Inhibitory effect of receptor for advanced glycation end product-specific small interfering RNAs on the development of hepatic fibrosis in primary rat hepatic stellate cells

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Abstract. Specific small interfering RNAs (siRNAs) targeting receptor for advanced glycation end products (RAGE) inhibit the expression of RAGE, α-smooth muscle actin and type I collagen in the T6 hepatic stellate cells (HSCs), indicating that RAGE is important for the activation of HSCs and the expression of collagen. The present study aimed to investigate the effect of specific siRNAs targeting RAGE on the development of hepatic fibrosis (HF), using primary rat HSCs, which were isolated and cultured *in vitro*. The expression vectors for specific siRNAs targeting RAGE were constructed and transfected into primary rat HSCs. Untreated and nonspecific siRNA-transfected primary rat HSCs served as controls. The expression levels of RAGE, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), transforming growth factor-β1 (TGF-β1), connective tissue growth factor (CTGF), laminin (LN), hyaluronic acid (HA) and N-terminal procollagen III propeptide (PIIINP) in primary HSCs were detected by reverse transcription quantitative polymerase chain reaction and western blotting. The mRNA and 42 kD protein expression of RAGE in the pAKD-GR126-transfected primary HSCs were significantly downregulated compared with those in the untreated and the pAKD-negative control (NC)-transfected controls. The mRNA and protein expression levels of IL-6, TNF-α, TGF-β1, CTGF, LN, HA and PIIINP in the pAKD-GR126-transfected primary HSCs were also markedly downregulated compared with those in the untreated and pAKD-NC-transfected controls. Therefore, RAGE-specific siRNAs inhibited the expression of RAGE in primary rat HSCs and inhibited the development of HF.

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Introduction

Liver cirrhosis is one of the most common illnesses compromising human health and no completely effective clinical treatment exists. The mechanisms underlying the development of hepatic fibrosis (HF) and effective treatments for this condition remain to be elucidated. Receptor for advanced glycation end products (RAGE), a member of the immunoglobulin superfamily (1), is found on the surface of various cell types, including mononuclear macrophages, neurons, renal mesangial cells and vascular endothelial cells. This protein contributes to various diseases, including type 2 diabetes, Alzheimer's disease, chronic kidney disease and atherosclerosis (2-5). AGEs are implicated in the pathogenesis of fibrosis in a number of tissue types (6,7) and evidence indicates that AGEs and RAGE contribute to the pathogenesis of chronic liver disease (6). RAGE can be significantly expressed on several types of liver cell, including hepatic stellate cells (HSCs) (8-10), which are the major effectors during hepatic fibrogenesis (7). The expression of RAGE is also increased in animal models exhibiting chronic liver disease (11,12). Therefore, RAGE may be important in the development of HF and inhibiting RAGE may be a method to prevent or reverse HF.

Specific small interfering RNA (siRNA) targeting RAGE, inhibits the expression levels of RAGE, α-smooth muscle actin and collagen type I in T6 HSCs, indicating that RAGE may be important for the activation of HSCs and the expression of collagen (11). The present study aimed to investigate the effect of RAGE-specific siRNAs on the development of HF in primary rat HSCs.

Materials and methods

Materials. Three Healthy male Sprague-Dawley rats, aged 15 months and weighing between 400 and 500 g, were purchased from the Nanjing Medical University Laboratory

Animal Center (Nanjing, China). The study was approved by the Animal Research Ethics Committee of Zhongda Hospital, Southeast University (Nanjing, China). The rats were fed a normal diet and had free access to food and water; in addition, the rats were maintained at a temperature of 22˚C under a 12-h light/dark cycle. Type IV collagenase, pronase E and Nycodenz were obtained from Sigma-Aldrich (St. Louis, MO, USA) and DNase I was obtained from Gibco Life Technologies (Carlsbad, CA, USA). The pAKD.CMV.bGlobin.egreen fluorescent protein (GFP).H1.short hairpin (sh)RNA vector was purchased from GenScript USA, Inc. (Piscataway, NJ, USA).

