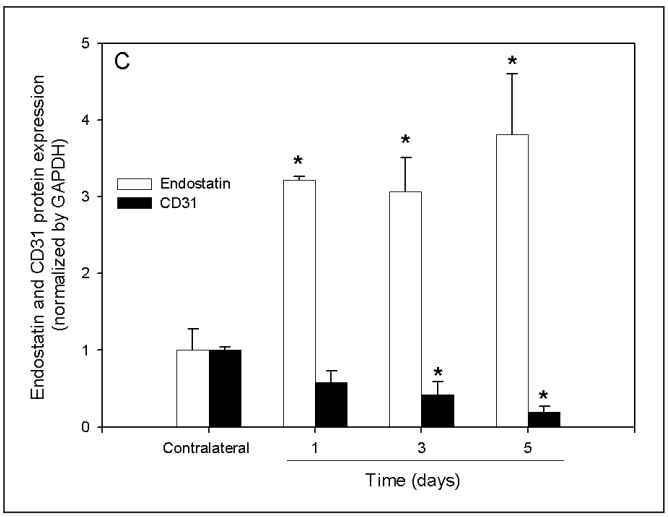


Fig. 2 - Endostatin expression after UUO. A) qPCR analysis of collagen XVIII relative expression in obstructed/contralateral kidney, normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each bar represents mean ± SEM, n=5; *p<0.05, **p<0.01, vs. contralateral. B) Western blot analysis of endostatin and CD31 protein levels in contralateral and obstructed kidneys. GAPDH protein expression was employed as loading control for the experiment. C) Densitometry of Western blot. Samples were quantified, normalized by GAPDH levels and obstructed to contralateral ratio was presented. Each bar represents mean ± SEM, n=3; *p<0.05, vs. contralateral.

shown). We performed Western blot analysis to confirm that endostatin was cleaved from collagen XVIII. A 30-kDa isoform of endostatin was recognized by polyclonal anti-endostatin antibody, demonstrating that endostatin was up-regulated at protein levels by UUO (Fig. 2B, C; maximum expression at day 5, 3.8 ± 0.8 fold change, n=3, $p<0.05$). Concurrently, we evaluated CD31 expression and, as expected, found it was significantly reduced during the course of injury (Fig. 2B, C; -5.2 ± 0.1 fold change at day 5, n=3, $p<0.05$). Immunohistochemistry revealed that endostatin was mainly expressed within tubular cells of obstructed kidney (Fig. 3). Endostatin signal was increased at day 1 (Fig. 3B) and maintained such expression levels 3 and 5 days after UUO (Fig. 3D, E, respectively). Of note, a slight basal expression of endostatin was observed in the contralateral kidney.

Endostatin do not modulate the expression of genes involved in progression of renal fibrosis

We demonstrated that collagen XVIII/endostatin mRNA and protein levels were up-regulated during progression of the UUO model. In this manner, we speculated that endostatin could contribute to development of renal fibrosis, increasing expression of genes involved with extracellular matrix. We employed in vitro stimulation of human proximal tubule cells with recombinant endostatin for 6 and 24 hours (Fig. 4). qPCR analysis demonstrated that endostatin failed to induce expression of TGF-β1, CTGF and osteopontin in HK2, suggesting that endostatin does not modulate genes involved with extracellular matrix and fibrosis.

TGF-β1 induces expression of endostatin in vitro

Collagen XVIII/endostatin was highly expressed in UUO. However, in vitro administration failed to trigger the expression of fibrosis-related genes. To confirm that collagen XVIII/endostatin expression was a consequence of

