Changes of Tissue Factor-Dependent Coagulant Activity Mediated by Adhesion Between Polymorphonuclear Leukocytes and Endothelial Cells

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ABSTRACT—The purpose of this study was to examine whether polymorphonuclear leukocytes (PMNs) facilitate a tissue factor, a physiologic initiator of coagulation in endothelial cells, -dependent coagulant activity (TF activity). The TF activity in bovine endothelial cells (BAECs) was significantly increased in a concentration-dependent manner by PMNs (1×10^5 – 1×10^7 cells/ml) without affecting the treatment of N-formyl-methionyl-leucyl-phenylalanine, a selective activator of PMNs, and the addition of PMNs finally resulted in cell damage as evaluated by the lactate dehydrogenase leakage method. In the same conditions, an increase of adhesion between PMNs and BAECs was also observed in a time-dependent manner. However, since direct adhesion of PMNs to BAECs was impossible by using the transwell, PMNs failed to induce any changes in the TF activity. Hence, the change of TF activity found here might be closely related to the PMNs adhesion to BAECs. Indeed, anti-intercellular adhesion molecule-1 (anti-ICAM-1) antibody blocked the increase of TF activity in BAECs. These findings suggest that PMNs could increase TF activity in endothelial cells, which is triggered by adhesion to endothelial cells through ICAM-1.

Keywords: Endothelial cell, Polymorphonuclear leukocyte, Tissue factor, Adhesion molecule

Tissue factor (TF) is a membrane integral glycoprotein tightly associated with generation and fibrin formation and is located in the brain, lungs, blood vessels and placental tissue (1). Although TF is not ordinarily found in endothelial cells under normal conditions, the TF-dependent coagulant activity (TF activity) is increased in several diseases, such as unstable angina (2), coronary atheroma (3), sepsis (4) and inflammatory diseases (5).

Many studies have reported that TF activity in endothelial cells is increased by reactive oxygen species (ROS) and/or cytokines released from inflammatory cells in ischemic and inflammatory disease (6 – 10). It has also been shown that polymorphonuclear leukocytes (PMNs), one type of inflammatory cells, contribute to endothelial cell dysfunction by the release of inflammatory mediators such as ROS and hydrolytic mediators (11). However, the physiological function of PMNs on TF activity in endothelial cells is still unclear.

In the present study, we clarified the direct effects of PMNs on the changes in TF activity in endothelial cells. The adhesion of PMNs to vascular endothelium activated during inflammation has been thought to occur in a coordinated sequence mediated by different classes of adhesion molecules (12). Furthermore, several consensus sequences for transcription factor binding have been identified in the promoter region of the intercellular adhesion molecule-1 (ICAM-1) gene (13). These include sequences for members of the immediate early gene family, whose activity may be controlled by changes in intracellular Ca^{2+} concentration ([Ca^{2+}]). Indeed, adhesion of PMNs to endothelial cells has been reported to increase [Ca^{2+}] in endothelial cells, suggesting that cell-cell adhesion plays an important role in the functional activation of endothelial cells (14). Hence, the involvement of the adhesion of PMNs to endothelial cells in the changes in TF activity were also evaluated using monoclonal antibodies against adhesion molecules, such as anti-ICAM-1 antibody.

MATERIALS AND METHODS

Cell isolation and culture

Vascular endothelial cells were isolated from the bovine thoracic aorta (BAECs) and cultured according to the
method of Yasuda et al. (15). Briefly, the endothelial cell layer of the blood vessel was removed by gently scraping the intimal surface with a scalpel. The cells were cultured in minimum essential medium (MEM) supplemented with 20% fetal bovine serum (FBS), 15 μg/ml gentamicin, 2 μg/ml amphotericin B, 1 μg/ml minocycline and 50 μg/ml ampicillin. When the cells reached confluence, they were trypsinized and then maintained in MEM containing 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. BAECs of 5 to 8 passages were used for all experiments.

**Measurement of TF activity in BAECs**

A measurement of TF activity in BAECs was performed in 12-well culture plates and 12-well culture transwells. BAECs were cultured at confluence, and various cell numbers of PMNs stimulated with or without 10^6 M N-formyl-methionyl-leucyl-phenylalanine (FMLP), were added to the BAECs. After incubation for the optimal time, each well was washed twice with phosphate-buffered saline (PBS), and 1.0 ml of Tris-buffered saline (50 mM Tris, 100 mM NaCl) containing 1 mg/ml bovine serum albumin was added to each well. The cells were mechanically scraped by a scraper and lysed by repeated freezing and thawing (−70°C/37°C) cycles. The measurement of TF activity was modified according to the method of Fukuda et al. (16). Briefly, a prothrombin complex (coagulation factor II, factor VII, factor IX and factor X) and TF were added to the cell lysate. The reaction was started by an addition of 10 mM CaCl₂, and the mixture was incubated for 15 min at 37°C. A further 20 μl chromogenic substrate was added to the reaction mixture, which is specific for factor Xa (S-2765; Kabi Diagnostica, Stockholm, Sweden). The absorbance of free chromophore (para-nitroaniline) generation was read by a spectrophotometer at 405 nm. However, to observe the direct influence of adhesion between PMNs and BAECs, on the changes in TF activity generation was read by a spectrophotometer at 405 nm.

