INHIBITORY EFFECT OF TCV-309, A NOVEL PLATELET ACTIVATING FACTOR (PAF) ANTAGONIST, ON ENDOTOXIN-INDUCED DISSEMINATED INTRAVASCULAR COAGULATION IN RATS: POSSIBLE ROLE OF PAF IN TISSUE FACTOR GENERATION

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Abstract The possible involvement of platelet activating factor (PAF) in the pathogenesis of endotoxin-induced disseminated intravascular coagulation (DIC) was investigated in rats using a novel potent PAF antagonist, TCV-309. TCV-309 (> 1 mg/kg, i.v.) showed beneficial effects in rats with experimental DIC induced by a 4-hour sustained infusion of endotoxin (1 mg/kg) in a dose-dependent manner. TCV-309 (1 mg/kg) significantly ameliorated the decrease in platelet count and plasma fibrinogen, the prolongation of prothrombin time (PT) and activated partial thromboplastin time (APTT) and the increase in fibrin and fibrinogen degradation products (FDP) and inhibited glomerular fibrin deposition. Furthermore, plasma tissue factor (TF) activity was greatly increased in the DIC rats, and this was also significantly decreased by TCV-309 (1 mg/kg). TCV-309 (1 mg/kg) did not affect these parameters in normal rats. A 4-hour sustained infusion of PAF (60 μg/kg) caused mild but significant changes in some DIC parameters such as PT, fibrinogen and FDP concentration and increased the plasma TF activity. TCV-309 (1 mg/kg) inhibited all these PAF-induced changes. TCV-309 (0.1 mM) itself had no direct in vitro effects on the blood coagulation system including TF activity. These results strongly suggest that PAF plays a role in the pathogenesis of endotoxin-induced DIC via the generation of TF. Prophylactic use of PAF antagonists may therefore be useful for the treatment of DIC with sepsis.

Key Words: PAF, endotoxin, DIC, TCV-309, tissue factor.
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Disseminated intravascular coagulation (DIC) is a clinico-pathological syndrome resulting from uncontrolled simultaneous activation of the coagulation and fibrinolytic systems (1). Diseases associated with DIC are sepsis, malignancy, liver diseases, and obstetrical complications. In the case of sepsis, DIC is commonly noted in patients with Gram-negative infection due to endotoxin release. Endotoxin has diverse biological effects such as activation of platelets, leukocytes and the blood coagulation-fibrinolytic system, synthesis of cytokines such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) and chemical mediators including platelet activating factor (PAF) (1). Moreover, endotoxin induces the expression of tissue factor (TF) activity in monocytes and vascular endothelial cells (1). TF is a membrane-bound procoagulant protein that activates the extrinsic blood coagulation pathway in the presence of factor VII. Thus, TF has been suggested to play a role in triggering endotoxin-induced DIC (1,2). Recently, in vivo evidence for a role of TF as a trigger of the coagulation cascade in septic shock was established by using anti-TF antibodies (3,4). Furthermore, Fukuda et al. demonstrated that the plasma TF activity increased in patients with DIC (5). However, the increase in plasma TF activity in the animal with DIC or septic shock has not been demonstrated. Furthermore, the responsible mediators for the in vivo induction of TF has not been fully elucidated.

PAF is a highly potent phospholipid mediator that has been implicated in the pathogenesis of inflammation, thrombosis, endotoxin shock and anaphylactic shock (6,7). In 1986, we reported the beneficial effects of CV-3988, a specific PAF antagonist, in endotoxin-induced DIC, and we proposed a hypothesis that PAF may be involved in DIC caused by endotoxin (8). However, the mechanism of the action of CV-3988 in DIC remains to be clarified. Recently, we obtained a new potent and selective PAF antagonist, TCV-309, which is over 100 times more potent than CV-3988 (9). This study was designed 1) to examine pharmacologically the involvement of PAF in endotoxin induced DIC in rats, 2) to determined whether plasma TF activity increases in DIC rats, 3) to clarify the contribution of PAF to TF production in vivo.

