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Effect of FR167653, a cytokine suppressive agent, on endotoxin-induced disseminated intravascular coagulation

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Abstract

R167653 (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate) is a low molecular weight inflammatory cytokine inhibitor that inhibits the production of interleukin-1α, interleukin-1β and tumor necrosis factor-α (TNF-α) in human monocytes stimulated with lipopolysaccharide, and in human lymphocytes stimulated with phytohemagglutinin-M. FR167653 inhibited these cytokines in a dose-dependent manner (IC_{50} values were 0.84, 0.088, 1.1 μM and 0.072, respectively). However, FR167653 did not inhibit even at 10 μM interleukin-6 production by human monocytes, and the production of interleukin-2 and interferon-γ by human lymphocytes. We evaluated the effect of FR167653 on lipopolysaccharide-induced disseminated intravascular coagulation in rats. FR167653 (0.032–0.32 mg/kg/h for 4 h, intravenous infusion) markedly improved thrombocytopenia and plasma coagulation parameters in a dose-dependent manner, but not leukopenia in this model. Plasma interleukin-1 and TNF-α levels were elevated by lipopolysaccharide administration and the treatment with FR167653 (0.32 mg/kg/h for 4 h) inhibited the increased plasma interleukin-1 (100.0%) and plasma TNF-α (89.2%) levels. These results suggest that interleukin-1 and TNF-α may play a pivotal role in the pathogenesis of DIC. FR167653 can act as a protective drug in lipopolysaccharide-induced DIC, and this protection is due to an inhibition of increased plasma interleukin-1 and TNF-α.

Keywords: Lipopolysaccharide; Disseminated intravascular coagulation; Interleukin-1; TNF-α (tumor necrosis factor-α)

1. Introduction

Abnormalities in coagulation and fibrinolysis are frequently observed in patients with septic shock (Fourrier et al., 1992). Widespread microvascular thrombosis in various organs substantially is seemed as a common feature of lethal septic shock (Carrico et al., 1986) and may considerably contribute to organ dysfunction (Tanaka et al., 1990; Leclerc et al., 1992). The most pronounced clinical manifestation is DIC. DIC is characterized by microvascular thrombosis, consumption of platelets and stimulation of fibrinolysis. Moreover, the mortality of patients with septic shock who develop DIC is significantly higher than that of patients without DIC (Fourrier et al., 1992; Coalson, 1986), the mortality rate being 77% compared with 32% in patients without DIC. Some clinical observations indicate that DIC is an important pathogenetic factor for the development of multiple organ failure (Fourrier et al., 1992).

The symptoms of septic shock are believed to be mediated by the release of cytokines, like as interleukin-1 (Okusawa et al., 1988; Dinarello, 1991) and TNF-α (Beutler et al., 1985). Several in vitro studies have demonstrated that endotoxin (Moore et al., 1987; Colucci et al., 1983), interleukin-1 (Bevilacqua et al., 1984) and TNF-α (Nawroth and Stern, 1986; Bevilacqua et al., 1986; Conway et al., 1989) enhance tissue factor expression on human endothelial cells in a few hours. These factors also can decrease thrombomodulin (Moore et al., 1989) and tissue type plasminogen activator expression (Emeis and Kooistra, 1986; Hanss and Collen, 1987; Schleef et al., 1988) in such an in vitro model. However, pathophysiological roles for these cytokines in DIC have been not clarified.

In this study, we investigated the effect of FR167653, (1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sul-

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fate monohydrate; Fig. 1), a dual inhibitor of interleukin-1 and TNF-α production, on the development of lipopolysaccharide-induced DIC in rats and assessed the interaction between inhibition of the production of these cytokines and improvement of DIC.

2. Materials and methods

2.1. Animals

Male Wistar rats (6–7 weeks old) were purchased from Japan SLC (Shizuoka, Japan) and were kept in a temperature-controlled environment. In the experiment with the DIC model, the rats were deprived of food 14 h before the experiment.

