Kangxian ruangan keli inhibits hepatic stellate cell proliferation mediated by PDGF

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INTRODUCTION
Hepatic fibrosis, a frequent pathologic change in a number of chronic liver diseases and an essential process during development of cirrhosis, is characterized by excessive proliferation of hepatic stellate cells (HSCs) and subsequent deposition of extracellular matrix (ECM)\(^\text{[1-6]}\). For this reason, HSC is regarded as one of the key cell types involved in progression of liver fibrosis, and is considered as a therapeutic target for treatment of hepatic fibrosis. Among several growth factors shown to be involved in the process, platelet-derived growth factor (PDGF) is the most potent mitogen for HSCs\(^\text{[7,8]}\). Hence, PDGF and its downstream growth factor (PDGF) is the most potent mitogen for HSCs and considered as a therapeutic target for treatment of hepatic fibrosis\(^\text{[9]}\).

Kangxian ruangan keli (KXR) is an authorized granular herbal preparation, which has been used in the clinical fields for almost 30 years and is believed to be able to promote blood circulation, phlegm elimination and to soften and resolve hard mass based on traditional Chinese medicine. Our clinical and experimental data have demonstrated its effect on chronic liver diseases\(^\text{[10]}\). It may also be preventive to the fibrogensis induced by CCL\(_4\) and inhibitory to HSC proliferation\(^\text{[11-14]}\). However, its mechanism is unknown. In this study, the effect of KXR on PDGF-mediated HSC proliferation was assessed in vitro using a serum-free culture system.

MATERIALS AND METHODS

Reagents
HSC-T6, a cell line from activated HSCs, was kindly provided by Prof. Liemin Xu in Institute of Liver Diseases, Shanghai University of Traditional Chinese Medicine. DMEM medium and fetal calf serum were purchased from Gibco (Life Technologies, Inc., Gaithersburg, MD, USA). PDGF-BB, propidium iodide and Fura-2/AM were purchased from Sigma (St. Louis, MO, USA). The antibody to phosphotyrosine was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). The SuperSignal West Pico chemiluminescent substrate was purchased from Pierce Chemicals (Rockford, IL, USA).

Cell culture
HSCs were grown in DMEM medium containing 100 mL/L fetal calf serum, 1×10\(^{-5}\) U/L penicillin, 100 mg/L streptomycin, 1 % L-glutamine and 0.1 mmol/L HEPES, and cultured at 37 °C in an incubator with 5 % CO\(_2\). KXR consists of several herbs including Radix Salviae Miltiorrhizae, Rhizoma Zedoariae, Sargassum, Carapax Trionycis\(^\text{[15]}\). The granular preparation was made in the Affiliated Hospital of Hubei Collage of Traditional Chinese Medicine, containing 2 g of crude herbs in each gram KXR. The preparation was dissolved in DMEM medium, and sterilized by filtration through a 0.45 µm filter. Following preincubation in the serum-free medium for 24 hours, the cells were grown and incubated for 48 hours, separately in the following media: 1) Only serum-free DMEM medium as a control, 2) The serum-free medium containing 10 ng/mL PDGF-BB, 3) before the addition of PDGF-BB.

RESULTS
The OD values for the HSCs growing in the media without and with addition of PDGF were 0.17±0.06 and 0.82±0.05, respectively. The PDGF-induced increase was hindered remarkably by KXR preparation in a dose-dependent manner. The reaction values for the systems with 5 mg/mL, 2.5 mg/mL and 1.25 mg/mL of KXR were 0.28±0.03, 0.37±0.02 and 0.43±0.04, respectively. Moreover, the percentages of S-phase cells in these KXR-containing culture systems were 10.95±1.35, 32.76±1.07 and 43.19±1.09, respectively, all of which were significantly lower than that in the culture free of KXR (68.24±2.72). In addition, the values for tyrosine-phosphorylated protein in HSCs treated with 5 mg/mL and 1.25 mg/mL of KXR were 0.139±0.0072 and 0.165±0.0025, respectively, which were smaller than that in the cells treated only with PDGF-BB (0.181±0.0117).

CONCLUSION: Within the dose range used in the present study, KXR preparation shows an inhibitory effect on HSC proliferation induced by PDGF. The mechanism of this process may involve interference with tyrosine phosphorylation mediated by PDGF.

