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Metalloproteinase inhibitor prevents hepatic injury in endotoxemic mice

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Abstract

This study was conducted to examine of [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(phenylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide (GI 129471), a matrix metalloproteinase inhibitor, for its effects on increase of serum pro-inflammatory cytokine levels as well as hepatic injury in D-galactosamine plus lipopolysaccharide-injected mice. In vitro experiments showed that GI 129471 was able to inhibit the elevation of tumor necrosis factor-α (TNF-α) in LPS-stimulated human and mouse whole blood with IC_{50} values of 370 nM and 260 nM, respectively. When administrated i.p. at 40 mg/kg, GI 129471 significantly reduced serum TNF-α level but not other pro-inflammatory cytokines in D-galactosamine plus lipopolysaccharide-injected mice. Treatment of mice with GI 129471 also reduced biochemical indices of hepatic injury to the normal level. Histopathological findings indicated that GI 129471 treatment can prevent severe centrilobular necrosis in liver. These results suggest that release of TNF-α from lipopolysaccharide-stimulated cells is the critical step leading to hepatic injury in endotoxemia and that a matrix metalloproteinase inhibitor with an inhibitory action on this step may be a promising drug for the clinical treatment of endotoxemia accompanied by hepatic injury. © 1998 Elsevier Science B.V.

Keywords: TNF-α (Tumor necrosis factor-α); Metalloproteinase inhibitor; Lipopolysaccharide; Hepatic injury; GI 129471

1. Introduction

Tumor necrosis factor-α (TNF-α) is a potent pro-inflammatory cytokine produced primarily by monocytes or macrophages in response to inflammatory stimuli such as bacterial lipopolysaccharide (Vassalli, 1992). Although TNF-α plays an important role in physiological defense responses, excessive or prolonged production of TNF-α is believed to cause severe damage to the host organism. Recently, TNF-α has emerged as a critical mediator of septic shock and of the systemic inflammatory response syndrome since sepsis and experimental endotoxemia are accompanied by a marked increase in plasma TNF-α levels (Michie et al., 1988; Damas et al., 1989; Calandra et al., 1990). More directly, administration of recombinant TNF-α to animals causes shock and tissue injury (Tracey et al., 1986; Remick et al., 1987). The physiological importance of TNF-α in endotoxemia is also supported by the finding that treatment of animals with anti-TNF-α antibodies protects the animals from LPS-induced lethality (Beutler et al., 1985; Tracey et al., 1987).

TNF-α is initially expressed as a 26 kDa cell-associated form which is then proteolytically processed to a 17 kDa secreted mature form (Kriegler et al., 1988). This secreted protein contains one intrachain disulfide bridge and exists as a dimer or trimer in circulation (Aggarwal et al., 1985). Although the proteolytic processing of TNF-α is well known, the enzyme that participates in this process is a matter of speculation. In recent years, it has been reported that matrix metalloproteinase inhibitors prevent the release of the soluble 17 kDa form of TNF-α in vitro and in vivo (Mohler et al., 1994; Gearing et al., 1994; McGeehan et al., 1994). Gearing et al. (1995) have reported that a variety of MMPs can cleave a recombinant pro-TNF-α substrate to yield mature TNF-α. More recently, TNF-α-converting enzyme (TACE) was identified as a membrane-bound disintegrin metalloproteinase (Black et al., 1997; Moss et al., 1997). Hence, it is expected that a matrix metalloproteinase inhibitor with selective inhibitory actions on TNF-α release will be a useful candidate for the treatment of TNF-α associated pathologies.

The present study was designed to assess the pharmacological efficacy of a matrix metalloproteinase inhibitor, [4-(*N*-hydroxyamino)-2*R*-isobutyl-3*S*-(phenylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide (GI 129471), on

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hepatic injury in endotoxemic mice. This compound is reported as a potent matrix metalloproteinase inhibitor (Campion et al., 1990) as well as a TNF-α processing inhibitor (McGeehan et al., 1994). We found that treatment with GI 129471 reduced serum TNF-α levels and not those of several pro-inflammatory cytokines, and improves hepatic injury in D-galactosamine plus lipopolysaccharide-injected mice.

