HSP47 regulates ECM accumulation in Renal Proximal Tubular cells
induced by TGF-β1 through ERK1/2 and JNK MAPK pathway

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ABSTRACT

Heat shock protein 47 (HSP47), a collagen-specific molecular chaperone, which is essential for the biosynthesis of collagen molecules. It is likely that increased levels of HSP47 contribute to the assembly of procollagen, and thereby cause excessive accumulation of collagens in disease processes associated with fibrosis. Although HSP47 promotes renal fibrosis, the underlying mechanism and associated signaling events have not been clearly delineated. We examined the role of HSP47 in renal fibrosis using a rat UUO model, and transforming growth factor-β1 (TGF-β1) treated human proximal tubular epithelial (HK-2) cells. An up-regulation of HSP47 in both in vivo and in vitro models was observed, which correlated with the increased synthesis of extracellular matrix (ECM) proteins and expression of tissue-type plasminogen activator inhibitor 1 (PAI-1). Blockade of HSP47 by siRNA suppressed the expression of ECM proteins and PAI-1. In addition, TGF-β1-induced HSP47 expression in HK-2 cells was attenuated by ERK1/2 and JNK MAPK inhibitors. These data suggest that ERK1/2 and JNK signaling events are involved in modulating the expression of HSP47, the chaperoning effect of which on TGF-β1 would ultimately contribute to renal fibrosis by enhancing the ECM proteins synthesis and deposition.
INTRODUCTION

In recent years numerous studies have focused to delineate the pathogenesis of renal fibrosis, which may be due to fact that there is a strong correlation between the degree of tubulo-interstitial disease and anticipated loss of renal functions. Renal interstitial fibrosis is characterized by excessive accumulation of extracellular matrix (ECM), and it is partly due to the uncontrolled synthesis and partly to the incomplete degradation of ECM proteins. Collagens, including type I - VI, are the major components of ECM proteins, and their uncontrolled synthesis and excessive deposition are frequently observed in various renal disease processes affecting man and experimental animals [32, 35]. It is conceivable that to ameliorate fibrosis measures to regulate the cellular synthesis of collagens need to be thoroughly understood. We speculate that some of the Heat shock proteins may modulate the intracellular processing of various types of collagens, and thus their pathobiology with respect to interstitial fibrosis need to be explored.

Heat shock proteins (HSPs) are expressed under the influence of a wide variety of stresses, and they are believed to be important modulators of various physiological and pathological processes [23]. Heat shock protein 47 (HSP47), a 47-kDa stress protein, is localized to the endoplasmic reticulum of cells synthesizing collagens. Interestingly, it is a collagen-specific molecular chaperone that is required for the maturation of various types of collagens while folding the procollagens in their appropriate molecular conformation [15, 17, 36]. It is therefore logical to infer that HSP47 is involved in the pathogenesis of chronic renal fibrosis, and the recent studies support this contention [25, 28, 32]. However, the signaling mechanism(s) by which HSP47 regulates renal fibrosis is not clear. To address this issue, we first investigated the role of HSP47 in renal fibrosis in an established animal model of tubulo-interstitial disease. The studies were then extended to adult human kidney (HK-2) cells, where the role of HSP47 in TGF-β1-induced ECM de novo synthesis in proximal tubular cells and relevant signaling events were delineated.
MATERIALS & METHODS

Animal Model: Male Wistar rats weighing (200 to 250 g) were used in this study. A unilateral ureteral obstruction (UUO) procedure was performed as described previously [41]. Under general anesthesia with isoflurane, the rats were subjected to UUO or a sham operation. In UUO animals, the left ureter was identified through a small suprapubic incision and was ligated with 5.0 silk at two points, and then severed between the ligatures to prevent retrograde urinary tract infection. The sham operation consisted of a similar suprapubic incision and identification of the left ureter, but ligation of the ureter was not performed. Rats were sacrificed after 2 weeks following the ureteral ligation. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Xiangya School of Medicine, Central South University.

