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ORIGINAL ARTICLE

A Targeted Multiple Antigenic Peptide Vaccine Augments the Immune Response to Self TGF-β1 and Suppresses Ongoing Hepatic Fibrosis

Yaping Li · Wenjun Wang · Xiaoli Jia · Song Zhai · Xiufang Wang · Yuan Wang · Shuangsuo Dang

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Abstract Transforming growth factor (TGF)-β1 expression is induced upon liver injury, and plays a critical role in hepatic fibrosis. Antibodies against TGF-β1 represent a novel approach in the treatment of hepatic fibrosis. However, TGF-β1 is not a suitable antigen due to immunological tolerance. In the current study, we synthesized a multiple antigenic peptide (MAP) vaccine against the dominant B-cell epitope of TGF-β1. The immunogenicity and potential therapeutic effects of this vaccine were examined using a rat model of hepatic fibrosis. Dominant B-cell epitopes of TGF-β1 were identified using bioinformatic program. An MAP vaccine corresponding to the 90–98 amino acid domain of TGF-β1 and containing four dendritic arms was synthesized using a 9-fluorenylmethoxycarbonyl solid phase method. Hepatic fibrosis which was induced in male Sprague-Dawley rats received a high-fat diet and ethanol (1.8 g/kg). Starting from the third week, rats were exposed to 40 % carbon tetrachloride (CCl_4; 150 μl/100 g body weight twice weekly, initially 200 μl/100 g) treatment for a duration of 8 weeks. Rats received the MAP vaccine (100 μg) or Freund's adjuvant at weeks 1, 3, 5. A group of rats receiving the fibrosis-inducing regimen alone and a group of healthy rats (receiving an olive oil vehicle alone) were included as controls. At the conclusion of the experiment, serum titre of TGF-β1 antibody was measured using ELISA and a standard liver functional test panel was examined. The extent of hepatic fibrosis was determined by measuring hydroxyproline content in the liver as well as hematoxylin–eosin (HE) and van Gieson (VG) staining. The expression of TGF-β1 and alpha-smooth muscle actin (α-SMA) was examined using immunohistochemistry, and presented as positive staining cells. The MAP purity was >90 % upon reverse phase high-performance liquid chromatography, with apparent molecular weight at 4.77 kDa. Serum TGF-β1 antibody titre was 1:256. The fibrosis-inducing treatment produced significant liver damage, as reflected by increases in liver functional test, HE and VG staining. The MAP vaccine attenuated such damage, as reflected by decreased alanine aminotransferase, aspartate aminotransferase, total bilirubin, and hepatic hydroxyproline (116.78 ± 23.76 vs. 282.71 ± 136.94 IU/L; 319.78 ± 82.48 vs. 495.29 ± 137.13 IU/L; 2.02 ± 0.27 vs. 4.01 ± 0.52 μmol/L; 263.67 ± 41.18 vs. 439.14 ± 43.29 μg/g vs. in model rats, respectively; $p < 0.01$), as well as fibrosis extent by HE and VG staining. The MAP vaccine reduced TGF-β1 and α-SMA expression in rats (0.325 ± 0.059 vs. 0.507 ± 0.044 IOD/area; 0.318 ± 0.058 vs. 0.489 ± 0.029 IOD/area vs. model rats, respectively; $p < 0.05$). The TGF-β1 MAP vaccine could generate sufficient antibody that suppresses the development of hepatic fibrosis.

Keywords Hepatic fibrosis · Transforming growth factor-β1 · Multiple antigenic peptides · Vaccine

Introduction

Hepatic fibrosis results from chronic damage to the liver in conjunction with the excessive accumulation of extracellular matrix (ECM) proteins including collagen, which is a characteristic of most types of chronic liver disease

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Y. Li · W. Wang · X. Jia · S. Zhai · X. Wang · Y. Wang · S. Dang (✉)
Department of Infectious Diseases, Second Affiliated Hospital of Medical School, Xi'an Jiaotong University, Xi'an, China
e-mail: dang212@126.com

(Bataller and Brenner 2005). The main causes of liver fibrosis include chronic hepatitis B and C virus infection, alcohol abuse, toxic/metabolic damage, autoimmune reactions, and non-alcoholic steatohepatitis (Pinzani and Rombouts 2004). Hepatic fibrosis is a pathological response to repeated damage and may progress to cirrhosis, portal hypertension and even liver failure. Prolonged liver injury results in hepatocyte damage, which triggers activation and proliferation of hepatic stellate cells (HSCs) and recruitment of massive inflammatory cells into the liver (Kisseleva and Brenner 2006). Activated HSC, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-producing cells in the acute and chronic injured liver tissue (Bataller and Brenner 2005). Activation and transdifferentiation of HSC to myofibroblasts is a key event in fibrogenesis (Gressner et al. 2008). HSC is activated by fibrogenic mediators platelet-derived growth factor, fibroblast growth factor, epidermal growth factor (EGF), connective tissue growth factor, insulin-like growth factor 1, vascular endothelial cell growth factor (Wynn 2008; Wynn and Barron 2010), and in particular, transforming growth factor (TGF)-β1 (Bataller and Brenner 2005; Gressner et al. 2002, 2008; Liu et al. 2006). The TGF-β/Smad signal pathway modulates transcription of important target genes including procollagen I and III, and by doing so, participates in the development of hepatic fibrosis (Wynn 2008).