2.2. Materials

FR167653 was synthesized in our laboratories. Actinomycin D, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lipopolysaccharide (from *Escherichia coli* 0111:B4) were purchased from Sigma (St. Louis, MO, USA). Ficoll-Paque was purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Lipopolysaccharide (from *Escherichia coli* 055:B5) and phytohemagglutinin-M were purchased from Difco (Detroit, MI, USA). Di-*n*-butyl phthalate and olive oil were purchased from nacali tesque (Kyoto, Japan). Human interleukin-1α, interleukin-1β, TNF-α and interleukin-2 ELISA Kits were purchased from Otsuka Pharmaceutical (Japan). Human interleukin-6 and interferon-γ ELISA kit, recombinant human interleukin-1β and TNF-α were purchased from Genzyme (Cambridge, MA, USA). [^{125}I]Interleukin-1β was purchased from Amersham (Arlington Heights, IL, USA). Fetal bovine serum was purchased from Gibco (Grand Island, NY, USA).

2.3. Cell culture

Murine Balb/3T3 fibroblasts and L929 fibroblasts (Dai Nippon Pharmaceutical Co., Japan) were grown in Dulbecco's modified Eagle's medium (DMEM; Flow Laboratories) containing 10% fetal bovine serum, 100 μg/ml streptomycin and 100 U/ml penicillin.

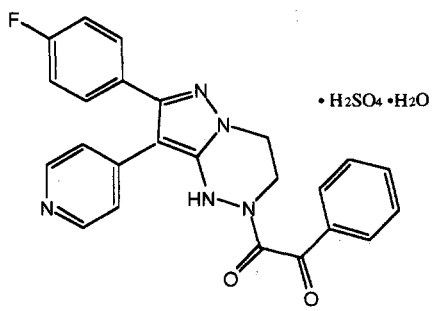

Fig. 1. Chemical structure of FR167653, 1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate.

2.4. Cytokine production by human lymphocytes and monocytes

Human lymphocytes were isolated from the blood of healthy volunteers by the centrifugation through Ficoll-Paque solution. Briefly, heparin-treated peripheral blood was layered on the Ficoll-Paque solution and centrifuged at 400 × *g* for 30 min. The lymphocytes were found at the interface between the plasma and the Ficoll-Paque solution. This fraction contained about 80% of mononuclear cells. The lymphocytes were suspended in RPMI 1640 supplemented with 10% Fetal bovine serum, 100 μg/ml streptomycin, 100 U/ml penicillin, and placed in a culture flask and incubated at 37°C for 30 min. Non-adherent cells were removed by rinsing and the remaining cells were used as the monocyte preparation. The fraction contained approximately 98% monocytes as scatter analyzed using a FACScan (Becton Dickinson). The monocytes were resuspended in RPMI 1640, seeded into 24-well plastic culture plates (5×10^4 cells/2 ml) and cultured with 1 μg/ml of lipopolysaccharide (from *Escherichia coli* 055:B5) in the presence or absence of FR167653 at 37°C for 18 h. Amounts of interleukin-1, TNF-α and interleukin-6 were measured by using ELISA kits. The lymphocytes were seeded into 24-well plastic culture plates (2×10^6 cells/2 ml) and cultured with 6.25% (w/v) of phytohemagglutinin-M in the presence or absence of FR167653 at 37°C for 72 h. Amounts of TNF-α, interleukin-2 and interferon-γ were measured by using ELISA kit. The inhibitory concentration that caused 50% inhibition (IC_{50}) was calculated by regression analysis of dose-response data.

