Paeoniflorin ameliorates schistosomiasis liver fibrosis through regulating IL-13 and its signalling molecules in mice

XIAOYUE LI[1,2,3,4], JILONG SHEN[1,2,3,4]*, ZHENGRONG ZHONG[1,2,3,4], JUN PENG[5], HUIQIN WEN[2,3,4], JING LI[2,3,4], QINGLI LUO[2,3,4] *and* WEI WEI[1]

[1] *Institute of Clinical Pharmacology, Anhui Medical University*
[2] *Provincial Laboratory of Zoonoses, Anhui Medical University*
[3] *Key Laboratory of Microbiology and Parasitology Anhui, Anhui Medical University*
[4] *Key Laboratory of Gene Resource Utilization for Severe Diseases, Ministry of Education of China, Hefei, P R China*
[5] *Department of Pathology, Anhui Medical University Hospital of An Qing, P R China*

(*Received 5 October 2009; revised 29 November and 10 December 2009; accepted 15 December 2009; first published online 19 April 2010*)

SUMMARY

Treatment of liver fibrosis associated with *Schistosoma japonicum* ova-induced granulomas remains a challenging proposition. Paeoniflorin (PAE, $C_{23}H_{28}O_{11}$) has anti-inflammatory, anti-allergic, and immunoregulatory effects and it is commonly used in Chinese Herbal prescriptions to treat hepatic disorders. The present study was carried out to investigate the effects of PAE on hepatic fibrosis of mice infected with *S. japonicum* and to explore its possible mechanism. Upon pathological examination of PAE-treated mice, the size of egg granuloma, fibrosis scores, the concentration of IL-13 and hydroxyproline in liver were significantly reduced compared with the model mice. In the primary culture of hepatic stellate cells (HSCs), PAE inhibited IL-13-induced collagen synthesis. These results suggested that PAE might alleviate the hepatic granulomas and fibrosis caused by *S. japonicum* and the inhibitory effect of PAE on hepatic fibrosis might be associated with its ability to decrease the level of IL-13 and to interfere with the IL-13 signalling molecule in HSCs.

Key words: paeoniflorin, schistosomiasis, liver fibrosis, hepatic stellate cell, IL-13, suppressor of cytokine signalling-1, STAT6.

INTRODUCTION

Schistosomiasis ranks second, behind malaria, among human parasitic diseases in terms of public health and socio-economic importance in tropical and subtropical areas (Greenwald, 2005). The egg-induced fibrosis can lead to portal hypertension, which causes much of the morbidity and mortality associated with this disease (Magalhaes *et al*. 2004; Wynn *et al*. 2004). However, the therapy for reversing liver fibrosis is not yet well established. Recently, research for new drugs has refocused on natural products (Sun *et al*. 2007). Traditional Chinese Medicine has been practiced widely in China and other Asian countries for thousands of years and is a potential source of pharmaceutical remedies (Sun *et al*. 2007). PAE, a monoterpene glucoside, is one of the main bioactive components of total glucosides of paeony extracted from the root of *Paeonia lactiflora* (Takagi and Harada, 1969; Kimura *et al*. 1984, 1985; Chu *et al*. 2007). Many studies (Chan *et al*. 2006; Chu *et al*. 2007; Hsu *et al*. 2007; Huang *et al*. 2008; Lee *et al*. 2008; Liu *et al*. 2006; Wu *et al*. 2007; Zhang *et al*. 2008*b*) suggested that PAE contribute to the main bioactivity of *Moutan cortex* such as anti-inflammatory, anti-allergic, and immunoregulatory effects. Our previous studies have confirmed that PAE can effectively attenuate hepatic fibrosis induced by CCL_4 in animals (Wang *et al*. 2005; Gui *et al*. 2006; Sun *et al*. 2007). These studies have resulted in considerable interest in PAE as a therapeutic agent in chronic liver disease caused by schistosomiasis.

HSCs are presently regarded as one of the key cell types involved in the progression of liver fibrosis. The activation of HSCs to a proliferative, myofibroblastic phenotype plays a key role in hepatic fibrogenesis, since these cells are the principal cellular source of the excess collagen synthesis during hepatic fibrosis. Hepatic fibrosis in *Schistosomiasis japonica* is initiated by egg deposition and followed by inflammation, which stimulates production of Th2 cytokines. The latter, in turn, activates 'resting' HSC, which secretes collagens (mainly collagen I and III)-rich matrix into the extracellular space. In the murine model of schistosomiasis, several Th2-associated cytokines, mainly IL-13, are associated

* Corresponding author: Provincial Laboratory of Zoonoses, Anhui Medical University, No. 81, Meishan Road, Hefei, China. Tel/Fax: +86 551 5113863. E-mail: shenjilong53@126.com

doi:10.1017/S003118201000003X

with an increased risk of fibrosis (Kaplan *et al*. 1998; Chiaramonte *et al*. 1999*b*; de Jesus *et al*. 2004; Magalhaes *et al*. 2004; Alves Oliveira *et al*. 2006; Talaat *et al*. 2007; Wilson *et al*. 2007). Furthermore, human *S. mansoni* and *S. japonicum* fibrosis is linked with high levels of IL-13 and thus IL-13 is a major regulator of liver fibrosis in *S. mansoni* and *S. japonicum*-infected people and perhaps other Th2-mediated inflammatory responses (de Jesus *et al*. 2004; Magalhaes *et al*. 2004; Alves Oliveira *et al*. 2006; Bartley *et al*. 2006; Coutinho *et al*. 2007). IL-13 mediates its effects via a complex receptor system IL-4Rα/ IL-13Rα1 and IL-13Rα2 (Hershey, 2003). IL-13 activates both IL-4Rα chain and IL-13Rα1 chain to stimulate JAK1, JAK2 and TYK2, leading to selective activation of STAT6 (Kelly-Welch *et al*. 2005; Wong and Leong, 2004). IL-13Rα2 acts as a decoy receptor and is a critical down-regulatory factor of IL-13–mediated tissue fibrosis induced by *S. mansoni* (Chiaramonte *et al*. 2003).

The aim of our present work was to evaluate the anti-fibrotic activity of PAE. It was designed to investigate the effects of PAE administration on *S. japonicum* egg-induced liver fibrosis in mice *in vivo*. In addition, the effects of PAE on proliferation and collagen synthesis in primary cultures of mouse HSCs were evaluated *in vitro*.

MATERIALS AND METHODS

Animals and treatment

Female BALB/c mice, 6–8 weeks old, were obtained from the Laboratory Animal Center of University of Science and Technology of China (Anhui, China). These mice were infected percutaneously with approximately 25 *S. japonicum* cercariae. The cercariae were obtained from laboratory-raised and infected *Oncomelania hupensis* (Jiangsu Institute of Parasitic Diseases, China). All animals for each experiment were infected on the same day with the same preparation of cercariae. These infected animals were divided into 3 groups randomly, group 1 were uninfected mice, group 2 were infected/untreated mice, and group 3 were infected/PAE-treated mice (Normal = Uninfected, Model = Untreated, Group 3 = Treated). In order to decrease the deaths of infected mice, each group, except for the normal group, was orally given praziquantel (PZQ, 500 mg/kg/day) on the 42nd day (2 days of successive administration) after infection. In the PAE (Nanjing Zelang Medical Technology Co. Ltd, China; purity, 98%; melting point, 196 °C; Voucher number: ZL080410) treated group, the PAE (30 mg/kg/day) was administrated orally on the 12th day (30 days of successive administration) after infection; the mice in the model group were synchronously given the same volume of solvent only. On the 102nd day following infection, the livers were obtained from mice under ether anaesthesia. Liver tissue was preserved for histological analysis in 4% paraformaldehyde, the remaining tissue was homogenized for analyses of IL-13 and hydroxyproline.

Liver histopathological examination

Measurements of granuloma and fibrosis. Liver tissue was fixed in 4% (v/v) paraformaldehyde in PBS and embedded in paraffin, and then processed for histology. From each liver tissue, serial sections (5 μm) were stained with haematoxylin and eosin (H&E) for light microscopic evaluation to examine the size of the granulomas. The size of each granuloma in the section was counted and the average granuloma size was calculated for each section. Masson trichrome staining for collagen in each liver tissue section was evaluated in order to score hepatic fibrosis. The severity of fibrosis was individually determined using the semi-quantitative grading system according to histological assessment of collagen staining described by Chen (2002). The scores of fibrosis in liver specimens were graded from − to +++ and correspondingly numbered from 0 to 3: Grade 0 ($2^0=1$), normal; Grade I ($2^1=2$): collagen around granulomas and penetrating into them; Grade II ($2^2=4$): liver fibrosis increases in the portal tracts, little collagen appears among interlobular tissue; Grade III ($2^3=8$): liver fibrosis forms interlobular tissue. The entire liver section was reviewed at a magnification of 100×. Each of the 5 random microscopic fields per section were detected and a score ranging from 0 to 3 was assigned. The results of fibrosis were determined as the mean of 5 different fields on each section, respectively. All assessments were performed in blind fashion by 3 independent investigators.

