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Protease-activated receptor-2 modulates hepatic stellate cell collagen release and apoptotic status

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ABSTRACT

The pathogenesis of hepatic fibrosis is to be further investigated. Protease-activated receptor-2 (PAR2) plays a role in hepatic fibrosis. This study aims to elucidate the role of activation of PAR2 in the regulation of hepatic stellate cell activities. In this study, the expression of PAR2, Fas and caveolin-1 in human hepatic stellate cell line, HHStec cell (HHStecs) was assessed by real time RT-PCR and Western blot. The levels of collagen were determined by enzyme-linked immunosorbent assay. The PAR2 gene was silenced in HHStecs using RNA interference. Apoptosis of HHStecs was assessed by flow cytometry. The results showed that HHStecs expressed PAR2, which was up regulated by activation with phorbol myristate acetate (PMA). Activation of PAR2 increased the release of collagen from HHStecs. Exposure to PMA induced HHStec apoptosis, which was significantly inhibited by activation of PAR2. The PAR2 activation also suppressed the expression of caveolin-1 and Fas in HHStecs. Over expression of caveolin-1 in HHStecs blocked PAR2-reduced apoptosis. We conclude that HHStecs express PAR2. Activation of PAR2 increases HHStecs to release collagen and reduces the activation-induced HHStec apoptosis, which can be inhibited by the over expression of caveolin-1.

Introduction

Hepatic fibrosis is featured by the over production and deposition of extracellular matrix, and excessive builds up connective tissue in the liver. Although the fibrosis does not cause symptoms, it leads to liver structure disruption and loss of liver function [1]. The current treatment of hepatic fibrosis is less satisfactory although some progresses promising in animal model studies have been reported [2].

Activated hepatic stellate cells, portal fibroblasts, and myofibroblasts of bone marrow origin have been identified as major collagen-producing cells in the injured liver; among the cells, hepatic stellate cells are more important in the development of hepatic fibrosis. The activation of the hepatic stellate cell causes accumulation of extracellular matrix and formation of scar, leading to deterioration in the hepatic functions [3]. The hepatic stellate cells are also called Ito cells, which store fat, produce excessive amounts of abnormal matrix including collagen, other glycoproteins and glycans, and matricellular proteins. Thus, stellate cell activation results in abnormal extracellular matrix, both in quantity and composition [4]. To date, the regulation of hepatic stellate cell activities is not fully understood yet.

Mast cells are being recognized as important constituents in the fibrotic processes [5]. The increases in mast cell number in the liver are found in hepatitis C [6], the latter is one of the major causes of hepatic fibrosis [7]. Mast cells release a number of chemical mediators upon activation; tryptase is one of the major mediators of mast cells that can cleave and activate the protease-activated receptor-2 (PAR2)[2] [8]. It is reported that PAR2 promotes experimental liver fibrosis in mice and activates human hepatic stellate cells [9]. Based on the above information, we hypothesize that activation of PAR2 modulates hepatic stellate cell activities. Thus, we carried out a cell culture study. The results showed that a hepatic cell line, HHStec cells (HHStec, in short), expressed PAR2. Activation of PAR2 increased the collagen release from HHStecs and interfered with the activation-induced HHStec apoptosis.

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[2] *Abbreviations used:* HHStec, human hepatic stellate cells; PAR2, protease-activated receptor-2; PMA, phorbol myristate acetate; PI, propidium iodide; AP, active peptides; CP, control peptides; AICD, activation-induced cell death.

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Materials and methods

Reagents

Antbodies of caveolin-1, PAR2, Fas, PAR2 siRNA and shRNA of PAR2 were purchased from Santa Cruz Biotech (Shanghai, China). Tryptase, PMA and Annexin V kit were purchased from Sigma Aldrich (Shanghai, China). The reagents of real time RT-PCR and caveolin-1 plasmid transfection were purchased from Invitrogen (Shanghai, China). Collagen ELISA kits were purchased from Alibaba Biotech (Guangzhou, China).

Cell culture

Human hepatic stellate cells (HHStec) were purchased from Bioon Group (Shanghai, China) and maintained in RPMI 1640 medium supplemented with 10% FBS and antibiotics (100 mg/L penicillin and 100 mg/L streptomycin). Cells were incubated in a humidified environment under 5% CO_2 at 37 °C. The medium was changed every 2–3 days. The cell viability was assessed by the trypan blue exclusion assay. Some cells were treated with tryptase, or active PAR2 peptides (SLIGKV-NH2), or control peptides VKGILS-NH2), in the culture.