Modulating TGF-β1 production and/or signaling pathways could attenuate hepatic fibrosis both in vivo and in vitro. Blockade of TGF-β1 activity in vivo in animal models inhibits fibrotic effect (Shek and Benyon 2004). Suppression of the TGF-β1 activity could be achieved by TGF-β1 antisense and ribozyme oligonucleotides that block TGF-β1 mRNA (Song et al. 2005; Wolff et al. 2005), neutralizing monoclonal antibody (metelimumab) (Bonafoux and Lee 2009; Pohlers et al. 2009), active immunization (Guo et al. 2006), soluble TGF-β type receptor (Ueno et al. 2000; Yata et al. 2002), and small interfering RNA for gene silencing of the TGF-β1/Smads pathway (Cheng et al. 2009; Xu et al. 2009).

Active immunization with vaccines could induce long-lasting and polyclonal antibodies and is more economical (Kopf et al. 2010). For example, interleukin (IL)-12/IL-23 p40 (Guan et al. 2009), IL-1 (Spohn et al. 2008), tumor necrosis factor-α (Chackerian et al. 2001; Delavallee et al. 2008), IL-17 (Sonderegger et al. 2006) and EGF (Neninger Vinageras et al. 2008) vaccines have shown promising results in the protection against chronic colitis, autoimmune arthritis, autoimmune myocarditis, and non-small-cell lung cancer.

Flanders et al. (1988) synthesized the 78–109 amino acid domain of TGF-β1 protein, and conjugated it with methylated bovine serum albumin (BSA) and achieved high titer of specific antibodies in rabbits. Purified TGF-β1 protein does not induce high specific antibody titers in vivo unless the dominant epitopes of TGF-β1 are modified. Synthetic peptides could induce antibodies reactive with their cognate sequences in the native proteins (Li et al. 2011; Tam 1988; Wang et al. 2010). The immunogenicity of synthetic peptides vaccines could be enhanced by multiple antigenic peptides (MAP) system (Tam 1996), conjugation to carrier proteins through the N terminus by glutaraldehyde to BSA or keyhole limpet hemocyanin (Cassidy Brady et al. 2012), and combination with immunoadjuvant (Leroux-Roels 2010). In this study, we synthesized an MAP vaccine against the TGF-β1 dominant antigens. The immunogenicity and potential therapeutic effects of this vaccine were examined using a rat model of hepatic fibrosis.

Materials and Methods

Experimental Animals and Laboratory Reagents

Male Sprague-Dawley rats (6–8 weeks of age, specific pathogen-free grade) were purchased from Central Animal Care Services, School of Medicine, Xi'an Jiaotong University. All protocols were approved by the University Animal Ethics Committee. Chemical reagents were obtained from Xi'an Chemical Re-agent Factory (Xi'an, China).

Antigenic Peptide

TGF-β1 amino acid sequences and structure were acquired and analyzed by the National Center for Biotechnology Information, based on information from the Protein Data Bank. The mature form of TGF-β1 (NP-000651), with a molecular weight of 12.5 kDa, consists of a 112 amino acid polypeptide (Tandon et al. 2010). B-cell epitope prediction of the TGF-β1 amino acid was made based on the characteristics of hydrophilicity, flexibility, mobility, accessibility, polarity and exposed surface, using a B-cell antigen epitope prediction software (Sun et al. 2009) (http://lifecenter.sgst.cn/seppa/index.php) (Fig. 1). Amino acids 90–98 were selected by bioinformatics approaches and relevant literatures (Grütter et al. 2008).

Synthesis and Characterization of MAP

To overcome the limitations of small linear peptides and improve immune response, we used MAP technology to produce high immunogenicity. The MAP approach uses a small peptidyl core matrix bearing radially branching synthetic peptides as dendrites arms (Tam 1988). The TGF-β1 MAP vaccine in the current study consists of a

1-50	ALDTNYCFSS TEKNccvRQL YIDFRKDLGW KWIHEPKGYH ANFCLGPCPY
51-100	IWSLDTQYsK VLaLYNQHNP GASAAPCCVP QALEPLPiVY YVGRKPKVEQ
101-150	LSNMIVRSCK cS

Fig. 1 The predicted epitope residues of TGF-β1 protein using B-cell antigen epitope prediction software (http://lifecenter.sgst.cn/seppa/index.php). Predicted epitope residues are marked. Core residues are presented in lower case

lysine-containing core and four dendritic arms (Fig. 2), and was synthesized using a 9-fluorenylmethoxycarbonyl solid phase method (Paramasivam Saravanan and Kumar 2009). Branching of the lysine residue was on Wang-resin. Following cleavage of MAP from Wang-resin, the crude peptide was desalted and purified by reverse phase high-performance liquid chromatography (Agilent TC-C18 reversed-phase column on Rili L300, Japan; Suppl. Fig. 1). The product was eluted, lyophilized, and verified with MALDI-TOF mass spectrographic analysis (Suppl. Fig. 2).

Induction of Hepatic Fibrosis in Rats and Vaccine Immunization

Forty male Sprague-Dawley rats were randomly assigned to four experimental groups as follows: the vaccine group was immunized with TGF-β1 MAP vaccine (100 μg in 250 μl sterile water and 250 μl of Freund's adjuvant (Sigma, St Louis, Mo., USA) at weeks 1, 3, 5, subcutaneously; 100 μg in 500 μl sterile water at weeks 6–10; $n = 10$); the adjuvant group received an injection of Freund's adjuvant at week 1, 3, and 5, and was given water and a standard chow diet ad libitum (vehicle for vaccine; $n = 10$); normal control received an injection of olive oil only, and was given water and a standard chow diet ad libitum ($n = 10$); model group was treated with carbon tetrachloride (CCl_4) for 8 weeks ($n = 10$). Staring from the third week, rats in the model and vaccine groups received subcutaneous injection with CCl_4 dissolved in 40 % v/v olive oil at 150 μl/100 g body weight (200 μl/100 g for the first time) twice weekly, fed with high-fat diet (79.5 % maizena, 20 % lard and 0.5 % cholesterol), and given ethanol (1.8 g/kg once every two days, orally) for a duration of 8 weeks. A flow diagram of the rat experiment is shown in Fig. 3.