2. Materials and methods

2.1. Animals

Male ddY mice were purchased from Japan SLC (Hamamatsu, Japan). They were housed in a temperature- and light-controlled room with free access to laboratory rodent chow and water.

2.2. Materials

GI 129471 was synthesized in our laboratories. Lipopolysaccharide (serotype 055: B5 from *Escherichia coli*) was purchased from Difco laboratory (Grand Island, NY); D-Galactosamine hydrochloride and Tween 20 were from Nacalai tesque (Kyoto); RPMI 1640 was from Nissui pharmaceutical (Tokyo); ELISA kits for mouse TNF-α and human TNF-α were from Amersham Japan (Tokyo); murine interleukin-1α ELISA kit, murine interleukin-1β ELISA kit, and murine interleukin-6 ELISA kit were from Endogen (Cambridge, MA); mouse macrophage inflammatory protein-2 (MIP-2) ELISA kit and mouse KC ELISA kit were from Immuno biological laboratories (Fujisawa); transaminase C-II test wako and bilirubin B-II test wako were from Wako pure chemical industries (Osaka).

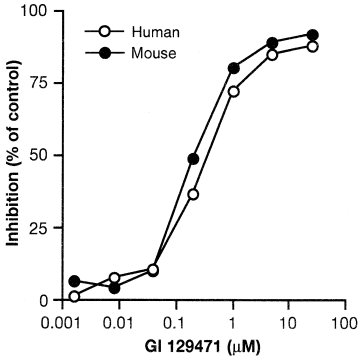

Fig. 1. Effect of GI 129471 on the increase in TNF-α concentration in human or mouse whole blood stimulated with lipopolysaccharide. Diluted whole blood from normal human volunteers or mice was stimulated with 1 μg/ml lipopolysaccharide for 24 h in the presence of indicated concentrations of GI 129471. TNF-α concentrations in the culture supernatants were measured by using ELISA kits. Data are the average of four determinations. The TNF-α levels of control group were 1.3 ± 0.13 ng/well for human and 160 ± 5.7 pg/well for mouse, respectively.