MATERIALS AND METHODS

Drugs: TCV-309[3-bromo-5-[N-phenyl-N-[2-[[2-(1,2,3,4-tetrahydro-2-isoquinolylcarbonyloxy)-ethyl]carbamoyl]-ethyl]carbamoyl]-1-propylpyridinium nitrate] was synthesized in the Chemistry Research Laboratories of Takeda Chemical Industries (Osaka, Japan) (10). Endotoxin (lipopolysaccharide from E. coli, 0111: B4) was purchased from Sigma (MO, USA), PAF(1-O-octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) from Funakoshi (Tokyo, Japan), Lyoplastin (lyophilized thromboplastin) from Mochida Pharmaceutical (Tokyo, Japan), fatty acid poor bovine serum albumin (BSA) from Miles (IL, USA), coagulation factor VII and factor X concentrates (PPSB) from Nippon
Pharmaceuticals (Osaka, Japan) and S-2222 (Bz-Ile-Glu-Gly-Arg-pNA) from Kabi Vitrum AB (Sweden). Clinical assay kits such as PT test Wako, APTT test Wako and Fibrinogen B Test Wako were purchased from Wako Pure Chemicals (Osaka, Japan) and FDPL test kit from Teikokuzouki (Tokyo, Japan). All other reagents were of the highest grade available commercially.

**Endotoxin-induced DIC in rats**: Experiments were done according to the methods in our previous report (8). In brief, male Sprague-Dawley rats, 6 to 9 weeks old, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and both femoral veins were cannulated for the infusion of endotoxin and TCV-309. Endotoxin (0.25 mg/kg/hour) was infused at a rate of 0.6 ml/hour for 4 hours using a syringe pump (model 2400-006, Harvard apparatus, MA, USA). High (1 mg/kg) and low (0.1 mg/kg) doses of TCV-309 were administered as follows. At first, 20% of the dose of TCV-309 was given as a bolus injection (1 ml/rat) 5 minutes before the endotoxin infusion, then the remaining 80% was infused concomitantly with the endotoxin at a rate of 0.6 ml/hour for 4 hours. In control rats, 0.9% saline was given instead of TCV-309. In normal rats, 0.9% saline was given instead of endotoxin and TCV-309. To examine the effects of TCV-309 on the DIC parameters, TCV-309 (1 mg/kg) was given in normal rats.

**PAF-induced DIC like symptoms in rats**: In another series of experiments, PAF (15 μg/kg/hour) was infused at a rate of 0.6 ml/hour for 4 hours. TCV-309 (1 mg/kg) was given as a bolus injection followed by a 4-hour infusion as described above. PAF was dissolved in 0.9% saline containing 0.25% BSA.

**Determination of DIC parameters**: At the end of the 4-hour infusion, 1.8 ml of blood was taken from the abdominal aorta using a plastic syringe containing 0.2 ml of 3.15% sodium citrate solution. This sample was used for the measurement of platelets, hematocrit, APTT, PT and fibrinogen. Another 1 ml of blood was taken using a syringe without any anticoagulant for the measurement of FDP. The platelet count and hematocrit value were measured using an automatic blood cell counter (Sysmex E2500, Toaiyoudenshi, Tokyo, Japan). Plasma was separated by centrifugation and stored at -70°C until the assay. Clotting time was measured with a fibrometer (Biomatic B10, Sarstedt, Germany), and the blood coagulation system assay was performed as previously described (6). The serum FDP level was determined using an immunoprecipitation test kit (FDPL test). If the FDP value was less than the detectable limit (2.5 μg/ml), it was regarded as zero.

**Determination of plasma TF activity**: In another series of experiments, the plasma TF activity was determined in addition to the DIC parameters. At the end of the 4-hour infusion of endotoxin or 0.9% saline, 1.8 ml of blood was taken from the abdominal aorta using a plastic syringe containing 0.2 ml of 3.15% sodium citrate solution. The plasma TF activity was measured according
to the method of Fukuda et al. (5). In brief, the euglobulin fraction was prepared to remove inhibitors such as antithrombin III, $\alpha_2$-plasmin inhibitor and $\alpha_2$-macroglobulin, heated to denature fibrinogen and factor Xa, and then incubated with both factor VII and X. The activity of the newly formed factor Xa was determined by the amidolytic activity using a chromogenic substrate, S-2222. Amounts of amidolysed S-2222 per minute were calculated from the optical density for paranitroaniline (11). If the amidolytic activity of the sample was less than the detectable limit (45 nkat/l), the TF activity was taken to be zero.

**Direct effects of TCV-309 on DIC parameters and TF activity**: The direct effect of TCV-309 on the DIC parameters were examined as follows. Normal rat plasma (0.99 ml) was mixed with 0.01 ml of TCV-309 (final concentration; 0.1 mM) or saline and TT, PT and APTT were measured as described above. The direct effect of TCV-309 on the TF activity was examined as follows. Lyophilized thromboplastin (dissolved in the barbital buffer, final concentration of 0.1 mg/ml) was mixed with TCV-309 (dissolved in the barbital buffer, a final concentration of 0.1 mM), and 0.075 ml of the mixture was incubated with 0.15 ml of Tris buffer containing 15 mM of CaCl$_2$ for 5 minutes at 37°C and then incubated with PPSB solution for 10 minutes to generate factor Xa. Its activity was determined as described above.

