Ultrasound in Med. & Biol., Vol. ■, No. ■, pp. 1–12, 2016

0301-5629/$ - see front matter

http://dx.doi.org/10.1016/j.ultrasmedbio.2016.07.022

● *Original Contribution*

SHORT HAIRPIN RNA KNOCKDOWN OF CONNECTIVE TISSUE GROWTH FACTOR BY ULTRASOUND-TARGETED MICROBUBBLE DESTRUCTION IMPROVES RENAL FIBROSIS

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(*Received* 5 *March* 2016; *revised* 14 *July* 2016; *in final form* 25 *July* 2016)

Abstract—The purpose of this study was to evaluate whether ultrasound-targeted microbubble destruction transfer of interfering RNA against connective tissue growth factor (CTGF) in the kidney would ameliorate renal fibrosis *in vivo*. A short hairpin RNA (shRNA) targeting CTGF was cloned into a tool plasmid and loaded onto the surface of a cationic microbubble product. A unilateral ureteral obstruction (UUO) model in mice was used to evaluate the effect of CTGF knockdown. Mice were administered the plasmid-carrying microbubble intravenously, and ultrasound was applied locally to the obstructed kidney. Mice undergoing a sham UUO surgery and untreated UUO mice were used as disease controls, and mice administered plasmid alone, plasmid with ultrasound treatment and microbubbles and plasmid without ultrasound were used as treatment controls. Mice were treated once and then evaluated at day 14. CTGF in the kidney was measured by quantitative reverse transcription polymerase chain reaction and Western blot. Expression of CTGF, transforming growth factor β1, α smooth muscle actin and type I collagen in the obstructed kidney was evaluated by immunohistochemistry. The cohort treated with plasmid-carrying microbubbles and ultrasound exhibited reduced mRNA and protein expression of CTGF ($p < 0.01$). Furthermore, CTGF gene silencing decreased the interstitial deposition of transforming growth factor β1, α smooth muscle actin and type I collagen as assessed in immunohistochemistry, as well as reduced renal fibrosis in pathologic alterations ($p < 0.01$). No significant changes in target mRNA, protein expression or disease pathology were observed in the control cohorts. A single treatment of ultrasound-targeted microbubble destruction is able to deliver sufficient shRNA to inhibit the expression of CTGF and provide a meaningful reduction in disease severity. This technique may be a potential therapy for treatment of renal fibrosis. (E-mail: yb12yx@hotmail.com) © **2016 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound-targeted microbubble destruction, Sonoporation, Cationic microbubbles, Renal fibrosis, Unilateral ureteral obstruction, Connective tissue growth factor, Transforming growth factor-β, Gene delivery, RNA interfering, Short hairpin RNA.**

INTRODUCTION

There is a high incidence of chronic kidney disease (CKD) worldwide, and a trend toward increasing frequency has been noted. Approximately 10–13% of the general population is affected by some degree of CKD (Coresh et al. 2007; de Jong et al. 2008; Zhang et al. 2012), which is not only a serious threat to human health, but also a severe social and economic problem (Ackland 2014; Essue et al. 2013). Renal fibrosis, which is characterized by excessive accumulation of extracellular matrix (ECM) and proliferation of myofibroblasts and fibroblasts, is widely regarded as the common histologic hallmark of CKD progressing to end-stage renal diseases (Klein et al. 2011; Tampe and Zeisberg 2014). This progression is considered to be effectively suppressed by anti-fibrotic treatment. α Smooth muscle actin (α-SMA) is a hallmark of myofibroblast differentiation and enables myofibroblasts to cause contraction of ECM (Rao et al. 2014). ECM production and cell proliferation are induced by several factors

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including transforming growth factor-β1 (TGF-β1). TGF-β1 is generally considered to be the key pro-fibrotic mediator in progressive renal fibrosis (Boor et al. 2010; Leask and Abraham 2004; Verrecchia and Mauviel 2002). Therapeutic strategies that involve blockage of TGF-β1 have resulted in attenuation of renal fibrosis in animal models (Feger et al. 2015). However, the multifunctional role of TGF-β1, which includes both anti-inflammatory and anti-proliferative effects, makes therapies using systemic blockade vulnerable to undesired adverse effects (Ihn 2008).