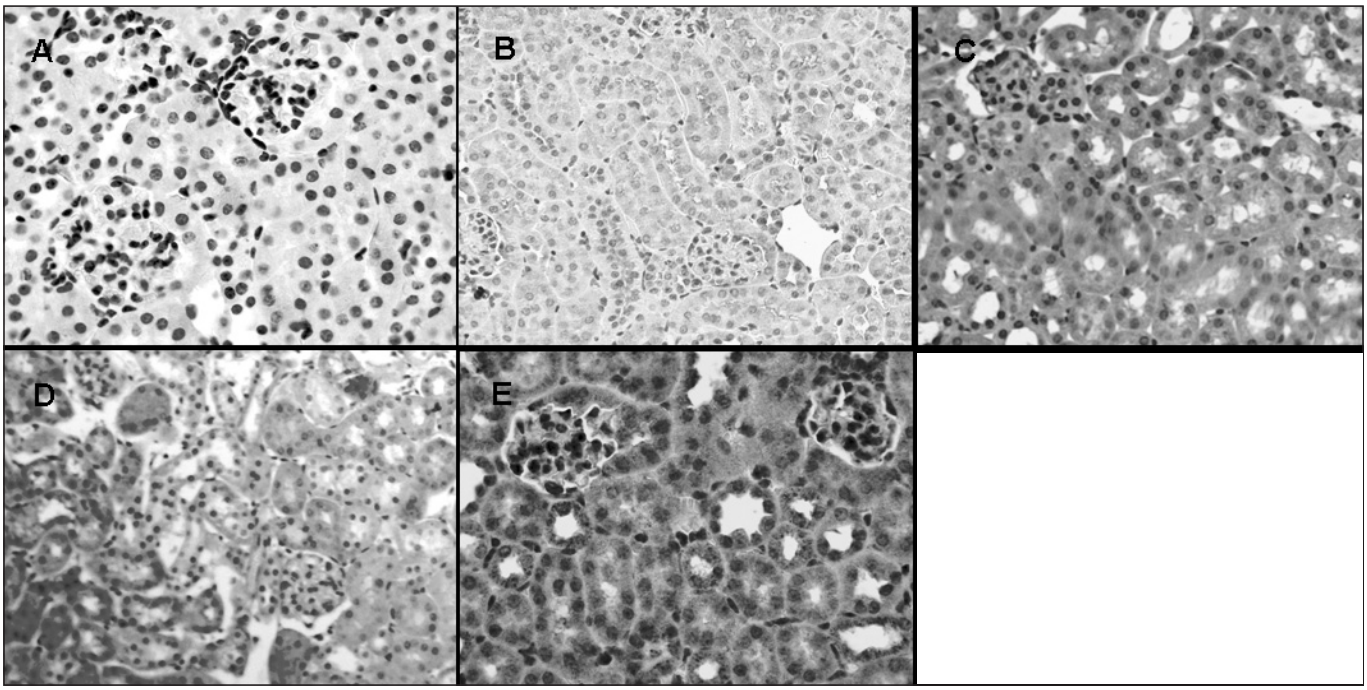


Fig. 3 - Immunohistochemistry for endostatin after UUO. Photomicrographs representing negative control staining using normal rabbit serum (A); contralateral (B) and kidney subjected to UUO (C) on day 1, day 3 (D) and day 5 (E). Each image was randomly acquired from the cortex area and is representative of 5 animals. Magnification ×200.

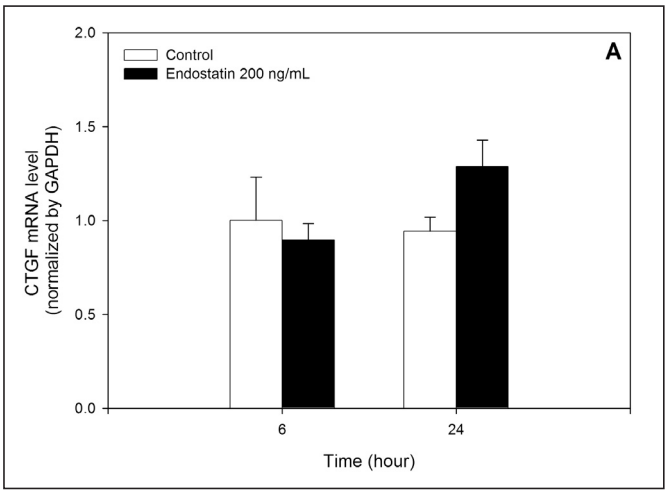
fibrosis, we employed in vitro stimulation of human proximal tubule cells with TGF-β1 for 6 or 24 hours (Fig. 5). qPCR analysis demonstrated that TGF-β1 induces collagen XVIII expression in proximal tubular cells in vitro (2.3 ± 0.2 fold change, n=4, $p<0.05$), corroborating the data observed in vivo.