**Histological examination of the kidney**: Two rats with DIC and two with DIC treated with TCV-309 (1 mg/kg) were used for histological examinations. The kidneys were removed just after the collection of blood for the measurement of DIC parameters, fixed with 10% neutrally buffered formalin and embedded in Tissue Prep (Fisher Scientific Corp., NJ, USA). Thin sections were made and stained with phosphotungstic acid hematoxylin.

**Statistical Analysis**: Data are expressed as the mean±SEM. Williams Wilcoxon's test was used for statistical analysis, and differences were considered significant when $p<0.05$

**RESULTS**

**Effects of TCV-309 on endotoxin-induced DIC**: A 4-hour infusion of endotoxin caused DIC symptoms in rats (Fig 1). The platelet count (PLT) was significantly decreased from the normal values of 70.5±2.7 to 24.2±2.8 $\times 10^4$ /µl (p<0.01), and PT and APTT were significantly prolonged from 11.4±0.2 and 34.4±1.2 to 18.8±1.5 (p<0.01) and 57.3±4.3 (p<0.01) seconds, respectively. Plasma fibrinogen was decreased from the normal values of 2.7±0.1 to 0.4±0.1 mg/ml (p<0.01), whereas serum FDP was increased from less than 2.5 to 16.8±2.9 µg/ml (p<0.01). TCV-309 showed beneficial effects in the endotoxin-induced DIC in a dose-dependent manner. A high dose of TCV-309 (0.2 mg/kg+0.2 mg/kg/hour for 4 hours) partially but significantly
improved all these DIC parameters (Fig 1): both platelet count and plasma fibrinogen were increased by 34%. PT and APTT were shortened by 65 and 63%, respectively, and the serum FDP level was decreased by 73%. The low dose of TCV-309 (0.02 mg/kg + 0.02 mg/kg/hour for 4 hours) tended to improve these DIC parameters. The high dose of TCV-309 in itself had no effect on DIC parameters in normal rats; PLT, 70.2 ± 3.4 × 10^4/μl; PT, 11.1 ± 0.2 seconds; APTT, 34.0 ± 0.7 seconds; plasma fibrinogen, 2.9 ± 0.3 mg/ml; and FDP, <2.5 μg/ml (n=5).

Plasma TF activity in DIC rats and the effects of TCV-309: Plasma TF activity was under the detectable limit in two out of six normal rats. The endotoxin infusion markedly increased plasma TF activity from the normal value of 59 ± 20 to 1453 ± 187 nkat/l (Fig 2). Thus, a 25-fold increase in plasma TF activity was observed in rats with DIC as compared with normal rats. The high dose of TCV-309 inhibited the increase in plasma TF activity induced by endotoxin by 47%, and the TF activity was 804 ± 147 nkat/l.

In vitro effects of TCV-309 on DIC parameters and TF activity: TCV-309 even at 0.1 mM had no in vitro effects on TT, APTT, PT or TF activity (Table 1).

Table 1

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>TCV-309</th>
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<tbody>
<tr>
<td>TT (sec)</td>
<td>9.7 ± 0.3</td>
<td>9.7 ± 0.2</td>
</tr>
<tr>
<td>PT (sec)</td>
<td>11.6 ± 0.2</td>
<td>11.5 ± 0.1</td>
</tr>
<tr>
<td>APTT (sec)</td>
<td>33.4 ± 0.7</td>
<td>33.6 ± 0.8</td>
</tr>
<tr>
<td>TF activity (μ kat/l)</td>
<td>2.8 ± 0.1</td>
<td>2.7 ± 0.1</td>
</tr>
</tbody>
</table>

Each result on TT, PT and APTT represents the mean ± SEM of 5 independent experiments. TF activity was measured in triplicate using lyophilized thromboplastin. TCV-309 was used at a final concentration of 0.1 mM.

