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Gremlin-mediated decrease in bone morphogenetic protein signaling promotes aristolochic acid-induced epithelial-to-mesenchymal transition (EMT) in HK-2 cells

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ABSTRACT

Ingestion of aristolochic acid (AA) is associated with the development of aristolochic acid nephropathy (AAN), which is characterized by progressive tubulointerstitial fibrosis, chronic renal failure and urothelial cancer. Our previous study showed that bone morphogenetic protein-7 (BMP-7) could attenuate AA-induced epithelial-to-mesenchymal transition (EMT) in human proximal tubule epithelial cells (PTEC). However, how gremlin (a BMP-7 antagonist) antagonizes the BMP-7 action in PTEC remained unsolved. The aim of the current study was to investigate the role of gremlin in AA-induced EMT in PTEC (HK-2 cells). HK-2 cells were treated with AA (10 μmol/L) for periods up to 72 h. Cell viability was determined by tetrazolium dye (MTT) assay. Morphological changes were assessed by phase-contrast microscopy. Markers of EMT, including E-cadherin and α-smooth muscle actin (α-SMA) were detected by indirect immunofluorescence stains. The BMP-7 and gremlin mRNA and protein expression in HK-2 cells were analyzed by quantitative real-time PCR (real-time RT-PCR) and western blotting after exposure to AA. The level of phosphorylated Smad1/5/8, a marker of BMP-7 activity, was also determined by western blot analysis. Cells were transfected with gremlin siRNA to determine the effects of gremlin knockdown on markers of EMT following treatment with AA. Our results indicated that AA-induced EMT was associated with acquisition of fibroblast-like cell shape, loss of E-cadherin, and increases of alpha-SMA and collagen type I. Interestingly, exposure of HK-2 cells to 10 μmol/L AA increased the mRNA and protein expression of gremlin in HK-2 cells. This increase was in parallel with a decrease in BMP-7 expression and a down-regulation of phosphorylated Smad1/5/8 protein levels. Moreover, transfection with siRNA to gremlin was able to recover BMP-7 signaling activity, and attenuate EMT-associated phenotypic changes induced by AA. Together, these observations strongly suggest that gremlin plays a critical role in the modulation of reno-protective action of BMP, and that inhibition of gremlin will be a promising means of developmenting novel treatments for AAN.

1. Introduction

Aristolochic acid, an active component of herbal drugs derived from plants of the Aristolochia species, is used as medicine in obstetrics and in the treatment of arthritis, gout, rheumatism, and many other disease (Cosyns, 2003). Since Belgian nephrologists first reported a rapidly progressive interstitial renal fibrosis induced by the Chinese herb containing AA in the early 1990s (Vanherweghem et al., 1993, 1996), the renal toxicity of Chinese herbs has received much attention in China (Liu et al., 2009; Zhu et al., 2005). Pathological examinations of kidneys from patients with AAN showed extensive hypocellular interstitial fibrosis with atrophy and loss of tubules (Debelle et al., 2002; Yang et al., 2007). The lesions of AAN are located in the cortex, primarily involving proximal tubular epithelial cells (Pozdzik et al., 2008a; Grollman et al., 2007). Both in vitro and in vivo studies have demonstrated that AA can induce PTEC apoptosis (Yang et al., 2011) and reduce the self-renewal of epithelial cells with the downexpression of epithelial growth factor (EGF) and bone morphogenetic protein-7 (BMP-7), transdifferentiation into a myofibroblastic phenotype, production of extracellular matrix components, and expression of the fibrotic cytokine transforming growth factor-β (TGF-β) along with connective tissue growth factor (CTGF) (Yang et al., 2007). However, the precise mechanisms leading to the pathogenesis of interstitial fibrosis remain largely unexplored, which has hampered the

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development of effective therapies for patients with AAN. Thus, discovery of the mechanisms leading to AAN and the development of effective therapies are urgently needed.