Immunohistochemistry (*IHC*). Col I protein was stained with goat anti-mouse polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA), then the IHC test was performed using a Histostain-Plus kit (Zhongshan Biotechnology Co., Beijing, China) according to the manufacturer's instructions. The α-SMA proteins were stained with anti-α-SMA monoclonal antibody (Sigma-Aldrich, Inc., St Louis, MO, USA), the IHC test was conducted following the instructions of the Powervision Two-Step detection system (Zhongshan Biotechnology Co., Beijing, China). By use of the point counting stereological method, quantitative histological analyses were made in a blinded manner under a light microscope and with a computer image analysis system (Nikon Corporation, Japan). Briefly, 5 photographs (200× magnification) were taken from each non-overlapping liver section per mouse. Then a unified image correction factor was set up, and the mean optical density value (MOD) was chosen. The results

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

of MOD were determined as the mean of 5 different fields on each section, respectively. All assessments were performed in blind fashion by 3 independent investigators.

Detection of hepatic collagen

The fresh liver was homogenized in 1 ml of PBS per 0·1 g tissue, and the supernatant of this homogenate was used for analysis of hydroxyproline content. A colourmetric assay was used to determine hepatic hydroxyproline content according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Results were expressed as μg/g wet tissue.

Detection of IL-13

Levels of IL-13 in liver homogenates were determined using commercial enzyme-linked immunosorbent assay (ELISA Kits, R&D system, Inc., Minneapolis, USA).

Isolation and cultivation of HSCs

HSCs were isolated from normal mice livers by sequential *in situ* perfusion with collagenase (Sigma-Aldrich, Inc., St Louis, MO, USA) and pronase (Merck, Chelles, France) as previously described (Chu *et al*. 2007) and were separated from the resulting cell suspension by density-gradient centrifugation through a single layer of 18% Nycodenz (Sigma-Aldrich, Inc., St Louis, MO, USA). After centrifugation (1200 ***g*** for 20 min at 4 °C), purified HSCs were collected from the top of the Nycodenz cushion and resuspended in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 20% fetal calf serum (FCS, Gibco, Grand Island, NY, USA), penicillin (100 U/ml), streptomycin (100 mg/ml). Then the isolated HSCs were seeded at a density of $1{\cdot}0 \times 10^5$ cells/ml in 96-well culture plates. After the cells became subconfluent (at 70–80% confluence), the cells were cultured with DMEM without FCS for 24 h (serum starvation) before the start of all experiments.

Measurement of HSC proliferation and Col I concentration

Effect of PAE on the proliferation of HSCs with rIL-13. HSCs in primary passage were plated at a density of $1{\cdot}0 \times 10^6$ cells/ml in 96-well culture plates. Subsequently, The confluent cells were incubated with 50 ng/ml rIL-13 (Sigma-Aldrich, Inc., St Louis, MO, USA) and various concentrations of PAE (0, 30, 60, 120 mg/l) or colchicine (1 μmol/l, Sigma-Aldrich, Inc., St Louis, MO, USA) in DMEM medium (DMEM containing 0·5% FCS) for 48 h. Colchicine is an inhibitor of cell proliferation and was used as the negative control in the experiment. HSC proliferation was detected by MTT (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colourimetric assay. The MTT cell proliferation test measures the reduction of a tetrazolium component (MTT) into an insoluble formazan product by the mitochondria of viable cells. The cells are read using a plate reader at a wavelength of 570 nm. The amount of colour produced is directly proportional to the number of viable cells. The absorbance was read on the reader at a wavelength of 570 nm. Three cultures in each group were examined.

Effect of PAE on ECM production in response to rIL-13. To evaluate ECM production, we measured the concentration of Col I in the HSC culture supernatant. The cells (1×10^5) were seeded into 96-well plates and incubated with various concentrations of PAE (0, 15, 30, 60, 120 mg/l) or AG490 (50 μmol/l, as a positive control, Sigma-Aldrich, Inc., St Louis, MO, USA) for 24 h in DMEM medium with 0·5% FCS. AG490 (Sigma-Aldrich, Inc., St Louis, MO, USA), the Janus-activated kinase (JAK)-selective inhibitor (Wong and Leong, 2004), tyrphostin AG490, was used to inhibit phosphorylation of JAK and the signal transducer and activator of transcription 6 (STAT6), and subsequently reduce the ECM produced by HSCs. Then the cells were incubated with rIL-13 (at a final concentration of 50 ng/ml) for 2 h. At the end of the experiment, cell culture media were collected and centrifuged for 20 min at 450 ***g***, at 4 °C. The Col I concentration in supernatants was then determined by ELISA using a Col I ELISA kit (R&D system, Inc., Minneapolis, USA) according to the instructions of the manufacturer.

RNA isolation and PCR

The purpose of PCR experiments is to examine the transcriptional response of HSCs to the treatment with PAE. Total RNA of all the HSCs was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instruction. The quantity of total RNA was assessed by spectrophotometry with an A_{260}/A_{280} ratio of more than 2·1 and the quality of RNA was determined by Sepharose gel electrophoresis. Then, DNase I digestion and first-strand cDNA synthesis (adjusting all total RNA to the same concentration) were performed using Reverse Transcription System (A3500, Promega Corporation, USA) according to the manufacturer's protocol. Next, the cDNAs were amplified with various specific primers (Table 1). All primers were designed by Primer Premier 5.0 software and PCR products were visualized on

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

Table 1. All primers for RT-PCR

mouse IL-4Rα (GenBank: AF016189)	forward, 5′-GCCCGGAAGAATACG-3′ reverse, 5′ACATCTGGGAAGCAAA3′	425 bp
mouse IL-13Rα1 (GenBank: NM008540)	forward, 5′GTGGCTGGTCGGAAAGG3′ reverse, 5′GTGCTGGTGGCGTTAGA3′	270 bp
mouse IL-13Rα2 (GenBank: AF015260)	forward, 5′ACTCGGTGCTCAAGAAACTC3′ reverse, 5′CCCAGGCTCCAGAAGAAG3′	479 bp
mouseCol Iα1 (GenBank: NM007742)	forward, 5′GCCCGGAAGAATACG3′ reverse, 5′-ACATCTGGGAAGCAAA3′	204 bp
mouse col Iα2 (GenBank: NM007743)	forward, 5′ CGGAGGTGGCTATGACTTT 3′ reverse, 5′CGGCTGTATGAGTTCTTCG3′	345 bp
SOCS-1 (GenBank: NM010754)	forward, 5′-GACTACACCCACTCCATTCC-3′ reverse, 5′-CACTTAGGCACTCAGCAAAC-3′	153 bp
mouse GAPDH (GenBank: BC095932)	forward, 5′-TCAACGGCACAGTCAAGG-3′ reverse, 5′-AAGTCGCAGGAGACAACC-3′	693 bp

agarose gels. Mouse glyceral dehyde-3-phosphate-dehydrogenase (GAPDH) was used as an internal control. The amplification was performed in thermal cycling with 30 cycles using a procedure of denaturation at 94 °C for 45 s, annealing at 50 °C for 45 s, and extension at 72 °C for 45 s followed by a final extension step for an additional 10 min. PCR products were quantified relative to GAPDH internal controls.

Western blotting analysis

The treated HSCs were lysed and the cell proteins were extracted using the method described previously (Chu *et al*. 2007). Total HSC protein concentrations in the supernatants were then measured by the Enhanced BCA (bicinchoninic acid) Protein Assay Kit (Nanjing Jiancheng Bioengineering Institute, China). The HSC proteins from each sample were analysed by SDS-polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane (Millipore, Billerica, MA, USA) by semi-dry transfer. Blots were probed overnight at 4 °C with the following primary antibodies: anti-Col I, anti-SOCS-1, anti-STAT6, anti-p-STAT6 and anti-β-actin (Santa Cruz Biotechnology, Inc., California, USA), followed by incubation with the appropriate horse-radish peroxidase-conjugated secondary antibody at various dilutions for 2 h. Detection was achieved by enhanced chemiluminescence using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific Inc., Rockford, USA) and exposed to film. Filters were quantified by using the JD-801 gel image analysis system (Jiangsu JEDA Science-Technology Development Co. Ltd, China).

Statistical analysis

Data were expressed as means ± S.D. Statistical significance of the difference between groups was determined by one-way analysis of variance (using SPSS 11.5 software package). $P<0{\cdot}05$ indicated a statistically significant difference.

RESULTS

Effect of PAE on liver histopathology

Histological examination showed a significant change in the profile of collagen fibre deposition in the liver sections of mice pre-treated with PAE compared to model mice. Liver sections from the normal mice showed no fibrosis. As shown in Fig. 1A and B, Masson trichrome and H&E staining revealed enormous schistosome granulomas surrounded by an inflammatory cell accumulation and fibril aggregation in the portal area of the livers. The cord-like fibril aggregation extended from the portal area to the hepatic sinus and dissected the hepatic lobule in the model group. In contrast, there were only scattered egg nodules of *S. japonicum* in the livers of the mice in the PAE-treatment group, with fewer and smaller granulomas and less significant fibrosis; moreover, the normal structure of hepatic lobules was maintained, with fibrogenesis confined to the portal area. As shown in Table 2, in the PAE-treatment group the mean area of granuloma and degree of fibrosis were reduced by nearly 53·4% and 38·5% compared with the model group.

