Y.-F. Shi, P.-Y. Cheung, C.-C. Fong, Y.O. Zhang, M. Yang

Effect of a porcine liver peptide extract on TGF-β induced activation of hepatic stellate cells

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Titolo
Effetto di un estratto peptidico di fegato di suino sull'attivazione indotta da TGF-β delle cellule stellate epatiche

Key words
Hepatic stellate cells, liver fibrosis, TGF-β1, decorin, fibrocorin

Parole chiave
Cellule epatiche stellate, fibrosi epatica, TGF-β1, decorina, fibrocorina

[1] Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon, Hong Kong SAR, P.R.C.
[2] Key Laboratory of Biochip Research, Shenzhen Research Institute of City University of Hong Kong, Shenzhen, China

Indirizzo per corrispondenza:
Prof. Yang Mengsu
Department of Biology and Chemistry,
City University of Hong Kong,
83 Tat Chee Avenue, Kowloon,
Hong Kong SAR, China
Tel. 852-27887797
Fax 852-27887406
E-mail: bhmyang@cityu.edu.hk

Summary
Liver fibrosis could lead to cirrhosis, liver failure, and even liver cancer. Activation of hepatic stellate cells (HSCs) is one of the major mechanisms of liver fibrogenesis. Inhibition of HSCs activation may provide promising therapies against liver fibrogenesis. Porcine liver peptide extracts were shown to have anti-fibrotic effect in clinical studies. The present study investigated the anti-fibrotic mechanism of a specifically prepared porcine liver peptide extract (PPE) through its effect on liver stellate cells LX-2. Transforming growth factor β1 (TGF-β1) was used to activate HSCs LX-2 cells. LX-2 cell proliferation was analyzed by 3-(4,5-dimethylthiazol 2-yl) 2,5-diphenyltetrazolium bromide (MTT) assay. Effect of PPE on the mRNA expression of matrix metallproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinase-1 (TIMP-1) genes, and protein levels of type I and type III collagens in the presence or absence of TGF-β1, were measured by reverse transcription polymerase chain reaction (RT-PCR) and western blotting analysis, respectively. In the presence of TGF-β1, LX-2 cells significantly proliferated, which could be reversed to basal level in the presence of PPE. Gene expression of MMP-2 and TIMP-1 and protein expression of type I and III collagen were increased in the presence of TGF-β1, but their expressions were reduced when the cells were co-treated with PPE. PPE alone did not affect the cell proliferation and the expression of the relevant genes and proteins. The results showed that PPE might have a beneficial effect reducing liver fibrosis through prevention of human HSCs activation and proliferation stimulated by TGF-β1.

Riassunto
La fibrosi epatica potrebbe portare a cirrosi, insufficienza epatica e persino al cancro al fegato. L'attivazione delle cellule stellate epatiche (HSC) è uno dei principali meccanismi di fibrogenesi del fegato. In studi clinici si è osservato che estratti peptidici di fegato di suino producevano un effetto anti-fibrotico. Questo studio ha esaminato il meccanismo anti-fibrotico di un estratto peptidico di fegato di suino (PPE) preparato in modo specifico attraverso i suoi effetti sulle cellule stellate epatiche LX-2. Il fattore di crescita trasformante β1 (TGF-β1) è stato utilizzato per

attivare le HSC LX-2. La proliferazione delle cellule LX-2 è stata analizzata con il test del 3-(4,5-dimethylthiazol 2-yl) 2,5-diphenyltetrazolium bromuro (MTT). Sono stati misurati mediante reazione a catena della polimerasi trascrittasi inversa (RT-PCR) e analisi Western blotting, rispettivamente, l'effetto del PPE sull'espressione dell'mRNA della metalloproteinasi-2 della matrice (MMP-2) e sui geni degli inibitori tissutali della metalloproteinasi-1 (TIMP-1), e i livelli di proteine collagene di tipo I e di tipo III in presenza o assenza di TGF-β1. In presenza di TGF-β1 le cellule LX-2 hanno proliferato in modo significativo; la proliferazione potrebbe essere riportata a livello basale in presenza di PPE. L'espressione genica di MMP-2 e TIMP-1 e l'espressione delle proteine collagene di tipo I e III erano aumentate in presenza di TGF-β1, ma le loro espressioni si sono ridotte quando le cellule sono state co-trattate con PPE. PPE da solo non ha influenzato la proliferazione cellulare e l'espressione dei geni e delle proteine in questione. I risultati hanno mostrato che PPE potrebbe avere un effetto benefico nel ridurre la fibrosi epatica attraverso la prevenzione dell'attivazione delle HSC umane e la proliferazione stimolata dal TGF-β1.

