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Schistosoma japonicum soluble egg antigens induce apoptosis and inhibit activation of hepatic stellate cells: a possible molecular mechanism

Yinong Duan [1,*], Xijuan Gu [1], Dandan Zhu, Wei Sun, Jinling Chen, Jinrong Feng, Ke Song, Feifan Xu, Xue He, Xingxin He

Department of Pathogen Biology, School of Medicine, Nantong University, 19 Qixiu Road, Nantong 226001, Jiangsu, People's Republic of China

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ABSTRACT

Hepatic stellate cells play a key role in the development of hepatic fibrosis. Activated hepatic stellate cells can be reversed to a quiescent-like state or apoptosis can be induced to reverse fibrosis. Some studies have recently shown that *Schistosoma mansoni* eggs could suppress the activation of hepatic stellate cells and that soluble egg antigens from schistosome eggs could promote immunocyte apoptosis. Hence, in this study, we attempt to assess the direct effects of *Schistosoma japonicum* soluble egg antigens on hepatic stellate cell apoptosis, and to explore the mechanism by which the apoptosis of activated hepatic stellate cells can be induced by soluble egg antigens, as well as the mechanism by which hepatic stellate cell activation is inhibited by soluble egg antigens. Here, it was shown that *S. japonicum*-infected mouse livers had increased apoptosis phenomena and a variability of peroxisome proliferator-activated receptor γ expression. Soluble egg antigens induce morphological changes in the hepatic stellate cell LX-2 cell line, inhibit cell proliferation and induce cell-cycle arrest at the G_1 phase. Soluble egg antigens also induce apoptosis in hepatic stellate cells through the TNF-related apoptosis-inducing ligand/death receptor 5 and caspase-dependent pathways. Additionally, soluble egg antigens could inhibit the activation of hepatic stellate cells through peroxisome proliferator-activated receptor γ and the transforming growth factor β signalling pathways. Therefore, our study provides new insights into the anti-fibrotic effects of *S. japonicum* soluble egg antigens on hepatic stellate cell apoptosis and the underlying mechanism by which the liver fibrosis could be attenuated by soluble egg antigens.

1. Introduction

Hepatic fibrosis is a common response to many chronic liver injuries induced by a variety of aetiological factors, such as hepatitis B and C viruses, alcoholism and schistosome infection (Wallace et al., 2008). The fibrotic process is characterised by an accumulation of excessive extracellular matrix (ECM) and depleted vitamin A storage. During the development of fibrosis, hepatic stellate cells (HSCs) are the key effector cells for hepatic fibrogenesis. In response to the liver injuries, HSCs often undergo cell activation from quiescent cells to myofibroblast-like cells, with an increased expression of α-smooth muscle actin (α-SMA) and collagen. This change promotes the migration and adhesion of HSCs, and decreases lipid (vitamin A) storage (Wallace et al., 2008).

Interestingly, liver fibrosis is considered to be reversible under some conditions. During the reversion of fibrosis, activated HSCs will return to a quiescent-like state or undergo apoptosis. This phenomenon of fibrosis reversion was first observed in patients with schistosomiasis, which is caused by infection with schistosomes (Katz and Brener, 1966; Wallace et al., 2008). Schistosomiasis is one of the most prevalent parasitic infections and often results in inflammatory granulomas and progressive chronic hepatic fibrosis. In 1966, Katz and Brenner found spontaneous regression of splenomegaly in several patients with schistosomiasis (Katz and Brener, 1966). In previous studies we also found that α-SMA expression was up-regulated in mice infected with *Schistosoma japonicum* at 12 weeks p.i. and down-regulated thereafter (Duan et al., 2011). A similar result has also been reported by Chen et al. (2013).

Recently, several studies have shown that *Schistosoma mansoni* eggs could suppress the activation of HSCs and soluble egg antigens (SEA) from schistosomes could promote immunocyte apoptosis. Specifically, SEA has been shown to induce apoptosis of granuloma T and splenic cells (Lundy et al., 2001). However, there are no prior published studies in which the direct effects of SEA on HSC apoptosis have been considered. Anthony et al. (2010) have reported that *S. mansoni* eggs blocked fibrogenesis and induced the conversion of LX-2 cells from an activated phenotype to a quiescent phenotype. More recently, Anthony et al. (2013) also reported

* Corresponding author. Tel.: +86 513 85051738; fax: +86 513 85051876.
E-mail address: yinongduan@gmail.com (Y. Duan).
[1] These authors contributed equally to this work.

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that eggs from *S. japonicum* can induce an anti-fibrogenic phenotype in LX-2 cells. However, the mechanism by which the activation of HSCs was inhibited by SEA is still unknown. In this study, we attempted to investigate the direct effects of *S. japonicum* SEA on the apoptosis of activated HSCs. We also attempted to explore the mechanisms by which this apoptosis is induced and by which the activation of HSCs is inhibited by SEA.

2. Materials and methods

2.1. Experimental animals and SEA preparation

Male Institute of Cancer Research (ICR) strain mice (each weighing 18–20 g), purchased from the Laboratory Animal Center of Nantong University, Jiangsu, People's Republic of China, were used in this study. All animal procedures were performed in accordance with the institutional ethical guidelines for laboratory animal care and use of Nantong University. Mouse models infected with *S. japonicum* were constructed by percutaneously infecting the mice with 20 ± 2 cercariae of *S. japonicum* (kindly provided by Jiangsu Institute of Parasitic Diseases, Wuxi, China), and euthanased at 6, 12, 18 and 24 weeks p.i. The liver tissue of each mouse was collected and prepared for immunofluorescence analysis and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labelling assay (TUNEL) analysis, or used to isolate the mouse HSCs. SEA from *S. japonicum* was prepared from purified eggs from the livers of *S. japonicum*-infected rabbits as previously described (Zhu et al., 2012), sterile-filtered and any endotoxins removed with Polymyxin B agarose beads (Sigma, USA) (Zaccone et al., 2010). A Limulus Amebocyte Lysate assay kit (Lonza, Switzerland) was used to confirm the removal of endotoxins from the SEA.