**Preparation of PMNs**

PMNs were collected from male Wistar rats (6–8-week-old; Saitama Animals Supply Co., Ltd., Saitama). Each rat was injected intraperitoneally (i.p.) 5.0 ml of 0.5% oyster glycogen in saline. After 4 h, the rat was i.p. injected with 20 ml of PBS containing 10% FBS. After centrifugation (170 × g) for 10 min at 4°C, the supernatant was discarded and the remaining red pellet underwent hypotonic lysis by the addition of 10% NaCl solution. After 30 s, the lysate underwent isotonicization by the addition of an equal volume of 1.6% NaC1 solution and was centrifuged at 170 × g for 10 min. The supernatant was discarded and the residual pellet was washed twice with 10 ml of PBS containing 2.0% FBS and then centrifuged at 170 × g for 10 min. The pellet was then suspended in 2.0 ml of MEM containing 2% FBS. The purity of PMNs was confirmed by May Grunwald-Giemsa staining (>95%). All animals, which were used in these experiments, were approved by the Animal Care and Use Committee of The Japanese Pharmacological Society.

**Adhesion assay**

Adhesion of PMNs to BAECs was performed by the modified method of Tonnesen et al. (18). Briefly, BAECs grown to confluence in 24-well plates were pretreated with or without FMLP (1 × 10^-6 M) for 5 min. The PMNs (1 × 10^6 cells/ml) were added to each well with or without anti-CD54 monoclonal antibody (anti-ICAM-1 antibody; Immunotech Co., Ltd., Marseille, France) and incubated for 30 min. After incubation, non-attached cells were washed three times with PBS and fixed by ethanol (500 μl). Adhesion of PMNs to BAECs was counted, and the findings were expressed as the number of PMNs adhesion per microscopic field.

**Cell injury assay**

Endothelial cell injury was estimated by leakage of lactate dehydrogenase (LDH). Confluent BAECs in 12-well plates were washed three times with PBS. The washed cells were then treated with PMNs (1 × 10^5 cells/ml) at 37°C in 0.5 ml of MEM containing 2% FBS. LDH activity was determined in cell supernatant and the fraction of the cells solubilized in 0.2% Tween-20. LDH activity was measured using on LDH assay kit (Kyokuto Pharmaceutical Co., Ltd., Tokyo). Fifty microliters of each sample was added to a 96-well plate. The reaction was carried out by addition of the chromogenic agent for 45 min and was terminated by addition of the stop solution. The absorbance at 560 nm was measured colorimetrically using a Nova Path™ mini reader (Nippon Bio-Rad Laboratories, Tokyo). The percentage of the total LDH activity (supernatant fraction plus cell lysate fraction) released into the supernatant fraction was then calculated.

**Statistical analyses**

Values were expressed as the means ± S.E.M. of n observations for each experiment. Differences between groups were assessed by Bonferroni’s test and ANOVA. Differences among means were considered significant at P<0.05.
RESULTS

The concentration-dependent changes of TF activity induced by PMNs

As shown in Fig. 1, TF activity was significantly increased by the PMNs (1 × 10⁴ to 1 × 10⁷ cells/ml) without affecting the presence or absence of FMLP in a concentration-dependent manner. The TF activity was maximum at the concentration of 1 × 10⁶ cells/ml. Addition of FMLP alone to BAECs did not induce any changes in TF activity (data not shown).

Time course of PMNs adhesion to BAECs and TF activity in BAECs

Adhesion of PMNs without FMLP to BAECs was significantly induced in a time-dependent manner within 30 min. The increase of adhesion was further enhanced by addition with FMLP (Fig. 2A). However, the increase in TF activity was induced by PMNs without relation to the presence of FMLP (Fig. 2B). Addition of FMLP alone to BAECs did not induce any changes in TF activity at each incubation time (data not shown).

The changes of TF activity induced by PMNs under differential conditions

Under the conditions, where PMNs could directly touch BAECs, TF activity was significantly increased by PMNs with or without FMLP (Fig. 3A). Changes in TF activity, however, were not observed under culture conditions with PMNs physically separated from BAECs by the transwell culture system (Fig. 3B).

Involvement of adhesion molecules on the changes of TF activity induced by PMNs

Adhesion of PMNs treated FMLP to BAECs was inhibited by anti-ICAM-1 antibody in a concentration-dependent manner (Fig. 4A). Similarly, anti-ICAM-1 antibody also reduced the increase in TF activity (Fig. 4B).