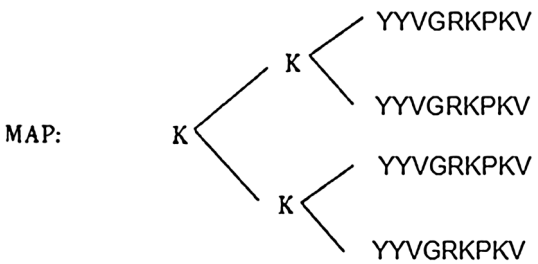

Fig. 2 Schematic diagram illustrating the structure of TGF-β1 MAP (multiple antigenic peptide)

The general condition of the rats including diet, weight, and hair was observed every day during the 10 weeks. Forty-eight hours after the last CCl_4 injection, the animals were anesthetized by 10 % chloral hydrate and blood samples were collected by peri-orbital bleeding. All animals were sacrificed by cervical dislocation and tissue specimens from spleen, liver and kidney were collected and weighed with an analytical balance. Liver, spleen and kidney coefficient was calculated and expressed as grams of organs per 100 gram of rats. Blood samples and some hepatic tissues were collected and stored at −70 °C, and some hepatic tissues were collected and fixed in 10 % (w/v) buffered formalin immediately.

Measurement of TGF-β1 Antibody by ELISA

Serum TGF-β1 antibody was measured using ELISA as previous described (Paramasivam Saravanan and Kumar 2009). 96-well plates were coated with TGF-β1 MAP (10 μg/ml) in coating buffer (Carbonate–bicarbonate buffer solution, pH 9.6), and incubated overnight at 4 °C. Wells were washed three times with phosphate buffer solution (PBS) to remove unbound antigens, and blocked with 10 % BSA in PBS for 2 h. Wells were washed five times with PBS-T (PBS + 0.05 % Tween) to remove excess blocking buffer. Sera from vaccinated rats were added to each well in twofold serial dilutions made using 5 % BSA in PBS-T and incubated for 3 h at 37 °C. Wells were washed five times with PBS-T. Goat anti-rat IgG horseradish peroxidase conjugate as secondary antibody was added and incubated for 30 min at 37 °C. After washing with PBS-T, the bound conjugate was reacted with ortho-phenylene diamine with hydrogen peroxide (Zhongshan Jinqiao Biotechnology, Beijing, China) and incubated for 10 min at 37 °C in dark. Optical densities (OD) were measured at 450 nm using ELISA reader. Increase in OD at >twofold over the negative control (pre-immune serum) was considered as positive (Paramasivam Saravanan and Kumar 2009).

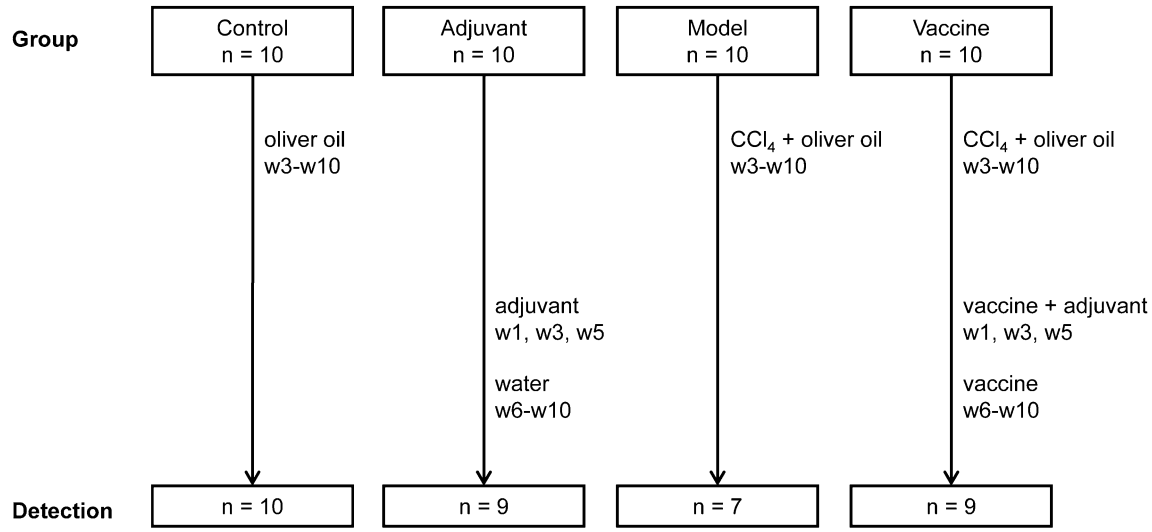

Fig. 3 Flow diagram illustrating the design of the experiment

Liver Function Examination and Measurement of Hydroxyproline in Hepatic Tissues

Serum alanine transferase (ALT), aspartate transferase (AST), total bilirubin (TBIL), albumin and globular protein were examined using a 7170-automatic biochemistry analyzer (Tokyo, Japan). Hepatic collagen content was evaluated by hydroxyproline quantification according to methods described by Edwards and O'Brien (Edwards and O'Brien 1980) with some modifications and the manufacturer's protocols by Nanjing Jiancheng Bioscience (Nanjing, China). The hepatic hydroxyproline content is expressed as micrograms of hydroxyproline per gram of hepatic tissue.

