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BASIC RESEARCH

Hepatic stellate cells may be potential effectors of platelet activating factor induced portal hypertension

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Abstract

AIM: To determine platelet activating factor (PAF) receptor expression in cirrhotic hepatic stellate cells.

METHODS: Hepatic stellate cells, isolated from the livers of control and CCl_4-induced cirrhotic rats, were placed in serum-free medium after overnight culture. We determined the PAF receptor in hepatic stellate cells by saturation binding technique and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR), and the effects of PAF and its antagonist BN52021 on prostaglandin E_2 (PGE_2) release by stellate cells.

RESULTS: Scatchard analysis indicated the presence of PAF receptor with dissociation constant (*Kd*) of 4.66 nmol/L and maximum binding capacity (Bmax) of 24.65 fmol/μg in cirrhotic stellate cells. Compared with the control, the maximum PAF binding capacity increased significantly (*Bmax*: 24.65 ± 1.96 fmol/μg. DNA, R = 0.982 *vs* 5.74 ± 1.55 fmol/μg. DNA, R = 0.93; $P < 0.01$), whereas receptor affinity had no significant difference (*Kd* of 4.66 ± 0.33 nmol/L for the cirrhosis and 3.51 ± 0.26 nmol/L for the control; $P > 0.05$). Consistent with the receptor binding data, the mRNA expression of PAF receptor was increased significantly in cirrhotic stellate cells. PAF in a concentration-dependent manner induced PGE_2 synthesis in cirrhotic hepatic stellate cells, but the effects were blocked significantly by BN52021.

CONCLUSION: Cirrhosis sensitizes hepatic stellate cells to PAF by elevating its receptor level and hepatic stellate cells maybe potential effectors of PAF induced portal hypertension.

Key words: Platelet activating factor; Hepatic stellate cells; Kupffer cells; Cirrhosis; Receptor

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INTRODUCTION

Platelet activating factor, a phospholipid, exhibits diverse biological activities[1-4]. Portal venous administration of platelet activating factor (PAF) in isolated perfused rat liver increases portal venous pressure and glycogenolysis[5,6]. Systemic (intravenous) administration of PAF to rats, *in vivo*, also induces these effects and, in addition, causes immediate decrease in arterial blood pressure[7]. As a potent hepatic vasoconstrictor and systemic vasodilator, PAF is implicated in portal hypertension and hyperdynamic circulation associated with liver cirrhosis. In a parallel study, we observed increased hepatic and circulating PAF concentrations in cirrhotic rats[8-11]. The increased hepatic synthesis of PAF is a major source of elevated circulating PAF and upregulates hepatic hemodynamic abnormalities, which contributes to portal hypertension[8-11]. The increased hepatic PAF in the cirrhotic liver due to its enhanced synthesis by Kupffer cells, Kupffer cells are a major source of increased platelet activating factor[12,13]. The increased portal pressure by endogenous PAF in cirrhosis can be decreased by BN52021 in some extent. Reduction in portal pressure by BN52021 measures the contribution of endogenous PAF[9,11]. In control rats, this contribution to basal pressure is zero but in cirrhotic rats endogenous PAF is responsible for 23% of the cirrhosis dependent increase in portal pressure[9]. The potential effectors of PAF induced increase in portal pressure remains undetermined. The smooth muscle component of the hepatic vasculature may contribute to PAF induced increase in portal pressure[14]. Hepatic stellate cells (HSCs), located in the space of Disse, play a crucial role in the development of liver fibrosis[15,16]. As PAF is one of only six mediators currently known to cause coupled Ca^{2+} increase and contraction in HSCs[17], we hypothesized the HSCs may be potential effectors of PAF induced increases in portal pressure. Therefore, PAF

receptors and PAF-induced prostaglandin E_2 release derived from cirrhotic hepatic stellate cells were investigated.

MATERIALS AND METHODS

Induction of cirrhosis

The experimental protocols were approved by the Institutional Animal Care and Use Committee of the Institute Infection Diseases of PLA in accordance with the guidelines of the National Institutes of Medicine. Cirrhosis was induced in male Sprague-Dawley rats (230-250 g) as described previously[13,18,19]. Briefly, intraperitoneal injections of CCl_4 (0.15 mL/kg WT twice a week for 8 wk) were combined with drinking water containing phenobarbital (0.4 g/L). Control rats received injections of the vehicle (peanut oil) and phenobarbital water.