Isolation and culture of primary rat HSCs. HSCs were obtained from rats as previously described (13). Primary rat HSCs were isolated by density gradient centrifugation at 400 x g for 5 min and cultured with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 10% fetal bovine serum *in vitro*. The cell viability was determined using trypan blue staining (Sigma-Aldrich) and the expression of desmin in cells was detected by immunohistochemistry. The primary HSCs were cultured for 5 days and divided at random into five groups as follows: Cells transfected with pAKD-GR125, pAKD-GR126, pAKD-GR127, pAKD-GR128 or pAKD-GR129.

Preparation of specific siRNAs targeting RAGE. Rat RAGE mRNA (GenBank accession no. NM-053336.1) was used as the target sequence. siRNA Target Finder design software (version 2.0; Ambion, Austin, TX, USA) was used to model the secondary structures of the rat RAGE mRNA and five pairs of 19 nt siRNA sequences were designed in accordance with the target sequences and their complementary sequences (the specificity of the sequences were confirmed using BLAST). These sequences were then converted into short RNA oligonucleotide sequences, which form hairpin structures. *Bgl*II and *Kpn*I restriction sites and a 9 bp hairpin structure were added to the two ends of the sequence. The final oligonucleotides were named GR125, GR126, GR127, GR128 and GR129 (Table 1).

The *Bgl*II and *Kpn*I restriction sites and the hairpin structure sequence were also added to the two ends of another pair of non-specific siRNAs (not homologous to rat RAGE mRNA, as confirmed by BLAST). This construct was termed negative control (NC; Table 1).

Construction of specific siRNA expression vectors. The pAKD.CMV.bGlobin.eGFP.H1.shRNA vector was linearized by restriction enzyme digestion using *Bgl*II and *Kpn*I and then ligated to the annealed double-stranded DNA fragments GR125, GR126, GR127, GR128 and GR129, forming the RAGE-specific siRNA expression vectors pAKD-GR125, pAKD-GR126, pAKD-GR127, pAKD-GR128 and pAKD-GR129, respectively. The non-specific siRNA expression vector, pAKD-NC, was constructed in the same way and was used as a control.

Cell transfection. The pAKD-GR125, pAKD-GR126, pAKD-GR127, pAKD-GR128 and pAKD-GR129 RAGE siRNA expression vectors, were transfected into primary rat HSCs individually at multiplicity of infections (MOIs) of 20, 100, 200 and 1,000. Untreated and nonspecific siRNA-transfected primary rat HSCs were used as controls. The medium was replaced with serum-free DMEM prior to transfection. The total RNA (0.5 μg) was extracted and the mRNA expression of RAGE was determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) following incubation for 48 h at 37˚C (11). RT reagent kit was purchased from Takara Bio, Inc. (Dalian, China) and the primers used were as follows: GR-129 sense, 5'-GATCCCCGTGAATCCTGCCTCT GAACTTCAAGAGAGTTCAGAGGCAGGATTCACTTTTT TGTAC-3' and antisense, 5'-AAAAAAGTGAATCCTGC CTCTGAACTCTCTTGAAGTTCAGAGGCAGGATTCAC GGG-3'. ABI-9700 PCR apparatus (Applied Biosystems, Waltham, MA, USA) was used and the cycling parameters were as follows: 94˚C for 3 min, followed by 40 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. β-actin served as the endogenous control, ΔΔCT method for relative quantification was used to calculate differences in the expression level of each target gene (14).

The specific siRNA expression vector pAKD-GR126 with the maximum ability to inhibit the expression of RAGE was selected and transfected into primary rat HSCs ($0.2x10^6$) cultured in DMEM for 5 days at 37˚C. Untreated and nonspecific siRNA-transfected primary rat HSCs served as controls. The cells were collected following incubation for 24, 48 and 72 h, and the total RNA was extracted. The efficiency of RAGE gene silencing was assessed by RT-qPCR, as described below.

