Peroxynitrite-mediated matrix metalloproteinase-2 activation in human hepatic stellate cells

Kiyoshi Migita\textsuperscript{a,*}, Yumi Maeda\textsuperscript{a}, Seigo Abiru\textsuperscript{a}, Atsumasa Komori\textsuperscript{a}, Terufumi Yokoyama\textsuperscript{a}, Yasushi Takii\textsuperscript{a}, Minoru Nakamura\textsuperscript{a}, Hiroshi Yatsuhashi\textsuperscript{a}, Katsumi Eguchi\textsuperscript{b}, Hiromi Ishibashi\textsuperscript{a}

\textsuperscript{a} Clinical Research Center, NHO Nagasaki Medical Center, Kabara 2-1001-1, Onura 856-8562, Japan
\textsuperscript{b} First Department of Internal Medicine, Nagasaki University School of Medicine, Japan

Received 24 December 2004; revised 29 March 2005; accepted 1 April 2005
Available online 12 May 2005

Abstract To investigate whether hepatic stellate cells (HSCs) alter their expression of MMPs after exposure to nitrogen oxide intermediate (NOI), a human hepatic stellate cell line, LI90 cells, was stimulated with an NO donor, SNAP, or a peroxynitrite donor, SIN-1, and the culture supernatants were analyzed by gelatin zymography or anti-MMPs immunoblot. Although SIN-1 did not enhance the secretions of MMP-1 and 13, SIN-1 induced the NF-\textit{k}B activation, MT1-MMP expression and the secretion of activated MMP-2 in LI90 cells. These results suggest that peroxynitrite may contribute to the remodeling of the extracellular matrix in liver by activating pro-MMP-2.

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Keywords: Hepatic stellate cell; Peroxynitrite; Matrix metalloproteinase; Nitric oxide

1. Introduction

Liver fibrosis occurs following most chronic liver injuries and results from changes in the balance between the synthesis and degradation of extracellular matrix (ECM) components [1,2]. Hepatic stellate cells (HSCs) play a pivotal role in the cellular and molecular events that lead to fibrosis [3]. Following liver injury, these cells undergo changes toward a myofibroblastic-like phenotype and secrete extracellular matrix components [4]. In addition, activated HSCs are involved in ECM degradation by providing MMPs [5]. During liver fibrogenesis, a number of MMPs, such as MMP-1, MMP-2, and MMP-13, are expressed in the liver by hepatic stellate cells [6]. Therefore, it has been suggested that the activation mechanism of HSCs and the secretion of MMPs may contribute to the process of liver fibrosis [7].

Evidence of oxidative stress has also been detected in clinical condition of liver fibrosis with different etiology [8]. It has become obvious that an increase in the levels of reactive oxygen species (ROS) occurs in the process of liver fibrosis [9]. ROS may represent direct or indirect pro-fibrogenic stimuli for HSCs [10]. Moreover, signs of oxidative stress and lipid peroxidation are concomitant or precede HSCs activation and collagen deposition [11]. It was demonstrated that HSCs express non-phagocytic NADPH oxidase and inducible NO synthase (iNOS) [12], which generate superoxide and NO, once exposed to effective stimuli. Furthermore, the simultaneous production of NO and superoxide by HSCs or Kupffer cells in liver may lead to the generation of peroxynitrite (ONOO\textsuperscript{−}) [13]. The present study was designed to more clearly elucidate the biological effects elicited by oxidative stress on HSCs. In particular, we investigated whether nitrogen oxide intermediate (NOI) may affect the synthesis of MMPs and their related inhibitors (TIMP-1 and TIMP2) in HSCs.

2. Materials and methods

2.1. Reagents

An NO donor, SNAP (\textit{S}-nitroso-\textit{N}-acetyl-DL-penicillamine), and a peroxynitrite donor, SIN-1 (\textit{N}-morpholino sydnonimine hydrochloride), were purchased from Dojindo Laboratories (Kumamoto, Japan). Mouse anti-human membrane-type 1 matrix metalloproteinase (MT1-MMP) monoclonal antibody and mouse anti-human tissue inhibitor metalloproteinase–1 (TIMP-1) and TIMP-2 antibodies were obtained from Sigma Chemical Co. (Takatsuki, Japan). Mouse monoclonal anti-\textit{k}B-smooth muscle actin antibody was purchased from Novacast Laboratories (Newcastle, UK). Mouse monoclonal anti-nitrotyrosine antibody (1A6) was purchased from UBI (Lake Placid, USA). Purified pro-MMP-2 was purchased from Calbiochem (San Diego, USA). Activation with p-aminophenylmercuric acetate (APMA, Sigma Chemical) was done by incubation of purified pro-MMP-2 with 1 mM APMA at 37 °C for 15 min [14]. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Cells

LI90 cells, human HSCs established by Dr. Murakami, were purchased by JCRB (Japanese Collection of Research Resources, Osaka, Japan). Cells were propagated in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). LI90 cells were stimulated with SIN-1 at different concentrations (0–500 \textmu M) for 24 h in serum-free DMEM. Culture supernatants were subjected to the analysis for gelatin zymography or immunoblot.