Morphologic Studies: For assessment of histopathologic changes, kidney tissues fixed with 4% buffered paraformaldehyde were embedded in paraffin, and 3 μm-thick sections were prepared. The sections were then stained with Hematoxylin & Eosin (H & E) or Masson’s trichrome stains [19]. Glomerular and tubulo-interstitial damage (score) was assessed by evaluating the degree of tubular atrophy, compression atrophy of glomeruli, interstitial fibrosis and influx of inflammatory cells. For immunohistochemical studies, Avidin-Biotin Complex method was used [34]. Three μm-thick kidney sections from UUO and control rats were prepared. They were deparaffinized and hydrated in graded series of decreasing concentrations of ethanol. The sections were then overlaid with primary monoclonal antibody directed against HSP47 at a dilution of 1:100 (StressGen Biotechnologies, Victoria, BC, Canada), fibronectin (FN) 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal mouse anti-collagen I antibody (Col.I) 1:100 (Abcam Biotechnology, Ltd, Hong Kong, China). The sections were then stained with avidin-biotin peroxidase by following the instructions provided by the vendor.
**In Vitro Cell Culture Studies:** An immortalized proximal tubule epithelial cell line derived from normal adult human kidney (HK-2) was obtained from American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in an atmosphere of 5% carbon dioxide in DME medium mixed 1:1 (vol:vol) with F12 medium (Invitrogen Life Technologies, Carlsbad, CA) and supplemented with 10% fetal bovine serum (FBS). Cells were grown to 70% to 80% confluency and subjected to serum deprivation for 24 hrs prior to further experimental procedures. Cells were incubated with TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) at a concentration range of 2.5 -10 ng/ml for a 12 - 48 hrs.

**siRNA transfection:** To analyze the mRNA and protein expression of HSP47, collagen I, collagen IV, FN and PAI-1 the HK-2 cells were transfected with siRNA directed against HSP47 (SI02777131, SI02777138) or control siRNA (SI03650318, SI04381048) (QIAGEN - Sample & Assay Technologies), as per manufacturer’s instructions. Control siRNA included a scrambled sequence that would cause degradation of any known cellular mRNA. For determination of the efficiency of HSP47 knockdown, Western blot analysis for HSP47 was performed. Several concentrations of HSP47-siRNA (1 to 10 nM) were used to evaluate the optimal gene-disruption conditions, as described previously. HiPerFect transfection reagent and siRNA were separately diluted in serum-free medium and incubated at room temperature for 5 minutes. They were then mixed and incubated at room temperature for 10 minutes. Aliquots of the transfection mixture were added to cell culture medium. The medium included DMEM/F12 supplemented with 10% FBS. Following a 3 hr of transfection the culture was maintained for another 21 hrs. The cells were then subjected to serum deprivation for 24 hrs prior to TGF-β1 treatment.

**Protein Expression Analyses:** Protein was isolated from cultured cell extracts and homogenized in lysis buffer (150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, 1mM EGTA, and 1% Triton X-100) containing a cocktail of protease inhibitors. The protein
concentration was measured with Bradford assay (Bio-Rad, Hercules, CA). After transfer onto the nitrocellulose membranes, the blots were probed overnight at 4°C with following primary antibodies: anti-HSP47 (1:500 dilution), -collagen I (1:1000), -collagen IV (1:1000), -fibronectin (FN) (1:400 dilution), -p-ERK1/2 (Santa Cruz Biotechnology, 1:300), -total ERK1/2 (Santa Cruz Biotechnology, 1:300), -p-JNK (Santa Cruz Biotechnology, 1:300), -total JNK (Santa Cruz Biotechnology, 1:300), -β-actin (Santa Cruz Biotechnology, 1:1000) and -GAPDH (ProMab Biotechnologies, 1:1000). After incubation with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies, the HRP reaction product was visualized by an enhanced chemiluminescence system (Kodak Medical X-Ray Processor, Rochester, NY). Swine anti-rabbit IgG or rabbit anti-goat IgG in PBS containing 1% normal goat serum or 1% Fetal calf serum served as negative control. The β-actin or GAPDH were used as internal gel loading controls.

mRNA Expression Analyses: Total RNA was isolated using High Pure RNA Isolation Kit (Roche, Switzerland) by following the manufacturer’s instructions. Purified RNA was quantified by absorption spectroscopy at 260 nm. Total RNA (100 µg) was reverse-transcribed and subjected to PCR analysis. I. For RT-PCR a Thermo Cycler (PERK1/2 in Elmer, USA) was used to assess the HSP47 and ECM gene mRNA expression in HK-2 cells as follows: 94°C for 2 min followed by 40 cycles of the following: 94°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, and a final extension at 72°C for 10min. The PCR-primer sequences of were as follows:

- Human HSP47:
  - sense  5'-AACTGCGAGCACTCCAAGA-3'
  - antisense  5'-ATGAAGCCACGGTTGTCC-3'