Effect of PAE on the expression of α*-SMA and Col I in liver*

IHC staining demonstrated that the content of α-SMA and Col I in the model group was dramatically increased compared with the normal mice. The content of α-SMA and Col I was found to extend from the portal area to the hepatic sinus, dissecting liver lobules, in the model mice. However, α-SMA and Col I were limited only to the portal area in the PAE-treatment group and the degree of α-SMA and Col I expression was markedly decreased by 30·3% and 46·6% compared with the model group (Fig. 1C and D, Table 2).

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

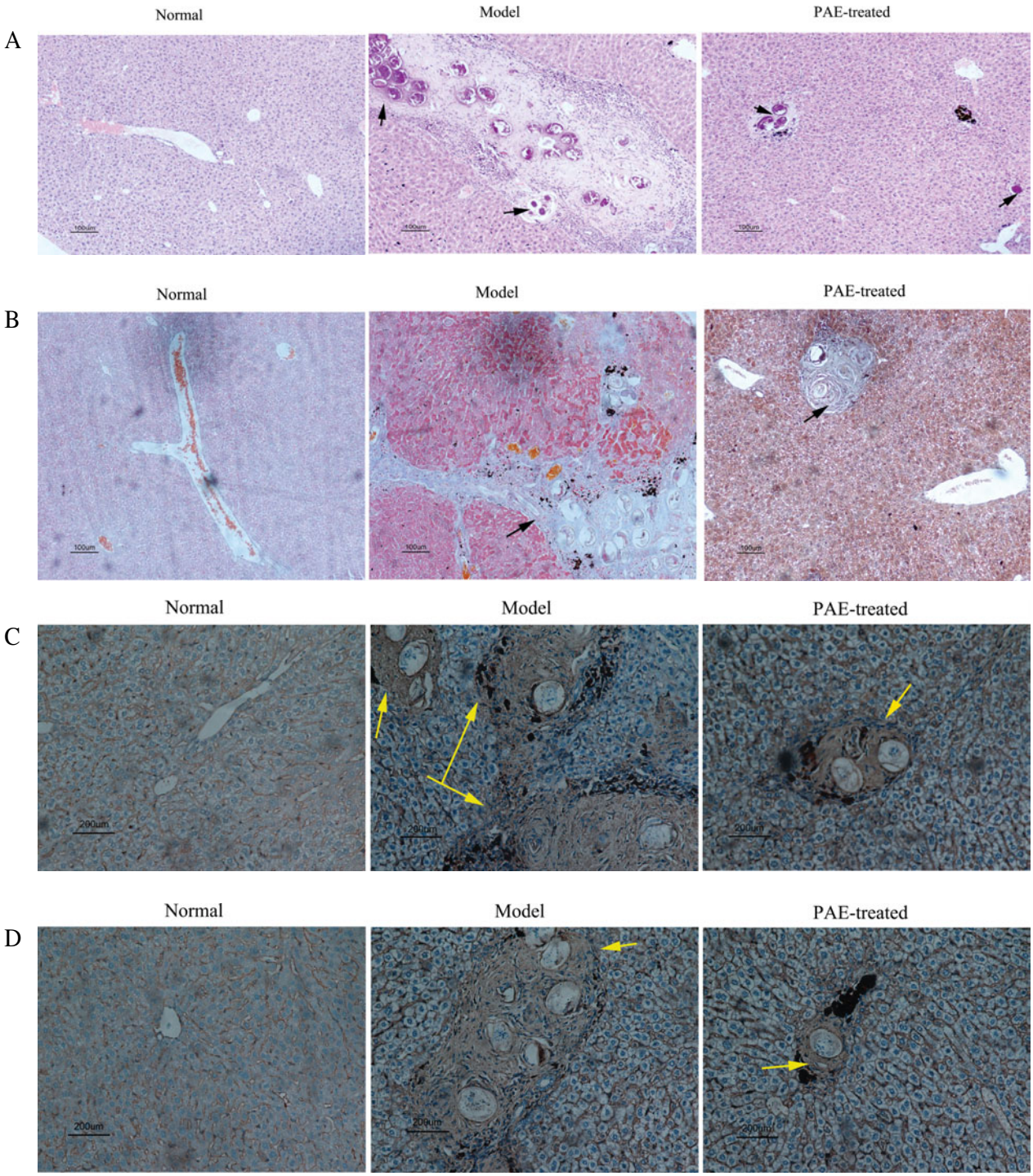

Fig. 1. Hepatic pathology in normal, model, and PAE pre-treatment mice infected with *Schistosoma japonicum*. (A) Haematoxylin and eosin-stained sections of livers from infected model mice show enormous schistosome granulomas surrounded by inflammatory cell accumulation. Only fewer and smaller egg granulomas were found in the livers of the mice in PAE pre-treatment group, ×100. The arrow identifies the egg. (B) Masson trichrome staining of sections of livers demonstrates that the cord-like fibril aggregation extended from the portal area to the hepatic sinus and dissected the hepatic lobule (blue staining) in model mice. There is less significant fibrosis in PAE-treated mice compared with wild-type model mice. Original magnifications, ×100. The arrow identifies deposition of collagen. (C) IHC staining of sections of liver shows the dramatically decreased content of Col I in the PAE group compared with the model group. The band of collagen fibrils extends from the portal area to the hepatic sinus and dissected liver lobules in model mice. Col I in the PAE group is confined to the portal area. Original magnifications, ×200. Col I proteins were stained brown in sections and the arrow identifies positive staining. (D) IHC staining of sections of liver demonstrates positive expression of α-SMA not only in the portal area, but also in the hepatic sinus in the model mice. In the PAE pre-treatment group, positive expression of α-SMA was clearly inhibited and limited only to the portal area. Original magnifications, ×200. α-SMA proteins were stained brown in sections and the arrow identifies positive staining.

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

Table 2. Effect of PAE on hepatic granuloma, fibrosis, hydroxyproline, Col I, α-SMA, and IL-13 ($\overline{x} \pm s$, $n = 24$)

Group	Granuloma (mm^2)	Fibrosis (score)	Hydroxyproline (μg/g liver)	IL-13 (ng/g liver)	α-SMA (MOD)	Col (MOD)
Normal	0	1·00 ± 0·00	110·167 ± 26·843	1·271 ± 0·722	1·269 ± 0·085	0·127 ± 0·030
Model	2·019 ± 0·661	6·00 ± 2·07	605·000 ± 110·459	2·906 ± 0·206	5·365 ± 1·744	0·635 ± 0·102
PAE	0·802 ± 0·191*	5·33 ± 2·19*	274·333 ± 46·817**	1·654 ± 0·137**	3·737 ± 1·101*	0·339 ± 0·042**

* $P < 0·05$; ** $P < 0·01$, compared with the corresponding model group.

Effect of PAE on hydroxyproline in liver homogenates

Analysis of hepatic hydroxyproline content was carried out as an index of liver fibrosis. Elevated hydroxyproline levels were measured in model animals with respect to normal mice (Table 2). Following treatment with PAE, hydroxyproline content in liver tissue was reduced by nearly 54·5% compared with model mice.

Effect of PAE on the IL-13 level in liver homogenates

In model mice, the level of IL-13 was dramatically increased in the hepatic homogenate whereas it was decreased by 43·0% in the PAE-treated mice (Table 2).

Effect of PAE on HSC proliferation stimulated by IL-13

Our study showed that (i) co-culture of HSCs with IL-13 induced a significant proliferative response as illustrated in Fig. 2; (ii) pre-incubation with increasing concentrations of PAE significantly reduced the proliferative response in a dose-dependent manner; (iii) no statistical significance was noted in reduction of the proliferative response between colchicines (1 μmol/l) and PAE ($P > 0·05$) when the concentration of PAE was increased to 120 mg/l.

PAE inhibits IL-13-induced collagen production in HSCs

To assess the effect of PAE on IL-13-induced Col I production, RT-PCR and Western blotting analysis were performed for Col I in HSCs, respectively. Pretreatment with PAE led to a dose-dependent suppression of Col I mRNA and protein, which was confirmed as shown in Fig. 4A and B, and this effect was significantly inhibited by the specific JAK kinase inhibitor, AG490. Furthermore, when HSCs were cultured with various concentrations of PAE, Col I secretion by IL-13-stimulated HSCs was inhibited significantly (Fig. 3C). In summary, Col I produced by IL-13-stimulated HSCs was suppressed by PAE at the gene and protein level in a concentration-dependent manner.