Histological Staining

The hematoxylin–eosin (HE) kit was purchased from Zhongshan Jinqiao Biotechnology. The van Gieson's (VG) staining kit was purchased from Xiamen Maiwei Biotechnology (Xiamen, China). Paraffin-embedded hepatic sections (4 μm) were deparaffinized, hydrated, and stained with HE and VG stains. The extent of hepatic fibrosis was assessed semi-quantitatively as described earlier (Zhang et al. 2007) using the following scale: 0—normal liver and no fibrosis; 1—thickened perivenular collagen and thin collagen septa; 2—thin septa with incomplete bridging between portal regions; 3—thin septa and extensive bridging; and 4—thickened septa with complete bridging of portal regions and complete pseudolobuli formation. Representative regions were photographed under bright field optics using an OLYMPUS BX51 light microscope.

Measurement of TGF-β1 and Alpha-Smooth Muscle Actin

Liver sections were quenched with 0.3 % hydrogen peroxide for 15 min at room temperature, subjected to standard antigen retrieval in 0.01 mmol/L citrate buffer, and blocked with 10 % normal goat serum for 15 min at 37 °C prior to incubation with primary antibody (Zhongshan Jinqiao Biotechnology) in concentrations of 1:50 (TGF-β1) or 1:100 (alpha-smooth muscle actin: α-SMA) for 2 h at 37 °C, respectively. After rinsing with PBS washing buffer, sections were incubated with biotinylated secondary antibody (goat anti-rabbit IgG, Zhongshan Jinqiao Biotechnology) for 15 min at 37 °C. After further washing with PBS, sections were incubated with complex/horseradish peroxidase (Zhongshan Jinqiao Biotechnology) for 15 min at 37 °C, and finally with diaminobenzidine (Zhongshan Jinqiao Biotechnology) for 5–10 min. Slides were counterstained with hematoxylin before dehydration and mounting. Incubation with PBS instead of the primary antibody was performed as a control for the background staining.

The intensity of positive staining of TGF-β1 and α-SMA and the changes of morphology in liver tissue sections were measured through Image-Proplus 6.0 software. All the images were taken using the same microscope and camera sets. The intensity of positive staining in tissue sections was analyzed by average optic density (oD). oD means integrated optical density (IOD) per stained area (μm^2) (IOD/area) for positive staining.

Statistical Analyses

SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA) was employed and differences were regarded as

statistically significant if $p < 0.05$. Continuous variables are presented as mean ± SD. ANOVA was used to evaluate the differences among multiple groups followed by a post hoc test (Student–Newman–Keuls) for continuous variables; RIDIT test was used for statistical analysis of categorical variables. Results of ranked data were statistically analyzed by the non-parametric Kruskal–Wallis H test followed by the Wilcoxon test for paired comparisons.

Results

The General Condition and the Comparison of Organ Coefficients of Sprague-Dawley Rats

The increased weight levels in the model group were significantly lower than those in the controls (74.00 ± 16.20 vs. 230.35 ± 28.50 g; $p < 0.05$). Compared with the model group, these levels were significantly high in the vaccine group (74.00 ± 16.20 vs. 174.67 ± 36.9 g; $p < 0.05$). There was no significant difference between the adjuvant and vaccine group (201.25 ± 39.22 vs. 174.67 ± 36.9 g; $p > 0.05$). During the experiment period, three rats in model group and one rat each in the adjuvant and vaccine groups died.

The value of the liver coefficient in model group was significantly higher than that in the control (Table 1; $p < 0.05$), but there was no significant difference between vaccine group and model group ($p > 0.05$). The values of the spleen and kidney coefficients showed no statistically significant difference among the groups ($p > 0.05$).

Serum Titers of Anti-Peptides

Serum TGF-β1 antibody titre was 1:256 in six rats, 1:128 in two rats and 1:64 in one rat. TGF-β1 antibody was not detected in rats not receiving the MAP vaccine.

TGF-β1 MAP Vaccine Decreased Liver Damage

CCl_4 exposure did not affect serum albumin and globular protein. CCl_4 exposure increased serum ALT, AST and TBIL (Table 2). TGF-β1 MAP vaccine attenuated such changes ($p < 0.01$ vs. model group). The hepatic hydroxyproline content in the model group was significantly higher than that in the control (439.14 ± 43.29 vs. 194.80 ± 25.96 μg/g; $p < 0.01$). The level of hydroxyproline was significantly reduced in vaccine group compared with model group (263.67 ± 41.18 vs. 439.14 ± 43.29 μg/g; $p < 0.01$). There was significant difference between the adjuvant group and the vaccine group (191.11 ± 38.24 vs. 263.67 ± 41.18 μg/g; $p < 0.01$). The results are shown in Fig. 4.

Representative liver tissues sections views are shown in Fig. 5a–h. As shown in tissue sections stained with HE, there were massive fibrotic hyperplasia and deposits in the model group (Fig. 5c), compared with control and adjuvant groups (Fig. 5a, b). The results indicated that the experimental liver injury model induced by CCl_4, ethanol and high fat diet was successful. Administration of TGF-β1 MAP vaccine apparently suppressed hepatic fibrosis by reducing the thickness of bridging fibrotic septa (Fig. 5d) compared with model group, but hepatic steatosis and inflammatory cells infiltration were still serious compared with control and adjuvant groups.