Morphometric analysis

An established histological grading system was employed for determination of pathological scores of liver injury[18-23]. Paraffin-embedded liver sections of 4 micron thickness were stained with hematoxylin-eosin and Masson's trichrome stain. Steatosis, inflammation, necrosis and fibrosis were scored in at least 3 random fields of view in each tissue section and a score for each specific parameter was estimated. Steatosis was assessed by estimating the percentage of cells with micro- and macrovesicular fat as follows: 0 (absent); 1 (1%-25%); 2 (26%-50%); 3 (51%-75%); 4 (76%-100%). Necrosis was scored as follows: 0 (absent); 1 (1-10 necrotic cells per view); 2 (11-20 necrotic cells per view); 3 (21-30 necrotic cells per view); 4 (31-40 necrotic cells per view); 5 (41-50 necrotic cells per view). Inflammation was assessed as follows: 0 (absent); 1 (rare); 2 (scattered); 3 (scatted with localized foci); 4 (abundant with foci); 5 (extensive). Architectural change, fibrosis and cirrhosis were estimated as follows: 0 (absent); 1 (rare); 2 (scattered deposition); 3 (scatted with localized deposition); 4 (abundant with minor bridging fibrosis); 5 (bridging fibrosis); 6 (cirrhosis). A total pathology score was calculated by combining and summing the score for the above pathological parameters, and the average score per animal/treatment group was calculated.

Preparation of hepatic stellate cells

Hepatic stellate cells were prepared essentially as described previously[24,25]. Briefly, cirrhotic rats (350-380 g) were digested with protease (0.05%) and collagenase (0.025%). After removal of hepatocytes and cell debris by low speed centrifugation, hepatic stellate cells were purified by density gradient centrifugation on a 28.7% (W/V) Nycodenz gradient. The cells were suspended in DEME containing 10% fetal bovine serum and 10% horse serum, antibiotics, and plated in at a density of 0.5×10^6 cells/cm^2. The cells were used for experiments after an overnight incubation. The purity of the cells was determined by light microscopy and vitamin A autofluorescence. Further characterization was conducted immunohistochemically with specific markers for stellate cells (anti-desmin and -α-smooth muscle actin antibodies; DAKO) as described previously[25].

Determination of PAF receptors mRNA in hepatic stellate cells

The levels of the low abundance mRNA transcripts of PAF receptor were determined by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) essentially as described previously[8-13,26] β-actin mRNA expression was determined using the same amount of cDNA. RNA was isolated from the livers using an RNA isolation kit (ToTALLY RNA™, Ambion, Austin, TX) as per the instructions of the manufacturer. The concentration of RNA was determined by measuring A260, and the purity was checked by the A260/A280 ratio (greater than 1.8). 2 μg total RNA obtained from hepatic stellate cells was used for the preparation of cDNA by reverse transcriptase-PCR. The PCR Primers for PAF cDNA: 5'-GCCACAACACAGAGGCTTGA-3'(F) and 5'-TCCATTGCTCTGGGCAGGAA-3'(R) (121 bp); β-actin cDNA: 5'-TTCTACAATGAGCTGCGTGTG-3' (F) and 5'-TTCATGGATGCCACAGGATTC-3'(R) (561 bp) were used. cDNA equivalent of 5 ng of original RNA was used in PCR. The reaction mixture (50 μL) contained 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTPs, 20 pmol of PCR primer and 2 units of Platinum Taq DNA polymerase (GIBCO-Invitrogen, Carlsbad, CA). The reaction for PAF receptor was run for 35 cycles as follow: denaturation at 94℃ for 1 min, annealing at 60℃ for 30 s, extension at 72℃ for 30 s. The PCR products were resolved in a 2.5% agarose gel and stained with 1X SYBR Green I (FMC Biproduct, Rockland, ME). The gels were scanned under blue fluorescence light using a phosphorimager and the band intensity was quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA).