Introduction

Liver fibrosis results from chronic damage to the liver in conjunction with accumulation of extracellular matrix (ECM) proteins, a characteristic of most types of chronic liver diseases (1). A variety of stimuli may trigger fibrogenesis, including viruses, toxins, alcohol, autoimmune disease, chronic biliary stasis, metabolic disorder, genetic defects, and hypoxia. Liver fibrosis is a major cause leading to cirrhosis, and its complications of portal hypertension, liver necrosis and hepatocellular carcinoma (2-4). Thus, the development of effective and well-tolerated antifibrotic therapies attracts much attention. An ideal antifibrotic drug should be liver specific and selectively decrease excessive ECM deposition. Efforts of antifibrotic drug development have been focused on scarring generating fibrogenic cells (5).

With increasing understanding on the molecular mechanism of liver fibrogenesis, it is known that the final outcome of the fibrotic process reflects the imbalance between ECM synthesis and degradation (6). Hepatic stellate cells (HSCs), which are the major source of ECM deposition, were identified as the primary cell type to mediate fibrogenesis (7). The activation of HSCs is the key event during fibrogenesis. Specific inhibition of the activation of HSCs seems to be a promising target for antifibrotic therapy. LX-2 is a low-passaged human hepatic stellate cell line derived from normal human stellate cells that are spontaneously immortalized (8). LX-2 cells were selected by their ability to grow under the low serum conditions (1% fetal bovine serum). The cells exhibit the typi-

cal features of HSCs in primary culture, expressing desmin, glial acidic fibrillary protein, and response to platelet-derived growth factor BB and transforming growth factor-β (TGF-β).

TGF-β, the mediator of liver fibrosis, has a pivotal role in the induction of ECM protein synthesis (9). TGF-β participates in several steps in initiation and maintenance of fibrogenesis including activation of HSCs, enhancement of survival, stimulation of ECM production, and overexpression of tissue inhibitor of metalloproteinase-1 (TIMP-1) (10). Stimulation of HSCs by TGF-β is widely accepted as the model for studying fibrogenic response in liver fibrosis (11).

Some traditional medicines used in treating hepatitis in China were based on extracts from natural sources and shown to exhibit certain effects on hepatocyte proliferation. Porcine liver peptide extracts were shown to benefit the treatment of severe hepatitis clinically (12), and also to be effective for cirrhosis. In this study, HSCs LX-2 was used as a cell model to evaluate the effect of a porcine liver peptide extract (PPE) on hepatic fibrosis through LX-2 cell proliferation, TIMP-1 and metalloproteinase-2 (MMP-2) gene expression, and type I and III collagen protein expression study under TGF-β1 stimulation.

Materials and methods

Materials

Immortalized human hepatic stellate cell line LX-2 was a gift of Dr. Alex Y. Hui, Division of Gastroenterology and Hepatology, Department of Medicine and Therapeutics, Prince of Wales Hospital, Hong Kong. Dulbecco's modified Eagle's medium (DMEM), Trizol reagent, penicillin-streptomycin, fetal bovine serum, trypsin-EDTA were purchased from Invitrogen Corporation (Carlsbad, CA). Human recombinant TGF-β1 was purchased from Merck (Wehreim, Germany). Mouse monoclonal anti-human Type I and Type III collagen antibody were from Calbiochem (California, USA). Peroxidase-conjugated goat anti-mouse secondary antibody was from Santa Cruz Biotechnoloy Inc. (Santa Cruz, CA).

Preparation for PPE

Livers from neonate pig were homogenized and mixed at 500 rpm for 1 h at room temperature. Then, sample was kept at 95°C for 15 min with low-speed stirring. After filtration and cooling, the sample was precipitated with 95% ethanol. The treated DEAE-52 Sepharose beads were added to the ethanol-precipitated sample at a ratio of 1:2 (W/V) and stirred slowly for a total of 1 hour with 2 minutes stirring and 5 minutes pausing. After binding, the above Sepharose beads were added to a 15x150 cm chromatography column and settled by gravity. The packed column was first eluted with solution A until OD_{280} was less than 0.5. Then, solution A was replaced with solution B and the eluted samples were collected. Desalting was performed using ultra-filtration membrane. Finally, the samples were sterilized by filtration with a 0.2 μm filter twice. SDS-PAGE gel electrophoresis was used to analyse the major components of PPE. Major bands were cut off for sequence analysis.

