Effect of TRK-100, a Prostacyclin Analogue, on Endotoxin-Induced Enhancement of Blood Coagulation in Rats

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Abstract—TRK-100 (0.03, 0.1 and 0.3 mg/kg, p.o.), a chemically stable analogue of prostacyclin, dose-dependently prevented blood coagulation and glomerular fibrin deposition which were enhanced by 4 hr infusion of endotoxin (100 mg/kg) in rats. In addition, TRK-100 suppressed the generation of endotoxin-induced tissue thromboplastin like activity in cultured human endothelial cells.

Vascular endothelial cells are considered to be anti-thrombogenic, while they participate in coagulation reactions when injured by various kinds of stimuli (1). Endotoxin is one of such injuring stimuli and known to cause shock and disseminated intravascular coagulation (DIC) in human and experimental animals (2, 3). The mechanism by which endotoxin induces DIC has not been fully elucidated yet. There are a number of reports suggesting the involvement of tissue factor. It is a procoagulant activity generated from injured endothelial cells, which might promote coagulation as a trigger in the onset of DIC (4, 5).

PGI₂ is known to possess diverse biological activities besides antiplatelet activity, but its chemical instability has restricted the detailed elucidation of these activities. TRK-100 is a stable analogue of PGI₂, and its actions are shown to mimic well the original PGI₂ (6–8). We studied the effect of TRK-100 on the endotoxin-induced blood coagulation and glomerular fibrin deposit in rats and the generation of tissue factor in cultured human endothelial cells. In addition, we compared the effects of TRK-100 with those of gabexate mesilate (GM) (Ono Pharmaceutical Co., Osaka, Japan), an anticoagulant with serine protease inhibitory activity (9).

Male Wistar rats weighing 191 to 225 g were used for the experiments. Rats were infused endotoxin (Lipopolysaccharide B, E. coli 055:B5, Difco, U.S.A.) at a dose of 100 mg/kg over 4 hr under urethane anesthesia (10). TRK-100, synthesized in the laboratory of Toray Industries, was administered p.o. at doses ranging from 0.03 to 0.3 mg/kg 30 min before and GM was injected i.p. at 10 mg/kg immediately before the infusion of endotoxin, respectively. In the normal group, the rats received i.v. the same volume of saline over 4 hr as the endotoxin infusion. Immediately after the infusion, the blood was collected from the abdominal aorta, and then, both kidneys were removed and fixed in a 10% buffered neutral formalin solution. Activated partial thromboplastin time (APTT), prothrombin time (PT), fibrinogen and fibrin degradation product (FDP), fibrinogen level and platelet count were measured to study the effect of endotoxin on blood parameters. FDP was determined by the latex aggregation reaction using the FDPL® test (Teikokuzoki Pharmaceutical Co., Tokyo, Japan). APTT and PT were determined using clinical assay kits (Toa lyo Densi, Kobe, Japan). Fibrinogen was also determined by measuring the clotting time to form fibrin clots from fibrinogen by addition of excess thrombin using the same kit. The number of platelets was counted with an automatic platelet counter (Toa lyo Densi, PL-100). The ratio of fibrin depositing glomeruli to 100 glomeruli observed was calculated for individual kidneys stained with phosphotungstic acid-hematoxylin. The
Data was represented as % of glomerulus fibrin deposit rate (GFD).

Human endothelial cells were obtained from the umbilical cord vein as described by Jaffe et al. (11). Endothelial cells were characterized by positive immunofluorescent staining with a specific anti-human Factor VIII antibody (MBL, Nagoya, Japan). Generation of procoagulant activity in endothelial cells was determined by the method of Colucci et al. (4), with minor modifications. In brief, endothelial cells at a density of 1.5×10⁴/cm² were cultured for 24 hr in Medium 199 supplemented with 10% fetal calf serum (FCS) in a 24-well plastic plate (Corning, U.S.A.). Then the cells were cultured in 2% FCS with or without TRK-100. After 24 hr, endotoxin was added to the cells at 0.1 µg/ml, and the cells were cultured for a further 6 hr. The cells were washed three times with phosphate-buffered saline (PBS), treated with 0.05% trypsin-0.02% EDTA and suspended with 0.5 ml of PBS. Procoagulant activity was determined by a one-stage clotting assay (4) after the cells were disrupted by three cycles of freezing and thawing. The clotting time was measured in duplicate with an automatic coagulation apparatus (Toa lyo Denshi, CA-100) using the following test system: 0.1 ml test sample, 0.1 ml human plasma and 0.1 ml 0.02 M CaCl₂. Results were expressed in arbitrary units by comparison of the clotting time of endothelial cells with a standard curve of clotting time produced by dilutions of rabbit brain thromboplastin (Kokusaishiyaku Co., Kobe, Japan). One hundred units of thromboplastin caused normal plasma to clot in 13 sec. The data, except GFD, represent the mean±S.E., and the results were statistically evaluated by Student’s t-test. The data of GFD represent the mean±S.D. and results were statistically evaluated by the Kruskal Wallis H-test and D'Annitt type test.

The changes in blood and histological parameters in endotoxin-treated rats and the effects of TRK-100 are shown in Fig. 1. The continuous infusion of endotoxin resulted in marked decreases in both platelet count and fibrinogen level. In contrast, there were an increase in FDP level and prolongation of APTT and PT in the endotoxin-treated group. The GFD rate was 17% in the endotoxin-treated group compared to 0% in the normal group.

Oral administration of TRK-100 dose-dependently prevented endotoxin-induced changes in blood parameters. At 0.1 mg/kg of TRK-100, the generation of FDP was significantly prevented and glomerular fibrin deposition was suppressed by nearly 70%. The fibrin deposit was completely prevented in the endotoxin-treated rats when TRK-100 was at 0.3 mg/kg. GM partly suppressed the deposition of fibrin, but the effect was not statistically significant.