Yang L, Zhang CZ, Zhu QJ. Kangxian ruangan keli inhibits hepatic stellate cell proliferation mediated by PDGF. World J Gastroenterol 2003; 9(9): 2050-2053
http://www.wjgnet.com/1007-9327/9/2050.asp
with cold ethanol at 4°C. After treated as above, the cells were incubated with DMEM containing 1 mg/mL 3-(4, 5-dimethylthiazol-2-yi)-2, 5-diphenyltetrazolium bromide (MTT) for 4 h at 37°C. The resulted precipitate was resolved by addition of dimethyl sulfoxide and incubation for 2 min. Cell growth was assessed using an enzyme-linked immunosorbent assay reader (Bio-Tek, Houston, USA) with the test wavelength at 570 nm and expressed as optical density (OD) values. The inhibition of HSC proliferation was expressed as inhibitory rate [(OD value of PDGF group - OD value of KXR group)/OD value of PDGF group]×100%.

The cell proliferation was also described by the growth curves. HSCs were plated in 24-well dishes at a density of 1×10³ cells/well in a complete culture medium. After 24 h (day 0), cells were washed twice with the serum-free medium and then divided and treated as described above. Cell counting was performed on triplicate wells on days 0, 2 and 4 following preparation of cell suspensions by digestion with trypsin. Fresh serum-free medium containing PDGF-BB (10 ng/ml) with or without KXR was added to the remaining wells on day 2.

The HSC proliferation kinetics was described by flow cytometry. The cells were plated in 10-cm dishes at a density of 1×10⁶ cells/L in the complete culture medium. After treated as described above, cells were harvested by digestion with trypsin and washed twice with PBS. After overnight fixation with cold ethanol at 4°C, cells were washed twice with PBS and suspended in the buffer containing 30 µg/mL propidium iodide, 0.1% TritonX-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/L NaCl, 1 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 500 µg/mL leupepin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 10 mmol/L Tris-HCl, pH 7.2[16] for 30 min on ice. The lysates were clarified by centrifugation at 16 000 g for 10 min at 4°C, and protein concentrations were determined using Bradford’s method[16].

Tyrosine phosphorylation assay

Subconfluent HSCs were cultured in a serum-free medium for 24 h, then in the medium with or without KXR for another 24 h. Following the culture with the presence of 10 ng/ml PDGF-BB for 24 h, cells were treated with KXR for 3 h, and incubated again in the medium containing 10 ng/ml PDGF-BB for 5 min. After washed with ice-cold PBS, the cell monolayers were harvested and suspended in a lysis buffer [1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mmol/L NaCl, 1 mmol/L ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 500 µg/mL leupepin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 10 mmol/L Tris-HCl, pH 7.2[16]] for 30 min on ice. The lysates were clarified by centrifugation at 16 000 g for 10 min at 4°C, and protein concentrations were determined using Bradford’s method[16].

Proteins were electrophoresed on a 0.1% SDS-7% polyacrylamide gel using a Mini apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The resolved proteins were transferred to a nitrocellulose filter by a transfer apparatus (Bio-Rad Laboratories, Hercules, CA, USA). After the blocking in a blocking buffer [5% BSA, 1% hen egg albumin, 10 mmol/L Tris.Cl, pH 7.4, 0.15 mol/L NaCl] for 60 min, the filter was incubated with a mouse monoclonal antibody to phosphotyrosine (1:2 000) for 120 min. Following the washing for 15 minutes respectively in a TN buffer (containing 10 mmol/L Tris pH 7.4, 0.15 mol/L NaCl) and a TN buffer containing 0.05% NP-40, the incubation was done with a peroxidase-conjugated goat antibody (1:2 000) against mouse IgG at room temperature for 60 min. The antigen-antibody complexes were visualized by an enhanced chemiluminescent (ECL) reaction[16]. The reaction intensities were assessed with an image analyzer (HPIAS-1000, Tongji Medical college Huazhong University of Science & Technology, Wuhan, China).

**Statistical analysis**

Results were expressed as mean ± standard deviation. Differences between groups were described by the Student t test. P<0.05 was considered significant.

**RESULTS**

**Effect of KXR on PDGF-mediated HSC Proliferation**

An inhibitory effect was observed by the MTT assay (Table 1) and cell counting (Figure 1) for KXR to PDGF-mediated HSC proliferation, which was in a dose-dependent manner. HSC growth response to PDGF stimulation with or without the presence of KXR was assessed by cell counting (Figure 1). PDGF-BB, with its concentration at 10 ng/mL, significantly increased HSC growth after 2 to 4 days of incubation, in comparison to the medium free of PDGF (P<0.01). This response was markedly inhibited by pretreatment with KXR (P<0.01), which was not associated with any detectable cytotoxic effects in the dose range used.