LX-2 cells culture

Human HSCs LX-2 cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% penicillin and 1% streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Effects of PPE on TGF-β1 stimulated LX-2 cell proliferation

LX-2 cells were seeded in 96-well microplates (2000 cells/well) in DMEM containing 10% FBS,

2mM L-glutamine, 1% penicillin and 1% streptomycin for 24 h. Cells were then starved in DMEM supplemented with 0.5% charcoal-stripped FBS for 24 h followed by addition of 26 μg/ml PPE in the presence or absence of TGF-β1 (2 ng/ml) for another 48 h in DMEM containing 2% FBS. Cells with PBS addition were used as control for comparison. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The method was based on the fact that living cells are capable of reducing light color tetrazolium salts into an intense color formazan derivative. This reduction process requires functional mitochondria, which are inactivated within a few minutes after cell death. Cell proliferation was measured by MTT assay based on the absorbance change at 570 nm.

Gene expression analysis of TIMP-1 and MMP-2

LX-2 cells were seeded in 6-well plates ($2x10^5$ cells/well) in DMEM containing 10% FBS, 2 mM L-glutamine, 1% penicillin and 1% streptomycin for 24 h. Cells were then starved in DMEM supplemented with 0.5% FBS for 24 h and allowed to replicate to 80% confluence. 26 μg/ml PPE in the presence or absence of TGF-β1 (2 ng/ml) were added to the cell for another 24 h in DMEM containing 2% FBS.

Total RNA was isolated from cells by using Trizol reagent (GIBCOL, MD, USA) according to manufacturer's protocol. Total RNA (2 μg) was used to generate cDNA in each sample using SuperScript II reverse transcriptase with oligo(dT) 12-18 primers (Invitrogen, Carlsbad, CA). cDNA were then purified using purification kit (Qiagen, USA).

Evaluation of TIMP-1 and MMP-2 expression was performed by semi-quantitative RT-PCR. Gene expression level of TIMP-1 and MMP-2 was normalized by a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GADPH). All primers were designed using the software Primer Express 1.0 (Applied Biosystems CA, USA). Primers used were 5'- TGT TGT TGC TGT GGC TGA TAG C-3' (TIMP-1 forward), 5'- TGT GCA TTC CTC ACA GCC AA-3' (TIMP-1 reverse), 5'-TTT CCA TTC CGC TTC CAG G-3' (MMP-2 forward), 5'-GCT CTC ATA TTT GTT GCC CAG G-3' (MMP-2 reverse), 5'-ACC ACA GTC CAT GCC TAC AC-3' (GADPH forward) and 5'-TTC ACC ACC CTG TTG CTG TA-3' (GADPH reverse). Semi-quantitative RT-PCR amplification with *Taq* polymerase was carried out with a denaturing step at 94°C for 45 s, an annealing step at 58°C for 45 s, and an extension step at 72°C for 60 s. The number of PCR cycles for MMP-2 and TIMP-1 gene was optimized to 35 cycles and 40 cycles, respectively. Samples were analyzed on 1.8% agarose gel and stained with ethidium bromide.

Western blotting analysis of type I and III collagen protein expression

Cells were grown as described in the above sections. Briefly, $2x10^5$ LX-2 cells were seeded in 6-well cell culture plate for 24 h. The culture medium was replaced with DMEM supplemented with 0.5% FBS and cells were starved for 24 h. After starvation, the medium was refreshed with DMEM containing 2% FBS. 26 μg/ml PPE in the presence or absence of TGF-β1 (2 ng/ml) were added to the cell for 48 h in DMEM containing 2% FBS before harvest.