Collagen deposition in liver tissues was evaluated using VG staining. As expected, the normal liver lobule was disrupted, and hepatic collagen deposition extending from central veins or portal tracts, with thick or thin fibrous septum formation and even pseudolobuli formation were evident in the liver injury model group (Fig. 5g) when compared with control (Fig. 5e). In contrast, the fibrotic hyperplasia and deposits were significantly reduced in vaccine group (Fig. 5h). These results indicated that the TGF-β1 MAP vaccine might prevent hepatic fibrosis in the experimental hepatic injury model. The degree of liver fibrosis is shown in Table 3.

Table 1 The comparison of organ coefficients of Sprague-Dawley rats (g/100 g)

Groups		Liver	Spleen	Left kidney	Right kidney
Control	10	2.841 ± 0.096	0.233 ± 0.052	0.438 ± 0.012	0.446 ± 0.005
Adjuvant	9	2.488 ± 0.256	0.210 ± 0.037	0.346 ± 0.022	0.348 ± 0.030
Model	7	4.518 ± 0.584[a]	0.264 ± 0.078	0.395 ± 0.031	0.383 ± 0.047
Vaccine	9	3.996 ± 0.446[b]	0.251 ± 0.058	0.363 ± 0.030	0.363 ± 0.032

Data presented as mean ± SD

[a] $p < 0.05$ vs. control

[b] $p < 0.05$ vs. adjuvant group

Table 2 Levels of serum ALT, AST, TBIL, GLB and ALB in different experimental groups

Groups	*n*	TBIL (μmol/L)	ALT (IU/L)	AST (IU/L)	ALB (g/L)	GLB (g/L)
Control	10	1.84 ± 0.03	85.40 ± 6.64	295.80 ± 9.59	33.21 ± 1.61	25.22 ± 0.82
Adjuvant	9	1.77 ± 0.41	72.78 ± 19.41	316.89 ± 40.15	34.40 ± 2.78	24.60 ± 1.90
Model	7	4.01 ± 0.52[a]	282.71 ± 136.94[a]	495.29 ± 137.13[a]	32.49 ± 1.38	23.93 ± 1.19
Vaccine	9	2.02 ± 0.27[b,c]	116.78 ± 23.76[b,c]	319.78 ± 82.48[b,c]	34.43 ± 1.49	24.62 ± 1.88

Data presented as mean ± SD

ALT alanine aminotransferase, *AST* aspartate aminotransferase, *TBIL* total bilirubin, *ALB* albumin, *GLB* globulin

[a] $p < 0.05$ vs. control

[b] $p < 0.05$ vs. model group

[c] $p < 0.05$ vs. adjuvant group

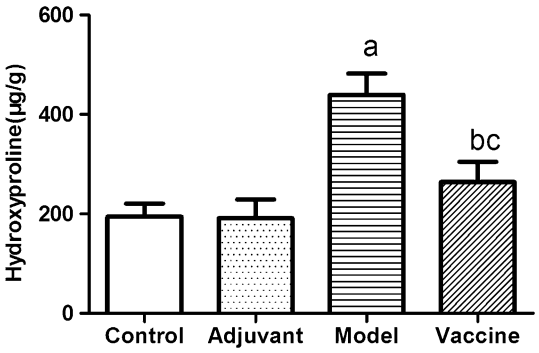

Fig. 4 Hydroxyproline contents in rat liver tissues. [a]$p < 0.05$ vs. control, [b]$p < 0.05$ vs. model group, [c]$p < 0.05$ vs. adjuvant group

TGF-β1 MAP Vaccine Reduced TGF-β1 and α-SMA Expression in Liver Tissues in the Rat Model

Immunohistochemical method was used to further evaluate the impact of vaccine on regulating the expression of TGF-β1 and α-SMA. Liver sections from each group were immunolabeled with TGF-β1 or α-SMA antibodies. As shown in Figs. 6 and 7, few positive staining cells were observed in the cytoplasm in the control and adjuvant groups. As expected, cells with brown granules in liver tissues were taken as positive staining. Administration of CCl_4 caused a significant increase in the number of positive staining cells observed by antibodies against TGF-β1 or α-SMA. The positive staining cells were predominantly distributed in the regions of fibroblasts, vascular endothelial cells, surrounding cells of the central vein, fibrous septum cells, and denatured hepatocytes. TGF-β1 MAP vaccine treatment significantly reduced the number of positive staining cells labeled with TGF-β1 or α-SMA, suggesting that the vaccine might inhibit HSC activation and reduce TGF-β1 expression in the rat liver injury model.

The degree of expression of TGF-β1 and α-SMA and the changes in morphology of liver tissues were measured using an image analysis system. Semi-quantitative IOD analysis indicated that there were more positive stainings against TGF-β1 and α-SMA in the liver tissues in the model group compared with the control (Table 4). The value of oD of vaccine treatment group was lower than that in the model group ($p < 0.05$). These results suggested that the TGF-β1 MAP vaccine might be able to reduce the expression of TGF-β1 and α-SMA in the rat liver injury model.

Fig. 5 Pathological changes of liver tissues in rats. HE staining of liver tissues (original magnification ×100): *A* control group, *B* adjuvant group, *C* model group, *D* vaccine group. van Gieson staining of liver tissues (original magnification ×100): *E* control group, *F* adjuvant group, *G* model group, *H* vaccine group

Discussion

In this study, we showed that a TGF-β1 MAP vaccine could protect the rat liver from CCl_4, ethanol and high fat diet induced experimental liver injury and fibrogenesis. The probable mechanism for this protective effect may relate to the fact that TGF-β1 MAP vaccine could induce special antibodies against TGF-β1 that could neutralize excessive TGF-β1 in hepatic fibrosis rats model.