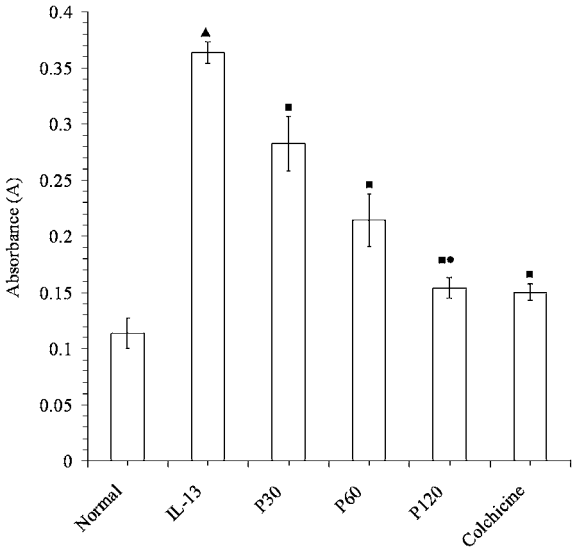

Fig. 2. Effect of PAE on proliferation of HSCs stimulated by rIL-13. HSCs were treated with PAE (0, 30, 60, 120 mg/l) or colchicine (1 μmol/l) for 48 h before being treated with IL-13 for 24 h, and then HSC proliferation was detected by the MTT colourimetric assay. Co-culture of HSCs with rIL-13 induced a significant proliferation of HSCs, and pre-incubation with increasing concentrations of PAE significantly reduced the proliferative response in a dose-dependent manner, especially when the concentration of PAE was increased to 120 mg/l, there is no statistical significance in the reduction of proliferation between colchicines (1 μmol/l) and PAE. The negative control contained PAE at 0 mg/l; $P < 0·01$ compared with the normal group; $P < 0·05$ compared with the IL-13-treated alone group; $P > 0·05$ compared with the colchicine group; Each bar represents the mean and standard deviation of triplicate determinations and data presented are representative of 3 independent experiments.

Analysis of IL-4/IL-13 receptor components on HSCs

To examine whether PAE exerts its inhibitory effects by down-regulating the expression of IL-13 receptor, we ran RT-PCR on the transcripts of IL-4Rα/IL-13Rα1 and IL-13Rα2. In this study, all experiments were performed with HSCs. By using the RT-PCR method, the transcripts of IL-4Rα and IL-13Rα1 on HSCs were induced by treatment

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

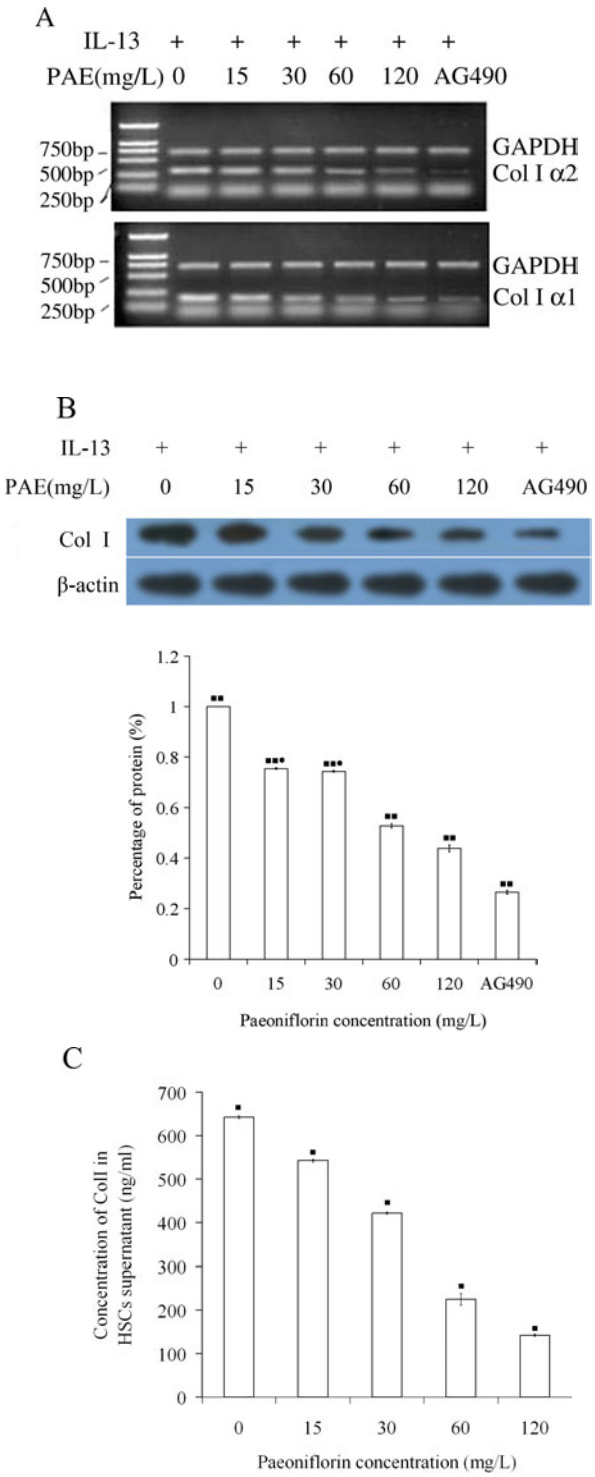

Fig. 3. Effect of PAE on rIL-13-induced Col I production in HSCs. Primary HSCs ($1{\cdot}0 \times 10^5$) were seeded in 24-well plates in growth medium containing 15% FCS for 24 h, then serum-starved for an additional 24 h. Subsequently, HSCs were pre-incubated with PAE (0, 15, 30, 60, 120 mg/l), or AG490 (at 50 μmol/l) for 24 h before being stimulated with rIL-13 (50 ng/ml) for 2 h. (A) RT-PCR analysis of Col I α1 and α2 gene expression in HSCs. Levels of Col I α1 and α2 mRNA from HSCs induced by rIL-13 were down-regulated by PAE in a concentration-dependent manner. (B) Western blotting analysis of Col I expression in HSCs. Levels of Col I protein from HSCs induced by rIL-13 were down-regulated by PAE in a concentration-dependent manner. (C) ELISA analysis of Col I protein secretion in HSCs culture media. Levels of Col I protein in HSC superatant induced by IL-13 were down-regulated by PAE in a concentration-dependent manner. ■■ There was statistical significance among these groups ($P<0{\cdot}01$); ■ there was statistical significance among these groups ($P<0{\cdot}05$). ● There was no statistical significance among the two groups ($P>0{\cdot}05$). Results are the mean ± S.D. from 3 independent experiments. Each bar represents the mean and standard deviation of triplicate determinations and data presented are representative of 3 independent experiments.

with IL-13 whereas IL-13Rα2, a critical down-regulatory factor of IL-13–mediated tissue fibrosis induced by *S. mansoni* and *S. japonicum*. (Chiaramonte *et al*. 2001; McKenzie and Fallon, 2003; Wynn *et al*. 2004), was not detectable (Fig. 4). Furthermore, the expression of IL-4Rα and IL-13Rα1 mRNA did not change in HSCs treated with increasing concentrations of PAE (data not shown). The results suggested that IL-13 could stimulate the expressions of IL-4Rα and IL-13Rα1 mRNA, but not IL-13Rα2. So, we presumed that IL-13 induces the expression of Col I mainly through the IL-4 Rα/IL-13Rα1 signal transduction pathway and PAE inhibits the production of collagens by interfering with the IL-13 signalling pathway. The following experiments were set up to test this hypothesis.

PAE inhibits IL-13-induced STAT6 phosphorylation

To determine whether PAE can inhibit activation of STAT-6 induced by IL-13 in HSCs, we examined the expression of STAT-6 and p-STAT6 by use of Western blotting. As shown in Fig. 5A and B, IL-13-induced STAT6 phosphorylation, and this effect did not peak until 2 h but was significantly attenuated at 4 h after addition of IL-13, and IL-13 had no effect on STAT6 protein expression in HSCs. PAE, however, effectively reduced phosphorylation of STAT6, but had no effect on STAT6 protein in HSCs. Furthermore, levels of STAT6 phosphorylation were markedly down-regulated by PAE in a concentration-dependent manner, and AG490, a inhibitor of JAKs, was shown to inhibit phosphorylation of STAT6, but had no effect on STAT6 protein.