2.2. Isolation and culture of HSCs

Primary HSCs were isolated from the livers of both normal and *S. japonicum*-infected ICR mice. In brief, livers were digested in 1640 medium (Invitrogen, USA) containing Type IV collagenase (0.08%; Invitrogen) and DNase I (4 U/ml; Thermo Fisher Scientific, USA) at 37 °C for 25 min. The harvested cells were then resuspended in 1640 medium and centrifuged through an OptiPrep cushion (Axis-Shield, Norway) (Liang et al., 2011). The HSC-enriched band was transferred into 1640 medium supplemented with 20% fetal bovine serum (FBS; Hyclone, USA) with 100 U/ml of penicillin and 100 μg/ml of streptomycin. The viability of the isolated cells was determined by a trypan blue dye exclusion assay. The purity was identified by retinoid autofluorescence, oil red O staining and immunofluorescence double staining with desmin and α-SMA. In order to obtain activated HSCs, primary HSCs isolated from normal livers were treated with transforming growth factor β1 (TGFβ1; Sigma) in vitro. Primary HSCs isolated from *S. japonicum*-infected mice were used as the target cells and they had been activated in vivo.

An 'immortalised' LX-2 human HSC line was purchased from Xiang Ya Central Experiment Laboratory, Hunan, China. This line has been shown to be the most similar to "activated" HSCs in vivo (Xu et al., 2005). LX-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, China) supplemented with 10% FBS, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

HSCs were treated with SEA at various concentrations and for various times (see Section 3). For the peroxisome proliferator-activated receptor γ (PPARγ)-associated studies, HSCs were cultured with PPARγ antagonist (GW9662, Sigma) for 6 h before SEA was added. For the caspase-associated studies, HSCs were pre-treated with caspase inhibitor Z-VAD-FMK (Beyotime, China) for 1 h before SEA was added. For the death receptor 5 (DR5)-associated apoptosis studies, LX-2 cells were transfected with DR5 short hairpin RNA (shRNA) plasmid (Genechem, China) or shRNA control plasmid for 48 h using FuGene reagent (Promega, USA).

2.3. Measurement of cell proliferation by (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) MTT assay

The effect of SEA on LX-2 cell proliferation was analysed by MTT (Sigma) assay. In brief, LX-2 cells were plated at a density of 1×10^4 cells/well in 96-well plates for 24 h before adding the stimulus. The cells were then cultured for 72 h in the presence of SEA (0, 5, 10 or 20 μg/ml). After being pulsed with MTT for 4 h, DMSO was added to dissolve the formazan products and the absorbance was measured at 570 nm with an ELISA reader (Bio-Tek, USA).

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted and reverse transcribed into cDNA as previously described (Duan et al., 2011). The cDNA products were then used as the template for qRT-PCR analysis with a SYBR Premix Ex Taq Kit (TAKARA, Japan) on the Eco Real-Time PCR Sequence Detection System (Illumina, USA). The thermal cycling conditions were comprised of an initial denaturation step at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 30 s and 72 °C for 30 s. The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control. The target gene values were normalised to the GAPDH values and expressed as a relative fold increase $2^{(-\Delta\Delta Ct)}$ over the non-treated samples. All experiments were repeated in triplicate using different samples each time and the data were represented as the mean ± S.E.M of all experiments.

2.5. Western blot

Proteins were extracted from HSCs with RIPA lysis buffer (Beyotime) and quantified by the Bradford method (Sangon, China). The protein solution was separated by 10% or 12% SDS–PAGE and electrotransferred on to polyvinylidene fluoride (PVDF) membrane (Merck, Germany) as previously described (Duan et al., 2011). The PVDF membranes were then incubated with primary antibodies against PPARγ (Santa Cruz Biotechnology, USA), α-SMA (Abcam, UK), Smad4 (Santa Cruz Biotechnology), caspase 3 (Cell Signaling Technology, USA), caspase 8 (Beyotime) or GAPDH (Goodhere, China). Horseradish peroxidase (HRP)-conjugated anti-rabbit or mouse IgG (Santa Cruz Biotechnology) was used as secondary antibody. The membranes were then visualised with enhanced chemiluminescence (ECL; Merck). GAPDH protein was used as the internal control. Band quantification analysis was performed by GeneTools software from Syngene (UK).

2.6. Cell cycle and apoptosis analysis

For cell cycle analysis, LX-2 cells were serum-starved for 24 h in DMEM before SEA treatment, and then the medium was replaced with DMEM containing 10% FBS and SEA (10 μg/ml). After being incubated with SEA for 72 h, the cells were harvested and resuspended in 0.3 ml of PBS, fixed with 0.7 ml of 100% ethanol and left at 4 °C overnight. The cell suspension was then incubated with RNase A (50 μg/ml, TAKARA,) at 37 °C for 20 min and labelled with propidium iodide (PI, 50 μg/ml, Biosharp, China) on ice for 30 min. The cell cycle distribution was analysed by flow cytometry using a FACSCalibur instrument (BD Biosciences, USA).

LX-2 cell apoptosis was determined by dual staining using an Annexin V-FITC Kit (Merck). The cells were serum-starved for 24 h in DMEM with 0.4% FBS and then treated with SEA (10 μg/