Cell treatment. Primary rat HSCs ($0.2x10^6$) cultured for 5 days at 37˚C were randomly divided into three groups (n=3/group): Group A, blank group, treated with AGE-bovine serum albumen (BSA, 200 mg/l); the pAKD-GR126 group, treated with AGE-BSA (200 mg/l) and pAKD-GR126 (MOI=1,000) and the pAKD-NC group, treated with AGE-BSA (200 mg/l) and pAKD-NC (MOI=1,000). Each group had three repeats. The medium was replaced with serum-free DMEM prior to transfection.

Total RNA extraction and RT-qPCR assay. The total RNA was extracted from the primary rat HSCs using TRIzol reagent (Sigma-Aldrich) according to the manufacturer's instructions. The purity and concentration of the RNA were determined prior to the RNA being reverse transcribed, as previously described (11). The cycling parameters were as follows: 94˚C for 3 min and 40 cycles of 94˚C for 1 min, 55˚C for 1 min and 72˚C for 1 min. β-actin served as an internal control. The $2^{-\Delta\Delta CT}$ method for relative quantification was used to calculate the differences in the expression level of each target gene (11,14).

Western blot analysis. The cellular proteins were extracted using modified radioimmunoprecipitation buffer (Sigma-Aldrich) containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% sodium dodecyl sulphate (SDS), 0.5% deoxycholate, 1 mM EDTA, 2 mg/l leupeptin and 1 mM phenylmethylsulfonyl fluoride. The protein concentration was determined using a bicinchoninic acid assay. The proteins (50 μg) were separated by electrophoresis on a 10% gradient SDS-polyacrylamide gel (Sigma-Aldrich) and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich). Following protein transfer, the membranes were blocked with 5% non-fat milk (Sigma-Aldrich) in Tris-buffered saline containing 0.1% Tween-20 (Sigma-Aldrich) for 1 h at room temperature and then incubated

Table I. Sequences of specific small interfering RNAs targeting receptor for advanced glycation end products.

Oligonucleotide	Sequence
GR125	
Sense	5'-GATCCCCGCCAACCCAGAAGCTAGAATTCAAGAGATTCTAGCTTCTGGGTTGGCTTTTTTGTAC-3'
Antisense	5'-AAAAAAGCCAACCCAGAAGCTAGAATCTCTTGAATTCTAGCTTCTGGGTTGGCGGG-3'
GR126	
Sense	5'-GATCCCCGTGAATCCTGCCTCTGAACTTCAAGAGAGTTCAGAGGCAGGATTCACTTTTTTGTAC-3'
Antisense	5'-AAAAAAGTGAATCCTGCCTCTGAACTCTCTTGAAGTTCAGAGGCAGGATTCACGGG-3'
GR127	
Sense	5'-GATCCCCGCCTCTGAACTCACAGCCATTCAAGAGATGGCTGTGAGTTCAGAGGCTTTTTTGTAC-3'
Antisense	5'-AAAAAAGCCTCTGAACTCACAGCCATCTCTTGAATGGCTGTGAGTTCAGAGGCGGG-3'
GR128	
Sense	5'-GATCCCCGAAGGTGGAACAGTCGCTCTTCAAGAGAGAGCGACTGTTCCACCTTCTTTTTTGTAC-3'
Antisense	5'-AAAAAAGAAGGTGGAACAGTCGCTCTCTCTTGAAGAGCGACTGTTCCACCTTCGGG-3'
GR129	
Sense	5'-GATCCCCGCGAAAACGACAACCCAGATTCAAGAGATCTGGGTTGTCGTTTTCGCTTTTTTGTAC-3'
Antisense	5'-AAAAAAGCGAAAACGACAACCCAGATCTCTTGAATCTGGGTTGTCGTTTTCGCGGG-3'
NC	
Sense	5'-GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTTGATC-3'
Antisense	5'-AAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAAGGG-3'

NC, negative control.