BMP-7 is a growth and differentiation factor of the TGF-superfamily (Lund et al., 2002). In the adult body, the kidney is the major source for BMP-7, and a decline in expression levels is associated with kidney injury and disease (Kalluri and Zeisberg, 2003; Zeisberg, 2006). Treatment with BMP-7 inhibited or reversed fibrosis caused by acute or chronic kidney injury, accelerating the restoration of kidney functions and improving survival (Klahr, 2003; Simic and Vukicevic, 2005). Our previous study demonstrated that BMP-7 was an inhibitor of AA-induced EMT in HK-2 cells, partially through reducing the activation of a myofibroblast phenotype and production TGF-β1 (Wang et al., 2010). The indispensable roles of BMP-7 in the kidney led us to postulate that BMP antagonist may modulate the renal activities of BMP-7. Gremlin heterodimerises with BMPs to prevent interaction with their receptors. This can inhibit BMP-2, -4 and -7 in several experimental models in vivo and in vitro (Glister et al., 2005; Myllarniemi et al., 2008; Zanotti et al., 2008). From these findings, we hypothesized that gremlin might regulate the renoprotective actions of BMP-7 in AAN.

To evaluate this hypothesis, we developed a cellular model of AAN and examined the expression of gremlin, BMP-7 and phosphorylation of Smad1/5/8 proteins to look at their relationship in the development of EMT.

2. Materials and methods

2.1. Cell culture and chemicals

Human HK-2 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). The HK-2 cells were grown in DMEM-F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 10 U/ml penicillin and 10 μg/ml streptomycin (Sigma, St. Louis, MO) at 37 °C and atmospheric condition of 95% and 5% CO_2. For experimental use, HK-2 cells were plated onto glass slides in medium containing 10% fetal bovine serum. At 80% confluence, the HK-2 cells were treated with medium-F12 containing 2% fetal bovine serum for 12 h, and then treated with 10 μmol/L AA in complete medium or complete medium alone (control). All experiments were performed on cells between passages 3 and 5. After treatment for 24 h, 48 h and 72 h, the cells were harvested for detection.

AA was obtained from the Institute of medicine (Sigma, USA), and dissolved in DMSO to reach stock solution (15 mM) for storage at 4 °C. The stock solution was diluted to a concentration of 10 μmol/L using the culture medium. During the experiment, the concentration of DMSO in the medium was no more than 0.5% (Zhang et al., 2011). In the preliminary experiment, we found that high doses of AA could lead to the death of HK-2 cells within a short time, but low doses in a long duration only induce HK-2 cells to transform into myofibroblast phenotype. Therefore, we chose AA at concentrations of 10 μmol/L as the stimulus dose for EMT studies.

2.2. MTT assay for cell viability

HK-2 cells were seeded into 96-well culture plates and were treated with various AA concentrations (10–40 μmol/L). Cell viability was determined by an MTT assay. Subsequently, cells were incubated with MTT solution (0.5 mg/ml) for 4 h at 37 °C. The purple formazan crystals derived from MTT were dissolved in DMSO and shook for 5 min. The absorbance at 570 nm was measured with a microplate reader (DTX880, Beckman, Germany).

2.3. Morphology observation

After incubation with AA (10 μmol/L) for 24 h, 48 h, and 72 h, cells were washed with phosphate buffed saline (PBS) twice. The morphological changes of HK-2 cells were observed under a phase-contrast photomicroscope (Olympus IMT-2, Tokyo, Japan) and photographed using a digital camera. Subsequently, all micrographs were processed by Adobe Photoshop software.

2.4. Quantitative real-time PCR

Total RNA was extracted from cell pellets using TRIzol reagent (Invitrogen, Carlsbad, CA) on the basis of manufacturer's instruction. Two micrograms of total RNA were reverse transcribed in a reaction volume of 20 μl cDNA according to the manufacturer's instructions. Then equal amounts of the reverse transcriptional products were subjected to PCR amplification. Primer sequences and PCR product sizes of primer pairs were provided in table. At the end of assay, melting curve analysis was performed. Each assay included a standard curve with serially dilution control points. The relative abundance of mRNAs was measured with β-actin mRNA levels as the control. The primers and their sequences used in the PCRs are listed in Table 1

Table 1
Nucleotide sequences of the primers used for reverse-transcription PCR.