Determination of PAF receptor in hepatic stellate cells

The assay was performed as previously described[8-13,27,28]. After hepatic stellate cells incubated overnight were washed twice time with HBSS containing 10 mmol/L HEPES, pH7.4, and 0.1% bovine serum albumin (HBSS/BSA), and then incubated in 50 mmol/L Tris-HCl (pH 7.2 containing 5 mmol/L $MgCl_2$, 125 mmol/L choline chloride, 0.25% BSA) and 0.0125-3.2 nmol/L 1-*O*-[^{3}H]octa-decyl-2-*O*-acetyl-sn-glycero-3-phosphocholine (151 Ci/mmol, 9.96 GBq/mg; Amersham) ± 10 μmol/L unlabelled PAF (1-*O*-hexadecyl-2-*O*-acetyl-sn-glycero-3-phosphocholine; Bachem Americas, King of Prussia, PA) at 22℃ for 3 h, the reaction was terminated with the addition of 5 mL ice-cold assay buffer. The cells were washed twice with assay buffer and then digested with 5% sodium dodecyl sulfate kept at 60℃ for 30 min. Radioactivity was determined in β-cintillation. The results were expressed as CPM of ^{3}H -PAF bound per μg DNA.

Determination of prostaglandin E_2 (PGE_2)

PGE_2 was extracted from the culture medium with or without BN52021, and analyzed by ELISA with a kit from Amersham Pharmacia biotech, Inc. NJ, USA., following protocol 1 of the manufacturer's instructions.

Statistical analysis

The values are presented as averages of triplicate

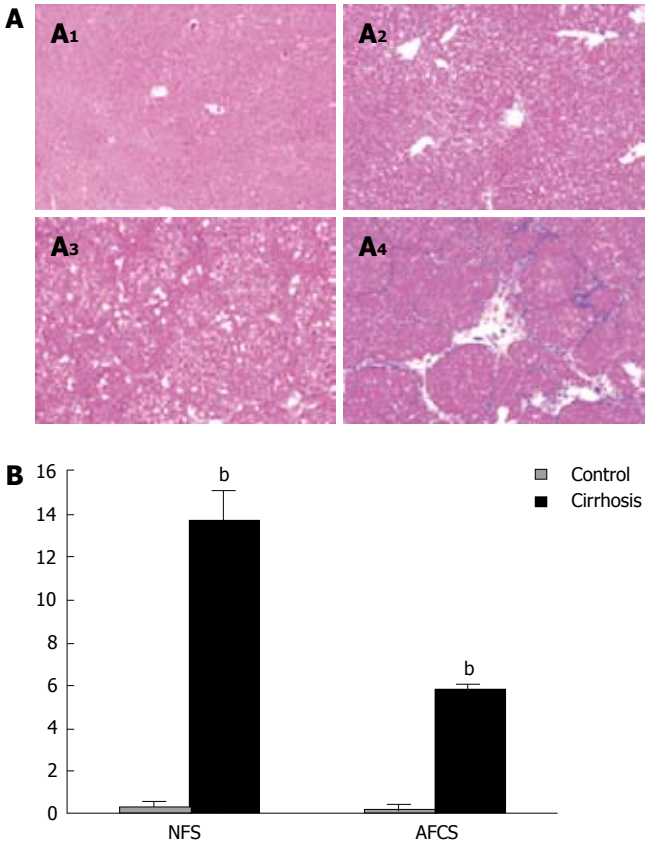

Figure 1 Morphometric analysis of the cirrhotic liver. **A**: After 8 wk of CCl_4 or vehicle treatment, liver tissue was fixed and stained with hematoxylin/eosin (**A1** and **A3**) as well as Masson's trichrome stain (**A2** and **A4**); **B**: Necroinflammatory score (NFS) and scores for architectural change, fibrosis and cirrhosis (AFCS) were determined as described in the Methods section. $^bP < 0.01$ *vs* control.

determinations ± SEM. Each experiment was repeated at least three times on cells from different animals. Student's *t*-test was employed for statistical comparison of the paired samples. A P value of < 0.05 was considered statistically significant.

RESULTS

General characteristics of cirrhosis

There were no notable changes in the liver histology through 8 wk in control rats (Figure $1A_1$). In contrast CCl_4-treated rats demonstrated extensive changes in liver morphology, including steatosis, inflammation, hepatocyte ballooning and necrosis. There was extensively distorted architecture due to excessive deposition of extracellular matrix that caused bridging fibrosis, and infiltration of inflammatory cells in the sinusoids and their accumulation around the islands of hepatocytes (Figure $1A_3$). Hepatic fibrosis was further validated histologically using Masson's trichrome stain (Figure $1A_2$ and $1A_4$).