In fibrotic diseases, the regions of ECM deposition show high expression of TGF-β1 (Branton and Kopp 1999). Recent advances in our understanding of the cytokine network in hepatic fibrosis, as well as technical progress in blocking TGF-β1 in vivo, are likely to provide options for new drugs that could control chronic hepatic fibrosis (Kopf et al. 2010). The previous studies have shown that either neutralizing antibodies or vaccine against cytokines can markedly inhibit fibrotic diseases (Liu et al. 2006; Szabo et al. 2010). However, there are challenging roadblocks ahead that must be overcome before any potential and effective treatment strategies can reach the clinic.

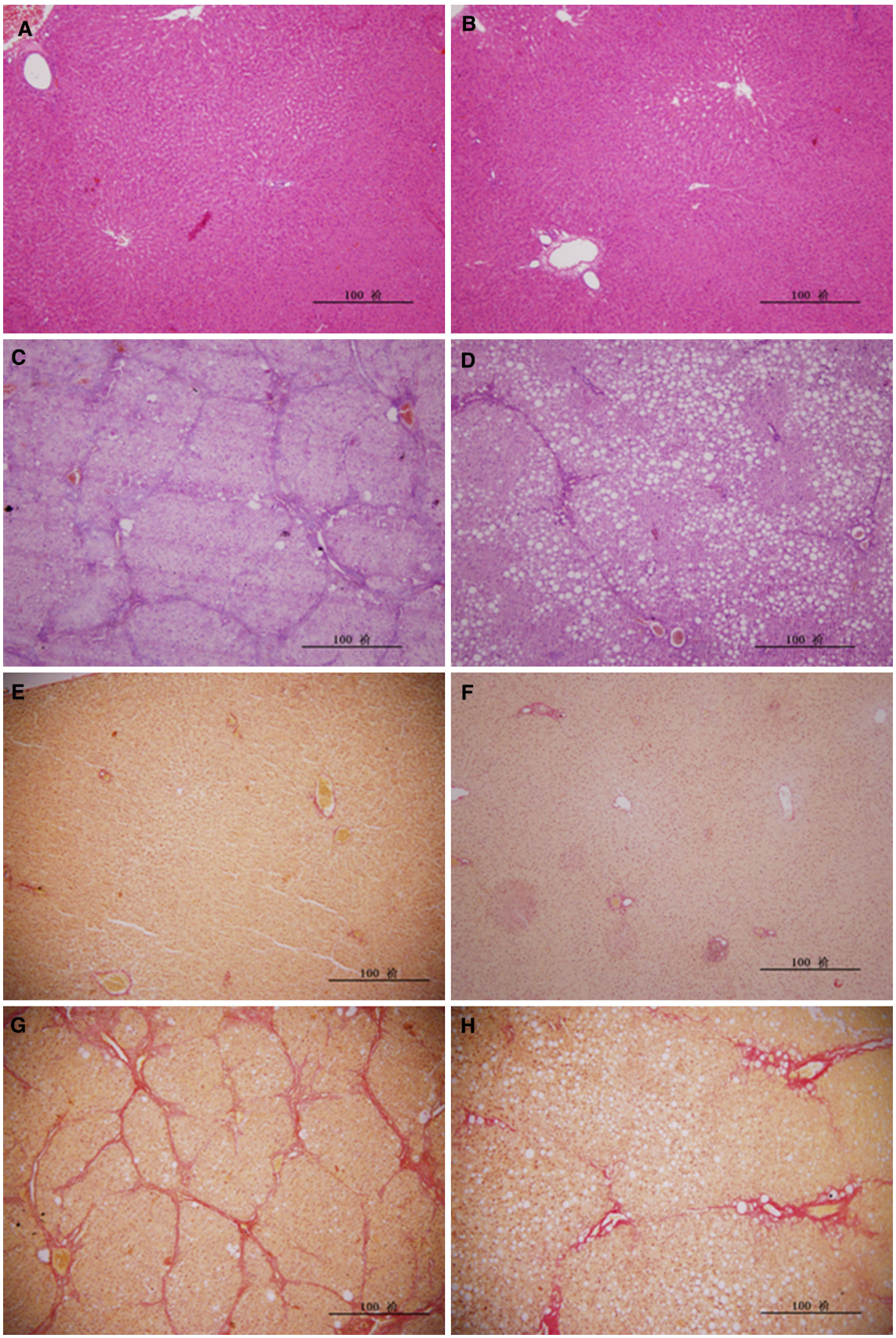
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Table 3 The degree of liver fibrosis in the groups

Groups	*n*	0	1	2	3	4
Control	10	10	0	0	0	0
Adjuvant	9	9	0	0	0	0
Model	7	0	0	0	2	5
Vaccine	9	0	4	3	2	0

$H = 16.771$. model group vs. control, $\chi^2 = 5.765$; $p < 0.05$; model group vs. vaccine group, $\chi^2 = 7.865$; $p < 0.05$; Vaccine group vs. adjuvant group, $\chi^2 = 4.625$; $p < 0.05$. The results were statistically analyzed by the non-parametric Kruskal–Wallis *H* test followed by Wilcoxon test for paired comparisons

A significant advantage of MAP as an immunogen is that in addition to inducing responses to predefined epitopes, the MAP contains higher concentration of the relevant antigen (Ciesielski et al. 2005). Moreover, the peptide vaccines are chemically more stable, easy to prepare, and have lower oncogenic potential (Berger et al. 2004). Consequently, we designed and synthesized TGF-β1 MAP vaccine containing a lysine core matrix linked to four copies of dominant B epitopes of TGF-β1 to induce special antibody titers by active immunotherapy. Our results showed that TGF-β1 MAP vaccine enhances immunogenicity and elicits immune response in rats, and induced low, but detectable anti-peptide antibody titers.