2.5. Experimental disseminated intravascular coagulation model

An experimental model of disseminated intravascular coagulation in rats was carried out according to the method of Imura et al. (1986) with slight modifications. Male Wistar rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and right femoral veins were cannulated for infusion of lipopolysaccharide (from *Escherichia coli* 0111:B4) and FR167653. Lipopolysaccharide at 0.25 mg/kg/h was infused at a flow rate of 0.5 ml/h for 4 h. Blood samples were withdrawn from the abdominal aorta at 4 h after the lipopolysaccharide infusion for measurement of the coagulation parameters of DIC, and collected from the right femoral artery at 1, 2, 3 and 4 h after the lipopolysaccharide infusion for the determination of interleukin-1 and TNF-α levels. The samples used for the measurement of coagulation parameters of DIC were anticoagulated with 3.8% sodium citrate (1:10 volume). The

coagulation parameters were estimated as described previously (Yokota et al., 1994): platelet counts, blood leukocyte counts, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrinogen/fibrin degradation products.

2.6. Detection of plasma interleukin-1 and TNF-α levels

Blood samples collected at various times were heparinized and centrifuged at $1200 \times g$ for 10 min. The obtained plasma was stored at −80°C until assayed. Plasma interleukin-1 levels were measured by a minor modification of the receptor binding assay described by Patricia et al. (1986). Briefly, murine Balb/3T3 cells maintained as described above were removed by addition of 0.2 mM EDTA and resuspended in binding buffer (RPMI 1640/5% fetal bovine serum/25 mM Hepes, pH 7.2). Each assay contained 1.3×10^6 cells and [^{125}I]interleukin-1β at 0.2 nM in 0.15 ml of binding buffer. Non-specific binding was determined by incubation of 5000 U/ml of unlabeled human interleukin-1β in the assay. Incubations were carried out in duplicate for 4 h at 4°C. Cell-bound radioactivity was separated from free [^{125}I] interleukin-1β by centrifugation of 60 μl of the assay mixture through 150 μl of an oil mixture (80% di-*n*-butyl phthalate/20% olive oil) at 4°C for 10 min. The tube tip containing the cell pellet was cut off, and cell-bound radioactivity was determined in a γ-counter. Recombinant human interleukin-1β was used as a standard in each assay. Plasma TNF-α levels were quantitated in the L929 cytotoxicity assay (Inamura et al., 1992). Rat plasma samples were assayed at a final dilutions of 1:100. Recombinant human TNF-α was used as a standard in each assay plate. The addition of FR167653 at concentrations expected from drug carry over had no effect in either the interleukin-1 or TNF assays.

2.7. Statistical analysis of data

The values are expressed as means ± S.E.M. The significance of differences was determined by Dunnett's multiple comparison test.

3. Results

3.1. Inhibitory effect of FR167653 on the production of cytokines by human leukocytes

The suppressive activity of FR167653 was assessed on the production of various cytokines by human peripheral blood leukocytes by using specific ELISA. As shown in Table 1, FR167653 inhibited the production of interleukin-1α, interleukin-1β and TNF-α by human monocytes stimulated with 1 μg/ml of lipopolysaccharide and the IC_{50} values were 0.84, 0.088 and 1.1 μM, respectively. FR167653 also inhibited TNF-α release from phytohemagglutinin-M (6.25%, w/v)-stimulated human lymphocytes and the IC50 values were 0.072 μM. However, FR167653 did not inhibit even at 10 μM interleukin-6 production by human monocytes stimulated with 1 μg/ml of lipopolysaccharide. In addition, FR167653 also did not block the production of other cytokines such as interleukin-2 and interferon-γ in response to phytohemagglutinin-M by human leukocytes.

Table 1
The suppressive effect of FR167653 on various cytokine production by human leukocytes

	IC_{50} value (μM)
Cytokines from monocytes	
Interleukin-1α	0.84
Interleukin-1β	0.088
TNF-α	1.1
Interleukin-6	> 10
Cytokines from lyumphocytes	
TNF-α	0.072
Interleukin-2	> 10
Interferon-γ	> 10

Interleukin-1α, interleukin-β, TNF-α and interleukin-6 were produced by human monocytes stimulated with lipopolysaccharide (1 μg/ml). Secretion of TNF-α, interleukin-2 and interferon-γ by lymphocytes were stimulated with phytohemagglutinin-M (6.25%, w/v). These cytokines were quantitated by using specific ELISA.