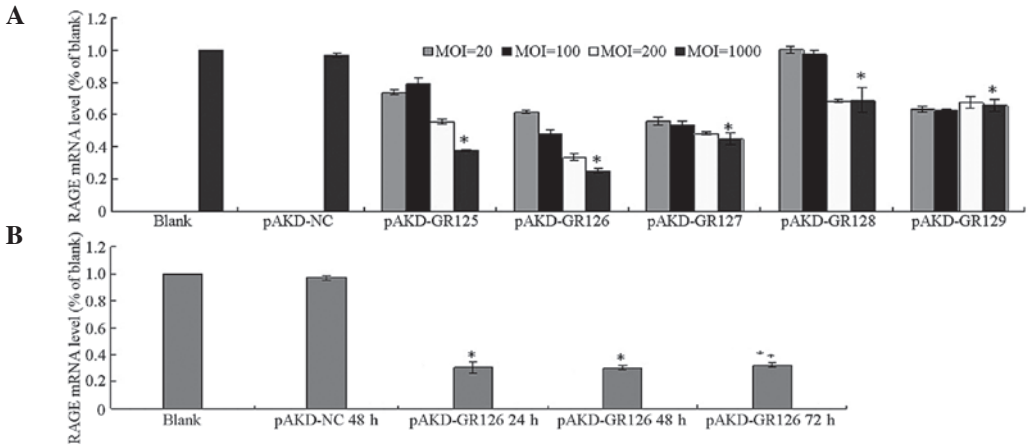

Figure 1. Inhibitory effect of RAGE-specific siRNAs on the expression of RAGE in primary rat HSCs. The mRNA expression of RAGE is presented as the ratio relative to the mRNA expression of β-actin. The mRNA expression levels were determined by reverse transcription quantitative polymerase chain reaction and the changes are expressed relative to that of β-actin (blank). (A) Effect of RAGE-specific siRNAs on the mRNA expression of RAGE in primary rat HSCs at different doses ($^*P<0.05$, vs. blank and pAKD-NC). (B) Effect of RAGE-specific siRNAs on the mRNA expression of RAGE in primary rat HSCs at various time-points. ($^*P<0.05$, vs. blank and pAKD-NC at 48 h). MOI, multiplicity of infection; RAGE, receptor for advanced glycation end products; si, small interfering; NC, negative control; HSC, hepatic stellate cell; blank, untransfected cells.

overnight at 4˚C with the following primary antibodies: Rabbit anti-rat polyclonal RAGE (ab3611), rabbit anti-rat polyclonal interleukin (IL)-6 (ab6672), mouse anti-rat monoclonal tumor necrosis factor (TNF)-α (ab92324), mouse anti-rat monoclonal transforming growth factor (TGF)-β1 (ab64715), rabbit anti-rat polyclonal connective tissue growth factor (CTGF; ab6992), mouse anti-rat monoclonal laminin (LN; ab8983), rabbit anti-rat polyclonal hyaluronic acid (HA; ab20084), mouse anti-rat polyclonal N-terminal procollagen III propeptide (PIIINP; ab169636) and mouse anti-rat monoclonal β-actin (ab8226), which were all purchased from Abcam (Cambridge, MA, USA). The membranes were subsequently washed with TBST and then exposed to a secondary horseradish peroxidase-labelled antibody [ab6721, goat polyclonal antibodies against rabbit IgG - H&L (HRP) and ab193651, rabbit polyclonal antibodies against mouse IgG - H&L (HRP)] in the blocking solution for 1 h at room temperature. The band intensities were measured using an enhanced chemiluminescent reagent (Western-Lightening Plus; PerkinElmer Life Sciences, Waltham, MA, USA) and the protein signals were normalized against β-actin.