- Human α2 type I collagen (COL1A2):
  - sense  5'-CCAGAGTGGAGCAGTGGTTACTACT-3'
  - antisense  5'-TTCTTGGCTGGGATGTTTTCA-3'

- Human Collagen type IV α2 (COL4A2):
• Human fibronectin (FN):
  sense  5'-ACACTGTGGACTTACCAGG-3'
  antisense  5'-CCAGGAAATCCAATGTCACC-3'

• Human Plasminogen Activator Inhibitor (PAI-1):
  sense  5'-GTGCTGGTGGAATGCCCCTCT-3'
  antisense  5'-GGTCGCAGCAACAACCTCC-3'

• Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH):
  sense  5'-GTGCTGGTGGAATGCCCCTCT-3'
  antisense  5'-GGCAGTTCCAGGATGTCGT-3'

Reaction specificity was confirmed by gel electrophoresis of PCR products. Ratios for HSP47/GAPDH, Collagen I/GAPDH, Collagen IV/GAPDH, fibronectin (FN)/GAPDH, PAI-1/GAPDH mRNA were calculated for each sample & expressed as Mean ± SEM.

II. Real time PCR was used to assess the transcription levels of genes in the kidney of rats as described quantitatively, as previously described [42]. The real time PCR primer sequences were as follows:

• Rat HSP47:
  sense  5'-AGAGGTCAACCAAGGATGAGGAG-3'
  antisense  5'-TTGGGCATGAGGATGAGATGAG-3'

• Rat COL1A2:
  sense  5'-TGTTCTGCTGCTCGTCACCAGG-3'
  antisense  5'-TTGGTCGAGGAGGTTCTTTG-3'

• Rat COL4A2:
  sense  5'-ACACTGTGGACTTACCAGG-3'
  antisense  5'-CCAGGAAATCCAATGTCACC-3'

• Rat FN
sense 5'-GGGATCAAAGGAAACACAG-3'
antisense 5'-AGACGGCAAAGAAAGCAG-3'

• Rat PAI-1:
sense 5'-CTTTATCCTGGGTCTCCCTG-3'
antisense 5'-TGATGCCTCCCTGACATACA-3'

• Rat GAPDH:
sense 5'-AGGACCA GGTGTCTCCTGT-3'
antisense 5'-TTACTCCTTGGAGGCCATGT-3'

**ELISA procedures:** Total plasminogen activator inhibitor-1 (PAI-1) in the cell culture supernatant was measured by specific ELISA methods (R&D Systems, Minneapolis, MN), as described [8]. A multi-well culture plate was prepared by coating it with a monoclonal mouse PAI-1 antibody and blocking with 4% BSA/PBS overnight at 4°C. The supernatant or PAI-1 standards (recombinant human PAI-1) were poured onto the plate and incubated for 2 hrs. After washing, each plate well was successively incubated with the biotinylated secondary antibody, the streptavidin-conjugated horseradish peroxidase detection reagent and the substrate solution. Absorbance was measured at 450 nm using a Wallace Victor V plate reader. The mean absorbance of each sample was plotted against PAI-1 standards to quantify of PAI-1, and expressed as pg/ml.

**Statistical Analyses:** The data were analyzed with t-test, where either the means between two groups were compared or with one-way ANOVA plus Tukey’s post hoc multiple comparison test was performed to compare mean values across multiple treatment groups. Analysis was performed with standard statistical software (SPSS for Windows, version 15.0). In all cases, $p < 0.05$ was considered statistically significant. All data were expressed as Mean ± SEM.
RESULTS

Morphologic Changes and Expression Profiles of HSP47 and ECM proteins in Kidneys of Rats subjected to Unilateral Ureteral Obstruction (UUO): Examination of renal tissues stained with H & E and Masson’s trichrome revealed notable tubular atrophy, tubular dilatation, interstitial fibrosis and inflammatory cell infiltration in kidneys of UUO rats (Figs. 1Ab and 1Ad). The glomerular damage (score) was ~10-fold higher compared to that of control kidneys, and tubulointerstitial damage (score) was also markedly increased in the UUO rats (Fig. 1B). By real-time PCR, the mRNA expressions of HSP47, Type I Collagen (Col.I), Type IV Collage (Col. IV), Fibronectin (FN) and PAI-1 were remarkably increased in the kidney of UUO rats on day 14 compared to that of control (Fig. 1C). The protein expression of HSP47 in the kidney of UUO rats was evaluated by immunohistochemical procedures. In control rats, the expression of HSP47 was quite low, and it was localized mainly in the renal tubular-interstitial area, However, an increased expression of HSP47 was noted in the tubular-interstitial compartment with expanded interstitial matrix in the kidneys of UUO rats on day 14, and similar increase was seen for the expression of FN (Figs. 1Db & 1Dd). In addition, Col.I was weakly positive in the tubulo-interstitium of control kidneys, while it increased notably in rats undergone UUO (Fig. 1Df). Immunoblotting analyses also showed an upregulation of HSP47 with a parallel increased expression of FN and Col.I in kidneys of UUO rats (Fig. 1E). Densitometric analyses confirmed the 2-3-fold increase of HSP47 and ECM proteins’ expression in kidneys of UUO rats compared with the controls (Fig. 1F).