These data suggest that FR167653 is a dual inhibitor of interleukin-1 and TNF-α production, and that the inhibitory effect is selective for interleukin-1 and TNF-α.

3.2. Effect of FR167653 on blood parameters of DIC-like syndrome caused by lipopolysaccharide infusion

Continuous infusion of lipopolysaccharide at 0.25 mg/kg per h for 4 h induced the development of DIC-like syndrome such as leukopenia, thrombocytopenia and changes of plasma coagulation parameters. The leukopenia was observed even at 1 h after infusion of lipopolysaccharide and the low levels of blood leukocytes was sustained for 4 h in this model (Yokota et al., 1994). The decrease in platelet counts occurred gradually after lipopolysaccharide infusion and was different from that of the leukocytes. As shown in Fig. 2, the treatment with FR167653 had no effect on the leukopenia at 4 h after the infusion of lipopolysaccharide. In contrast, the decrease of platelet counts induced by lipopolysaccharide administration was reversed by FR167653 (0.32 mg/kg per h) in a dose-dependent manner.

The changes in plasma coagulation parameters occurred at 2 h and peaked at 4 h after lipopolysaccharide exposure. There was a significant prolongation of plasma coagulation parameters such as prothrombin time and activated partial thromboplastin time, and a significant decrease in plasma fibrinogen and fibrin degradation product levels in the lipopolysaccharide-treated group compared with the saline-treated group (Table 2). These abnormalities of

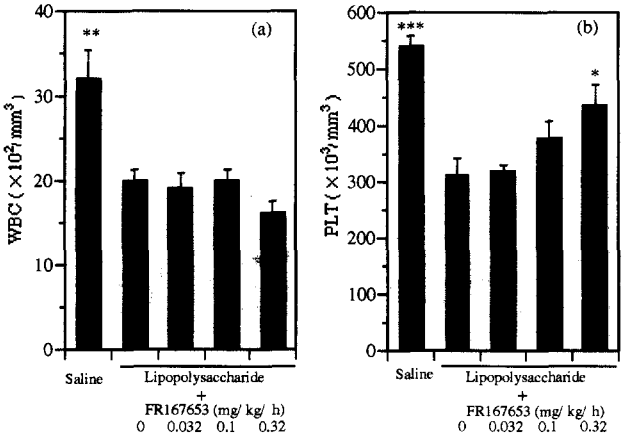

Fig. 2. Effect of FR167653 on leukopenia and thrombocytopenia induced by lipopolysaccharide infusion in rats. Lipopolysaccharide at 0.25 mg/kg per h was infused continuously for 4 h and simultaneously with FR167653 or not. Blood samples were collected at the end of the infusion. The results are expressed as means ± S.E.M. of ten experiments per group. Asterisks show the significance of the differences from the lipopolysaccharide-treated group (* $P < 0.05$, ** $P < 0.01$).

coagulation parameters were significantly reversed by the treatment with FR167653 (0.1–0.32 mg/kg per h). Fibrinolytic parameter such as fibrin degradation products was also improved by FR167653 at a similar dose (0.1–0.32 mg/kg per h) as for the coagulation parameters.

3.3. Effect of FR167653 on plasma interleukin-1 and TNF-α levels in rats

Circulating levels of plasma interleukin-1 induced by lipopolysaccharide administration in rats were elevated together with similarly elevated levels of TNF-α. Peak plasma TNF-α level was observed at 2 h after lipopolysaccharide infusion (57.5 U/ml) and the levels had declined to undetectable levels at 4 h (Fig. 3a,b). This was followed by an elevation of plasma interleukin-1 levels

Table 2
Effect of FR167653 on the coagulation and fibrinolytic parameters of DIC induced by lipopolysaccharide in rats