Effect of PAF infusion on DIC parameters: As shown in Table 2, a 4-hour infusion of PAF (15 μg/kg/hour) caused significant changes in some DIC parameters; such as PT, FDP and plasma fibrinogen. Moreover, PAF slightly but significantly increased the plasma TF activity. TCV-309 (0.2 mg/kg + 0.2 mg/kg/hour for 4 hours) inhibited the PAF-induced changes in FDP, fibrinogen and TF and tended to inhibit the prolongation of the PT.
Fig 1
Inhibitory effects of TCV-309 on endotoxin-induced DIC in rats. Saline or endotoxin (1 mg/kg) was infused for 4 hours. A total dose of 0.1 or 1 mg/kg of TCV-309 was given with the endotoxin (see methods). □; normal (n=8), ■; control (n=11), ●; TCV-309 0.1 mg/kg (n=10), ▲; TCV-309 1 mg/kg (n=10).
Table 2
Effects of TCV-309 on the changes in DIC parameters and plasma tissue factor activity in rats receiving PAF

<table>
<thead>
<tr>
<th></th>
<th>Normal(n=4)</th>
<th>Control(n=8)</th>
<th>TCV-309(n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count(×10⁴/µl)</td>
<td>67.7±1.5</td>
<td>91.4±3.8 **</td>
<td>75.5±3.2 #</td>
</tr>
<tr>
<td>Hematocrit value(%)</td>
<td>38.6±0.4</td>
<td>53.1±2.3 **</td>
<td>40.3±0.5 #</td>
</tr>
<tr>
<td>PT(sec)</td>
<td>11.7±0.2</td>
<td>13.7±0.5 **</td>
<td>12.6±0.2</td>
</tr>
<tr>
<td>APTTT(sec)</td>
<td>34.4±1.0</td>
<td>33.7±4.9</td>
<td>34.1±0.6</td>
</tr>
<tr>
<td>Fibrinogen(mg/ml • plasma)</td>
<td>3.1±0.3</td>
<td>2.2±0.2 *</td>
<td>3.1±0.2 #</td>
</tr>
<tr>
<td>FDP(µg/ml • serum)</td>
<td>&lt;2.5 (4/4)</td>
<td>5.6±2.5 * (3/8)</td>
<td>&lt;2.5 # (8/8)</td>
</tr>
<tr>
<td>TF activity(nkat/l • plasma)</td>
<td>27±27 (3/4)</td>
<td>113±27 * (2/8)</td>
<td>8±8 # (7/8)</td>
</tr>
</tbody>
</table>

Saline containing 0.25% BSA or PAF (60 µg/kg) was infused for 4 hours. A total dose of 1 mg/kg of TCV-309 was given with PAF (see methods). Numerator shows the number of rats with a value under the detectable limit. * and **, significant difference between normal and control at p<0.05 and p<0.01. # and # #, significant difference between TCV-309 and control p<0.05 and p<0.01
Fig 3
Phosphotungstic acid hematoxylin staining of a paraffin section of the kidney from control (A) and TCV-309 (1 mg/kg) treated (B) rats. Prominent fibrin depositions within the glomerular capillaries were observed in the control (arrows). TCV-309 inhibited the fibrin deposition. Original magnification × 520.

Histopathological Examination: In the kidney from DIC rats, massive fibrin deposits were observed in the glomerular capillaries (Fig 3A). The high dose of TCV-309 clearly inhibited the glomerular fibrin formation in the DIC rats (Fig 3B).

DISCUSSION
At a total dose of 1 mg/kg, TCV-309 significantly ameliorated endotoxin-induced DIC symptoms such as thrombocytopenia, prolongation of PT and APTT, hypofibrinogenemia, an increase in FDP and glomerular fibrin deposit in the rat (Figs 1 and 3). Thus, we have confirmed the beneficial effects of PAF antagonists in endotoxin-induced DIC in rats using TCV-309, an agent chemically different from CV-3988 (8).

Though rat platelets lack the PAF receptor (12), TCV-309 as well as CV-3988 significantly attenuated the decrease in platelet count in endotoxin-induced DIC. Rabinovici et al. reported that PAF primed endotoxin-induced thrombocytopenia via the production of TNF α in rats (13). But a part of the thrombocytopenia in the experimental DIC may be caused by the incorporation of platelets into thrombi during blood coagulation. TCV-309 completely inhibited the glomerular fibrin deposit (Fig 3); however, the effects of TCV-309 on the blood DIC parameters were
significant but partial (Fig 1). At the present time, we cannot explain these differential effects of TCV-309.