PAE elevated the expression of SOCS-1

To determine whether IL-13 causes expression of SOCS-1 (a suppressor of cytokine signalling-1) in HSCs, SOCS-1 mRNA and protein were semi-quantified by RT-PCR and Western blotting analysis in our study. Our results demonstrated that

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

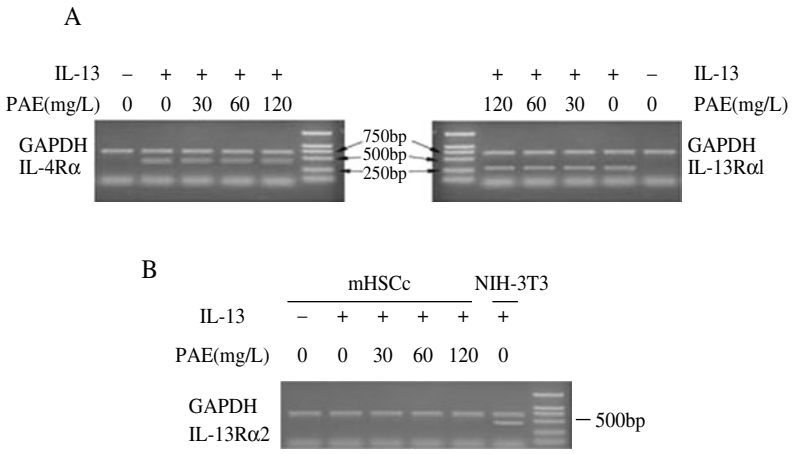

Fig. 4. Composition of the receptor on mouse hepatic stellate cells that were treated with different PAE concentrations for 24 h before rIL-13 incubation for RT-PCR. Amplification of IL-4Rα, IL-13Rα1, and IL-13Rα2 mRNA was performed on total RNA from HSCs. (A) PAE did not alter IL-4Rα and IL-13Rα1 levels induced by rIL-13. (B) Neither rIL-13 nor PAE treatment induced the transcript of IL-13Rα2. Experiments were set up in triplicate (data not shown). Amplified PCR products for IL-4Rα and IL-13Rα1 are shown in their expected positions/sizes.

SOCS-1 mRNA levels peaked after 1 h in comparison with the control. After a long incubation of the cells, SOCS-1 mRNA and protein expression were still at elevated levels (data not shown). Moreover, we detected whether SOCS-1 could be affected by PAE at various concentrations in all groups. As shown in Fig. 6A and 6B, levels of SOCS-1 mRNA and SOCS-1 protein were up-regulated following PAE treatment relative to IL-13 treatment alone. Additionally, levels of SOCS-1 mRNA protein were up-regulated by PAE in a concentration-dependent manner.

DISCUSSION

Natural drugs have made a significant contribution to the treatment of liver fibrosis. Use of herbal drugs in the treatment of liver diseases (Sun, 2007) has a long tradition, especially in Eastern medicine. PAE is an active monomer extracted from paeony, one of the well-known traditional herbs in China (Takagi and Harada, 1969). PAE has immunoregulatory (Takayama *et al*. 2006; Chu *et al*. 2007; Hung *et al*. 2008) and anti-inflammatory effects (Zhang *et al*. 2008*a*); Huang *et al*. 2008; Smith, 2008). Preparations of many traditional Chinese herbs used in anti-hepatic fibrosis contain paeony root (Li *et al*. 2003; Chu *et al*. 2007). The present study demonstrated that PAE had therapeutic effects on liver fibrosis caused by *S. japonicum* in mice. We studied the effect of PAE on the formation of hepatic granuloma and fibrosis, and the changes of fibrotic markers in liver tissues including hydroxyproline, α-SMA and Col I. The results indicated that PAE, administrated in a safe dosage with minimal side effects (Chu *et al*. 2008), not only diminishes the dimension and degree of hepatic granuloma and fibrosis, but also decreases the level of hydroxyproline, α-SMA and Col I in liver. Furthermore, our study also revealed that no impact of PAE on the worm and egg burden was seen in the infected mice with or without PAE treatment, suggesting that the lower hepatic hydroxyproline and collagen deposition in PAE-treated mice was not associated with parasite killing.

Interleukin 13 is an immunoregulatory cytokine predominantly secreted by activated Th2 cells (Wynn, 2003). It has similar functions to IL-4 and both of them share a common receptor. However, unlike IL-4, IL-13 appears to be necessary in the effector phase of inflammation and fibrogenesis (McKenzie *et al*. 1993, 1998; Minty *et al*. 1993; Granel *et al*. 2007). IL-13 ablation experiments and studies with IL-13–deficient mice as well as IL-13 antagonist in wild mice demonstrated that IL-13 and IL-13-/IL-4Rα, and the Stat6-dependent pathway represented a central mechanism regulating fibrosis in murine schistosomiasis (Chiaramonte *et al*. 1999*a*, 2001; Magalhaes *et al*. 2004; Alves Oliveira *et al*. 2006; Ta, 2008).

In our study, we found that the expression of IL-13 in liver homogenate was elevated significantly more in model mice than in normal mice and that PAE could markedly lower the level of IL-13. Therefore, one of the mechanisms of the suppressive effects of PAE on collagen synthesis may be a direct decrease of IL-13 production in liver, presumably related to the suppression of Th2 cells. Our hypothesis is that the anti-fibrotic activity of PAE might be associated with regulating the IL-13 signal pathway and simultaneously suppressing the proliferation of HSC.

HSCs are the primary cell-type in the liver responsible for excessive collagen synthesis during

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

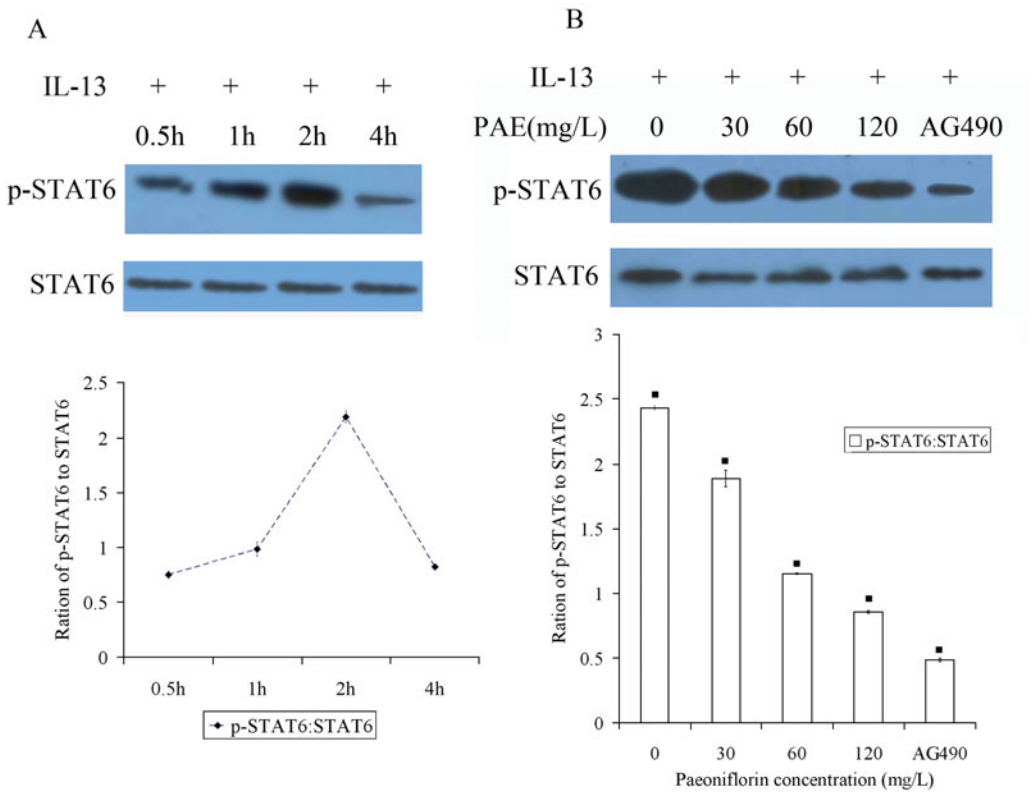

Fig. 5. Effect of PAE on rIL-13-induced STAT6 activation in HSCs. Primary HSCs ($1{\cdot}0 \times 10^5$) were seeded in 24-well plates in growth medium containing 15% FCS for 24 h, then serum-starved for an additional 24 h. Subsequently, HSCs were either left alone as a control or pre-incubated with PAE or AG490 for 24 h before being stimulated with rIL-13 for 2 h. (A) Time-course of STAT6 phosphorylation stimulated by rIL-13. rIL-13 caused transient STAT6 phosphorylation, and STAT6 phosphorylation did not peak until 2 h but significantly attenuated at 4 h after addition of rIL-13. rIL-13 had no effect on STAT6 protein expression in HSCs. (B) Effects of PAE and AG490 on rIL-13-induced STAT6 phosphorylation. PAE effectively reduced the phosphorylation of STAT6, but had no effect on STAT6 protein in HSCs. Levels of STAT6 phosphorylation were down-regulated by PAE in a concentration-dependent manner, and AG490, an inhibitor of JAKs, was shown to inhibit phosphorylation of STAT6, but had no effect on STAT6 protein. For Fig. 5A and B, cell extracts were subjected to Western blotting using phospho-specific antibodies. The band intensities of phosphorylation of STAT6 were normalized to that of the corresponding non-phosphorylated. ■ There was statistical significance among these groups ($P < 0{\cdot}05$). Results are the mean ± S.D. from 3 independent experiments. Each bar represents the mean and standard deviation of triplicate determinations and data presented are representative of 3 independent experiments.

hepatic fibrosis (Bartley *et al*. 2006; Tsukada *et al*. 2006). Following liver injury, the HSC undergoes a complex transformation or activation process in which the cell changes from a quiescent, vitamin A-storing cell to that of an activated, myofibroblast-like cell with remarkable proliferative, contractile, pro-inflammatory, and fibrogenic properties, which is characterized by *de novo* expression of α-smooth muscle actin (α-SMA). Metabolically, an increase in DNA synthesis and cellular proliferation occurs following HSC activation (Bartley *et al*. 2006; Chang *et al*. 2006; Tsukada *et al*. 2006). HSCs play a crucial role in liver fibrosis, as they are responsible for excessive deposition of extracellular matrix proteins, of which Col I predominates. Activated HSCs have now been clearly identified as the primary cellular source of ECM components, thus HSCs have been identified as a 'signal target' of hepatic fibrosis (Gressner, 1998). Because IL-13 was the primary fibrogenic mediator in schistosomiasis (Chiaramonte *et al*. 2001, 1999*b*) and IL-13 was shown to directly stimulate Col I production by fibroblasts and LI-90 HSCs cell lines *in vitro* (Chiaramonte *et al*. 1999*b*; Jinnin *et al*. 2004; Sugimoto *et al*. 2005). Some studies reported that PAE significantly inhibited HSC proliferation and collagen synthesis stimulated by TGF-β *in vitro* (Chu *et al*. 2007; Sun, 2007). So we here intended to explore whether PAE could directly interfere with collagen production induced by IL-13 from isolated murine primary HSCs.