TGF-β1 induced increased expression of HSP47, Collagen I, Collagen IV, FN, PAI-1 in HK-2 cells: The above in vivo studies indicated that the expression of HSP47, Col.I and FN was notably increased following UUO. To delineate the mechanisms involved in the increased expression of these proteins in vitro studies utilizing proximal tubular cells (HK-2 cells) were performed with the premise that pro-fibrogenic cytokine TGF-β may be responsible for the changes observed in rats with UUO.
To assess whether the changes are induced by TGF-β, the latter was added into the culture medium with increasing concentrations of cytokine of (0, 5 and 10 ng/ml) and HK-2 cells maintained for 12 - 48 hrs. Following which, the expression of HSP47, Col.I, Col.IV, FN and PAI-1 in response to TGF-β1 in HK-2 cells was assessed after 12 - 48 hrs. RT-PCR analyses and densitometric tracings demonstrated a dose- and time-dependent increase of HSP47, Col.I, Col.IV, FN and PAI-1 (Fig. 2A). A maximal increase of Col.I, Col IV, FN and PAI-1 in response to TGF-β1 was observed when 10 ng/ml of cytokine was included in the culture medium for 48 hrs (Figs. 2A & 2A.1). ELISA methods also confirmed the time- and dose-dependent increase in protein expression of PAI-1 with the treatment of TGF-β1 (0-10 ng/ml) for 12-48 hrs (Fig. 2E). Furthermore, Western blot analyses revealed TGF-β1 induced increase of HSP47 protein in HK-2 cells treated with varying concentrations of profibrogenic cytokine (0, 2.5, 5 and 10 ng/ml) for (12 - 48 hrs) (Fig. 2B). Similarly, an increase in the protein expression of Col.IV, Col.I and FN in HK-2 cells treated with TGF-β1 (Figs. 2C & 2D). The concomitant increase HSP47 and ECM protein expression following TGF-β1 treatment suggests that this cytokine may mediate profibrogenic effect via modulating the biology of HSP47, and thus could play a role in the evolution of renal interstitial fibrosis.

Attenuation of TGF-β1-induced expression of collagen I, collagen IV, FN, PAI-1 by transfection of HK-2 cells with HSP47-siRNA: To determine whether the increase of HSP47 expression was specifically related to TGF-β1-induced ECM protein de novo synthesis, HK-2 cells were transfected with HSP47-siRNA prior to TGF-β1 inclusion in the culture media. HSP47-siRNA resulted in a significant reduction in the TGF-β1-induced mRNA expression of HSP47, Col.I, Col.IV, FN and PAI-1 in HK-2 cells, as analyzed by RT-PCR (Figs. 3A & 3A.1). Also, similar reduced protein expression of HSP47, Col.I, Col.IV and FN was observed by Western blotting procedures in HK2 cells treated with TGF-β1 (Figs. 3B, 3B.1-B.4). Transfection of scrambled siRNA (control) had no discernible effect on the expression of various ECM proteins (Figs. 3B, 3B.1-B.4). In addition, as assessed by ELISA, HSP47-siRNA
also inhibited PAI-1 protein expression in HK-2 cells induced by TGF-β1 (Fig. 3C).

These data thus support the contention that HSP47 specifically modulates TGF-β1-induced ECM proteins’ synthesis in HK-2 cells.

**Induction of HSP47 expression by TGF-β1 involves ERK1/2 and JNK MAP Kinase signaling pathway:** Extracellular signal-regulated kinase (ERK)1/2 and c-Jun N-terminal kinase (JNK) are two main members of MAPK family which apparently play an important role in regulating stress responses and the expression of heat shock proteins [11, 27, 37, 40]. Furthermore, both ERK1/2 and JNK have been shown to be involved in pathogenesis of interstitial fibrosis stemming from a wide variety of mechanisms in different renal disease processes [12, 20, 22]. This led us to investigate whether the TGF-β1-induced HSP47 expression is regulated by ERK1/2 and JNK kinases.