Connective tissue growth factor (CTGF) was found to be an important downstream mediator of TGF-β1, and controls pro-fibrotic activities in renal fibrosis (Grotendorst 1997; Nguyen and Goldschmeding 2008). CTGF induced by TGF-β1 also enhances the biological activity of the latter (Crean et al. 2004; Riser et al. 2003). The expression of CTGF is low in normal renal tissue, whereas it is strongly upregulated both in experimental models of CKD and in human patients with a variety of chronic renal disease (de las Heras et al. 2006; Ito et al. 1998; Kanemoto et al. 2004). Because of its unique function in mediating fibrogenic activity, this novel modulator is considered to be a more suitable therapeutic target than direct inhibition of TGF-β1. Multiple animal and clinical studies have confirmed that inhibition of CTGF, by either blocking antibodies or gene therapy, reduces renal fibrosis (Adler et al. 2010; Guha et al. 2007; Yokoi et al. 2004).

Gene therapy holds enormous potential in the treatment of renal disease. Use of a tissue-specific delivery technology would enable therapeutic genes to be targeted selectively to the kidney, potentially increasing efficacy and reducing off-target effects (van der Wouden et al. 2004). Existing methods of gene delivery generally suffer from low efficacy or poor tolerability in the kidney, which motivated our investigation of a novel kidney-specific approach to gene delivery.

Ultrasound is widely used in clinical diagnostic imaging, both with and without microbubble contrast agents. Low-frequency ultrasound, in combination with some microbubble formulations, has been explored as a means for targeted delivery of biomolecules through a mechanism known as sonoporation (Delalande et al. 2015; Fan et al. 2014; Sirsi and Borden 2012) or ultrasound-targeted microbubble delivery (UTMD). Although the precise mechanism underlying sonoporation is not known, it is believed that oscillation of the microbubble in the ultrasound field induces transient poration in adjacent cells. The size of the pores can be large, on the order of 5 μm (Hu et al. 2013), which enables cytosolic delivery of a wide range of bio-active molecules. Various payloads have been delivered, including small molecules (Zhang et al. 2014), antibodies (Togtema et al. 2012), plasmid DNA (Tlaxca et al. 2013), viral particles (Warram et al. 2012) and small interfering RNA (Li et al. 2013). Sonoporation provides a method for localized and non-invasive intracellular delivery of therapeutic payloads. In addition, the microbubbles used for sonoporation are generally detectable using contrast ultrasound imaging, which provides a non-invasive means for precise guidance of the payload delivery (Carson et al. 2011).

In the present study, we sought to determine whether UTMD could be used to deliver a short hairpin RNA (shRNA) specifically to the kidney and mediate knockdown of CTGF sufficiently to induce a meaningful therapeutic response. We used a commercially available sonoporation microbubble and designed an acoustic treatment protocol using a conventional ultrasound scanner to deliver a shRNA against CTGF in a mouse model of renal fibrosis.

METHODS

Construction of shRNA expression plasmids

Three shRNAs targeting CTGF were synthesized and cloned into GV102 tool plasmid (Genechem, Shanghai, China). The sequences used were:

shRNA1: 5′- CTTCCAAAGCAGTTGCAAA-3′
shRNA2: 5′- ATACCTTCTGCAGGCTGGA-3′
shRNA3: 5′-AAGCTGACCTAGAGGAAAA-3′.

Expression was driven by the U6 promoter. All inserted sequences were verified through sequencing. The plasmids were amplified in *Escherichia coli* and purified using a plasmid DNA purification kit (Tiangen Biotech, Beijing, China) according to the manufacturer's protocol. The plasmid concentration was measured by photometric absorption at 260 nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Knockdown efficiency of three CTGF-targeted shRNAs was verified in NRK-49 F cells (Cell Resource Center of Shanghai Life Sciences Institute, Chinese Academy of Sciences, Shanghai, China). shRNA sequence 1 had the highest silencing efficiency and was selected for subsequent use with UTMD.

Microbubble preparation

Cationic microbubbles (Targesphere) were purchased from Targeson (San Diego, CA, USA; distributed by Origin Biosciences in China). Targesphere is a dispersion of lipid/polymer microspheres encapsulating a core of decafluorobutane gas. The microbubble shell contains a slight positive charge to enable electrostatic binding of nucleic acids (Tlaxca et al. 2010). All microbubble products used here were from a single Targesphere lot. The size distribution of the microbubble product was