The present study demonstrated that PAE could inhibit the HSC proliferation induced by IL-13, suggesting that PAE may decrease collagen production by suppressing HSC activation. Furthermore, the HSCs used in this study expressed one type of receptor complex for IL-13, IL-4Rα/IL-13Rα1 heterodimer, and did not express IL-13Rα2 under the experimental conditions. Moreover, the

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

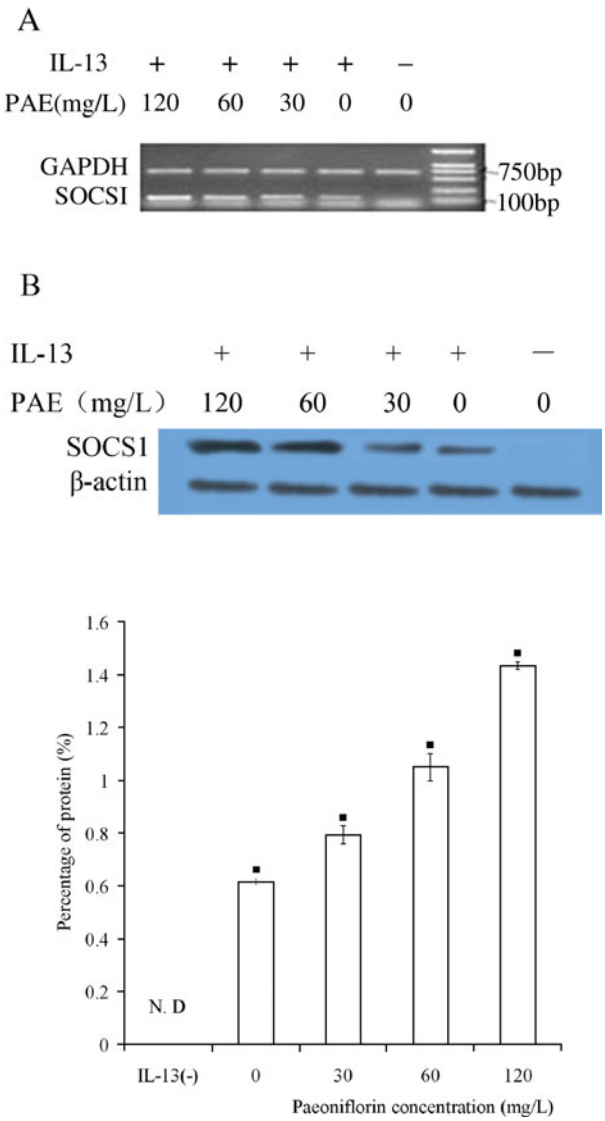

Fig. 6. Effect of PAE on rIL-13-induced gene and protein levels of SOCS-1 in HSCs. Primary HSCs ($1{\cdot}0\times10^5$) were seeded in 24-well plates in growth medium containing 15% FCS for 24 h, then serum-starved for an additional 24 h. Subsequently, HSCs were either left alone as a control or pre-incubated with PAE (0, 30, 60, 120 mg/l) for 24 h before being stimulated with rIL-13 (50 ng/ml) for 1 h when various concentrations of PAE (0, 30, 60 and 120 mg/l) were added to medium containing rIL-13. Levels of SOCS-1 mRNA and protein were markedly up-regulated by PAE in a concentration-dependent manner. (A) RT-PCR analysis of SOCS-1 gene expression in HSCs. (B) Western blotting of SOCS-1 protein expression in HSCs. ■ there was statistical significance among these groups ($P<0{\cdot}05$). N.D.: not detected. Results are the mean ± S.D. from 3 independent experiments. Each bar represents the mean and standard deviation of triplicate determinations and data presented are representative of 3 independent experiments.

expression of IL-4Rα/IL-13Rα1 was not affected by PAE, indicating that it is a constitutive expression and is not up-regulated. The IL-4Rα/IL-13Rα1 complex responded to IL-13 in HSCs and was probably linked to intracellular signalling pathways in our study. Additionally, we found that treatment of HSCs with IL-13 could induce STAT-6 phosphorylation, but had no effect on expression of STAT6. Pre-treatment with PAE decreased the amount of total p-STAT6 observed in response to IL-13, without altering the total level of STAT6 protein in the cells. The decoy receptor, IL-13Rα2, acts as a negative feedback inhibitor of IL-13 by reducing its interaction with the signalling type-2 IL-4 receptor. In the progression of schistosomiasis-induced liver fibrosis, IL-13Rα2 played a critical role in the inhibition of IL-13–mediated tissue fibrosis. Moreover, the decoy IL-13Rα2 can also regulate the magnitude of Th2-type cytokine production *in vivo* (Chiaramonte *et al*. 2003; Wynn *et al*. 2004). Our results showed that neither IL-13 nor PAE treatment induced the expression of the decoy receptor IL-13Rα2 and, thus, this receptor does not seem to contribute to the inhibitory effects of PAE. However, the reason why PAE can reduce the amount of p-STAT6 in the absence of the decoy receptor IL-13Rα2 remains unknown.

Proteins of the suppressors of cytokine signalling (SOCSs) family have important functions as negative regulators of cytokine signalling (Yoshimura and Kubo, 2007). Another study revealed that higher amounts of Col I and lower levels of SOCS-1 mRNA were produced by fibroblasts from lungs of patients with IPF compared to healthy lungs (Nakashima *et al*. 2008). Furthermore, the deficiency of SOCS-1 in murine fibroblasts resulted in increased collagen production, whereas over expression of SOCS-1 suppressed collagen production *in vitro* (Hiroyasu Shoda *et al*. 2007). Previous investigators (Hebenstreit *et al*. 2003) reported that SOCS-1 inhibits pulmonary inflammation and fibrosis, and SOCS-1 expression can be induced in human epithelial lung cell line A549 by IL-4 and IL-13. Other reports (Yoshida *et al*. 2004; Zhi-Xin Zhao *et al*. 2008) demonstrated that expression of SOCS-1, a suppressor of liver fibrosis, had obvious correlations to the hepatic fibrosis stage. The investigation of SOCS-1 molecule has given important insights into the regulatory mechanisms of cytokine signalling. These regulatory systems offer a fascinating explanation for our results, and the interaction between these cytokines and the SOCS family is warranted.

To date, however, no published studies have examined the down-regulation of SOCS-1 expression by IL-13 in HSCs. We here report for the first time, that IL-13 could induce expression of SOCS-1 both at mRNA and protein levels in HSCs. Meanwhile, we found that PAE pre-treatment induces higher levels of SOCS-1 expression in HSCs in a concentration-dependent manner, and the elevated expression of this protein coincides with decreased STAT6 phosphorylation. SOCS-1 can bind

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

to the Jak kinase domain and inhibit the kinase activity (Dickensheets *et al*. 1999; Yoshimura, 2007) and SOCS-1 promoter contains 3 functional STAT6 binding sites (Hebenstreit *et al*. 2003). It suggested that PAE's attenuating impact on the level of p-STAT6 protein might be partly through the elevation of SOCS-1 gene expression. However, the reason for PAE's elevation effect on the expression of SOCS-1 gene in the presence of IL-13 in HSCs remains to be clarified. This also suggested that SOCS-1 may be a potential therapeutic targeting molecule against fibrogenesis. Additionally, PAE obviously suppresses the proliferation of HSCs caused by IL-13 in our study. This is another reason for the decreased expression of Col I in HSCs.

We could conclude from our work that the level of p-STAT6 inversely coincides with SOCS-1 and that PAE inhibits IL-13-induced activation of STAT6 and Col I expression at least in part by increasing expression of SOCS-1. So we hypothesize that increased expression of SOCS-1 may attenuate STAT6 phosphorylation in our experimental model, which in turn leads to decreased proliferation and collagen production in response to IL-13 in HSCs. PAE has marked anti-fibrotic effects in the liver of mice with chronic *Schistosomiasis japonica*. Investigation of the potential of PAE therapy for management of various stages in the progress of fibrosis-associated chronic schistosomiasis is currently in progress.