2.3. Gelatin zymography

LI90 cells-conditioned culture media (serum-free) were incubated at 37 °C for 20 min in sodium dodecyl sulfate (SDS) sample buffer free of reducing agents and then electrophoresed on 8% polyacrylamide gels containing 0.5 mg/ml gelatin at 4 °C. After electrophoresis, gels were...
washed in 2.5% Triton-X 100 to remove SDS, and incubated with 50 mM Tris–HCl buffer (pH 7.5) containing 0.15 M NaCl, 10 mM CaCl₂, and 0.02% NaN₃ for 16 h at 37 °C, and stained with 0.1% Coomassie Blue R250.

2.4. Immunoblot analysis

The expression of MT1-MMP on L910 cells and the secretions of MMP-1, MMP-13 and TIMPs from synovial cells were analyzed by immunoblot. For this purpose, cells were washed with cold PBS and lysed by the addition of a lysis buffer containing 1% Nonidet P-40, 50 mM Tris (pH 7.5), 100 mM NaCl, 5 mM EDTA, 10 μg/ml apro- tinin, and 10 μg/ml leupeptin for 20 min at 4 °C. Insoluble materials were removed by centrifugation at 1500 × g for 15 min at 4 °C. The supernatant was saved and the protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). An identical amount of protein (30 μg) from each lysate or the culture supernatant was subjected to 8% SDS-polyacrylamide gel electrophoresis (PAGE). The fractionated proteins were transferred to nitrocellulose membranes (Amersham, Arlington Heights, IL), and the filters were blocked for 1.5 h using non-fat dried milk in Tris-buffered saline (TBS: 50 mM Tris, 0.15 M NaCl, pH 7.5) containing 0.1% Tween 20, washed with TBS, and incubated at room temperature for 2 h at a 1:150 dilution of mouse anti-MT1-MMP or MMP-13 monoclonal antibodies (mAbs). The membranes were further incubated with a 1:2000 dilution of horseradish peroxidase-conjugated donkey anti-mouse immunoglobulin G (IgG) antibody (Promega, Madison, WI) for 20 min. An enhanced chemiluminescence (ECL) system (Amersham) was used for detection. The blots were analyzed by densitometer (Fuji Film, Tokyo, Japan). Images from densitometer were transferred to a personal computer and analyzed using Science Lab software (Fuji Film). The density of the target protein band of untreated cells was assigned the value of 1.0 and each result was calculated as relative units.

2.5. RNA preparation and quantitative PCR assay

Total cellular RNA was extracted from L910 cells using guani- dium thiocyanate and phenol (RNAzol B, Cinna/Biotek Labs Int., Inc., Friendswood, TX). First-strand cDNA was synthesized by reverse transcription at 45 °C for 45 min in a 50 μl reaction mixture containing 1 μg of total RNA and M-MuLV reverse transcriptase (Invitrogen). After denaturing at 99 °C for 5 min followed by cooling at 5 °C, the cDNA was amplified using PCR. The quantification of MT1-MMP transcripts in L910 cells was carried out with the LightCycler PCR system (Roche Diagnostics, Meylan, France) using the DNA binding SYBR Green I dye for the detection of PCR products. A serial dilution was used to generate each standard curve. For real time quantitative PCR, each reaction contained: 1× of LightCycler DNA master SYBR Green I, specific primer pair (Roche), and 2 μl of cDNA matrix, in a final volume of 20 μl. After 2 min of denaturation at 95 °C, the reactions were cycled 40 times for 5 s at 95 °C, 10 s at the annealing temperature, and 15 s or 7 s at 72 °C for MT1-MMP and GAPDH, respectively. Product specificity was determined by melting curve analysis as described in the LightCycler handbook. The amount of transcripts in each sample is given as copy number. The results are expressed as ratios of MT1-MMP transcripts to GAPDH transcripts.

2.6. Plasmids and transfection

pNFX-b-SEAP (CLONTECH Laboratories, Inc., Palo Alto, CA) is designed to monitor the activation of NF-κB and NF-κB-mediated signal transduction pathways. pNFX-b-SEAP contains four tandem copies of the κB4 enhancer fused to the HSV-TK promoter. pTAL-SEAP (CLONTECH Laboratories) was used as a negative control to determine the background signals associated with the culture medium. Cells were grown to approximately 50% confluence on 30-mm plates. Transfections were done using the calcium phosphate reagents and following the manufacturer’s instructions (CLONTECH Laboratories), and cells were treated as described above. Chemiluminescence detection of SEAP activity was performed according to the manufacturer’s instructions (CLONTECH Laboratories) using a plate fluorometer (Berthold, Bad Wildbach, Germany).