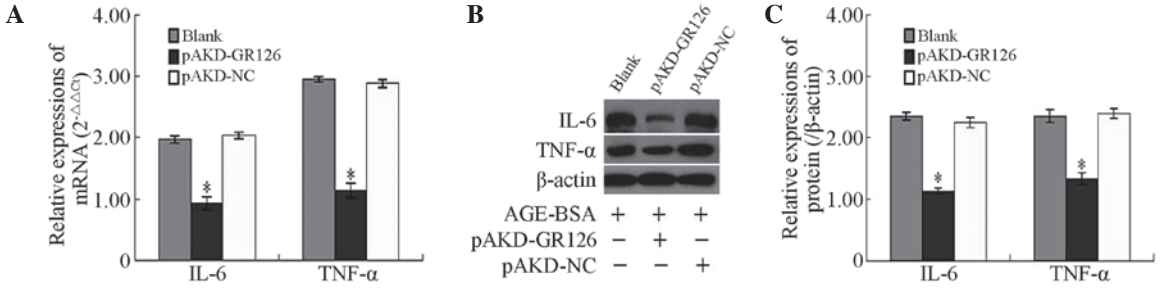

Figure 2. Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the IL-6 and TNF-α pro-inflammatory cytokines in primary HSCs. (A) mRNA expression levels of IL-6 and TNF-α were determined by reverse transcription quantitative polymerase chain reaction. (B) Protein expression levels of IL-6 and TNF-α were determined by western blot analysis. (C) Protein expression levels of IL-6 and TNF-α are expressed as ratios relative to the expression of β-actin and were determined using densitometric scanning (*P<0.05, vs. blank and pAKD-NC). IL, interleukin; TNF, tumor necrosis factor; NC, negative control; BSA, bovine serum albumen; Blank, untransfected cells; Ct, cycle threshold.

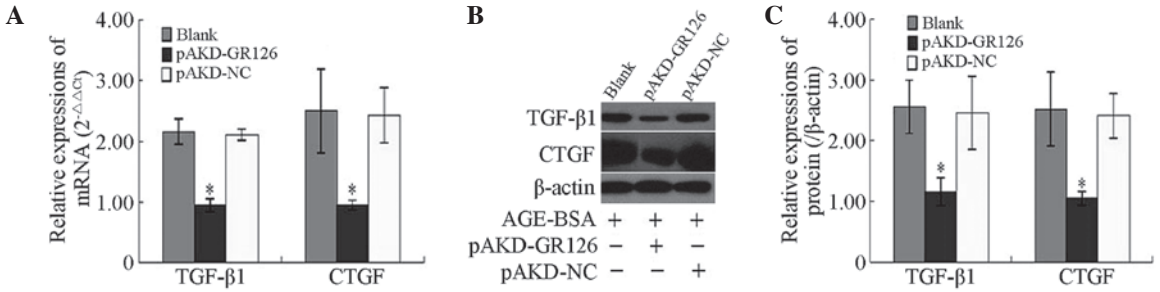

Figure 3. Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the TGF-β1 and CTGF profibrogenic cytokines in primary HSCs. (A) mRNA expression levels of TGF-β1 and CTGF were determined by reverse transcription quantitative polymerase chain reaction. (B) Protein expression levels of TGF-β1 and CTGF were determined by western blot analysis. (C) Protein expression levels of TGF-β1 and CTGF are expressed as ratios relative to the protein expression of β-actin and were determined by densitometric scanning (*P<0.05, vs. blank and pAKD-NC). TGF, transforming growth factor; CTGF, connective tissue growth factor; HSC, hepatic stellate cell; BSA, bovine serum albumin; Blank, untransfected cells; Ct, cycle threshold; NC, negative control.