ACKNOWLEDGEMENTS

The present work conforms to Chinese legal requirements and accepted international ethical standards, including those relating to animal conservation and welfare, and to the journal's policy on these matters. We thank Dr Deyong Chu at the Department of Parasitology, Anhui Medical University (Anhui, China) for his technical assistance.

FINANCIAL SUPPORT

The project was funded by the Natural Science Foundation of China (Nos 30571631 and 30872209), Doctoral Program of Higher Education of China (No. 200803660003), and Natural Science Foundation of Anhui (No. 090413089).

REFERENCES

Alves Oliveira, L. F., Moreno, E. C., Gazzinelli, G., Martins-Filho, O. A., Silveira, A. M., Gazzinelli, A., Malaquias, L. C., Loverde, P., Leite, P. M. and Correa-Oliveira, R. (2006). Cytokine production associated with periportal fibrosis during chronic schistosomiasis mansoni in humans. *Infection and Immunity* **74**, 1215–1221.

Bartley, P. B., Ramm, G. A., Jones, M. K., Ruddell, R. G., Li, Y. and Mcmanus, D. P. (2006). A contributory role for activated hepatic stellate cells in the dynamics of *Schistosoma japonicum* egg-induced fibrosis. *International Journal for Parasitology* **36**, 993–1001.

Chan, K., Liu, Z. Q., Jiang, Z. H., Zhou, H., Wong, Y. F., Xu, H. X. and Liu, L. (2006). The effects of sinomenine on intestinal absorption of paeoniflorin by the everted rat gut sac model. *Journal of Ethnopharmacology* **103**, 425–432.

Chang, D., Ramalho, L. N., Ramalho, F. S., Martinelli, A. L. and Zucoloto, S. (2006). Hepatic stellate cells in human schistosomiasis mansoni: a comparative immunohistochemical study with liver cirrhosis. *Acta Tropica* **97**, 318–323.

Chen, F., Cai, W., Chen, Z., Chen, X. and Liu, R. (2002). Dynamic changes in the collagen metabolism of liver fibrosis at the transcription level in rabbits with *Schistosomiasis japonica*. *Chinese Medicine Journal* (*English*) **115**, 1637–1640.

Chiaramonte, M. G., Cheever, A. W., Malley, J. D., Donaldson, D. D. and Wynn, T. A. (2001). Studies of murine schistosomiasis reveal interleukin-13 blockade as a treatment for established and progressive liver fibrosis. *Hepatology* **34**, 273–282.

Chiaramonte, M. G., Donaldson, D. D., Cheever, A. W. and Wynn, T. A. (1999*a*). An IL-13 inhibitor blocks the development of hepatic fibrosis during a T-helper type 2-dominated inflammatory response. *Journal of Clinical Investigation,* **104**, 777–785.

Chiaramonte, M. G., Mentink-Kane, M., Jacobson, B. A., Cheever, A. W., Whitters, M. J., Goad, M. E., Wong, A., Collins, M., Donaldson, D. D., Grusby, M. J. and Wynn, T. A. (2003). Regulation and function of the interleukin 13 receptor alpha 2 during a T helper cell type 2-dominant immune response. *Journal of Experimental Medicine* **197**, 687–701.

Chiaramonte, M. G., Schopf, L. R., Neben, T. Y., Cheever, A. W., Donaldson, D. D. and Wynn, T. A. (1999*b*). IL-13 is a key regulatory cytokine for Th2 cell-mediated pulmonary granuloma formation and IgE responses induced by *Schistosoma mansoni* eggs. *Journal of Immunology* **162**, 920–930.

Chu, D., Luo, Q., Li, C., Gao, Y., Yu, L., Wei, W., Wu, Q. and Shen, J. (2007). Paeoniflorin inhibits TGF-beta1-mediated collagen production by *Schistosoma japonicum* soluble egg antigen *in vitro*. *Parasitology* **134**, 1611–1621.

Chu, D. Y., Li, C. L., Yang, F., Wu, Q., Li, J., Ding, X. D., Luo, Q. L. and Shen, J. L. (2008). [Effect of paeoniflorin on hepatic immunopathogenesis in mice with *Schistosoma japonicum* infection]. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi* **26**, 10–15, 20.

Coutinho, H. M., Acosta, L. P., Wu, H. W., Mcgarvey, S. T., Su, L., Langdon, G. C., Jiz, M. A., Jarilla, B., Olveda, R. M., Friedman, J. F. and Kurtis, J. D. (2007). Th2 cytokines are associated with persistent hepatic fibrosis in human *Schistosoma japonicum* infection. *Journal of Infectious Diseases* **195**, 288–295.

De Jesus, A. R., Magalhaes, A., Miranda, D. G., Miranda, R. G., Araujo, M. I., De Jesus, A. A., Silva, A., Santana, L. B., Pearce, E. and Carvalho, E. M. (2004). Association of type 2 cytokines with hepatic fibrosis in human *Schistosoma mansoni* infection. *Infection and Immunity* **72**, 3391–3397.

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

Dickensheets, H. L., Venkataraman, C., Schindler, U. and Donnelly, R. P. (1999). Interferons inhibit activation of STAT6 by interleukin 4 in human monocytes by inducing SOCS-1 gene expression. *Proceedings of the National Academy of Sciences, USA* **96**, 10800–10805.

Ga., R. (1998). Isolation and culture of rat hepatic stellate cells. *Journal of Gastroenterology and Hepatology* **13**, 846–851.

Granel, B., Chevillard, C. and Dessein, A. (2007). [Interleukin 13 and interleukin 13 receptor involvement in systemic sclerosis]. *Revue de Medicne Interne* **28**, 613–622.

Greenwald, B. (2005). Schistosomiasis: implications for world travelers and healthcare providers. *Gastroenterology Nursing* **28**, 203–205; quiz 206–207.

Gressner, A. M. (1998). The cell biology of liver fibrogenesis – an imbalance of proliferation, growth arrest and apoptosis of myofibroblasts. *Cell and Tissue Research* **292**, 447–452.

Gui, S. Y., Wei, W., Wang, H., Wu, L., Sun, W. Y., Chen, W. B. and Wu, C. Y. (2006). Effects and mechanisms of crude astragalosides fraction on liver fibrosis in rats. *Journal of Ethnopharmacology* **103**, 154–159.

Hebenstreit, D., Luft, P., Schmiedlechner, A., Regl, G., Frischauf, A. M., Aberger, F., Duschl, A. and Horejs-Hoeck, J. (2003). IL-4 and IL-13 induce SOCS-1 gene expression in A549 cells by three functional STAT6-binding motifs located upstream of the transcription initiation site. *Journal of Immunology* **171**, 5901–5907.

Hershey, G. K. (2003). IL-13 receptors and signaling pathways: an evolving web. *Journal of Allergy and Clinical Immunology* **111**, 677–690; quiz 691.

Hiroyasu Shoda, A. Y., Ryouhei Nishino, Taku Nakashima, Nobuhisa Ishikawa, Y. H., Noboru Hattori and Tetsuji Naka, N. K. (2007). Overproduction of collagen and diminished SOCS1 expression are causally linked in fibroblasts from idiopathic pulmonary fibrosis. *Biochemical and Biophysical Research Communications* **353**, 1004–1010.

Hsu, Y. C., Chiu, Y. T., Cheng, C. C., Wu, C. F., Lin, Y. L. and Huang, Y. T. (2007). Antifibrotic effects of tetrandrine on hepatic stellate cells and rats with liver fibrosis. *Journal of Gastroenterology and Hepatology* **22**, 99–111.

Huang, H., Chang, E. J., Lee, Y., Kim, J. S., Kang, S. S. and Kim, H. H. (2008). A genome-wide microarray analysis reveals anti-inflammatory target genes of paeonol in macrophages. *Inflammation Research* **57**, 189–198.

Hung, J. Y., Yang, C. J., Tsai, Y. M., Huang, H. W. and Huang, M. S. (2008). Antiproliferative activity of paeoniflorin is through cell cycle arrest and the Fas/Fas ligand-mediated apoptotic pathway in human non-small cell lung cancer A549 cells. *Clinical and Experimental Pharmacology and Physiology* **35**, 141–147.

Jinnin, M., Ihn, H., Yamane, K. and Tamaki, K. (2004). Interleukin-13 stimulates the transcription of the human alpha2(I) collagen gene in human dermal fibroblasts. *Journal of Biological Chemistry* **279**, 41783–41791.

Kaplan, M. H., Whitfield, J. R., Boros, D. L. and Grusby, M. J. (1998). Th2 cells are required for the *Schistosoma mansoni* egg-induced granulomatous response. *Journal of Immunology* **160**, 1850–1856.

Kelly-Welch, A., Hanson, E. M. and Keegan, A. D. (2005). Interleukin-13 (IL-13) pathway. *Science's STKE: Signal Transduction Knowledge Environment* **293**, cm8.