The pathology of the animal model used in the current study is similar to the human cirrhosis (Dong et al. 2009). CCl_4 is a hepatotoxin and can cause profound hepatocyte damages, including hepatocyte necrosis, steatosis, inflammation, and fibrosis and cirrhosis upon repeated exposure. Ethanol promotes the generation of reactive oxygen species and disrupts physiological defense mechanisms, particularly in the liver. Oxidative stress plays a major role in liver injury caused by ethanol exposure (Wu and Cederbaum 2009). Ethanol could increase collagen and soluble proteins in the liver (Häkkinen et al. 1975). In the current study, rats were placed on a high-fat diet to accelerate the development of fatty liver, and to enhance the sensitivity to CCl_4 damage. Such an experimental design is consistent with the "two-hit theory" of liver fibrosis (James and Day 1998; Willemin et al. 2013). The first hit is an infiltration of fat into the hepatocytes (hepatic steatosis) and the second hit is characterized by an infiltration of immune cells such as monocytes and lymphocytes because of abnormal oxidative stress.

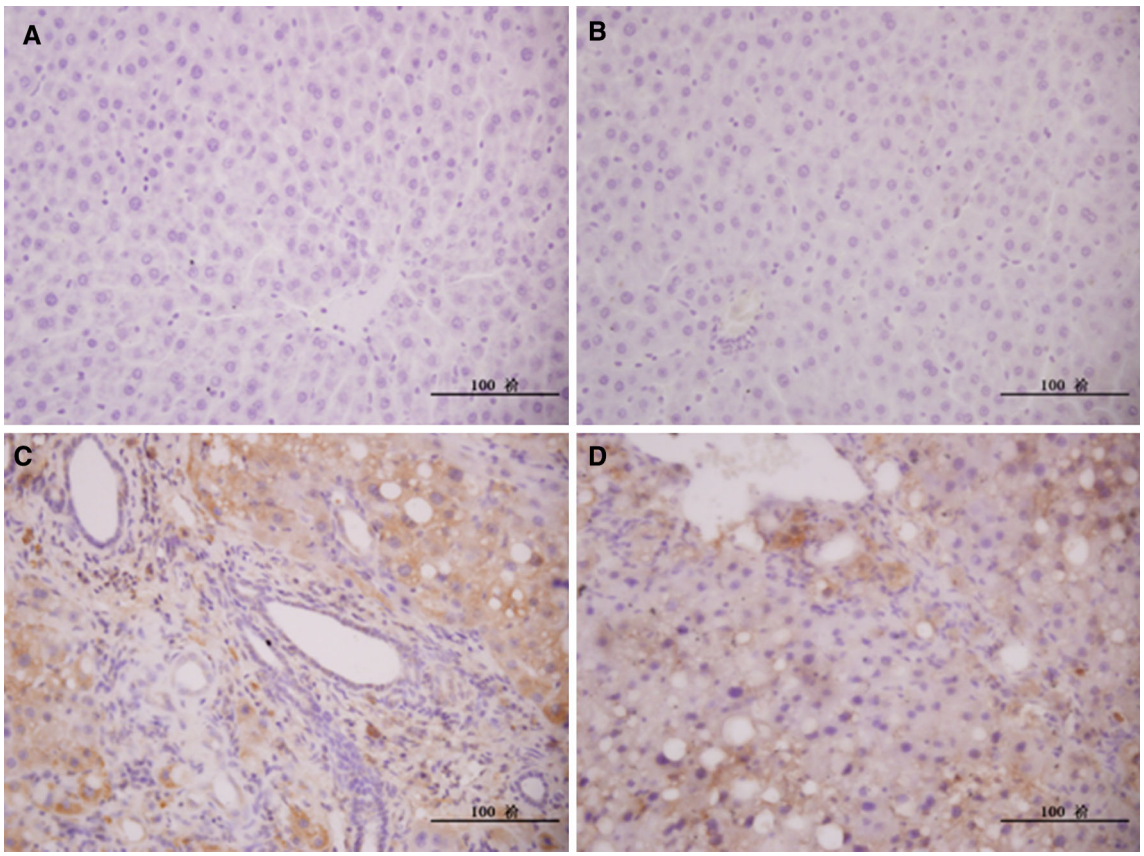

Fig. 6 Immunohistochemical staining of TGF-β1 in rat liver tissues (original magnification ×400). *A* control group, *B* adjuvant group, *C* model group, *D* vaccine group