First, ERK1/2 and JNK phosphorylation status was investigated to assess if TGF-β1 activates the JNK and ERK1/2 pathway. The results of time course studies revealed a transient increase in phosphorylation of ERK1/2 and JNK in HK-2 cells treated with TGF-β1 (10 ng/ml). A ~4-fold increase in ERK1/2 phosphorylation and a ~3.5-fold increase in JNK phosphorylation in HK-2 cells in response to 10 ng/ml TGF-β1 treatment was observed at 60 min (Figs. 4A, 4A.1 & 4B, 4B.1). Total expression of ERK (t-ERK) and JNK (t-JNK) was not significantly altered (Figs. 4A, 4A.2 & 4B, 4B.2).

To ensure if TGF-β1 induced increase in HSP47 expression is directly related to the ERK1/2 and JNK activation, HK-2 cells were individually pretreated with ERK1/2 inhibitor PD98059 (25 – 75 μM) or JNK inhibitor SP600125 (10-30 μM) for 60 min, followed by treatment with TGF-β1 (10ng/ml) for 24 hrs. Compared to the treatment with TGF-β1 alone, RT-PCR analysis revealed that PD98059 and SP600125 attenuated the mRNA expression of HSP47 in a dose-dependent manner in HK-2 cells (Figs. 5 A.1, A.1a & A.2, A.2a). Similar results were seen for the protein expression of HSP47 by Western blot analyses following the treatment of ERK1/2...
and JNK inhibitors (Figs. B.1, B.1a & B.2, B.2a). The HSP47 expression was unaffected with the treatment of SB202474, an inactive congener of PD98059 or with the inactive analog of SP600125 (SP-ve) (Figs. B.3, B.3a & B.4 and B.4a), suggesting that the effects of inhibitors of ERK1/2 and JNK are specific. These results indicate that the ERK1/2 and JNK pathways, most likely, are responsible for modulating the HSP47 expression in HK-2 cells treated with TGF-β1.

**DISCUSSION**

Literature reports relevant to the mechanism(s) of HSP47 in renal fibrosis have focused mainly on its regulation of collagens. Studies have shown that there is a close association between increased expression of HSP47 and excessive accumulation of collagens in various human and experimental diseases affecting the kidney, such as, murine anti-thymocyte serum (ATS)-induced glomerulonephritis, streptozotocin-induced diabetic nephropathy and UUO; and human diabetic nephropathy, IgA nephropathy and hypertensive nephrosclerosis [7, 24, 26, 30, 31]. There are other reports which indicate that suppression of HSP47 expression with antisense HSP47-ODNs or HSP47-siRNA not only reduce the accumulation of collagens to delay the progression of fibrosis in experimental animal models but also abrogate α-SMA expression, reduce the influx of macrophages, macrophage infiltration in blood vessels and MCP-1 expression in rats [28, 39]. The reports also indicate that in UUO kidneys up-regulation of HSP47 mRNA preceded the increased expression of type I collagen mRNA and tubulointerstitial fibrosis. Also, in the angiotensin-converting enzyme inhibitors (ACEI) treated mice, alterations in HSP47 mRNA expression preceded the changes in type I collagen mRNA. The sequence of changes in HSP47 mRNA, type I collagen mRNA and interstitial fibrosis strongly suggest that overexpression of HSP47 was an upstream event, and it may play a role in regulating pathogenesis of renal fibrosis [00]. However, these studies do not describe the changes in the other ECM components, such as, FN and laminin. These studies indicated that the role of HSP47 may be limited to regulating collagen
synthesis alone to promote renal fibrosis. We contend that HSP47, an upstream regulator in the progression of renal fibrosis, besides modulating expression of collagen, has the potential to regulate the expression of other ECM proteins, such as, FN and laminin, and also other inflammatory cytokines.