2.7. Statistical analysis

Differences in the data were analyzed by 1-way ANOVA combined with the Bonferroni’s test, and all values were expressed as means ± S.D. The differences between groups were considered to be significant at P < 0.05.

3. Results

The effects of NO to human hepatic stellate cells were investigated by incubating L910 cells with an NO donor, SNAP, which generates NO in vitro. As shown in Fig. 1, SNAP treatment did not modulate the gelatinase secretions from L910 cells. Next, we incubated the cells with SIN-1, which generates ONOO⁻ by releasing NO and O₂⁻ simultaneously for 24 h and the culture supernatants were subjected to gelatin zymography. SIN-1 was not toxic to L910 cells at the concentrations (~500 μM) used in this experiment (data not shown). Gelatin zymography demonstrated that in addition to the secretion of a late form of MMP-2 (Mᵦ = 72 kd), the activated form of MMP-2 (Mᵦ = 66 kd) was detected when L910 cells were treated with SIN-1 (Fig. 2A and B). This gelatinolytic band with lower molecular weight (Mᵦ = 66 kd) was similar to APMA-activated MMP-2 in that it migrated at 66 kd (Fig. 2C). Also, these gelatinolytic activities were completely inhibited by EDTA demonstrating that these are metalloproteinases (Fig. 2D). In contrast, the SIN-1 treatment did not significantly affect the secretion of MMP-1 (Fig. 3A and B) and MMP-13 (Fig. 3C and D).

It was demonstrated that peroxynitrite is capable of degrading TIMP-1, a process which could potentiate MMP-2 activation [15]. Therefore, we examined the effects of SIN-1 on TIMP-1 secretion from L910 cells. The molecular forms of TIMP-1 and its secretion levels were not affected by SIN-1 treatment (Fig. 3E and F). TIMP-2 was not detected in L910-conditioned media with or without SIN-1 treatment by anti-TIMP-2 immunoblot analysis (data not shown).

Because peroxynitrite has the functional property of inducing nitration, we examined whether SIN-1-treated cells contain proteins with nitrated tyrosine residues [16]. The level of tyrosine nitration was clearly increased after 4 h of treatment with SIN-1. This effect was smaller in SNAP-treated L910 cells (Fig. 4A). Expression of α-smooth muscle actin (α-SMA) is considered as an indicator of HSCs activation [17]. The expression of α-SMA was also examined using L910 cells treated with SIN-1.

Fig. 1. Effects of SNAP on gelatinase secretions from L910 cells. L910 cells were cultured with SANP in serum-free culture media for 24 h. The conditioned media were analyzed by gelatin zymography. The data shown are representatives of at least three independent experiments.
for 24 h. SIN-1 treatment resulted in an increased α-SMA expression on LI90 cells (Fig. 4B and C).

As shown in Fig. 5, the incubation of LI90 cells-conditioned media containing pro-MMP-2 with SIN-1 for 24 h did not result in the activation of pro-MMP-2. This result indicates that SIN-1-induced pro-MMP-2 activation requires the presence of pro-MMP-2 producing IL90 cells. It has been suggested that the conversion of pro-MMP-2 to active MMP-2 requires cell membrane-associated processing by membrane-type matrix metalloproteinase (MT-MMP) [18]. Therefore, we examined whether MT-MMP is involved in the SIN-1-induced pro-MMP-2 activation. LI90 cells were cultured with SIN-1 for
24 h. The cells were lysed and the lysates were subjected to immunoblot using antibodies to human MT1-MMP. The band of MT1-MMP of untreated cells was assigned the value of 1.0 and data were expressed as the relative units. (B) MT1-MMP bands were calculated as a relative unit by densitometer. The band of untreated cells was assigned the value of 1.0 and data were expressed as the relative units. These data represent means ± S.D. of three independent experiments. *P < 0.01 versus untreated LI90 cells.

We further investigated the peroxynitrite-mediated signaling pathway in LI90 cells. We examined the effects of SIN-1 on the state of ERK1/2, p38 and JNK1/2. The phosphorylation of these MAPKs was examined by immunoblot using anti-phospho-specific MAPKs antibodies. The phosphorylation of ERK1/2, p38 and JNK1/2 was not increased but rather decreased in SIN-1-treated LI90 cells (Fig. 8A–F). Immunoblot of β-actin is shown as a control for equal loading of samples (Fig. 8G). We also evaluated IkB-α proteolysis. The protein levels of IkB-α in SIN-1-treated LI90 were measured by immunoblot analysis (Fig. 9A and B). The SIN-1 activated form of MMP-2 in SIN-1-treated LI90 cells-conditioned media (Fig. 2A and B).