Characterization of HSCs isolated from normal and cirrhotic livers

HSC_S viability was more than 95%. The desmin positive cell rate was (94.3% ± 3.8%). HSCs isolated from cirrhotic livers showed morphological characteristics of myofibroblasts with a spindle-like shape and low vitamin A droplets content. These cells highly expressed aSMA (Figure 2). In contrast, HSCs freshly isolated from normal rat livers showed a quiescent phenotype consisting of an oval shape and numerous vitamin A-rich droplets. These cells barely expressed aSMA.

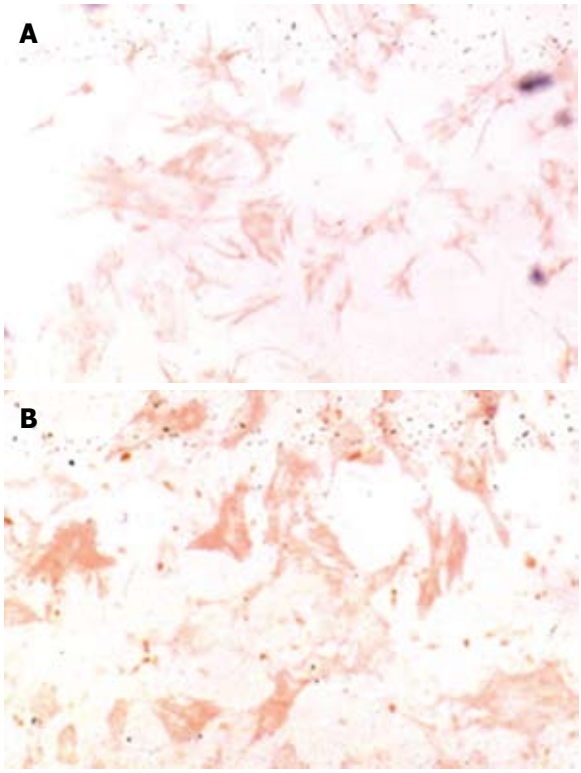

Figure 2 Expression of desmin and aSMA in HSCs freshly isolated from cirrhotic livers. HSCs from cirrhotic livers show immunostaining for desmin (**A**) and aSMA (**B**) (× 400).

Characters of PAF receptor expression in cirrhotic hepatic stellate cells

Scatchard plot analysis of the saturation binding data (Figure 3) revealed an about four-fold increase in ^{3}H-PAF binding capacity in hepatic stellate cells from cirrhotic rats as compared to that of the control (Bmax: 24.65 ± 1.96 fmol/μg. DNA, R = 0.982 *vs* 5.74 ± 1.55 fmol/μg. DNA, R = 0.93; $P < 0.01$). There was no significant difference in the PAF receptors affinity between the cells from control and those from cirrhotic rats (Kd of 4.66 ± 0.33 nmol/L for the cirrhosis and 3.51 ± 0.26 nmol/L for the control; $P > 0.05$). The data showed that the number of PAF receptor in cirrhotic hepatic stellate cells had a dramatic increase of about 3.3 folds compared to that of the control (0.40 ± 0.09 *vs* 0.12 ± 0.03, $P < 0.05$) (Figure 3). Consistent with hepatic PAF receptor saturation binding data, the mRNA expression of PAF receptor was also increased in the cirrhotic hepatic stellate cells (Figure 4).

Effect of PAF and its antagonist BN52021 on synthesis of prostaglandin E2 in cirrhotic hepatic stellate cells

PAF stimulated PGE_2 synthesis in a concentration-dependent manner both in control and cirrhotic hepatic stellate cells; the effect being more manifest in the latter. The date revealed an about 1.8-fold increase in hepatic