The culture medium was collected for analyzing the protein expression of type I and III collagen. Equal amount of each sample was separated by SDS-PAGE on 7% reducing gels at constant voltage and transblotted onto PVDF membranes (Immobilin-P, Millipore Corp., MA, U.S.A.). Immuno-detection was performed after blocking non-specific binding sites on the membrane with 5%-skimmed milk. The blots were

probed with monoclonal mouse anti-human Type I (5 μg/ml) or Type III collagen (1 μg/ml) as the primary antibody (Calbiochem, California, USA) which was followed by incubation with goat anti-mouse antibody conjugated with horseradish peroxidase (1:2000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as the secondary antibody. The antigen-antibody complexes were then detected with enhanced chemiluminescence (ECL) reagent and visualized by Lumi-Imager using Lumi Analyst version 3.10 software (Roche, Mannheim, Germany). Results are expressed as intensity fold change.

Statistical analysis

Data are reported as the mean ± SEM. Significance of difference between group means was determined by One-way analysis of variance (ANOVA). The independent student's *t* test was used to calculate statistical significance between the control group and each treatment group in MTT assay. *p*-value < 0.05 was considered statistically significant.

Results

Studies showed that porcine liver peptide extracts could act as a therapeutic agent in the treatment of hepatitis and cirrhosis (12). In this study, we have prepared a peptide extract from liver of neonate porcine based on the purification protocol described in the Materials and methods section. Preliminary analysis of the major components of PPE was carried out. Five major bands shown by SDS-PAGE were detected (Fig. 1) and cut off for N-terminal sequence analysis (Tab. 1). It was hypothesized that decorin may play an important role in the effect of PPE on fibrosis, although the functions of other peptide components could not be ruled out. Human HSCs LX-2 activated by TGF-β1 was used as cell model to investigate the effect of PPE on liver fibrosis. LX-2

Figure 1 - Preliminary analysis of PPE major components by SDS-PAGE

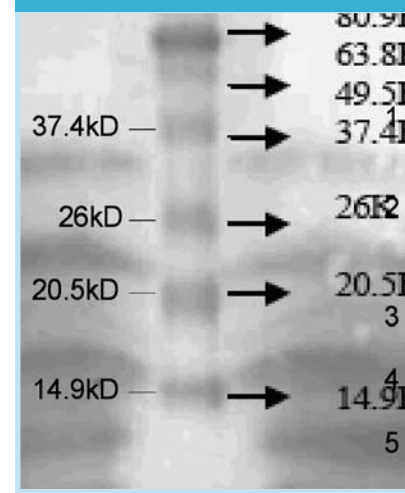


Table 1 - N-terminal sequences of major peptides in PPE

Sample	MW(kDa)	Amino Acid Sequence (1)	Amino Acid Sequence (2)	Protein Identification
1	39.180	Block	SGVDI	Calponin 2
2	24.923	DEAAGIGP	DEAAGIAP	Decorin
3	20.650	KAPAKK	SAPAKK	Non-histone chromosomal Protein HMG-17
4	14.175	PEPAKKAPAA		Hepatoma-derived growth factor-related protein (HRP-1)
5	10.199	AKKRKKK	AVKRKKK	Ubiquitin

cells were induced by TGF-β1 followed by the treatment of PPE. The effect of PPE on fibrogenesis was evaluated through cell viability, the expression of gene and protein in LX-2 cells. LX-2 cells without TGF-β1 activation and treatment of PPE were used as controls for comparison.

Cell proliferation assay

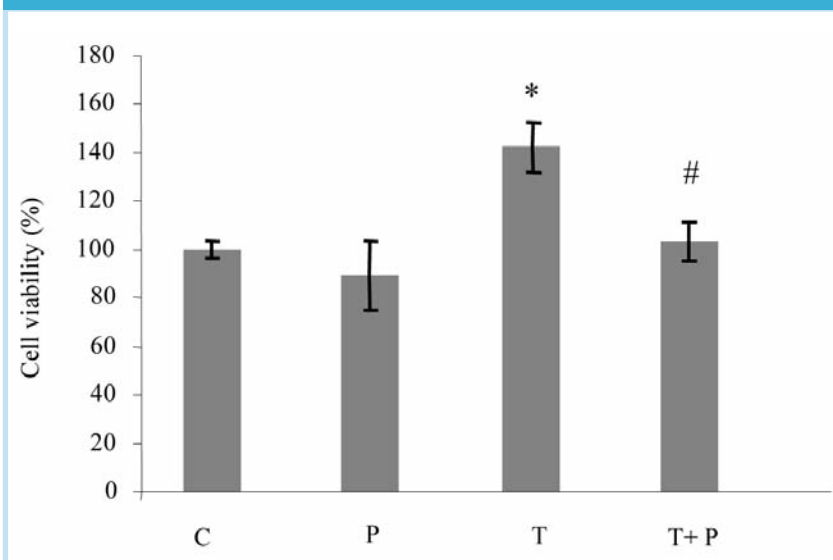
The effect of PPE on the proliferation of human HSCs LX-2 cells was examined. Cell viability was evaluated by MTT assay. The result was shown in Fig. 2. In the presence of 2 ng/ml TGF-β1, the proliferation of LX-2 cells was significantly increased. However, such proliferation could be reduced to basal level by simultaneous addition of 26 μg/ml PPE, indicating PPE significantly inhibited activated HSCs proliferation. PPE alone, however, had no effect on LX-2 proliferation.