We investigated the in vitro effects of SIN-1 on gelatinase. LI90 cells-conditioned media were incubated with SIN-1 for 24 h at 37 °C. Incubated media were analyzed by gelatin zymography. The data shown are representatives of at least three independent experiments.
stimulation induced substantial IκB-α degradation in a time-dependent manner, suggesting the activation of NF-κB in LI90 cells. However, the SNAP treatment did not affect the protein levels of IκB-α. To determine whether SIN-1 treatment modulates the transcriptional activity of NF-κB, LI90 cells were transiently transfected with a reporter gene construct, pNFκB-SEAP. Cells were subsequently treated with SIN-1, and SEAP in the culture supernatants was assayed. As shown in Fig. 10A, SIN-1 treatment increased the SEAP levels compared to those of control cells. SNAP treatment did not activate the NF-κB transcriptional activity (Fig. 10B).

4. Discussion

Hepatic stellate cells (HSCs) play a central role in the development of liver fibrosis [3]. After chronic liver injury, HSCs proliferate and transform into myofibroblastic cells (activated HSCs) that are the major source of collagens that accumulate in the fibrotic liver [4]. This process is promoted by a variety of cytokines and soluble factors released by Kupffer cells and inflammatory cells as well as HSCs [19]. Mayoral et al. [20] demonstrated an increased hepatic expression of iNOS in bile duct ligated rats, model animals for hepatic fibrosis. Furthermore, stimulation of NO synthesis with L-arginine treatment augmented hepatic fibrosis. These results suggest that NO may be involved in the development of fibrosis. We found that peroxynitrite induces the secretion of activated MMP-2 in cultured human HSCs.

A number of studies have emphasized the profibrogenic functions of MMP-2 [21,22]. However, the exact mechanisms leading to MMP-2 activation have not been clearly delineated. The MMPs activity is closely regulated at 3 levels, namely gene transcription, proteolytic activation, and inhibition by endogenous inhibitors [23]. As well, proteolytic activation of MMP-2 is induced by membrane-type MMPs (MT-MMP) [24]. Previous reports suggest that MT1-MMP-activated MMP-2 exerted a profibrogenic role during hepatic fibrosis [25]. It is possible...
that peroxynitrite generated by SIN-1 directly nitrates MMP-2 and induces its enzymatic activation. However, SIN-1 did not activate pro-MMP-2 in the absence of HSCs in vitro, suggesting that the interaction between peroxynitrite and HSCs is required for this MMP-2 activation.

The hepatic expression of MMP-2 and MT1-MMP is increased in human fibrotic livers, and activated HSCs have been identified as a source of both proteins [26,27]. Therefore, we investigated whether the SIN-1 treatment increases MT1-MMP expression in HSCs. Our data showed that MMP-2 activation in SIN-1-treated HSCs is associated with the increased expression of MT1-MMP by these cells. In response to peroxynitrite, HSCs may express MT1-MMP on their surface thereby stimulating pro-MMP-2 activation. The mechanism leading to MT1-MMP expression in SIN-1-treated HSCs is not known. The finding that SIN-1 leads to the rapid degradation of IκBα suggests that the peroxynitrite-mediated NF-κB dependent pathway might be involved in the MT1-MMP induction as reported previously [28,29].

Oxidative stress has been detected in fibrotic liver and acts as a mediator of the ECM remodeling responsible for liver fibrosis [30]. In liver injury, iNOS is upregulated in liver cell populations including HSCs, and the NO generation is increased [31]. The pathogenic action of NO seems to occur as the interaction of NO with O₂⁻ to generate peroxynitrite. The effects of peroxynitrite can be deleterious, since it induces the oxidation and nitration of biological macromolecules [32]. Our data suggest that peroxynitrite may activate MMP-2 secreted from HSCs by inducing MT1-MMP. ECM remodeling in fibrotic liver could be accelerated by MMP-2 activation, because this enzyme degrades the normal subendothelial matrix, hastening its replacement by collagens [33]. The result of this investigation may help to elucidate the mechanism by which oxidative stress activates MMP-2 in the development of liver fibrosis.

In conclusion, our study shows that peroxynitrite promotes the activation of MMP-2 secreted by human hepatic stellate cell (HSC) line and that this activation is at least in part a result of MT1-MMP induction. These findings suggest that oxidative stress represents pro-fibrogenic stimuli for HSCs leading to MT1-MMP expression, thereby stimulating MMP-2 activation and ECM remodeling in hepatic fibrosis development.

References