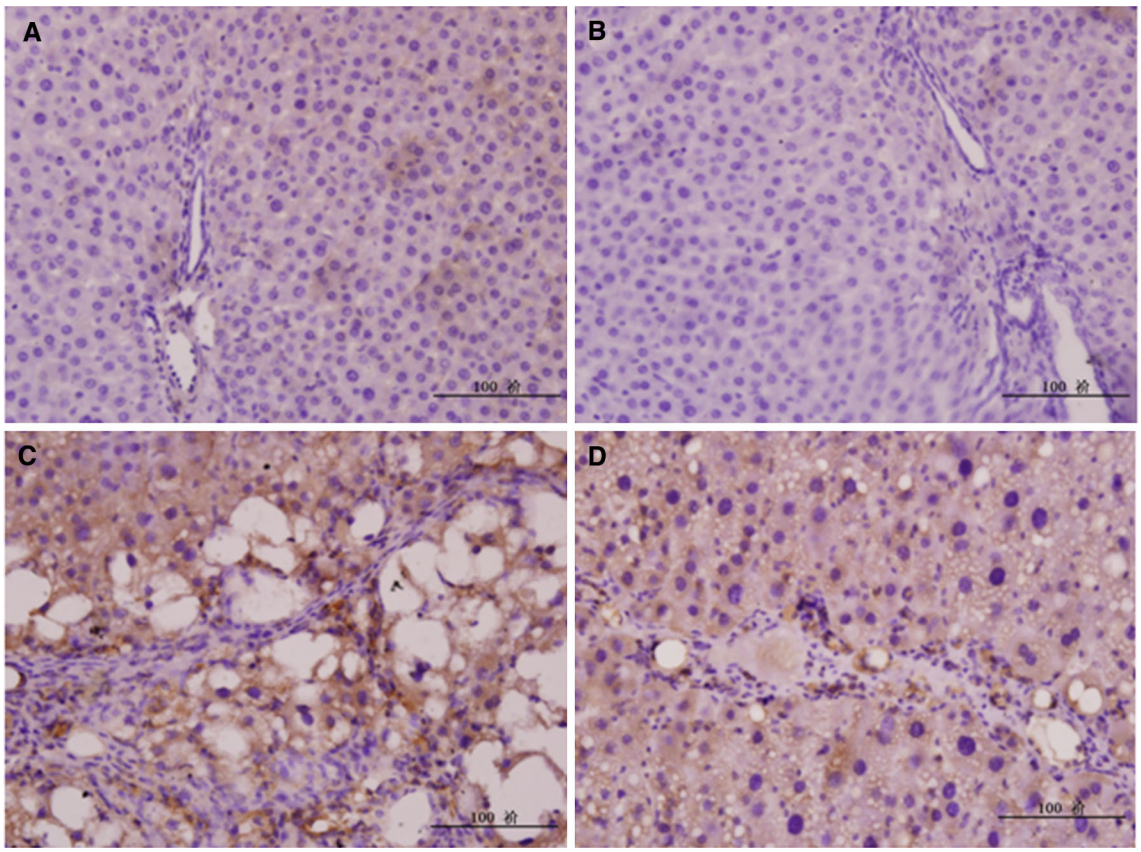

Fig. 7 Immunohistochemical staining of α-SMA in rat liver tissues (original magnification ×400). *A* control group, *B* adjuvant group, *C* model group, *D* vaccine group

Table 4 The values of average optic density of TGF-β1 and α-SMA in liver tissues in different experimental groups (IOD/area)

Groups	*n*	TGF-β1	α-SMA
Control	10	0.087 ± 0.002	0.074 ± 0.006
Adjuvant	9	0.093 ± 0.006	0.095 ± 0.004
Model	7	0.507 ± 0.044[a]	0.489 ± 0.029[a]
Vaccine	9	0.325 ± 0.059[b,c]	0.318 ± 0.058[b,c]

Data presented as mean ± SD

TGF-β1 transforming growth factor-β1, *α-SMA* alpha-smooth muscle actin

[a] $p < 0.05$ vs. control

[b] $p < 0.05$ vs. model group

[c] $p < 0.05$ vs. adjuvant group

In our study, ALT and AST levels were dramatically elevated by the fibrosis-inducing regimen. Our results showed that CCl_4 caused hepatic fibrosis, as evidence by HE and VG staining, and higher levels of ALT, AST, TBIL and hydroxyproline. These results demonstrated that we successfully established hepatic injury model by administration of CCl_4, combined with ethanol and high fat diet.

Hepatic fibrosis is associated with major alternation in both the quantity and composition of ECM (Bataller and Brenner 2005). It is now widely accepted that the dominant stimulus for ECM deposition is cytokine TGF-β1 (Bataller and Brenner 2005). It had been suggested that sustained overexpression of TGF-β1 could activate HSCs and induce massive ECM deposition in the progression of hepatic fibrosis (Bataller and Brenner 2005). TGF-β1 antagonists could block TGF-β1 activity (Liu et al. 2006). In this study, TGF-β1 MAP vaccine was used to induce antibodies targeting TGF-β1 (amino acids 90–98). The immunohistochemistry results showed low expression of TGF-β1 in hepatic tissues in normal rats, and markedly elevated expression after exposure to CCl_4. TGF-β1 MAP vaccine down-regulated TGF-β1 in hepatic fibrosis tissues. The results also demonstrated that TGF-β1 MAP vaccine could attenuate hepatic fibrosis through down-regulation of TGF-β1 expression.

Activated HSCs are the main ECM-producing cells and critically implicated in hepatic fibrogenesis. Thus, inhibition of activated HSCs is an important therapeutic strategy (Tu et al. 2007). Our results showed that the expression of α-SMA (a maker of HSCs activation) in hepatic tissues was

increased after injection of CCl_4. The results demonstrated that TGF-β1 MAP vaccine might inhibit HSC activation in hepatic tissues in rat model.

One specific detail merits consideration in our study. The steatosis and the ballooning necrosis are more severe in the vaccine group than in model group. As fatty degeneration is a reversible process during the early phase of liver fibrosis, it is theoretically possible that apparent increase in fat vacuolization could represent the attenuation and reversal of the more severe damage. Observations through the entire intervention course may be considered in future studies.

In summary, TGF-β1 MAP vaccine exhibits significant partial protection on hepatotoxicity as evidenced from the reversal of various biochemical indices and histopathology. The results suggest that active immunization is a promising approach to ameliorate the destructive effects of chronic hepatic fibrosis.

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Conflict of interest The authors declare that they have no conflict of interest.

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