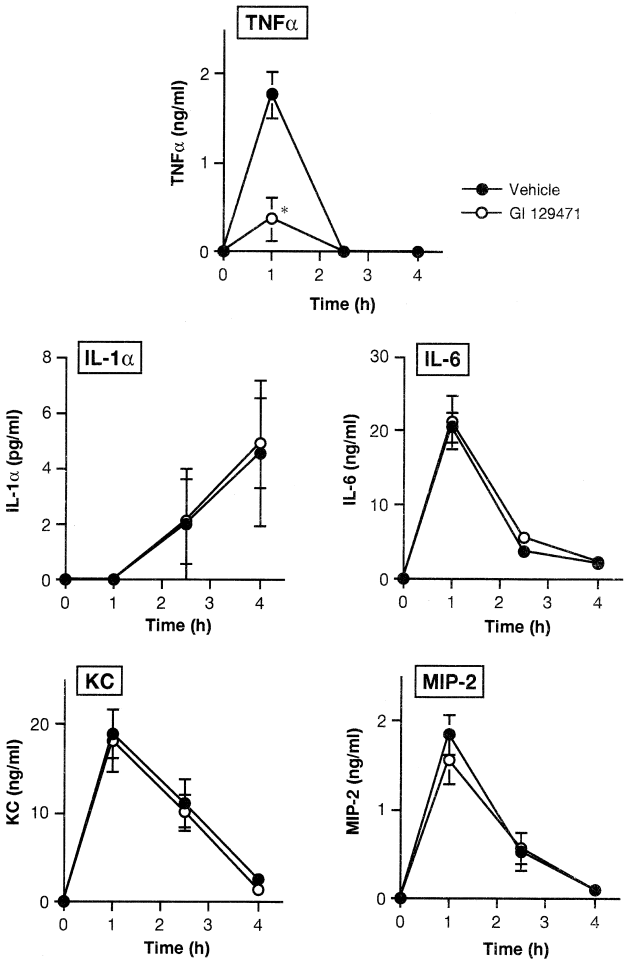

Fig. 2. Effect of GI 129471 on the increase in serum pro-inflammatory cytokine levels in D-galactosamine plus lipopolysaccharide-injected mice. Either GI 129471 (40 mg/kg) or vehicle (saline containing 1% Tween 20) was injected i.p. 10 min before D-galactosamine (600 mg/kg)/lipopolysaccharide (4 μg/kg) injection. At the time indicated, serum was obtained for cytokine measurements. Data are expressed as mean $\pm$ S.E.M. of 7–8 animals. $^{*}P < 0.05$: statistically different from vehicle group (Student's *t*-test).

2.3. In vitro studies

Whole blood from human volunteers and mice was anticoagulated with heparin and diluted to 4-fold with RPMI 1640. Diluted blood (100 μl) was plated in 96-well microplates and then incubated for 1 h or more at 37°C under 5% carbon dioxide in air. GI 129471 dissolved in DMSO (final concentration; 0.1%) was added into the wells in increasing concentrations ranging from 0.0016 to 25 μM. Immediately after the addition of GI 129471, the reaction was initiated by adding LPS solution at a final concentration of 1 μg/ml. Cell supernatants were harvested 24 h later for determination of TNF-α levels.

2.4. Endotoxemic model

To induce endotoxemia, 600 mg/kg of D-galactosamine and 4 μg/kg of lipopolysaccharide were simultaneously injected into the tail veins of mice weighing from 25 to 30 g. GI 129471 dissolved in saline containing 1% Tween 20 was administered i.p. to the animals at 40 mg/kg 10 min before the D-galactosamine plus lipopolysaccharide injection. Saline containing 1% Tween 20 (vehicle) was administrated to the control mice. Blood samples were taken at 1, 2.5, and 4 h after D-galactosamine plus lipopolysaccharide injection for the measurement of various cytokines, and were taken at 18 h for the measurement of biochemical markers of liver function. The concentrations of various cytokines in serum were measured using commercial ELISA kits. Serum levels of glutamate-oxaloacetate-transaminase, glutamate-pyruvate-transaminase and bilirubin were measured enzymatically, using commercial diagnostic kits. For histophathological examination, five mice in each treatment groups were killed 18 h after D-galactosamine (600 mg/kg)/lipopolysaccharide (4 μg/kg) injection. Livers were excised, fixed in 10% neutral buffered formalin, embedded in paraffin, stained with hematoxylin and eosin, and then examined microscopically.

In additional studies, protective effect of GI 129471 against death was tested in mice received D-galactosamine plus lipopolysaccharide. GI 129471 (40 mg/kg) or vehicle was i.p. injected into mice. Immediately thereafter, they were received i.v. injections of 600 mg/kg of D-galactosamine and 25 μg/kg of lipopolysaccharide. The animals were then monitored for 48 h to determine the survival rate.

2.5. Statistics

Student's *t*-test was performed for differences in serum cytokine levels between vehicle- and GI 129471-treated groups. Tukey–Kramer multiple test was used to evaluate the effects of GI 129471 on the changes in marker enzymes. $P < 0.05$ was considered to be statistically significant. The data are expressed as mean ± S.E.M.

Table 1
Effect of GI 129471 on increases in serum glutamate-oxalacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT) and bilirubin levels in D-GalN/LPS-injected mice

Groups	GOT (K.U.)	GPT (K.U.)	Bilirubin (mg/dl)
Normal	40 ± 1[a]	11 ± 3[a]	1.8 ± 0.9
Control	2493 ± 723	3089 ± 895	6.4 ± 2.7
GI 129471	80 ± 9[a]	114 ± 35[a]	2.0 ± 1.3

Enzymes activities were measured in serum obtained from normal mice (Normal), D-GalN/LPS-injected mice (Control) and D-GalN/LPS plus GI 129471 (40 mg/kg)-injected mice (GI 129471). GOT and GPT activities are expressed in Karmen units (K.U.) Data are expressed as means ± S.E.M. for 5 animals.
[a] $P < 0.01$: statistically different from the control (Tukey–Kramer test).