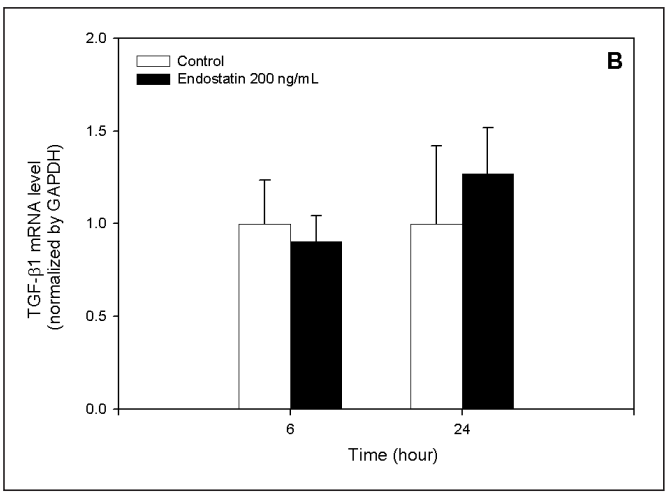
Discussion

This study describes the expression of endostatin, an antiangiogenic protein, in progression of tubulointerstitial fibrosis induced by UUO in mice. Progressive renal disorders are accompanied by development of tubulointerstitial modifications characterized by interstitial infiltration of mononuclear cells, epithelial-to-mesenchymal transition and accumulation of matrix proteins, leading to fibrosis (1). Decreased peritubular capillary density correlates with progression of renal disease (12). Of note, peritubular capillary regression occurs and might contribute to progressive tubulointerstitial fibrosis (13). During the progression of tubulointerstitial fibrosis, prominent regression of peritubular capillary networks take place toward the end of the disease period.

One of the key players in angiogenesis is endostatin, which inhibits proliferation and migration and induces cell-cycle arrest of endothelial cells in vitro (10). Endostatin's role in adult tissue includes inhibition of tumor development and angiogenesis by suppressing endogenous expression of VEGF-A (14) and blocking VEGF-mediated proangiogenic signaling (15).

In the present study, we employed the UUO model to demonstrate the expression of endostatin in the progression of tubulointerstitial fibrosis. It is reasonable to confirm that progression of kidney injury induced by UUO involves endostatin modulation and release in the fibrotic milieu, as confirmed by collagen XVIII mRNA expression and endostatin protein levels. In this context, we hypothesized that endostatin does not allow the recovery of blood flow through formation of new peritubular capillaries, which possibly enhances hypoxia triggered by injury. CD31 analysis by Western blot corroborated our theory, demonstrating that expression of this endothelial cell surface marker decreased in UUO, while the endostatin fragment is significantly increased at the same time points evaluated. We do not address the question of whether endostatin changes CD31 expression or if that capillary density actually changes in consequence of endostatin up-regulation





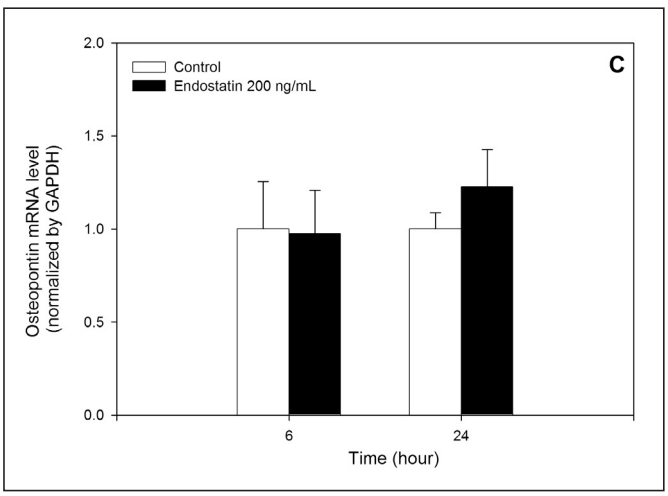


Fig. 4 - Expression of fibrosis markers after endostatin administration. qPCR analysis for CTGF (A), TGF-β1 (B) and osteopontin (C) mRNA levels in HK2 cells treated with recombinant endostatin (200 ng/mL) for 6 or 24 hours. Expression levels were normalized by GAPDH and compared with baseline. Each bar represents mean ± SEM, n=4-8. Results were not statistically significant vs. control.