The antagonistic effect of TCV-309 is specific for PAF. TCV-309 specifically inhibits in vitro PAF-induced platelet aggregation without affecting the aggregation induced by ADP, collagen and arachidonic acid: and furthermore, TCV-309 shows no inhibitory effects on the activity of phospholipase A₂ or enzymes involved in arachidonic acid metabolism such as cyclooxygenase, thromboxane A₂ synthetase, 5-lipoxygenase and 12-lipoxygenase (9). TCV-309 at a dose of 1 mg/kg (i.v.) specifically inhibits hypotension induced by PAF without affecting changes in blood pressure induced by various vasoactive substances including arachidonic acid, bradykinin, leukotriene C₄, prostacyclin and a thromboxane A₂ agonist, U-46619 (9). TCV-309 at a high concentration of 0.1 mM did not affect TF activity or coagulation parameters such as TT, PT and APTT in vitro (Table 1). Furthermore, TCV-309 (1 mg/kg) showed no effects on blood parameters such as APTT and PT in normal rats. TCV-309, therefore, exhibited beneficial effects in endotoxin-induced DIC through its PAF antagonistic action. Thus, we have shown the involvement of PAF in the pathogenesis of endotoxin-induced DIC in rats. To clarify the mechanism of the action of PAF antagonists, we focused on the plasma TF activity in rats with DIC. Recently, Fukuda et al. established a method for the measurement of plasma TF activity using a chromogenic substrate, S-2222, and demonstrated a marked increase in plasma TF activity in patients with DIC (5). We applied their method to the plasma of rats with endotoxin-induced DIC. We found that plasma TF activity in the DIC rats was much higher than that in normal rats, and TCV-309 (1 mg/kg) significantly decreased plasma TF activity in the DIC rats (Fig 2). The infusion of PAF caused a slight but significant increase in plasma TF activity and changes in some DIC parameters, which were ameliorated by the treatment with TCV-309 (Table 2). These results lead to a hypothesis that PAF is involved in the endotoxin-induced DIC via the enhancement of TF generation.

The origin of the TF responsible for the plasma TF increase in the DIC rats remains to be clarified. Possible cells are monocytes and endothelial cells. TF is not normally present in monocytes or endothelial cells, and a variety of stimuli such as endotoxin and cytokines including IL-1 and TNF can cause the expression of TF in these cells (13,14,15). Little is known about the cellular mechanism by which endotoxin induces TF generation. Most recently, however, Hirata et al. showed that PAF and an active component of endotoxin, lipid A, increased TF in macrophages, and that a PAF antagonist (ONO-6240) inhibited the TF generation induced by them (16). Furthermore, endotoxin stimulates biosynthesis of PAF in monocytes, and PAF potentiates the endotoxin-induced generation of cytokines such as IL-1 and TNF (17,18,19). PAF itself even at 0.1 mM has no direct effects on PT and APTT (8). These findings strongly suggest that PAF, that production is stimulated by endotoxin, contributes to the blood coagulation system through the generation of TF. On the other hand, PAF indirectly activates the fibrinolytic system through the release of tissue-type plasminogen activator (tPA) (20,21). Thus, PAF can activate the blood
coagulation-fibrinolytic system via the generation of TF and the release of tPA and can contribute to the pathogenesis of DIC.

The PAF antagonistic action of TCV-309 in platelets is over 100 times more potent than that of CV-3988 (9). However, TCV-309 is about 10 times more potent than CV-3988 in the beneficial effect in DIC rats: Effective doses of TCV-309 and CV-3988 are 1 mg/kg (Fig 1) and 10 mg/kg (8), respectively. Though the precise reason for this contradiction remains to be clarified, the following reasons would explain it. First, the relative potency of the PAF antagonistic action of TCV-309 to that of CV-3988 in monocytes and endothelium may be different from that in platelets. Second, the membrane of these cells may be less permeable to TCV-309 than CV-3988, which masks the 100-fold difference in their PAF antagonistic actions at the intracellular PAF receptor. Stewart et al. reported the existence of an intracellular PAF receptor which is linked to signal transduction in leukocytes and endothelial cells (22).

Our findings do not indicate that PAF is the sole chemical mediator involved in endotoxin-induced DIC, since TCV-309 significantly but partially improved the DIC parameters. Endotoxin causes activation of Hageman's factor and the complement system, platelet aggregation, generation of TF and cell injury (23). These diverse biological actions of endotoxin could explain the quantitative differences in the DIC parameters induced by endotoxin and PAF administration (Fig 1 and Table 1).

We did not measure PAF levels in plasma or tissues, specifically monocytes and endothelium, in this experimental DIC. Endotoxin increases the plasma level of PAF in pigs (24), and causes the release of PAF from monocytes (17). Furthermore, Sakaguchi et al. showed direct evidence that PAF in the blood increased in a patient with DIC using gas chromatography-mass spectrometry (25).

In conclusion, we suggest that PAF plays a role in the pathogenesis of endotoxin-induced DIC via the generation of TF. PAF antagonists may therefore be useful for the treatment of DIC with sepsis.

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