3. Results

3.1. Effect of GI 129471 on release of TNF-α from whole blood in vitro

GI 129471 was tested for its inhibitory actions on TNF-α production in human and mouse whole blood

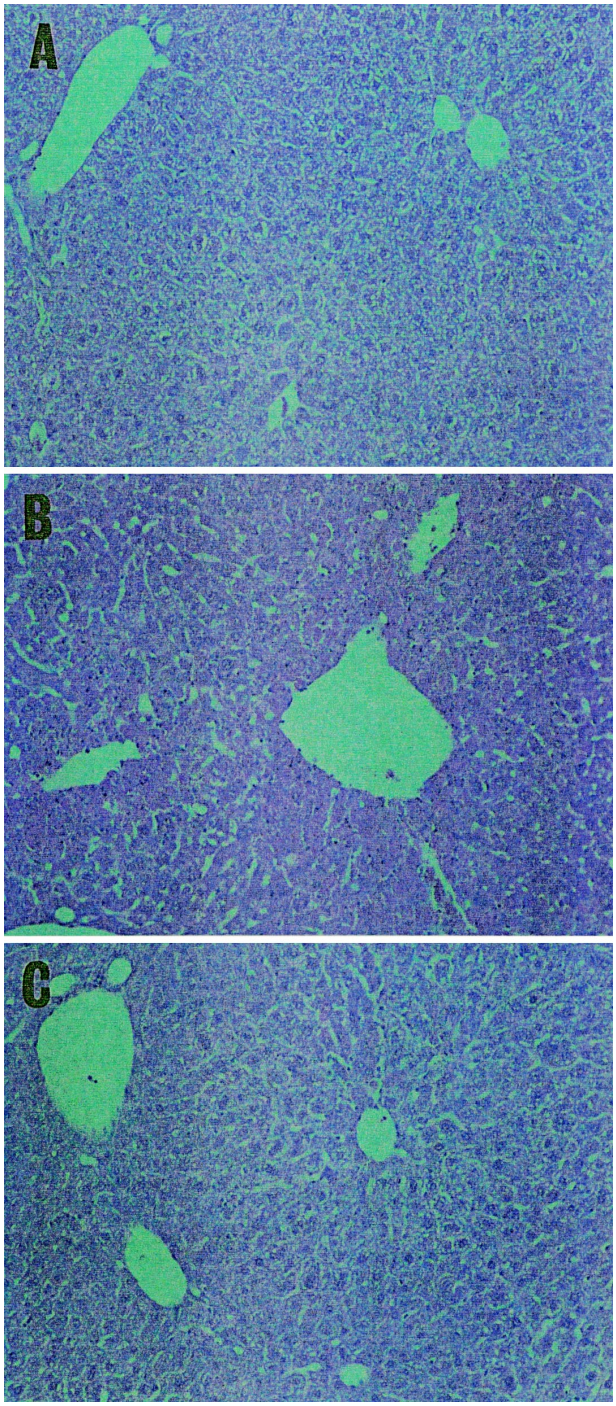

Fig. 3. Section of liver from a normal mouse (A), a D-galactosamine plus lipopolysaccharide-injected mouse (B), and D-galactosamine plus lipopolysaccharide plus GI 129471 (40 mg/kg)-injected mouse (C). Sections were stained with hematoxylin and eosin, and photographed at ×100 magnification.

stimulated with lipopolysaccharide (Fig. 1). GI 129471 caused a dose-dependent inhibition of the increase in TNF-α in the supernatants of both human and mouse whole blood. The IC_{50} values were 370 nM for human and 260 nM for mice, respectively. In contrast, interleukin-8 production in human blood was unaffected by GI-129471 (data not shown).