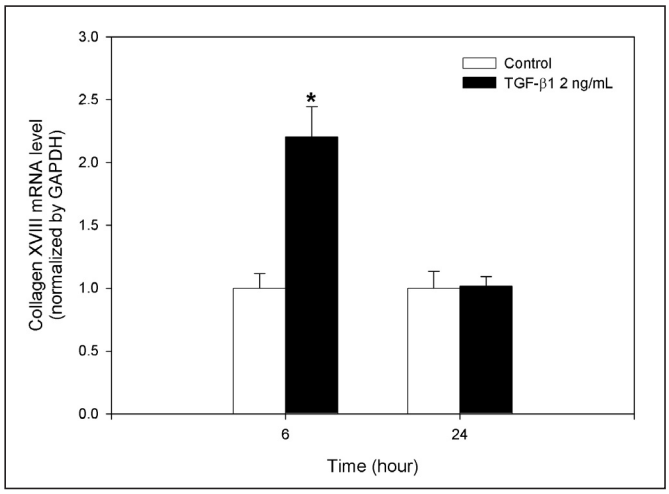


Fig. 5 - Collagen XVIII mRNA expression in vitro. qPCR analysis of collagen XVIII mRNA expression in HK2 cells treated with TGF-β1 (2 ng/mL) for 6 or 24 hours. Expression levels were normalized by GAPDH and compared with baseline. Each bar represents mean ± SEM, n=4; *p<0.05, vs. control.

after UUO. We are currently engaged in pursuing that question, and we presume that chronic ischemia in later stages of the UUO model could be in part explained by the expression and release of endostatin.

In addition to the aforementioned theory regarding endostatin, a recent study demonstrated that collagen (I and IV), laminin and fibronectin improve endostatin-mediated inhibition of VEGF (16). We have not addressed this question. However, if we consider that extracellular matrix components and endostatin levels were increased in UUO, blockade of peritubular capillary recovery could be improved in kidney disease.

Others have already indicated that the primary mechanism of UUO is related to tubular injury and subsequent activation of vasoactive factors, initiating fibrosis (17). In UUO, fibrosis somewhat preceded peritubular capillary regression in early stages of the disease; however, as mentioned before, peritubular capillary loss was often correlated with degree of tubulointerstitial injury. This finding suggests that expression of endostatin even at early stages contributes to accelerate or trigger the regression of peritubular capillaries.

Several studies demonstrated that hypoxia stimulates tubular cell proliferation, osteopontin expression, TGF-β1 synthesis and CTGF release (18, 19). However, in vitro administration of endostatin in tubular cells did not modify expression of the genes mentioned above, suggesting that endostatin did not play a role in the expression of

fibrosis-related genes. Interestingly, TGF-β1 was significantly up-regulated in vivo, and stimulation of tubular cells with TGF-β1 in vitro increased expression of collagen XVIII, supporting evidence that modulation of endostatin took place as a consequence of renal fibrosis development.

Our group has recently published a study of the expression of endostatin in the murine model of ischemia/reperfusion-induced acute renal failure (20). Other reports have demonstrated that endostatin reduces fibrosis in models of peritoneal sclerosis and diabetic nephropathy (21, 22), and elevated serum endostatin levels were observed in patients with systemic sclerosis accompanying pulmonary fibrosis (23). However, endostatin effects in renal fibrosis still have to be evaluated in knockout mice or interventions that block endostatin activity.

In summary, we confirmed the expression and release of endostatin during the progression of tubulointerstitial injury induced by the UUO model. Our findings suggest that synthesis of collagen XVIII and subsequent release of endostatin may inhibit neovascularization of damaged kidney and renewal of adjacent peritubular capillaries, resulting in antiangiogenic changes and a poor prognostic for kidney disease. We are currently working on the analysis of endostatin-releasing proteases and endostatin receptors in the UUO model, as well as an attempt to demonstrate the mechanism of endostatin-mediated peritubular capillary regression.

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Conflict of interest statement: None declared.

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