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Induced secretion of tissue inhibitor of metalloproteinases-1 (TIMP-1) in vivo and in vitro by hepatotoxin rubratoxin B

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Abstract

To elucidate the mechanism of rubratoxin B toxicity, we investigated rubratoxin B-induced secretion of tissue inhibitor of metalloproteinases-1 (TIMP-1) in mice and cultured cells; we also documented the involvement of stress-activated MAP kinases (c-Jun-N-terminal kinases [JNKs] and p38s) in this process. Rubratoxin B significantly ($P < 0.05$) induced serum TIMP-1 levels in mice. Because TIMP-1 is thought to play a crucial role in the process of liver fibrosis, rubratoxin B may cause liver fibrosis. Rubratoxin B enhanced TIMP-1 secretion in HepG2 cells to a peak level of ~40 μg/ml. The amount of TIMP-1 mRNA increased with the duration of rubratoxin B treatment; and this hepatotoxin appears to induce TIMP-1 secretion through a transcriptional control mechanism. Unlike similar treatment with rubratoxin B and JNK inhibitor, concomitant treatment with rubratoxin B and p38 inhibitor increased rubratoxin B-induced TIMP-1 secretion, suggesting that p38s (but not JNKs) antagonize this process. In addition, treatment with p38 inhibitor slightly increased the amount of rubratoxin B-induced TIMP-1 mRNA, suggesting that p38s control rubratoxin B-induced TIMP-1 secretion chiefly post-transcriptionally. In this study, we showed that rubratoxin B induces TIMP-1 production in vivo and in vitro and that p38s antagonize rubratoxin B-induced TIMP-1 secretion.

Keywords: Rubratoxin B; Tissue inhibitor of metalloproteinases (TIMP)-1; Biomarker; Acetaminophen; Stress-activated MAP kinase (SAPK)

1. Introduction

Rubratoxin B is a potent hepatotoxic mycotoxin (Burnside et al., 1957; Natori et al., 1970; Richer et al., 1997) produced by certain *Penicillium* fungi (Natori et al., 1970; Richer et al., 1997; Moss et al., 1968; Wilson and Harbison, 1973). Human rubratoxicosis has been reported (Richer et al., 1997), indicating that rubratoxin B can be a threat to human health. We previously reported that rubratoxin B hinders cell proliferation (Nagashima, 1996; Nagashima and Goto, 2000) and induces apoptosis (Nagashima and Goto, 1998; Nagashima et al., 1998, 2004). In addition to its cytotoxic effects, rubratoxin B induces the secretion of several cytokines into the media of HL60, HepG2, and HuH-7 cells (Nagashima et al., 2001b, 2003). Regarding in vivo studies, treatment with rubratoxin B led to increased serum levels of interleukin (IL)-6 in mice (Nagashima et al., 2001a; Iwashita and Nagashima, 2005), and we recently found that rubratoxin B causes fatty change in liver in mice (Iwashita and Nagashima, 2005).

Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a 28-kDa glycoprotein that originally was characterized as a specific inhibitor of matrix metalloproteinases (MMPs) (Cawston et al., 1981) but is now known to be a multifunctional protein. TIMP-1 has numerous physiological effects, including inhibition of metastasis and invasion of cancer cells (Schultz et al., 1988), promotion of cell proliferation

Abbreviations: DMSO, dimethyl sulfoxide; IL-6, interleukin-6; IL-8, interleukin-8; JNK, c-Jun-N-terminal kinase; MMP, matrix metalloproteinase; RT-PCR, reverse transcription-polymerase chain reaction; SAPK, stress-activated MAP kinase; TIMP-1, tissue inhibitor of metalloproteinases-1.

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(Hayakawa et al., 1992), inhibition of angiogenesis (Johnson et al., 1994), and inhibition of apoptosis (Guedez et al., 1998). In addition, TIMP-1 is considered to contribute to liver fibrosis (Arthur, 1995; Olaso and Friedman, 1998).

Because it is well known that fatty liver disease often proceeds to liver fibrosis, rubratoxin B-treated mice may show some of the early warning signs of liver fibrosis. In the present study, therefore, to elucidate the molecular mechanism of rubratoxin B toxicity, we investigated the effect of rubratoxin B on TIMP-1 levels in mice and cultured cells. Moreover, we documented the involvement of stress-activated MAP kinases (SAPKs), which mediate numerous biological responses to external stressors (Davis, 2000; Chang and Karin, 2001), in the rubratoxin B-associated induction of TIMP-1.

2. Materials and methods

2.1. Chemicals and cells

Rubratoxin B, acetaminophen, and SB203580 (Cuenda et al., 1995) were purchased from Sigma–Aldrich (St. Louis, MO, USA). SP600125 (Bennett et al., 2001) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). These four chemicals were dissolved in dimethyl sulfoxide. The human hepatoblastoma line HepG2 was purchased from the RIKEN Cell Bank (Tsukuba, Japan) and cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal calf serum.