Expression of TIMP-1 and MMP-2 in LX-2 cells activated by TGF-β1

MMP-2 and TIMP-1 are the important genes that participate in ECM synthesis and degradation pathways. MMP-2 is well known to regulate cell-matrix composition by degrading components of the ECM whereas TIMP-1 is a specific inhibitor of matrix metalloproteinases which is the key regulator of MMP activity and ECM degradation. To further demonstrate the effect of PPE on liver fibrosis, mRNA expression of MMP-2 and TIMP-1 in LX-2 cells under the treatment of TGF-β1 was investigated by semi-quantitative RT-PCR (Fig. 3).

$2x10^5$ LX-2 cells were seeded in 6-well cell culture plate and exposed to 26 μg/ml PPE, either alone, or in the presence of 2 ng/ml TGF-β1 for 24 h. Gene expression of MMP-2 and TIMP-1 was up-regulated in the presence of TGF-β1. However, the mRNA expression levels of both genes in the activated HSCs were attenuated by the PPE. PPE alone had no effect on the expression of these genes.

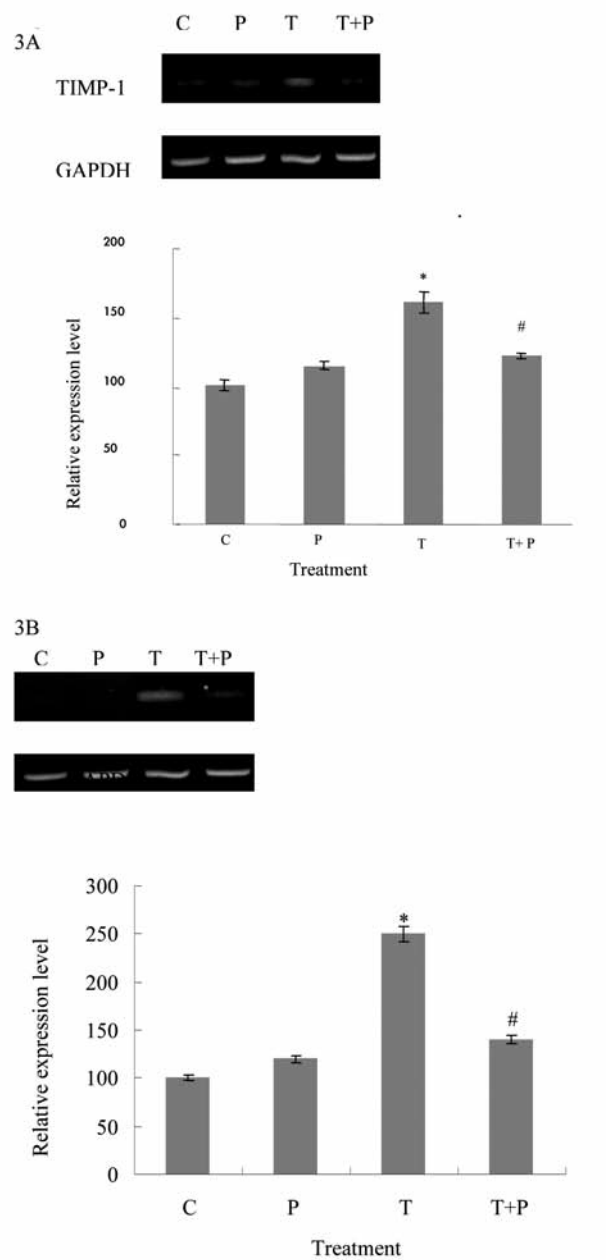
Figure 2 - Effect of PPE on LX-2 proliferation. LX-2 cells were treated with PPE (P, 26 μg/ml) either alone, or in the presence of 2 ng/ml TGF-β1 (T) for 48 h, followed by MTT assay as described in materials and methods section. Sample with PBS addition was used as control (C). Results are expressed as mean ± SD. * $p<0.05$ vs. C, # $p<0.05$ vs. T, n = 3