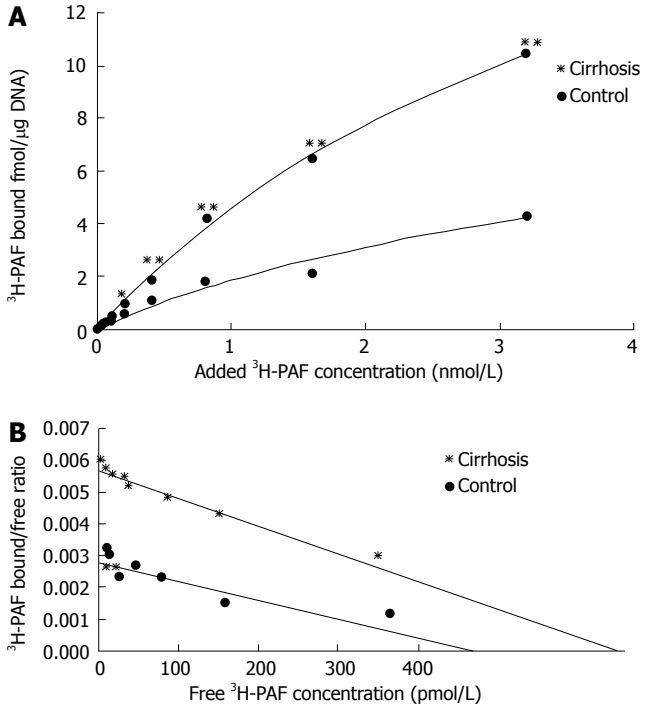

Figure 3 **A**: Saturation curve of ^{3}H-PAF binding to cultured cirrhotic hepatic stellate cells. ^{3}H-PAF in the concentration between 0.125 and 3.2 nmol/L,in presence or the absence of 5 μmol/L unlabeled incubated at 25℃ for 3 h; **B**: Scatchard plot analysis of binding of ^{3}H-PAF to cirrhotic hepatic stellate cells. Cirrhosis: Kd: 4.66 nmol/L, Bax: 24.65 ± 1.96 fmol/μg DNA, R = 0.982; Control: Kd: 3.51 nmol/L, Bax: 5.74 ± 1.55 fmol/μg DNA, R = 0.93.

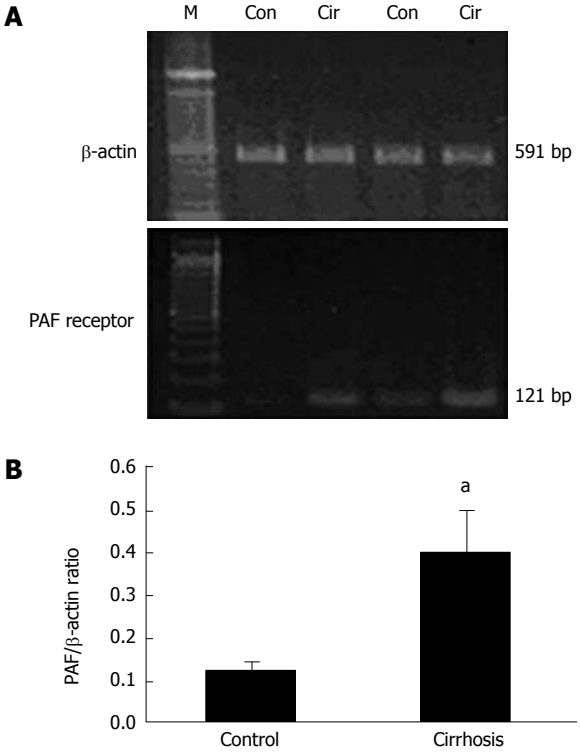

Figure 4 PAF receptor expression in cirrhotic hepatic stellate cells. RT-PCR of PAF receptor mRNA was performed with cDNA prepared from RNA samples of control and cirrhotic HSCs. Expression of β-actin mRNA was assessed using the same amout of cDNA. **A**: PCR products of PAF and β-actin from control (Con) and cirrhotic (Cir) rat livers are shown; **B**: Ratio of the PAF receptor and β-actin mRNA. $^aP < 0.05$ *vs* control.

stellate cells from cirrhotic rats compared with that from the control (3.89 ± 0.21 pg/μg. DNA *vs* 2.08 ± 0.13 pg/μg. DNA, $P < 0.01$) in 100 Nm (Figure 5).

Cells were placed in serum-free medium after overnight culture, and were challenged with PAF(100 nmol/L) ± BN52021 (5 nmol/L) 24 h later. PGE_2 synthesis in the medium was decreased with BN52021 both in control (2.08 ± 0.13 pg/μg DNA *vs* 1.02 ± 0.10 pg/μg. DNA, $P < 0.01$) and cirrhotic hepatic stellate cells (3.89 ± 0.21 pg/μg. DNA *vs* 1.28 ± 0.14 pg/μg. DNA, $P < 0.01$).