Apoptosis assay

The apoptosis of HHStecs was assessed by flow cytometry using Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions. Briefly, the cells were stained with Annexin V-FITC and propidium iodide (PI). The frequency of apoptotic HHStec was determined by using a FACSCanto II flow cytometer (BD Bioscience, Shanghai, China) at 488 nm.

Quantitative real time RT-PCR (qRT-PCR)

The total RNA from HHStecs was extracted by Trizol reagent according to the manufacturer's protocol. The cDNA was synthesized using a reverse transcription kit. qPCR was performed on a Real-time PCR system (MiniOpticon, Bio-Rad Life Science. Shanghai, China). The primers include: PAR2 (AY336105): Forward, ttcatgacctgcctcagtgt; reverse, agatggtctgcttcacgaca. Caveolin-1 (AF095591): Forward, tctcatccctccctctgtga; reverse, tgttttggcagggc ttcttc. Fas (NM_000043): Forward, ccggacccagaataccaagt; reverse, gaagacaaagccaccccaag.

Western-blot

Protein samples were extracted from HHStecs by cell extraction buffer. The equivalent aliquots of proteins were electrophoresed on a 10% SDS/polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were blocked by incubation with 5% nonfat milk for 1 h before incubation with primary antibodies (200–500 ng/ml) overnight. Following incubated with secondary antibody (horseradish peroxidase-conjugated), the immune complexes on the membranes were detected by enhanced chemoluminescence substrate; the results were recorded with X-ray films.

Enzyme-linked immunosorbent assay (ELISA)

Collagen levels in the culture supernatants were determined by ELISA with a commercial ELISA kit following the manufacturer's instruction.

RNA interference (RNAi)

HHStecs were treated with commercial reagent kits of PAR2 shRNA, PAR2 siRNA, or control shRNA, following the manufacturer's instruction. On day 6 after the transduction, the expression of PAR2 in the cells was below detectable levels. The cells were used for further experiments.

Over expression of caveolin-1 in HHStecs

The whole length of caveolin-1 gene fragment was cloned by Tianqi Biotech (Guangzhou, China), which was carried by pIRES vector. The pIRES vector was replicated for propagation in *E. coli*, and then transfected into HHStecs using a lipofectamine kit following the manufacturer's instruction. The caveolin-1 expression in the HHStecs was assessed by Western blotting. Some HHStecs were transfected with an empty vector using as controls.

Statistics

Data are presented as mean ± SD. Differences between two groups were determined by student *t* test or ANOVA if more than two groups. $P < 0.05$ was set as a criterion of significance.

Results

HHStecs express protease-activated receptor 2

Published data indicate that stellate cells play a critical role in liver fibrosis [10]. PAR2 is also involved in the process of fibrosis [11]. Whether PAR2 modulates stellate cell activities is unclear. We firstly observed the expression of PAR2 in HHStecs. As shown by qRT-PCR (Fig. 1A) and Western blotting (Fig. 1B and C), the expression of PAR2 was detected in HHStecs at low levels; exposure to phorbol myristate acetate (PMA) markedly increased the expression of PAR2 in HHStecs (Fig. 1A–C). The results indicate that HHStecs express PAR2; activation by PMA can up regulate the expression of PAR2 in HHStecs.

Activation of PAR2 increases the release of collagen from HHStecs

Data of Fig. 1 indicate that HHStecs express PAR2. We inferred that activation of PAR2 could modulate functions of HHStecs. To this end, we cultured HHStecs in the presence of tryptase, or PAR2 active peptides (AP), or control peptides (CP). The culture supernatants were collected 48 h later and analyzed by ELISA. The results showed that collagen was detected in the culture supernatants in HHstecs cultured with medium alone, which was markedly increased in the HHstecs treated with tryptase in a tryptase dose-dependent manner (Fig. 2A). Knockdown of the PAR2 gene (Fig. 2B and C) abolished the increase in collagen release from HHStecs. Exposure to AP, but not CP, also markedly increased the collagen release (Fig. 2A). The results indicate that activation of PAR2 can increase collagen release from HHStecs.

PAR2 interferes with the activation-induced HHStec apoptosis

The activation-induced cell death (AICD) is an important mechanism to maintain the homeostasis in the body. We next observed if the activation induced HHStec apoptosis. Using PMA as a stimulator, we activated HHStecs in the culture. As shown by flow cytometry data, PMA significantly increased the frequency of apoptotic HHStecs (Fig. 3A, B and I). Considering that PAR2 also can activate target cells, such as activation of PAR2 increases intestinal epithelial barrier permeability, we observed the effect of activation