3.2. Effect of GI 129471 in endotoxemic mice

Fig. 2 shows the effects of GI 129471 on the increase in various cytokines in D-galactosamine plus lipopolysaccharide-injected mice. Serum TNF-α increased transiently with a peak of approximately 1.8 ng/ml at 1 h. Serum interleukin-6, KC and MIP-2 increased rapidly, peaked at 1 h, and then decreased. The peak levels of interleukin-6, KC and MIP-2 were approximately 20 ng/ml, 19 ng/ml and 1.8 ng/ml, respectively. Serum interleukin-1α began to increase at 2.5 h. Interleukin-1β was not detected under our experimental conditions (data not shown). GI 129471 injected i.p. at 40 mg/kg reduced serum TNF-α level by 79% at 1 h after D-galactosamine plus lipopolysaccharide injection. In contrast, the levels of other pro-inflammatory cytokines were not affected by GI 129471.

At 18 h of D-galactosamine plus lipopolysaccharide injection, there was acute hepatocellular injury, as evidenced by marked increases in serum glutamate-oxaloacetate-transaminase, glutamate-pyruvate-transaminase and bilirubin levels (Table 1). Treatment with GI 129471 (40 mg/kg, i.p.) reduced these biochemical indices to the normal level. Histopathological examination of the livers revealed large regional areas of severe centrilobular necrosis. Importantly, no remarkable alterations were observed with the livers of GI 129471-treated mice (Fig. 3). In addition, we tested whether GI 129471 treatment prevented death caused by D-galactosamine plus lipopolysaccharide. At 48 h after D-galactosamine plus lipopolysaccharide i.v. injection, 7 of 20 control mice survived while 14 of 20 GI 129471 (40 mg/kg, i.p.)-treated mice survived (data not shown). In both groups death occurred within 24 h.

4. Discussion

An excessive release 17 kDa soluble TNF-α is thought to be a key pathological process in septic shock (Vassalli, 1992). In recent years, it has been shown that GI 129471, a matrix metalloproteinase inhibitor, prevents the release of 17 kDa soluble form of TNF-α in vitro and in vivo (McGeehan et al., 1994). However, it remains unknown whether this compound is effective in experimental animal models of sepsis. The present study was undertaken to determine whether GI 129471 treatment has beneficial effects in endotoxemic mice.

The results of this study indicated that GI 129471 reduces the concentration of TNF-α in both human and mouse whole blood in vitro. In addition, intraperitoneal administration of GI 129471 markedly reduced serum TNF-α levels in D-galactosamine plus lipopolysaccharide-injected mice. These results were comparable with the results of a previous report (McGeehan et al., 1994). However, our results provided further information that the effects of this compound may not be species dependent since GI 129471 reduced TNF-α concentrations in both human and mouse whole blood at almost the same concentration. These findings also suggest that active site of TACE may not differ greatly between human and mice.

It is well known that TNF-α is able to stimulate production of other cytokines in vitro and in vivo (Tracey and Cerami, 1993). These facts suggest that TNF-α plays a central role in the cascade of cytokine production. This notion is supported by the fact that administration of anti-TNF-α antibodies is effective in lowering the levels of other cytokines in lipopolysaccharide-injected baboons (Fong et al., 1989). However, the previous in vitro experiment demonstrated that GI 129471 reduces specifically the concentration of TNF-α but not interleukin-1β, interleukin-2 and interleukin-6 in vitro (McGeehan et al., 1994). In our study, administration of GI 129471 reduced serum TNF-α levels but not those of other pro-inflammatory cytokines, including interleukin-1, interleukin-6 and mouse interleukin-8 (KC and MIP-2), in D-galactosamine plus lipopolysaccharide-injected mice. The reason for this discrepancy is not clear, however it is possible that cell membrane-bound TNF-α is important in stimulate the production of other cytokines. Indeed, it has been reported that cell membrane-bound TNF-α has biological activity (Kriegler et al., 1988).