Expression of type I and III collagen in LX-2 activated by TGF-β1

Deposition of excess ECM is a pathologic process during fibrogenesis. Activated HSCs secrete large amount of ECM including collagen type I and type III. To investigate its activity, PPE was used to treat LX-2 cells with or without the stimulation of TGF-β1. Expression of type I and III collagen in the cultured medium was analyzed by western blotting. As shown

Figure 3 - Effect of PPE treatment on TIMP-1 and MMP-2 gene expression. LX-2 cells were treated with PPE (P, 26 μg/ml) either alone, or in the presence of 2 ng/ml TGF-β1 (T) for 24 h. Sample with PBS addition was used as control (C). Cells were harvested for total RNA extraction. RT-PCR was carried out to detect the effect of PPE on TIMP-1 and MMP-2 mRNA expression using GADPH as control for normalization. The mRNA expression level was expressed as a ratio to the expression of GAPDH. 3A. Gene expression of TIMP-1. 3B. Gene expression of MMP-2. Graphic results shown are representative of four independent experiments and expressed as mean ± SD. * p<0.05 vs. C, # p< 0.05 vs. T, n = 4



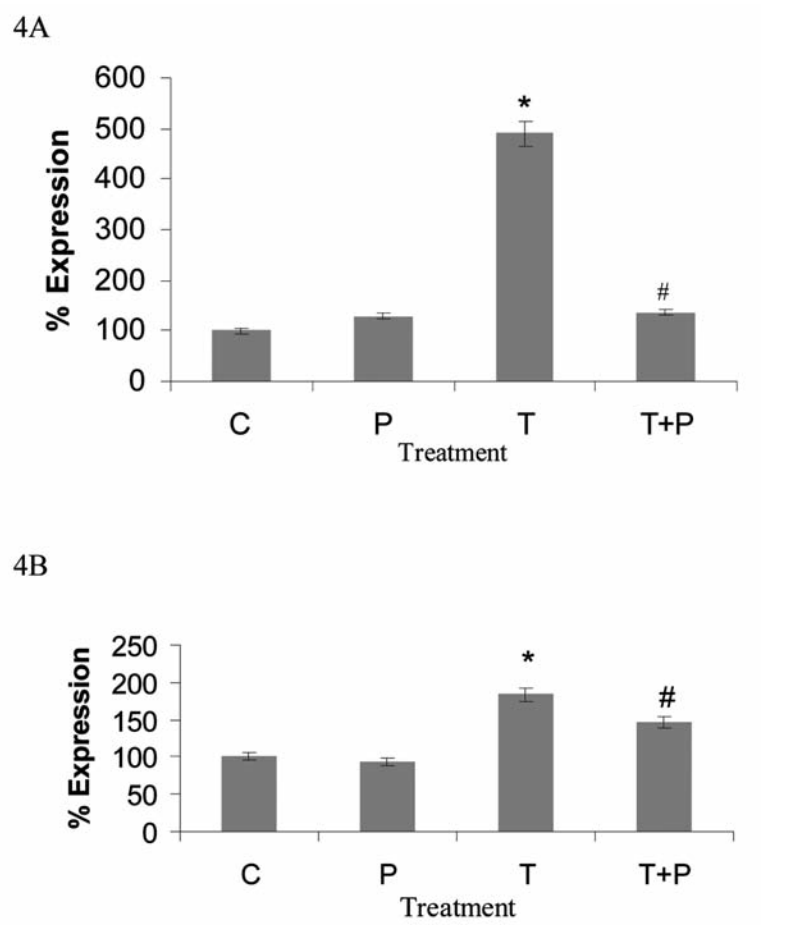
in Fig. 4, TGF-β1 stimulation can significantly increase the expression of type I and III collagen in comparison with the control. With the addition of PPE, the increase in protein expression level was reduced. PPE alone had no the effect on collagen protein expression in LX-2 cells.