Dose (mg/kg per h)	PT (s)	APTT (s)	Fibrinogen (mg/dl)	FDP (mg/dl)
Saline	14.3 ± 0.2 ^{b}	20.7 ± 0.4 ^{b}	207 ± 4.4 ^{b}	0.3 ± 0.1 ^{b}
Lipopolysaccharide-treated				
0	21.7 ± 1.3	46.2 ± 3.0	42 ± 6.2	3.3 ± 0.4
0.032	19.6 ± 0.9	42.1 ± 2.9	54 ± 10.0	3.1 ± 0.4
0.1	17.5 ± 0.7 ^{b}	37.0 ± 2.3	70 ± 8.6	2.8 ± 0.4
0.32	16.5 ± 1.0 ^{b}	32.5 ± 3.7 ^{b}	106 ± 15.3 ^{a}	1.5 ± 0.2 ^{b}

Lipopolysaccharide was infused for 4 h with or without simultaneous treatment with FR167653. Blood samples were collected at the end of the infusion and analyzed PT (prothrombin time), APTT (activated partial thromboplastin time), fibrinogen and FDP (fibrinogen and fibrin degradation products). The results were expressed as means ± S.E.M.; significance of the differences from lipopolysaccharide-treated group: ^{a} $P < 0.05$, ^{b} $P < 0.01$.

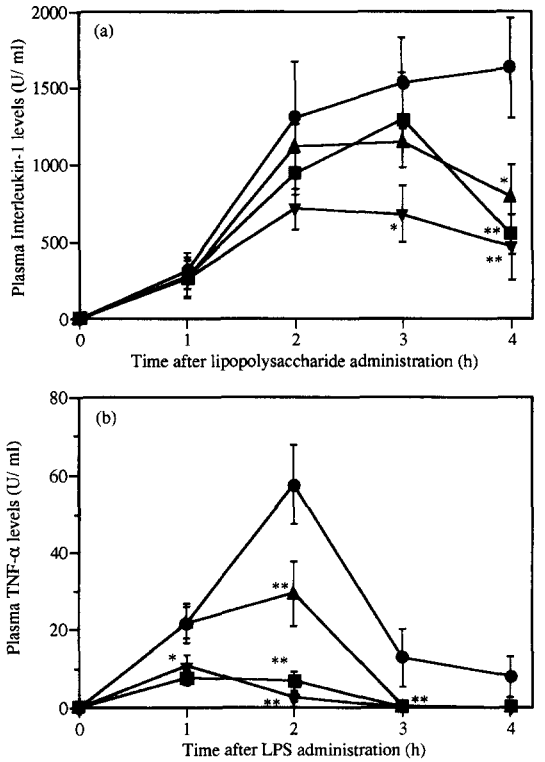

Fig. 3. Effect of FR167653 on plasma interleukin-1 (a) and TNF-α (b) levels in rats treated with lipopolysaccharide. Blood samples at various time were collected and assayed in the receptor binding assay and L929 bioassay, respectively. The results are expressed as means ± S.E.M. for lipopolysaccharide-treated control group (●) and control rats treated with 0.032 (▲), 0.1 (■) or 0.32 (▼) mg/kg per h of FR167653 (ten rats per group). Asterisks show the significance of the differences from lipopolysaccharide-treated group (* $P < 0.05$, ** $P < 0.01$).

(1634.0 U/ml), which continued to increase gradually up to 4 h. Thus, systemic infusion of lipopolysaccharide brought about the production of large amounts of interleukin-1 and TNF-α, and the high levels of these circulating cytokines were maintained between 2 and 4 h after lipopolysaccharide exposure. There was a similarity between the pattern of the changes in the levels of these cytokines and that of the changes in coagulation parameters.