Gene	Sequence	Primer sequence 5′–3′	Size bp
BMP-7	Forward	5′-AACTCCTACATGAACGCCAC-3′	126
	Reverse	5′-GAAGTAGAGGACGGAGATGG-3′	
Gremlin	Forward	5′-GCAAATACCTGAAGCGAGAC-3′	218
	Reverse	5′-GCAGTTGAGTGTGACCATCA-3′	
β-Actin	Forward	5′-GTCCACCGCAAATGCTTCTA-3′	190
	Reverse	5′-TGCTGTCACCTTCACCGTTC-3′	

2.5. Western blotting

The cells were harvested, washed twice with ice-cold PBS and resuspended in lysis buffer and sonicated briefly. After centrifugation at 12,000 × *g* for 10 min, the supernatant was prepared as protein extract, and protein concentrations were measured (Pierce BCA protein assay reagent kit; Pierce Biotechnology). Equal amounts of protein were separated on 12 or 15% sodium dodecy sulfate polyacrylamide gels. The proteins were electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA). The blots were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% Tween-20 at pH 7.5) for 1 h. The BMP-7 antibody, Gremlin antibody, p-Smad1/5/8 and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies were diluted in a blocking buffer and incubated with the blots overnight at 4 °C. Bound antibodies were then detected with a 1:1000 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ).

2.6. Gremlin siRNA synthesis and transfection

The target sequences for human Gremlin small interfering siRNA were designed with Web-based criteria and generated with a siRNA construction kit.

The primer of this gremlin siRNA were:

Forward: 5′-TGCTGACACGTGTGACTCTCTTCTTCGTTTTGGCCACTGACTGACGAAGAAGAGTCACACGTGT-3, Reverse: 5′-CCTGACACGTGTGACTCTTCTTCGTCAGTCAGTGGCCAAAACGAAGAAGAGAGTCACACGTGTC.

Control siRNA nucleotide sequences were:

Forward: 5′-TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3′, Reverse: 5′-CCTGAAATGTACTGCGTGGAGACGTCAGTCAGTGGCCAAAACGTCTCCACGCGCAGTACATTTC-3′.

According to the manufacturer's instructions (Invitrogen, Inc., Carlsbad, CA, USA), the best results were acquired by transfecting 10 nm of the gremlin (130-3) with the transfection reagent. HK-2 cells were seeded in 12-well plates allowed to grow to 80% confluence and transfected with non-silencing control siRNA or 20 nm gremlin siRNA using lipofectamine 2000 reagent (Invitrogen Inc.). After transfected 24 h, fresh medium with 10% FBS was added to the plates. Then the cells were incubated with complete medium containing 10 μmol/L AA for 72 h before they were harvested.

2.7. Immunofluorescent

The cells were collected and fixed in 4% paraformaldehyde for 30 min and subsequently washed twice with PBS. Then cells were incubated with serum for 30 min to block non-specific binding at room temperature and rewashed with PBS. To permeabilize, the cells were washed with 0.1% Triton X-100 for 20 min at 4 °C, and then incubated with α-SMA antibody (Abcam, UK) or E-cadherin antibody (Santa Cruz, USA) overnight at 4 °C. Thereafter, the cells were incubated with goat anti-rabbit secondary antibody for 30 min at the ambient temperature and they were stained with the nuclear-specific stain DAPI (Gibco, USA) for 30 min at room temperature. Finally, cells were covered with a water-soluble mounting medium, and were viewed with a Leica Dfc480 fluorescent microscopy (Leica, Germany).

2.8. Statistical analysis

All data were reported as means ± standard deviation (SD). Statistical analysis was performed using the statistical package SPSS for Windows version 13.0 (SPSS, Inc., Chicago, IL, USA). The multiple comparisons between different treatment groups were made using One-way ANOVA and significant differences between two groups were analyzed by Tukey's test. Values of $P < 0.05$ were considered as statistically significant.