Endotoxin-induced procoagulant activity in endothelial cells was considered to be a tissue thromboplastin-like factor (TLF), because it failed to shorten the coagulation time of Factor VII-deficient plasma but did shorten the coagulation time of Factor VIII-deficient plasma (Table 1). Endotoxin caused a dose-dependent generation of TLF, and the maximal response was detected at 0.1 µg/ml. The generation reached a maximum 4 to 8 hr after the endotoxin addition (data not shown). There were no microscopic changes in morphology of endothelial cells treated with endotoxin at concentrations ranging from 0.01 to 10 µg/ml for 6 hr. Pretreatment of endothelial cells with TRK-100 resulted in significant suppression of endotoxin-induced TLF generation at a maximum response of 10 nM (Table 1). In contrast, GM, a protease inhibitor, did not suppress it.

We reported here that TRK-100 was effective in inhibiting endotoxin-induced promotion of blood coagulation and glomerular fibrin deposition in rats. In addition, TRK-100 suppressed TLF generation induced by endotoxin when added directly to cultured human endothelial cells. As TRK-100 is known to mimic well PG12 in the activation of platelet adenylate cyclase (8) and in the inhibition of platelet aggregation (6, 7), the present findings suggest a protective role of PG12 in enhancement of coagulation induced by endotoxin. GM is known to be effective in prevention of DIC (12), but there was no significant improvement in blood parameters and glomerular fibrin deposit in the present experiment.

There is no evidence at the moment in-
Fig. 1. Effect of TRK-100 on endotoxin-induced changes in blood parameters (A, B, C, D and E) and in glomerular fibrin deposition (F). Each value represents the mean ± S.E. for A, B, C, D and E and the mean ± S.D. for F of eight rats. Values were significantly changed after the endotoxin infusion in comparison with normal rats: ***P < 0.001. The values after endotoxin were compared with those after pretreatment with TRK-100 or gabexate mesilate (GM). *P < 0.05, **P < 0.01, ***P < 0.001. APTT, activated partial thromboplastin time; PT, prothrombin time; FDP, fibrinogen and fibrin degradation product; GFD, glomerular fibrin deposit rate.

Table 1. Effect of TRK-100 and gabexate mesilate (GM) on generation of tissue thromboplastin like factor (TLF) activity in cultured human endothelial cells

<table>
<thead>
<tr>
<th>Cell treatment</th>
<th>TLF activity (units/3×10⁴ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp. 1: Characterization of TLF</td>
<td></td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) in human normal plasma</td>
<td>1.68±0.13</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) in human Factor VII deficient plasma</td>
<td>0.16±0.02</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) in human Factor VIII deficient plasma</td>
<td>1.02±0.09</td>
</tr>
<tr>
<td>Exp. 2: Effect of TRK-100</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.30±0.02</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + TRK-100 1 nM</td>
<td>0.94±0.05</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + TRK-100 10 nM</td>
<td>0.88±0.07</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + TRK-100 100 nM</td>
<td>0.62±0.07*</td>
</tr>
<tr>
<td>Exp. 3: Effect of GM</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.36±0.03</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + GM 1 μM</td>
<td>1.20±0.11</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + GM 10 μM</td>
<td>1.25±0.13</td>
</tr>
<tr>
<td>Endotoxin (0.1 μg/ml) + GM 100 μM</td>
<td>1.16±0.07</td>
</tr>
</tbody>
</table>

The results are from three separate experiments in duplicate and are means±S.E. *Effect of TRK-100 is significant at P<0.05.

indicating whether the ability of TRK-100 to decrease TLF in endothelial cells contributes to inhibition of endotoxin-induced changes in the blood and glomerulus, but particular attention has been given to the pathological roles of endothelial cells as a trigger in the onset of DIC. The cells participate in the enhancement of coagulation through a direct or other cell mediated mechanism of injuring stimuli (3, 13, 14). Generation of tissue procoagulant factor in the injured endothelial cells might induce promotion of coagulation. So the suppressive effect of TRK-100 on TLF generation suggests the beneficial role of TRK-100 in the enhancement of coagulation induced by endotoxin. The reason why TRK-100 suppressed TLF generation has remained unclear. It is speculated that the increased level of cAMP might be responsible for the suppression. Galdal et al. (15) reported that phosphodiesterase inhibitors suppressed endotoxin-induced tissue thromboplastin generation in cultured human endothelial cells. TRK-100 possibly stimulates adenylate cyclase via PGI₂ receptors in endothelial cells as it does in platelets.

Another possible mechanism for how TRK-100 inhibits endotoxin induced enhancement of coagulation may involve its antiplatelet activity. Aggregated platelets would form clots on the impaired vascular walls to accelerate the development of DIC. TRK-100 has been shown to inhibit collagen induced decrease in platelet count over 5 hr when administered orally to rats at doses ranging from 0.1 to 3 mg/kg (6). In the present experiments, TRK-100 significantly suppressed endotoxin-induced decrease in platelet count at the similar dose range. The inhibition of platelet loss would be partly due to suppression of aggregation through the antiplatelet activity of TRK-100. TRK-100 has neither anticoagulant nor fibrinolytic activity in vitro (T. Murata et al., unpublished observation).

In conclusion, the antiplatelet activity and suppressive effect on TLF generation would be responsible for the protective effect of TRK-100 against endotoxin-induced enhancement of coagulation and glomelular
fibrin deposition. These findings suggest the beneficial role of TRK-100 in prevention of DIC.

References