There are two types of TNF receptors, 55 kDa TNF receptor and 75 kDa TNF receptor. Of these receptors, 55 kDa TNF receptor can be selectively stimulated by 17 kDa soluble TNF-α (Grell et al., 1995). The triggering of this receptor is considered to be a crucial step in the pathogenesis of hepatocyte necrosis and in the lethal outcome of the toxic shock syndrome (Pfeffer et al., 1993). TNF-α causes hepatocyte damage and death in vitro (Wang et al., 1995). Furthermore, administration of recombinant TNF-α causes hepatocellular dysfunction and liver injury in vivo (Wang et al., 1993; Tiegs et al., 1989). On the basis of these facts, it is speculated that soluble TNF-α plays an important role in hepatic injury through activation of 55 kDa TNF receptor. In the present study, we demonstrated that treatment with GI 129471 reduces specifically serum TNF-α levels and prevents hepatic injury in D-galactosamine-injected mice. Histopathological finding showed that the areas of necrosis mainly surrounded the central hepatic veins, suggesting that circulating TNF-α may act on hepatic cells directly. Again, our findings strongly suggested that the production of 17 kDa soluble TNF-α is a crucial step leading to hepatic injury in endotoxemia.

Interleukin-1α, interleukin-6, KC and MIP-2 are known

to be pro-inflammatory cytokines and are considered to be involved in various inflammatory diseases. However, our results suggested that these cytokines are not important for the development of liver injury in D-galactosamine plus lipopolysaccharide-injected mice since GI 129471 almost completely attenuated liver injury biochemically and histopathologically without affecting the increase in these cytokines. Some organs other than liver may be the target of these cytokines.

In contrast to our results, recent study by Solorzano et al. (1997) demonstrated that matrix metalloproteinase inhibitor (GM-6001) is not effective in preventing hepatic injury in endotoxemic mice. Their experiments suggested that D-galactosamine plus lipopolysaccharide-induced hepatic injury is dependent upon the production of 26 kDa cell-associated TNF-α but not 17 kDa soluble TNF-α. The conflicting results seem to be caused by differences in the experimental conditions, e.g., animal species, dose of D-galactosamine plus lipopolysaccharide, matrix metalloproteinase inhibitor and serum TNF-α assay system. Most importantly, the activity of GM-6001 is far lower than that of GI 129471. In addition, Solorzano et al. assayed TNF-α by a bioassay.

The Fas/Fas ligand system has been shown to be involved in the pathogenesis of hepatitis (Ogasawara et al., 1993). It has also been reported that Fas ligand is a type II integral membrane protein homologous with TNF, and that Fas belongs to TNF receptor family (Nagata and Golstein, 1995). Recently, Kayagaki et al. (1995) reported that matrix metalloproteinase inhibitors prevent the release of soluble Fas ligand from human Fas ligand cDNA transfectants. Hence, we cannot exclude the possibility that GI 129471 caused improvement of hepatic injury through the inhibition on Fas ligand release. To access this point, we should examine whether the level of Fas ligand in serum increases in D-galactosamine plus lipopolysaccharide injected animals and whether GI 129471 attenuates this response.

It has been reported that matrix metalloproteinases such as stromelysin and gelatinase A are expressed in the early phase of liver injury (Arthur, 1995). GI 129471 was originally designed as a matrix metalloproteinase inhibitor. Therefore, we cannot rule out the possibility that GI 129471 might provide protection against hepatic injury induced by D-galactosamine plus lipopolysaccharide by inhibiting certain matrix metalloproteinases.

In conclusion, our findings demonstrated that treatment with matrix metalloproteinase inhibitor (GI 129471) reduces serum TNF-α level as well as hepatic injury in D-galactosamine plus lipopolysaccharide-injected mice. Therefore, it is suggested that soluble TNF-α plays an important role in the hepatic injury of endotoxemic mice. It is also suggested that a matrix metalloproteinase inhibitor may be a useful therapeutic agent for endotoxemia. The development of more specific TACE inhibitors will help to clarify the role of 17 kDa soluble TNF-α in endotoxemia since GI 129471 was originally synthesized as a collagenase inhibitor.

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