To assess the effect of FR167653 on circulating interleukin-1 and TNF-α levels in lipopolysaccharide-induced DIC, the concentrations of plasma interleukin-1 and TNF-α were measured. As shown in Fig. 3, treatment with FR167653 (0.032–0.32 mg/kg per h) significantly suppressed the lipopolysaccharide-induced increase in plasma interleukin-1 and TNF-α levels in a dose-dependent manner at all time points as compared with those of the lipopolysaccharide-treated group. Especially, the maximal elevations of plasma interleukin-1 and TNF-α levels were markedly attenuated by the infusion of FR167653 at 0.32 mg/kg per h.

4. Discussion

Coagulation disorders and abnormalities of fibrinolysis are frequently observed in septic shock (Fourrier et al., 1992). The most pronounced clinical feature is DIC which is considered to be an important cause of circulatory shock and death evoked by endotoxin. Indeed, patients with septic shock who develop DIC have a higher mortality than those who have no signs of DIC. Some clinical observations indicate that DIC is an important pathogenic factor for the development of multiple organ dysfunction. Widespread microvascular thrombosis in various organs is a common feature of lethal septic shock and may substantially contribute to organ dysfunction. In addition, plasma interleukin-1β (Wada et al., 1991a), TNF-α (Wada et al., 1991b) and interleukin-6 (Wada et al., 1991c) levels in patients with DIC have been reported to be higher than those of patients without DIC. The levels of cytokines in patients with organ failure are also higher than in those without organ failure (Fourrier et al., 1992).

Interleukin-1 and TNF-α are thought to be major factors on the development of thrombosis, for example by inducing tissue factor activity on human endothelial cells and monocytes. In the *Escherichia coli*-induced septic shock baboon model, administration of anti-tissue factor monoclonal antibody protects against the lethality and attenuates the coagulopathy (Taylor et al., 1991). These data strongly suggest that interleukin-1 and TNF-α are key mediators of microvascular thrombosis and these cytokines contribute to the development of DIC.

In the present study, we examined the effect of FR167653, a novel cytokine inhibitor, on the onset of a DIC-like syndrome induced by continuous i.v. infusion of lipopolysaccharide in rats. The rats exhibited changes in coagulation and fibrinolytic parameters such as platelet counts, prothrombin time, activated partial thromboplastin time, fibrinogen and fibrin degradation products, and an elevation of plasma interleukin-1 and TNF-α levels. These changes started 2 h after lipopolysaccharide administration and plasma TNF-α levels also increased at the same time point.

Subsequent changes followed the increase in plasma interleukin-1 levels, which started at 2 h and remained elevated up to 4 h after lipopolysaccharide infusion. This pattern of changes in cytokines and in parameters of coagulation/fibrinolysis lead us to speculate that the onset of DIC in this model is mainly related to plasma TNF-α levels, and that the development of DIC is due to the increase in plasma interleukin-1 levels. However, we cannot eliminate the possibility that an additional TNF-α/interleukin-1-independent component is involved in the development of DIC.

FR167653 had no effect against leukopenia in this model. The decrease in the number of leukocytes may be produced by other inflammatory mediators, because an interleukin-1 receptor antagonist failed to inhibit the granulocytopenia in a primate model of endotoxemia induced by live *Escherichia coli* or lipopolysaccharide (Hawes et al., 1993), and FR167653, which inhibits interleukin-1 and TNF-α, also did not prevent leukopenia in the lipopolysaccharide-induced DIC model.

Our data showed that FR167653 inhibited the development of DIC induced by intravenous infusion of lipopolysaccharide in a dose-dependent manner and also suppressed the increase in plasma levels of interleukin-1β and TNF-α. The inhibitory effect of FR167653 on the development of DIC in rats is thought to be mediated via the inhibition of interleukin-1 and TNF-α production, and the inhibition of these cytokines may result in the suppression of coagulation factors such as tissue factor and PAI-1, and in the restoration of the down-regulation of fibrinolytic factors such as thrombomodulin.

In conclusion, we propose that the inhibition of interleukin-1 and TNF-α production is necessary to prevent the onset and development of DIC, and that FR167653 may be highly useful for the therapy of DIC or lethal septic shock.

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