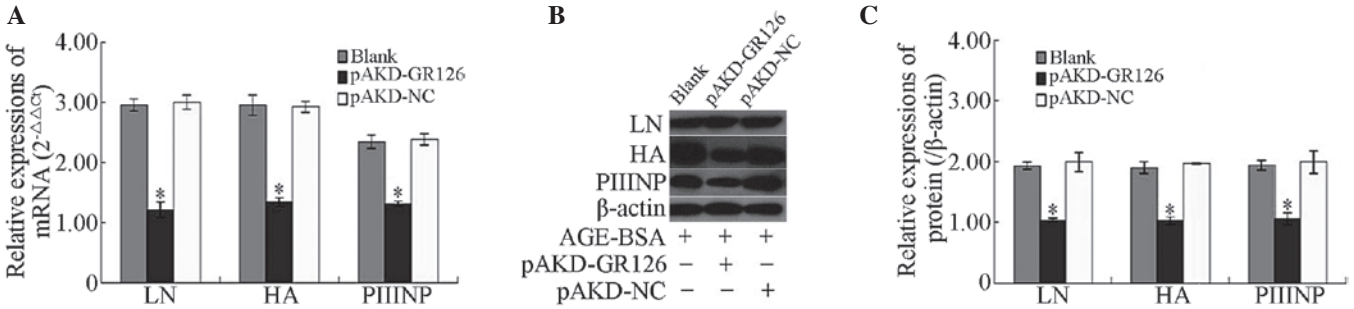

Figure 4. Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the LN, HA and PIIINP fibrosis markers in primary HSCs. (A) mRNA expression levels of LN, HA and PIIINP were determined by reverse transcription quantitative polymerase chain reaction. (B) Protein expression levels of LN, HA and PIIINP were determined by western blot analysis. (C) Protein expression levels of LN, HA and PIIINP are expressed as ratios relative to the protein expression of β-actin and were determined by densitometric scanning (*P<0.05, vs. blank and pAKD-NC). LN, laminin; HA, hyaluronic acid; PIIINP, procollagen III propeptide; NC, control; BSA, bovine serum albumen; HSC, hepatic stem cell; Blank, untransfected cells; Ct, cycle threshold.

Statistical analysis. Statistical analyses were performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). The data were analyzed using one-way analysis of variance and Student-Newman-Keuls multiple comparison test. P<0.05 was considered to indicate a statistically significant difference. The data are expressed as the mean ± standard deviation.

Results

Inhibitory effect of RAGE-specific siRNAs on the expression of RAGE in primary rat HSCs. The mRNA expression of RAGE was significantly downregulated in the primary rat HSCs treated with pAKD-GR125, pAKD-GR126, pAKD-GR127, pAKD-GR128 and pAKD-GR129 (all P<0.05) compared with the untreated primary rat HSCs and the HSCs treated with pAKD-NC. The mRNA expression of RAGE decreased in a dose-dependent manner in the MOI range of between 20 and 1,000. The most marked decrease was observed in the HSCs treated with pAKD-GR126 at an MOI of 1,000 (Fig. 1A). The mRNA expression of RAGE was downregulated in the primary rat HSCs treated with pAKD-GR126 at 24, 48 and 72 h (all P<0.05) compared with the untreated cells. However, inhibition in the mRNA expression of RAGE exhibited no differences between 24 and 72 h

(P>0.05; Fig. 1B). These results indicated that the RAGE siRNA expressed by pAKD-GR126 effectively inhibited the expression of RAGE.

Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the IL-6 and TNF-α pro-inflammatory cytokines in primary HSCs. The mRNA and protein expression levels of the IL-6 and TNF-α pro-inflammatory cytokines, were significantly downregulated in the primary rat HSCs treated with pAKD-GR126 (P<0.05) compared with the untreated primary rat HSCs and those treated with the pAKD-NC (Fig. 2).

Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the TGF-β1 and CTGF profibrogenic cytokines in primary HSCs. The mRNA and protein expression levels of the TGF-β1 and CTGF profibrogenic cytokines were significantly downregulated in the primary rat HSCs treated with pAKD-GR126 (P<0.05), compared with the untreated primary rat HSCs and those treated with the pAKD-NC (Fig. 3).

Inhibitory effect of pAKD-GR126 on the mRNA and protein expression levels of the LN, HA and PIIINP fibrosis markers in primary HSCs. The mRNA and protein expression levels of the LN, HA and PIIINP fibrosis markers were significantly downregulated in the primary rat HSCs treated with pAKD-GR126 (P<0.05), compared with the untreated primary rat HSCs and those treated with the pAKD-NC (Fig. 4).