Unilateral ureteral obstruction (UUO) has been widely used to study various pathogenetic mechanisms that lead to tubulointerstitial fibrosis [18]. Previously, we reported that the low-dose administration of paclitaxel in a rat model of UUO significantly reduces tubulointerstitial fibrosis [42]. In order to attest to our contention that the other ECM proteins are involved in the pathogenesis of renal tubulointerstitial fibrosis and the phosphorylation of signaling molecules induced by HSP47 are essential for this process, we employed the UUO model for studies described in this investigation. In accordance with our contention, our data indicated that increases in mRNA and protein expression of type-I and -IV collagens, fibronectin and PAI-1 correlated with the increased expression of their molecular chaperone-HSP47 in the kidneys of UUO. Next, we investigated if the overexpression of HSP47 promotes excessive collagen synthesis which could explain the pathogenesis of renal tubulo-interstitial fibrosis. In order to delineate the mechanism by which HSP47 would increase the expression of collagen, *in vitro* experiments using HK-2 cells treated with TGF-β1 were employed. TGF-β1 is known to induce fibrosis in kidneys and also has been described to increase the ECM expression [2, 13]. We demonstrated that TGF-β1 increased type I collagen, type IV collagen, FN and PAI-1 expression in HK-2 cells. Normally, HSP47 is expressed at low levels in the absence of TGF-β1 in HK-2 cells. Treatment with TGF-β1 significantly increased the protein and mRNA levels of HSP47. The question that arises here is what is the specific role of HSP47 in TGF-β1 induced ECM synthesis in HK-2 cells? To address this question, gene-specific inhibition of inducible HSP47 by means of siRNA was carried out. First, we established that HSP47 induction can be effectively and specifically silenced by the use of HSP47-siRNA. Following the successful down-regulation of HSP47 with siRNA *in vitro* a remarkable decrease in the *de novo* synthesis of type-1 and -IV collagens was observed, meaning thereby also that its secretion into the extracellular
space would be reflective of its accumulation in the interstitial compartment \textit{in vivo}.

From these findings one may infer that the suppression of HSP47 may be a therapeutic tool to ameliorate fibrosis in the kidney. Besides, modulating collagens' expression our data suggest that HSP47-siRNA also effectively inhibited FN expression. The probable reason that more than one ECM components are affected by HSP47-siRNA may be that various ECM proteins form an interlacing network of assembly in the tubulo-interstium compartment whose expression is modulated in an inter-dependent manner [6, 8]. This may mean that changes in the expression of one ECM protein would be reflected in the expression of others at the same time. However, it is also conceivable that HSP47 may regulate FN expression independent that of the collagens.

To explore whether HSP47 plays a role in regulating ECM degradation, we investigated PAI-1 expression following HSP47-siRNA treatment of HK-2 cells. PAI-1 is a powerful fibrosis-promoting molecule by inhibiting fibrinolysis and upregulating ECM genes [5]. Our data show that HSP47-siRNA remarkably decrease PAI-1 expression that is induced by TGF-$\beta$1 treatment of HK-2 cells. The precise mechanism by which HSP47 exerts its modulatory effect on PAI-1 is not clearly defined. The HSP47 is mainly localized in the endoplasmic reticulum; and FN and PAI-1 are both secreted proteins which ought to be processed within the endoplasmic reticulum before secreted into the exterior. In view of this, so we speculate that HSP47 may regulate FN and PAI-1 expression by certain yet to be characterized mechanism (chaperone?) within the endoplasmic reticulum before getting excreted out of the cells to form the assembled matrix extracellularly. Nevertheless, further investigations are needed to delineate the mechanism(s) responsible for the interplay among these molecules.

Another significant aspect of the current study was to investigate various signaling pathways, such as, MAPK, that relate to the biology of HSP47 modulated by TGF-$\beta$1. The mitogen-activated protein kinases (MAPK) are intricately linked to the pathobiology of various kidney diseases, including renal fibrosis. It is also known that there is a considerable crosstalk between TGF-$\beta$1 and MAPK signalling pathways in
the synthesis and turnover of extracellular matrix by fibroblast-like cells in the kidney.