**Table 1 Effects of KXR on PDGF-mediated HSC proliferation (mean ± s)**

| Groups               | KXR concentration (mg/mL) | OD values (mean ± s) | Inhibitory rate (%) |
|----------------------|---------------------------|----------------------|---------------------|
| Control              |                           | 0.17±0.06            | -                   |
| PDGF                 |                           | 0.82±0.05            | 0.00                |
| KXR treatment before | 5                         | 0.28±0.03            | 65.9                |
| PDGF stimulation     | 2.5                       | 0.37±0.02            | 54.9                |
| 1.25                 | 0.43±0.04                | 47.6                 |

*P <0.01 vs PDGF group.*

**Figure 1 Effect of Kangxian Ruangan Granule on PDGF-induced cell proliferation in HSC-T6 cell line.**

KXR affects cell cycle of HSC

Flow cytometry showed a reduction in G0/G1-phase cell fraction and an increase in the S-phase cell fraction for the HSCs exposed to PDGF. Pretreatment with KXR inhibited the PDGF-mediated response for the cells in S phase, the result was in agreement with the data obtained by MTT assay. However, the G0/G1-phase cell fraction was similar to that without KXR pretreatment (Table 2). It indicated that KXR treatment was inhibitory to the PDGF-induced progression of...
the cell cycle beyond the G1 phase. The effect seemed to be dose-dependent (P<0.01).

Table 2 Effect of KXR on cell cycle progression of HSCs mediated by PDGF (mean ±SD)

| Groups               | n  | G0-G1 phase | S phase | G2-M phase |
|----------------------|----|-------------|---------|------------|
| Control              | 6  | 94.6±2.17   | 4.13±0.92 | 1.20±0.47  |
| PDGF 10 ng/ml        | 6  | 20.18±1.12  | 68.24±2.72 | 11.18±1.93 |
| KXR 5 mg/ml          | 6  | 83.64±3.68  | 10.95±1.39 | 5.41±0.98  |
| PDGF 2.5 mg/ml + KXR| 6  | 62.58±4.52  | 32.76±1.07 | 4.66±0.81  |
| KXR 1.25 mg/ml + PDGF| 6  | 48.18±3.37  | 43.19±1.09 | 8.63±0.71  |

**Effect of KXR on PDGF-induced tyrosine phosphorylation**

As shown in Figure 2 and Table 3, treatment of KXR resulted in a reduction in the content of tyrosine-phosphorylated proteins, the result was in accordance with its effects on cell proliferation and kinetics.

**DISCUSSION**

KXR is a granular herb preparation mainly composed of *Salviae Miltiorrhiza*, *Rhizoma Zedoariae*, *Sargassum* and *Carapax Trionycis*. It has been reported that *Salviae Miltiorrhiza* exhibits a series of important effects including anti-inflammation, anti-oxidation and retardation of HSC proliferation[17,18]. Its extract has been shown to induce HSC apoptosis[19-20]. The oil from *Rhizoma Zedoariae* was reported to be inhibitory to the proliferation of fibroblast cells and hepatocellular carcinoma cells[21,22]. It was pointed out that *Sargassum* could be helpful for the elimination of reactive oxygen species which were believed to be important factors in the progression of fibrosis[23]. Our previous clinical and experimental observations[10,14] have approved these effects using decoction preparation of KXR in patients with chronic hepatitis or cirrhosis and in rats with CCLα-induced liver fibrosis.

HSCs are a key cell type during liver fibrogenesis. Its activation is characterized by the myofibroblast-like phenotypes including an elevated proliferation rate, expression of α-smooth muscle actin, synthesis and excretion of extracellular matrix components[4,24,31]. Recently, the phenotypic transformation has been linked to some cytokines, including PDGF, and their intracellular signal transduction pathways[6,25-30].

Among many polypeptide growth factors potentially involved in chronic hepatitis, PDGF, a dimer of two chains referred to as A-chain and B-chain, has been shown to be the most potent mitogen for cultured HSCs isolated from rat, mouse or human liver. Of the three possible dimeric forms, including AA, AB and BB, PDGF-BB has been shown to be most effective[1,6,26,30]. Tyrosine protein kinase plays an important role in the PDGF-mediated activation and proliferation of HSCs[27,31]. PDGF binds to the extracellular domain of its receptor, PDGFR-, results in the receptor molecule autophosphorylation, which allows the docking of numerous signaling cascades, such as PI3-K, Raf-MEK-MAPK, Na+/H+ and Ca2+, leading to cell division and chemotaxis[26,28-30]. In the current study, we showed that KXR blocked PDGF-stimulated mitogenesis and DNA synthesis, and inhibited PDGF-induced autophosphorylation of tyrosine. These results suggest KXR may have the effects as those of tyrosine phosphorylation inhibitors. Clearly, more data are needed to elucidate the effect of KXR on tyrosine phosphorylation and its underlying mechanism.

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Edited by Su Q