Kimura, M., Kimura, I. and Nojima, H. (1985). Depolarizing neuromuscular blocking action induced by electropharmacological coupling in the combined effect of paeoniflorin and glycyrrhizin. *Japanese Journal of Pharmacology* **37**, 395–399.

Kimura, M., Kimura, I., Takahashi, K., Muroi, M., Yoshizaki, M., Kanaoka, M. and Kitagawa, I. (1984). Blocking effects of blended paeoniflorin or its related compounds with glycyrrhizin on neuromuscular junctions in frog and mouse. *Japanese Journal of Pharmacology* **36**, 275–282.

Lee, B., Shin, Y. W., Bae, E. A., Han, S. J., Kim, J. S., Kang, S. S. and Kim, D. H. (2008). Antiallergic effect of the root of *Paeonia lactiflora* and its constituents paeoniflorin and paeonol. *Archives of Pharmacology Research* **31**, 445–450.

Li, C., Luo, J., Li, L., Cheng, M., Huang, N., Liu, J. and Waalkes, M. P. (2003). The collagenolytic effects of the traditional Chinese medicine preparation, Han-Dan-Gan-Le, contribute to reversal of chemical-induced liver fibrosis in rats. *Life Science* **72**, 1563–1571.

Liu, D. F., Wei, W. and Song, L. H. (2006). Protective effect of paeoniflorin on immunological liver injury induced by bacillus Calmette-Guerin plus lipopolysaccharide: modulation of tumour necrosis factor-alpha and interleukin-6 MRNA. *Clinical and Experimental Pharmacology and Physiology* **33**, 332–339.

Magalhaes, A., Miranda, D. G., Miranda, R. G., Araujo, M. I., Jesus, A. A., Silva, A., Santana, L. B., Pearce, E., Carvalho, E. M. and Jesus, A. R. (2004). Cytokine profile associated with human chronic *Schistosomiasis mansoni*. *Memórias do Instituto Oswaldo Cruz* **99**, 21–26.

Mckenzie, G. J., Bancroft, A., Grencis, R. K. and Mckenzie, A. N. (1998). A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Current Biology* **8**, 339–342.

Mckenzie, A. N., Culpepper, J. A., De Waal Malefyt, R., Briere, F., Punnonen, J., Aversa, G., Sato, A., Dang, W., Cocks, B. G., Menon, S., De Vries, J. E., Banchereau, J. and Zurawski, G. (1993). Interleukin 13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proceedings of the National Academy of Sciences, USA* **90**, 3735–3739.

Mckenzie, A. N. and Fallon, P. G. (2003). Decoy receptors in the regulation of T helper cell type 2 responses. *Journal of Experimental Medicine* **197**, 675–679.

Minty, A., Chalon, P., Derocq, J. M., Dumont, X., Guillemot, J. C., Kaghad, M., Labit, C., Leplatois, P., Liauzun, P., Miloux, B., Minty, C., Casellas, P., Loison, G., Lupker, J., Shire, D., Ferrara, P. and Caput, D. (1993). Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature, London* **362**, 248–250.

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press

Nakashima, T., Yokoyama, A., Onari, Y., Shoda, H., Haruta, Y., Hattori, N., Naka, T. and Kohno, N. (2008). Suppressor of cytokine signaling 1 inhibits pulmonary inflammation and fibrosis. *Journal of Allergy and Clinical Immunology* **121**, 1269–1276.

Smith, P. F. (2008). Inflammation in Parkinson's disease: an update. *Current Opinion in Investigational Drugs* **9**, 478–484.

Sugimoto, R., Enjoji, M., Nakamuta, M., Ohta, S., Kohjima, M., Fukushima, M., Kuniyoshi, M., Arimura, E., Morizono, S., Kotoh, K. and Nawata, H. (2005). Effect of IL-4 and IL-13 on collagen production in cultured LI90 human hepatic stellate cells. *Liver International* **25**, 420–428.

Sun, W. Y., Wei, W., Wu, L., Gui, S. Y. and Wang, H. (2007). Effects and mechanisms of extract from *Paeonia lactiflora* and *Astragalus membranaceus* on liver fibrosis induced by carbon tetrachloride in rats. *Journal of Ethnopharmacology* **112**, 514–523.

Ta, W. (2008). Cellular and molecular mechanisms of fibrosis. *Journal of Pathology* **214**, 199–210.

Takagi, K. and Harada, M. (1969). [Pharmacological studies on herb paeony root. II. Anti-inflammatory effect, inhibitory effect on gastric juice secretion, preventive effect on stress ulcer, antidiuretic effect of paeoniflorin and combined effects with licorice component Fm 100]. *Yakugaku Zasshi: Journal of the Pharmaceutical Society of Japan* **89**, 887–892.

Takayama, G., Arima, K., Kanaji, T., Toda, S., Tanaka, H., Shoji, S., Mckenzie, A. N., Nagai, H., Hotokebuchi, T. and Izuhara, K. (2006). Periostin: a novel component of subepithelial fibrosis of bronchial asthma downstream of IL-4 and IL-13 signals. *Journal of Allergy and Clinical Immunology* **118**, 98–104.

Talaat, R. M., El-Bassiouny, A. I., Osman, A. M., Yossif, M., Charmy, R. and Al-Sherbiny, M. M. (2007). Cytokine secretion profile associated with periportal fibrosis in *S. mansoni*-infected Egyptian patients. *Parasitology Research* **101**, 289–299.

Tsukada, S., Parsons, C. J. and Rippe, R. A. (2006). Mechanisms of liver fibrosis. *Clinica Chimica Acta* **364**, 33–60.

Wang, H., Wei, W., Wang, N. P., Wu, C. Y., Yan, S. X., Yue, L., Zhang, L. L. and Xu, S. Y. (2005). Effects of total glucosides of peony on immunological hepatic fibrosis in rats. *World Journal of Gastroenterology* **11**, 2124–2129.

Wilson, M. S., Mentink-Kane, M. M., Pesce, J. T., Ramalingam, T. R., Thompson, R. and Wynn, T. A. (2007). Immunopathology of schistosomiasis. *Immunology and Cell Biology* **85**, 148–154.

Wong, W. S. and Leong, K. P. (2004). Tyrosine kinase inhibitors: a new approach for asthma. *Biochimica et Biophysica Acta* **1697**, 53–69.

Wu, H., Wei, W., Song, L., Zhang, L., Chen, Y. and Hu, X. (2007). Paeoniflorin induced immune tolerance of mesenteric lymph node lymphocytes via enhancing beta 2-adrenergic receptor desensitization in rats with adjuvant arthritis. *International Immunopharmacology* **7**, 662–673.

Wynn, T. A. (2003). IL-13 effector functions. *Annual Review of Immunology* **21**, 425–456.

Wynn, T. A., Hesse, M., Sandler, N. G., Kaviratne, M., Hoffmann, K. F., Chiaramonte, M. G., Reiman, R., Cheever, A. W., Sypek, J. P. and Mentink-Kane, M. M. (2004). P-selectin suppresses hepatic inflammation and fibrosis in mice by regulating interferon gamma and the IL-13 decoy receptor. *Hepatology* **39**, 676–687.

Yata, Y. E. S., Suzuki, S., Li, X. K., Tamura, A., Kimura, H., Takahara, T. and Watanabe, A. (1999). An improved method for the purification of stellate cells from rat liver with dichloromethylene diphosphate (CL2MDP). *Methods in Cell Science* **21**, 19–24.

Yoshida, T., Ogata, H., Kamio, M., Joo, A., Shiraishi, H., Tokunaga, Y., Sata, M., Nagai, H. and Yoshimura, A. (2004). SOCS1 is a suppressor of liver fibrosis and hepatitis-induced carcinogenesis. *Journal of Experimental Medicine* **199**, 1701–1707.

Yoshimura, A. N. T. and Kubo, M. (2007). SOCS proteins, cytokine signalling and immune regulation. *Nature Reviews Immunology* **7**, 454–465.

Zhang, L. L., Wei, W., Wang, N. P., Wang, Q. T., Chen, J. Y., Chen, Y., Wu, H. and Hu, X. Y. (2008*a*). Paeoniflorin suppresses inflammatory mediator production and regulates G protein-coupled signaling in fibroblast – like synoviocytes of collagen induced arthritic rats. *Inflammation Research* **57**, 388–395.

Zhang, X. J., Li, Z., Leung, W. M., Liu, L., Xu, H. X. and Bian, Z. X. (2008*b*). The analgesic effect of paeoniflorin on neonatal maternal separation-induced visceral hyperalgesia in rats. *Journal of Pain* **9**, 497–505.

Zhi-Xin Zhao, Q.-X. C., Xiao-Mou Peng, Yu-Tian Chong, Zhi-Liang Gao (2008). Expression of SOCS-1 in the liver tissues of chronic hepatitis B and its clinical significance. *World Journal of Gastroenterology* **14**, 607–611.

https://doi.org/10.1017/S003118201000003X Published online by Cambridge University Press