Damage of endothelial cells induced by PMNs

Leakage of LDH from BAECs was not changed by the addition of PMNs with FMLP within 60 min, compared with the control untreated PMNs. However, 120 min after PMNs addition with FMLP, LDH leakage, indicating cell damage, was significantly increased (Fig. 5).

Fig. 1. Effects of PMNs on TF activity in BAECs. BAECs were incubated with various concentrations of PMNs (1 × 10⁴ to 1 × 10⁷ cells/ml) in the presence or absence of FMLP (1 × 10⁻⁶ M) at 37°C for 30 min. TF activity in BAECs was measured as described in Materials and Methods. Each value represents the mean ± S.E.M. of three experiments. ***P<0.001, compared with BAECs alone.

Fig. 2. Time course of PMNs adhesion to BAECs and TF activity in BAECs. A) PMNs adhesion was evaluated by counting adhesive PMNs (1 × 10⁶ cells/well) in the presence or absence of FMLP (1 × 10⁻⁶ M). B) The changes in TF activity in BAECs induced by PMNs in the presence or absence of FMLP was measured as described in Materials and Methods. Measurement was conducted at 0, 15, 20 and 30 min after PMNs addition. Each value represents the mean ± S.E.M. of three experiments. *P<0.05, compared with BAECs alone (the absence of PMNs).
DISCUSSION

TF, a biologic initiator of coagulation, is tightly controlled in its function of accelerating factor VIIa-mediated activation of factors X and IX. TF release in plasma has been reported to increase under various diseases, such as acute myocardial infarction (19), and unstable angina (2, 9), suggesting the involvement of thrombus formation.
Recently, we have reported that TF plays an important role in endothelial cell injury after ischemia-reperfusion in vivo (20). In addition, endothelial cell injury under inflammation has been understood to be partially involved in an adhesion of inflammatory cells (21, 22). In the present study, we tried to directly demonstrate that PMNs could increase TF activity and its activity was closely related to the adhesion of PMNs to endothelial cells.

It has been reported that the increase in TF activity in endothelial cells is induced by ROS or cytokines released from inflammatory cells (5, 7–9). PMNs stimulated by FMLP enhance the adhesion to endothelial cells and release ROS and cytokines (23, 24). Therefore, the influence of FMLP on TF activity increased by PMNs was examined. In the present study, however, TF activity increased by PMNs was not influenced by FMLP treatment, while the hyper-adhesion of PMNs to endothelial cells was observed by FMLP treatment. These findings indicate that the TF activity increased by PMNs is not dependent on the release of ROS and cytokines. However, in these experiments, as PMNs were prepared from rats, the possibility that PMNs were originally being activated without treatment of FMLP could not be excluded in the present time. Furthermore, under conditions where adhesion between PMNs and endothelial cells was impossible using the transwell, PMNs failed to increase the TF activity, suggesting that the adhesion might be one of the important factors for the increase of TF activity. These hypotheses were further confirmed by the following experiments, which were conducted by the use of adhesion molecule antibodies, such as anti-ICAM-1 antibody. Interestingly, the PMNs-increased TF activity was reduced by this antibody in parallel with the inhibition of adhesion of PMNs to endothelial cells. These findings strongly suggest that the changes of TF activity induced by PMNs are closely related to the adhesion of PMNs to endothelial cells through ICAM-1. In this case, however, anti-ICAM-1 antibody was not able to inhibit completely both phenomena. As it is well known that PMNs contain, in addition to ICAM-1, the adhesion molecules P- and E-selectin, involvement of these selectins could not be excluded.

Recently Schmid et al. have been reported evidence for E-selectin- and ICAM-1-linked signal pathways via an autocrine feedback loop involving PAF and TNF-alpha secretion (10). We speculate that the TF activity induced by the adhesion of PMNs to endothelial cells may amplify ICAM-1-linked signal pathways through an autocrine feedback.

In a previous study, we showed an increase in TF activity leading to endothelial cell injury under the ischemia-reperfusion as evaluated by LDH method (20). In the present study, TF activity increased by PMNs was observed within 30 min after addition of the PMNs, while LDH release in endothelial cells was observed after more than 120 min. It has recently been reported that apoptosis (programmed cell death) leads to an increase in TF activity and the induction of ICAM-1 and VCAM-1, indicating the close relationship between apoptosis and endothelial adhesion (25, 26). These findings appear to support the present findings that there is an intimate connection between TF activity and endothelial adhesion. Furthermore, the coupling of TF activity with the apoptosis-mediated endothelial adhesion system might lead to the death of dispensable cells within tissues being remodeled by apoptosis.

In conclusion, PMNs could increase TF activity in endothelial cells. Moreover, the adhesion of PMNs to endo-
Endothelial cells through ICAM-1 may play an important role in the changes in TF activity.

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