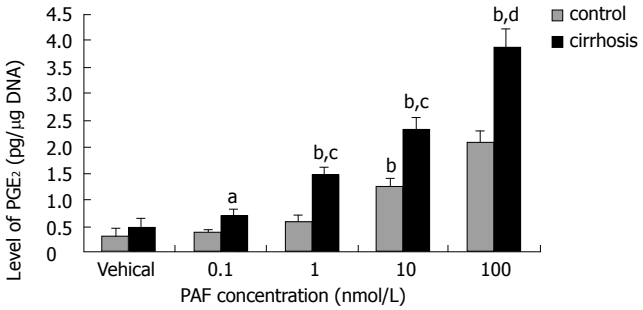

Figure 5 Effect of PAF concentration on PGE_2 synthesis in cirrhotic hepatic stellate cells. Cell were placed in serum-free medium after overnight culture, and were challenged with PAF 24 h later. PGE_2 in the medium was measured by ELISA after 15 min incubation. $^aP < 0.05$, $^bP < 0.01$ *vs* vehical; $^cP < 0.05$, $^dP < 0.01$ *vs* control.

DISCUSSION

In the present study, stellate cells from cirrhotic liver had a 3.3-fold increase in PAF receptor density and a 1.8-fold increase in PAF induced PGE_2 synthesis relative to control cells, suggesting cirrhosis sensitizes hepatic stellate cells to PAF and HSCs may be potential effectors of PAF induced portal hypertension. One of the major characteristics of liver cirrhosis is proliferation and activation of stellate cells, which are responsible for excessive deposition of fibrous tissue and the contractile component of portal hypertension. After acute or chronic liver injury, HSCs transdifferentiate to a transforming myofibroblastic phenotype, increasing contractility, proliferating, accumulating, and expressing a number of fibrogenic and proliferative cytokines and their cognate receptors[29-31]. Previous work showed PAF cause contraction in HSCs[17], but as there is on reliable assay system for normal stellate cell contractility, we measured PAF dependent PGE_2 synthesis in HSCs, which were increased in cirrhotic HSCs including PAF stimulated and basal synthesis. The existence of increased PAF receptor density in cirrhotic HSCs and PAF induced PGE_2 synthesis was blocked by its antagonist BN52021 suggest PAF act its biological effects by its specific receptors. It has been shown that PGE_2, a hepatoprotective eicosanoid, can inhibit growth factor induced proliferation of activated stellate cells by causing inhibition of MAP kinase activity[32]. So it is reasonable to assume that enhanced synthesis of PGE_2 (basal as well as PAF stimulated) in cirrhosis may be a homeostatic mechanism limiting the contractile response.