Discussion

Accumulation of ECM proteins is a significant phenomenon during fibrosis that distorts hepatic architecture by forming fibrotic scar, and subsequent development of nodules of regenerating hepatocytes leads to cirrhosis (1). Liver fibrosis is reversible, whereas cirrhosis, the end-stage consequence of fibrosis, is generally irreversible. Thus, efforts have been focused on the understanding of the early events of fibrogenesis in the hope of identifying therapeutic targets to slow its progression (13). HSCs as the major ECM-producing source in liver is regarded as a promising target for antifibrosis drug development (14, 15).

PPE has the beneficial effect for the clinical treatment of severe hepatitis, and was also found to be effective for cirrhosis. However, its action mechanism is still unclear. Since cirrhosis results from advanced liver fibrosis, we hypothesized that PPE, which contains decorin

Figure 4 - Effect of PPE treatment on collagen protein expression. LX-2 cells were treated with PPE (P, 26 μg/ml) either alone, or in the presence of 2 ng/ml TGF-β1 (T) for 24 h. Sample with PBS addition was used as control (C). Cells were harvested for type I and type III collagen protein expression analysis. 4A. Protein expression of type I collagen. 4B. Protein expression of type III collagen. Graphic results shown are representative of three independent experiments and expressed as mean ± SD. * $p<0.05$ vs. C, # $p<0.05$ vs. T, n = 3



peptide, might have the therapeutic effect on liver fibrosis. Human HSCs LX-2 activated by TGF-β1 was used to evaluate the effect of PPE in this study.

In normal liver, HSCs constitute a small percentage (about 5% to 10%) of total resident liver cells and locate in the subendothelial space between hepatocytes and sinusoidal endothelial cells (5, 16). Several functional roles were assigned to HSCs in normal liver, such as metabolism and storage of retinoids, synthesis of hepatocyte growth factor and pericyte-like role in regulation of the sinusoidal blood flow (17, 18). Quiescent HSCs have a low proliferation rate, low fibrogenic activity, and no contractile property (16). However, these cells are progressively activated from the quiescent state and undergo phenotypic changes, lose retinoid, and synthesize large amount of ECM components including collagen, proteoglycan, and adhesive glycoproteins, especially type I and III collagen after liver injury (19). Activated HSCs also participate in the modulation of ECM degradation through synthesizing and secreting TIMPs to inhibit the activity of MMPs, which is responsible for the degradation of ECM. Specific inhibition of the activation of HSCs seems to be a promising target for antifibrotic therapy.

To understand the action of PPE in fibrogenesis, we have examined the effect of PPE on activation of LX-2 cells, a human hepatic stellate cells line, stimulated by TGF-β1. HSCs activation is an essential process of liver fibrogenesis. The proliferation of LX-2 cells stimulated by TGF-β1 was significantly reduced by PPE. However, PPE alone had no effect on normal LX-2 cells, indicating that PPE prevented liver fibrosis by inhibiting the proliferation of HSCs through the regulation of TGF-β1 related pathway.

Hepatic stellate cells express a wide range of MMPs as well as MMPs activator that cleave pro-MMP into their active form. In addition, they also produce specific TIMPs. Production of MMPs and TIMPs is tightly regulated ac-

cording to the activation state of HSCs, and reflects ECM remodelling during chronic liver injury (1). As shown in Fig. 2, TGF-β1 activated HSCs and increased the expression of MMP-2 and TIMP-1 genes. Their expressions were reduced when the cells were co-treated with PPE. PPE also directly participates in the alteration of ECM component and quantity, which is associated with liver fibrosis (20). Collagen type I and III are major composition of ECM during hepatic fibrosis and their percentage is gradually increased with fibrogenesis. Their expression was steadily reduced by the co-treatment of PPE. These results demonstrated that PPE exerted anti-fibrosis effect through regulation of enzymes responsible for ECM remodeling.

Since PPE is a peptide mixture from neonate porcine liver, it is necessary to identify all the major proteins in order to discover the active component. Preliminary results showed that PPE was composed of five major peptides that might include proteins related to improvement of liver functions and degradation of ECM, such as decorin and HRP-1. Further study is needed for identification and characterization of PPE's components in order to develop a more effective therapeutic agent for liver fibrosis through inhibiting HSCs activation and proliferation

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