In addition, MAPK signalling is believed to contribute in TGF-β1-induced transition of tubular epithelial cells into myofibroblasts [10, 16, 21, 29, 33]. Moreover, ERK1/2 and JNK can regulate heat shock tranbscription factor [3, 16, 29], which in turn can regulate the synthesis of nearly all of the heat shock proteins [1]. Interestingly, MAPK is involved in the synthesis of heat shock protein in response to various stimuli [4, 38]. For instance, ERK1/2 is known to participate in the pathways that relate to TGF-β1-stimulated HSP27 induction in osteoblasts [11]. As HSP47 is a member of heat shock proteins, we speculate that ERK1/2 and JNK would participate in the pathways of the TGF-β1-stimulated HSP47 induction in HK-2 cells. In line with this contention, we demonstrated that treatment of HK-2 cells with TGF-β1 activates the ERK1/2 and JNK pathway as evidenced by ERK1/2 and JNK phosphorylation. The kinetics of ERK1/2 and JNK phosphorylation is in agreement with previous reports [14]. Further support for the role of these signaling molecules was derived from the experiments in which PD98059 and SP600125 were shown to inhibit ERK1/2 and JNK signaling as well as suppression of the TGF-β1-stimulated HSP47 expression. The results clearly suggest that TGF-β1 induced HSP47 expression in HK-2 cells in vitro mainly utilizes ERK1/2 and JNK pathways as mediators. However, increased HSP47 expression induced by TGF-β1 could not be completely abrogated by blocking ERK1/2 or JNK signaling in HK-2 cells, this would suggest that other pathways may also be involved that contribute to the increase of HSP47 expression.

In summary, the data provided in this study suggest that HSP47 mediated TGF-β1-induced ECM accumulation in human proximal tubular cells. Two novel observations made in this investigation include: First, HSP47-siRNA attenuates TGF-β1-induced collagen I, collagen IV, FN and PAI-1 expression in HK-2 cells, suggesting the functionality of HSP47 in regulating ECM synthesis and degradation in processes related to renal tubulointerstitial fibrosis. Second, ERK1/2 and JNK pathways are involved in the enhancement of HSP47 expression in HK-2 cells induced by TGF-β1. Overall, our study establishes the role of HSP47 in the
pathogenesis of tubulointerstitial fibrosis, and it is anticipated that this chaperone may serve as an important therapeutic tool in future investigations.

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REFERENCES


34. **Sun L, Sahai A, Chugh SS, Pan X, Wallner EI, Danesh FR, Lomasney JW,**


FIGURE LEGENDS

Figure 1.  Morphologic Changes and Expression Profiles of HSP47 and ECM proteins in Kidneys of Rats subjected to Unilateral Ureteral Obstruction (UUO).  Panel A: Kidney sections stained with Hematoxylin & Eosin (a & b) and Masson (c & d) show a notable tubular dilatation and expansion of the interstitial compartment due to fibrosis in rats undergone unilateral ureteral obstruction (UUO).  Panel B:  Semiquantitative score indicating marked glomerular and tubulointerstitial damage in the renal cortex of rats with UUO.  Panel C:  Real-time PCR analyses reveal a significant increase in mRNA expression of HSP47, Collagen I (Col.I), Collagen IV (Col.IV), Fibronectin (FN), and PAI-1 in the kidney cortices of UUO model compared to the sham group (p < 0.01, N = 6).  Panel D:  A significant increase in the expression of HSP47 and FN in tubular and interstitial compartments of the kidneys in UUO rats (b & d) compared to the control (a & c), as assessed by Immunohistochemistry. Also, Col.I expression was noted to be remarkably increased in the kidney of rat subjected to UUO (f) compared to the control (e).  Panel E:  Western blot analyses revealed an increased protein expression of HSP47, Col I and FN in kidneys of UUO group.  Panel F: Densitometric analysis of Western blot bands of HSP47, Col.I and FN confirmed the increased expression of the respective proteins (N=4, *P < 0.01 versus Control ).

Figure 2.  TGF-β1-induced increased expression of HSP47, Col.I, Col.IV, FN and PAI-1 in HK-2 cells.  HK-2 cells were maintained for 12 - 48 hrs and treated with various concentrations of TGF-β1 (0-10 ng/ml), and expression of HSP47, Col.I, Col.IV, FN and PAI-1 was evaluated.  Panel A:  RT-PCR analyses revealed increased mRNA expression of HSP47, Col.I, Col.IV, FN and PAI-1 with the
treatment of TGF-β1. No discernible change in the GAPDH expression was observed.

**Panel A.1:** The bar graphs represent the mRNA expression of HSP47, Col.I, Col.IV, FN and PAI-1 relative to GAPDH, as depicted in panel A. The values are expressed as Mean±SEM (N=4. *P< 0.01 versus control). **Panels B, B.1-B.3:** Western blots and their respective densitometric analyses of the bands indicate dose- and time-dependent increased in the expression of HSP47 in HK-2 cells following TGF-β1 treatment. The values are expressed as Mean±SEM (N=5. *P <0.01 compared with control). **Panels C & D:** TGF-β1 treatment also increased the protein expression of Col.IV, Col.I and FN in HK-2 cells in a dose- and time-dependent manner. The bar graphs in panels C.1, D.1 and D.2 represent the densitometric analyses of the above Western blots. The values were normalized against GAPDH control and expressed as Mean±SEM (N=5. *P <0.01). **Panels E:** ELISA methods revealed that TGF-β1 increases the PAI-1 expression in a dose- and time-dependent manner in HK-2 cells. N=6, *P < 0.01 versus that of treated for 12 hrs.