2.2. Mice and in vivo assays

Inbred 9-week-old male C3H/HeNCrj (C3H/He) mice were purchased from Charles River Japan (Yokohama, Japan). The animals were delivered to the specific pathogen-free facility at the National Food Research Institute (Tsukuba, Japan), where they were acclimated for 5 days prior to experiments. The mice were housed three per cage in an air-conditioned room maintained at 23–25 °C and 50–60% relative humidity and on a 12:12-h light:dark cycle (lights on, 08:00). The animals were given standard rodent chow (NMF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum. The experiments were conducted according to institutional ethical guidelines for animal experiments.

Rubratoxin B (dose, 1.5 mg/kg) and acetaminophen (dose, 500 mg/kg) were injected intraperitoneally into mice (weight, 20–25 g); mice treated with vehicle only served as controls. Serum samples were collected 24 h after treatment, and their TIMP-1 levels were quantified using the Quantikine Mouse TIMP-1 Immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.3. TIMP-1 secretion from HepG2 cells

Approximately 1×10^5 cells in 0.5 ml of medium (with or without chemical[s]) were cultured in each well of a 24-well culture plate for 24 h. Media then were collected and centrifuged at $5000 \times g$ for 5 min to remove cells and debris, and the levels of TIMP-1 in the supernatants were quantified using the TIMP-1 Kit (Daiichi Fine Chemical Co. Ltd., Takaoka, Japan) according to the manufacturer's recommended procedure.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

HepG2 cells (2×10^6 cells) in 10 ml of medium (with or without chemical[s]) were cultured in 10-cm plates. RNA was extracted with an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Total RNA (1 μg) underwent RT-PCR amplification by using the SuperScript One-Step RT-PCR with Platinum *Taq* kit (Invitrogen Corporation, Carlsbad, CA, USA). Primers used were those for human TIMP-1 (R&D Systems) and human β-actin (β-actin Primer Set for RT-PCR, Stratagene, La Jolla, CA, USA).

The conditions for RT-PCR amplification of TIMP-1 mRNA were 20 min at 50 °C for reverse transcription; 4 min at 94 °C for initial denaturation; 30 cycles of 45 s at 94 °C for denaturation, 45 s at 60 °C for primer annealing, and 45 s at 70 °C for primer extension; and 10 min at 72 °C for final extension. The conditions for RT-PCR amplification of β-actin mRNA were 20 min at 50 °C for reverse transcription; 2 min at 94 °C for initial denaturation; 25 cycles of 15 s at 94 °C for denaturation, 30 s at 60 °C for primer annealing, and 50 s at 70 °C for primer extension; and 10 min at 72 °C for final extension. Manufacturer of β-actin primers recommends 35 cycles of PCR reaction. However, we repeated RT-PCR experiments and set optimum number of cycle (25 cycles) for β-actin mRNA. The TIMP-1 product was 396 bp long, and that for β-actin was 661 bp. RT-PCR products were electrophoresed on 2% agarose gels and visualized by ethidium bromide staining. For quantification, the appropriate bands were analyzed with a densitometer (Densitograph AE-6920, Atto Corporation, Tokyo, Japan).

2.5. Statistics

Data are expressed as mean ± 1 standard deviation. Differences between groups were analyzed using the two-tailed Student's *t*-test, Tukey's test, or Dunnett's test, and synergism was evaluated using two-way analysis of variance with replication. If statistically significant interaction between groups was present, we judged that the effects of two chemicals were synergistic. A P value of <0.05 was considered to be significant.

3. Results

3.1. Rubratoxin B-induced serum TIMP-1 levels in mice

Rubratoxin B significantly ($P = 1.45 \times 10^{-6}$) induced serum levels of TIMP-1 in mice (Table 1). To evaluate whether TIMP-1 might be used as a biomarker of hepatic injury, we investigated the effect of a sub-lethal dose of acetaminophen (500 mg/kg), one of the most common hepatotoxic chemicals, on serum TIMP-1 levels. Acetaminophen significantly ($P = 1.44 \times 10^{-4}$) induced serum levels of TIMP-1 (Table 1), similar to those seen with rubratoxin B. To our knowledge, this is the first report of acetaminophen-associated induction of serum TIMP-1 in animals.

3.2. Rubratoxin B-induced TIMP-1 secretion from HepG2 cells

To explore the mechanism of rubratoxin B-associated induction of TIMP-1 production in detail, we investigated the effect of rubratoxin B by using the hepatoma line

Table 1
Secretion of TIMP-1 in rubratoxin B- and acetaminophen-treated mice

	TIMP-1 (ng/ml)	
	Rubratoxin B	Acetaminophen
Control	5.8 ± 5.4	1.9 ± 0.4
Treated	44.1 ± 7.7*	12.2 ± 3.6*

Results are means ± SD ($n = 6$). The doses of rubratoxin B and acetaminophen were 1.5 mg/kg and 500 mg/kg, respectively. Differences were analyzed using the two-tailed Student's *t*-test. The symbol * represents statistical significance ($P < 0.05$).