Discussion

The present study demonstrated for the first time, to the best of our knowledge, that specific siRNAs inhibited the effect of RAGE on the development of HF in primary rat HSCs. Increasing investigations are being performed on the biological effects of AGEs and their receptors (15-18) and the role of the AGE-RAGE axis as a cofactor in the development of liver fibrosis (15-18). Honsawek *et al* revealed that serum RAGE is associated with the severity of biliary atresia and, therefore, may serve as an indicator reflecting the severity and development of HF in individuals with postoperative biliary atresia (19). Goodwin *et al* demonstrated that AGEs were damaging to the liver and augmented HF in an animal model exhibiting chronic liver disease, and these effects of which were associated with the activation of myofibroblasts (20). The upregulation of RAGE, induced by AGE administration, may be important in mediating these effects. Additionally, the serum AGE levels are significantly increased in patients with liver cirrhosis (21-23). RAGE is exclusively expressed in HSCs and myofibroblasts in the rat liver and its expression is upregulated during HSC activation and transdifferentiation into myofibroblasts (24), during which, the expression of TGF-β1 is significantly increased (8). These previous findings suggest that RAGE may be a major receptor involved in the activation and transdifferentiation of HSCs into myofibroblasts and that the expression of RAGE in the liver is important in liver fibrosis.

Several methods to inhibit RAGE in liver cells have been developed. Lin *et al* revealed that curcumin can suppress the gene expression of RAGE by increasing the activity of PPARγ and attenuating oxidative stress, leading to the elimination of the effects of AGEs on the activation of HSCs (25). Our previous study demonstrated that a specific siRNA targeting RAGE inhibited HF in a rat model (5). The present study constructed expression vectors for specific siRNAs targeting RAGE and transfected these into primary rat HSCs. The results revealed that the mRNA expression of RAGE was downregulated in the primary rat HSCs treated with pAKD-GR126 compared with the untreated primary rat HSCs and those treated with pAKD-NC. These results indicated that the RAGE-specific siRNA expressed by pAKD-GR126 effectively inhibited the gene expression of RAGE.

Furthermore, the activation of HSCs is important in HF (26-30) and several cytokines are important in the activation of HSCs (31-34). Pro-inflammatory cytokines, including IL-6 and TNF-α, promote the activity and proliferation of HSCs (35,36). TGF-β1 and its downstream target, CTGF, are potent activators of HSCs and important profibrogenic cytokines (37-40). These cytokines promote the activation and transdifferentiation of HSCs into myofibroblasts (41) and promote the synthesis and secretion of extracellular matrix (ECM) components (42,43). The ECM and its degradation products, including LN, HA and PIIINP, are useful fibrosis markers and the expression of these products are closely associated with the degree of HF (18). The present study indicated that the mRNA and protein expression levels of RAGE, IL-6, TNF-α, TGF-β1, CTGF, LN, HA and PIIINP were downregulated in primary rat HSCs treated with pAKD-GR126 compared with the untreated primary rat HSCs and those treated with pAKD-NC. These results demonstrated that the RAGE-specific siRNA expressed by pAKD-GR126 effectively inhibited gene expression of RAGE and also inhibited the expression levels of IL-6, TNF-α, TGF-β1, CTGF, LN, HA and PIIINP. Although the effect of RAGE on the development of HF remains to be fully elucidated, these findings suggested that RAGE may be a novel target for treating liver fibrosis and that RAGE-specific siRNA may be an effective candidate for the prevention of liver fibrogenesis.

In conclusion, the present study revealed for the first time, to the best of our knowledge, that a RAGE-specific siRNA can inhibit the effect of RAGE on the development of HF in primary rat HSCs. The results demonstrated that the mRNA and protein expression levels of pro-inflammatory cytokines, profibrogenic cytokines and fibrosis markers were significantly downregulated in cells treated with RAGE-specific siRNA, indicating that RAGE may be a novel target for treatment of liver fibrosis, and that RAGE-specific siRNA may be an effective candidate to prevent liver fibrogenesis. However, there were limitations to the present study, including the number of control groups and the lack of repeats, therefore, the results obtained are not sufficient to provide a firm conclusion.

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