Previous study showed the increased PAF in the

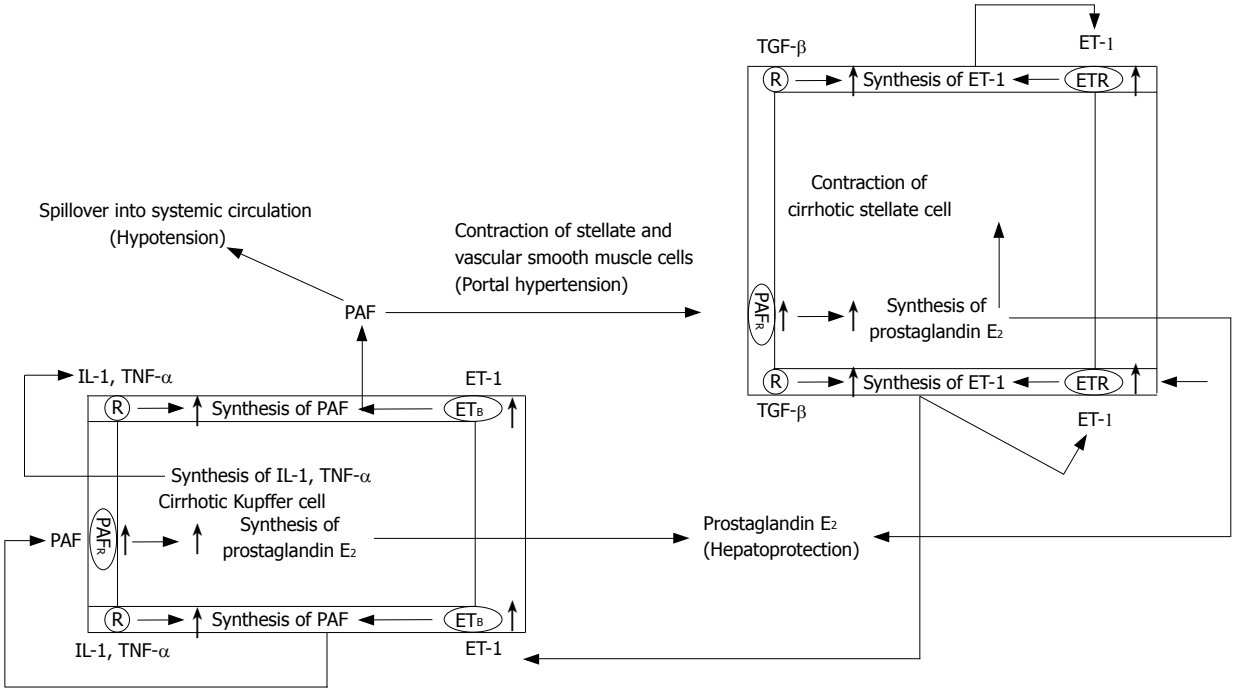

Figure 6 Implication of platelate-activating factor, ET-1, Kupffer cells and hepatic stellates cells in the pathology of liver cirrhosis. Increased ET-1 released by stellates and endothelial cells in the cirrhosis liver acts on upregulated ET_B receptors in Kupffer cells and stimulates the synthesis of PAF. Likewise, autocrine actions of proinflammatory mediator such as IL-1 and TNF-α may also stimulate PAF synthesis by Kupffer cells. PAF then acts on hepatic vascular smooth muscle cells and stellate cells and contributes to portal hypertension by causing their contraction. Its spillover into systemic circulation is likely a cause of hypertension associated with cirrhosis. Autocrine PAF and paracrine ET-1 can act on their respective upregulated receptors in Kupffer cells and stellate cells to cause the synthesis and release of prostaglandin E_2, which in turn may act to limit the liver injury.

cirrhotic liver due to its enhanced synthesis by the Kupffer cells, with ET-1 playing a major role. Endothelin-1 (ET-1) stimulates PAF synthesis by Kupffer cells[13,33]. Cirrhotic Kupffer cells also had elevated densities of functional receptors for both PAF and ET-1 (exclusively ETB)[12,13,33]. By respective receptor, both ET-1 and PAF were shown to stimulate PGE_2 synthesis in Kupffer cells[34,35]. Since both PAF and ET-1 individually cause portal hypertension, an increase in ET-1-stimulated PAF synthesis in Kupffer cells was to exacerbate the hepatic and extrahepatic complications of cirrhosis. As activated stellate cells also synthesize greater amounts of ET-1[36,37], the implication of platelate-activating factor, ET-1, Kupffer Cells and hepatic stellate cells in the pathology of liver cirrhosis is summarized in a schematics (Figure 6).

In conclusion, the findings of this paper shows upregulated PAF receptors in cirrhotic HSCs, by which PAF induce PGE_2 synthesis, suggesting HSCs may be potential effectors of PAF induced portal hypertension. PAF is an integral part of portal hypertension.

COMMENTS

Background

Platelet activating factor (PAF) is a potent hepatic vasoconstrictor and systemic vasodilator. In a parallel study, the increased hepatic and circulating PAF concentrations in cirrhotic rats was observed, which contributes to portal hypertension. The potential effectors of PAF induced increase in portal pressure remains undetermined. Hepatic stellate cells play a crucial role in the development of liver fibrosis. So we hypothesized the HSCs may be potential effectors of PAF induced increases in portal pressure. Therefore, PAF receptors and PAF-induced prostaglandin E_2 release derived from cirrhotic hepatic stellate cells were investigated.

Research frontiers

This study demonstrated upregulated PAF receptors in cirrhotic HSCs, suggesting HSCs may be potential effectors of PAF induced portal hypertension.

Innovations and breakthroughs

This study explained one of the possible mechanisms of development of portal hypertension in CCl_4-induced cirrhotic rats, showing that hepatic stellate cells may be potential effectors of PAF induced portal hypertension.

Applications

In this study, the implication of Platelate-activating Factor and Hepatic Stellate Cells in the pathology of liver cirrhosis was explored. Upregulated PAF receptors in cirrhotic HSCs were determined. So it is a strategy to block the interaction of PAF and HSCs for treatment of portal hypertension.

Terminology

The existence of PAF receptor in HSCs suggests HSCs may be potential effectors of PAF induced portal hypertension. PAF acts its biological activities by its receptor.

Peer review

This study determined upregulated PAF receptors in cirrhotic HSCs and explained the implication of PAF and HSCs in the pathology of liver cirrhosis. It is a strategy to block the interaction of PAF and HSCs for treatment of portal hypertension. It was thought to be very innovative and deserved to be published.

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