**Figure 3.** HSP47siRNA inhibits TGF-β1-induced ECM gene and protein expression in HK-2 cell. HK-2 cells were transiently transfected with human HSP47-siRNA or control-siRNA for 24 hrs then treated with 10 ng/ml TGF-β1 for additional 48 hrs.

**Panels A & A.1:** RT-PCR and densitometric analyses indicate that the treatment of HSP47-siRNA significantly decreases the mRNA expression of Col.I, Col.IV, FN, HSP47 and PAI-1, while that GAPDH was unaffected. **Panels B & B.1-B.4:** Similarly, immunoblotting procedures revealed a reduction in the protein expression of HSP47, Col.I, Col.IV and FN following the HSP47-siRNA transfection. No change was observed in cells treated with the control siRNA, and GAPDH served as a loading control. The densitometric analyses are expressed as Mean±SEM (N=4, *P < 0.01).

**Panels C:** ELISA methods showed that the PAI-1 expression was increased in HK-2 cells induced by TGF-β1, while the effect was abolished with the treatment of HSP47-siRNA. N=6, *P < 0.01 versus control, #P < 0.01 versus TGF-β1 group.
Figure 4. Effect of TGF-β1 on the phosphorylation of ERK1/2 and JNK MAPK in HK-2 cells. The cells were exposed to TGF-β1 (10 ng/ml) for varying periods, the cellular extracts were subjected to SDS-PAGE, and their protein blots were probed with various antibodies. Panels A & B: The TGF-β1 treatment increased ERK1/2 and JNK phosphorylation in a dose-dependent manner with a peak effect around 60 min following the cytokine exposure. No change was observed in total ERK1/2 and JNK. Panels A.1, A.2, B.1 & B.2: The bar graphs representing the density of the relative bands detected by immunoblotting procedures relative to that of GAPDH. Values are expressed as Mean±SEM (N=5. *P <0.05, **P <0.01 compared with control).

Figure 5. Effect of ERK1/2 and JNK inhibitors on the HSP47 expression following the treatment of HK-2 cells with TGF-β1. The HK-2 cells were pretreated with varying doses of PD98059 (ERK1/2 inhibitor) or SP600125 (JNK inhibitor) for 60 min and then treated with TGF-β1 (10 ng/ml) for 24 hrs. Panels A: RT-PCR analyses revealed that the expression of HSP47 was markedly decreased in a dose-dependent manner in cells treated with PD98059 or SP600125. Panels B.1 & B.2: Western blot analyses confirmed the results seen by RT-PCR analyses. Panels B.3 & B.4: There was no change in cells treated with SB202474, a negative control for PD98059 and SP-ve (inactive analog of SP600125). Panels A1.a, A2.a, B.1a, B.2a, B3.a & B4.a: The bar graphs representing the density analyses of the bands of RT-PCR and Western blots. N=6, *P < 0.05 versus control (0 µM), # P < 0.05 versus 25 µM PD98059 or 10 µM SP600125; $ P < 0.05 versus 50 µM PD98058 or 20 µM SP00125.
**FIGURE 1**

A. Comparison of HE and Masson staining between Control and UUO groups.

B.1 Glomerular damage

B.2 Tubulointerstitial damage

- Score values with asterisk (*) indicating a significant difference (p<0.01) compared to Control.

C. Relative mRNA expression levels for HSP47, Col.I, Col.IV, FN, and PAI-1 in Control and UUO groups.

D. Appearance of HSP47, FN, and Col.I staining in Control and UUO groups.

E. Western blot analysis showing protein expression levels for HSP47, Col.I, FN, and GAPDH.

F. Relative band density for HSP47, Col.I, and FN in Control and UUO groups.
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* p < 0.01 vs TGF-β group

C (By ELISA)

Relative PAI-1 Protein expression

B

HSP47 siRNA
Control siRNA

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B.1

Ratio (HSP47/GAPDH)

B.2

Ratio (Col.I/GAPDH)

B.3

Ratio (Col.IV/GAPDH)

B.4

Ratio (FN/GAPDH)
**FIGURE 4**

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**FIGURE 5**