Involvement of Adenosine A₂ Receptors in the Changes of Tissue Factor-Dependent Coagulant Activity Induced by Polymorphonuclear Leukocytes in Endothelial Cells

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Received July 30, 2001 Accepted December 28, 2001

ABSTRACT—We have already reported that polymorphonuclear leukocytes (PMNs) could increase tissue factor-dependent coagulant activity (TF activity) in endothelial cells mediated by adhesion of PMNs to endothelial cells. In the present study, the role of adenosine receptors in the changes of TF activity and of adhesion between PMNs and endothelial cells was examined. The increases of the TF activity and adhesion were significantly reduced in a concentration-dependent manner by pretreatment of adenosine (0.1 and 1.0 mM); an adenosine A₁/A₂-receptor agonist, CGS-21680 (5, 10 and 50 μM); and an adenosine A₂-receptor agonist, 5′-(N-cyclopropyl)-carboxamidoadenosine (CPCA; 1.0, 10 and 100 nM). An adenosine A₂-receptor antagonist, 3,7-dimethyl-1-(2-propynyl) xanthine (DMPX; 1.0 and 100 nM), antagonized significantly the reduction of the TF activity and the adhesion induced by adenosine (1.0 mM), while 8-cyclopentyl-1,3-dimethylxanthine (CPDMX; 1.0 and 100 nM), an adenosine A₁-receptor antagonist, did not affect it. On the other hand, the TF activity and the adhesion were not changed by N⁶-cyclohexyladenosine (CHA; 10 and 100 nM) and 2-chloro-N⁶-cyclopentyladenosine (CCPA; 10 and 100 nM), adenosine A₁-receptor agonists in the same conditions. These results suggest that the reduction in the TF activity stimulated by PMNs is closely related to the adhesive inhibition between PMNs and endothelial cells through the adenosine A₂-receptor-mediated system.

Keywords: Tissue factor, Endothelial cell, Adenosine receptor, Polymorphonuclear cell, Cell adhesion

In our previous report (1), polymorphonuclear leukocytes (PMNs) have been shown to lead to the increase of tissue factor-dependent coagulant activity (TF activity) in endothelial cells, which is triggered by adhesion of PMNs to endothelial cells.

Adenosine receptors classified into three types, A₁, A₂ and A₃ are known to be located on the membrane of PMNs (2 – 4). It has been shown that the blockade of adenosine A₂ receptors induces an anti-inflammatory action mediated through the inhibition of adhesion of PMNs to endothelial cells (5) and of reactive oxygen species (ROS) release from PMNs stimulated by adenosine (6). Cronstein et al. (7) have reported that adenosine A₁ receptors also play an important role in the PMNs chemotaxis, phagocytosis, and adhesion activity. Furthermore, it has been shown that the cell adhesion between PMNs and endothelial cells is inhibited by adenosine deaminase inhibitor, suggesting the intimate participation of adenosine receptors on the modulation of adhesion between PMNs and endothelial cells (8). Hence in the present study, the role of adenosine receptors on the changes of TF activity after PMNs stimulation in bovine aorta endothelial cell (BAECs) was examined by the use of agonists and antagonists for adenosine receptors.

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. (9). 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

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10% FBS, 100 units/ml penicillin G, 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 to confluence, and various cell numbers of PMNs stimulated with or without 10^{-6} M N-formyl-Met-Leu-Phe (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. (10). 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 CaCl_2, 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.

**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 4.0 ml of 100 U/ml heparin. The cells infiltrated in the abdominal cavity were collected with 50 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 0.2% NaCl solution. After 30 s, the lysate underwent isotonization by the addition of an equal volume of 1.6% NaCl 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% fetal calf serum (FCS) 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 Grünwald-Giemsa staining (>95%). Procedures for animal experiments were conducted according to the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

**Adhesion assay**

Adhesion of PMNs to BAECs was performed by accord-
Adenosine on TF Activity and Adhesion

Fig. 1. Effects of adenosine on the increase of TF activity induced by PMNs (A) and cell adhesion between BAECs and PMNs (B). Adenosine (0.1 and 1.0 mM) was incubated with PMNs (1 x 10^6 cells/ml) in the presence of FMLP (1 x 10^-6 M) at 37°C for 30 min. TF activity in BAECs was measured as described in “Materials and Methods”. The control (C) was incubated with MEM instead of PMNs. Each value represents the mean ± S.E.M. ###P<0.001, compared with control group. *P<0.05, **P<0.01 and ***P<0.001, compared with PMN-treated group without adenosine.

Fig. 2. Antagonistic effects of CPDMX on the decrease of TF activity induced by adenosine (A) and cell adhesion between BAECs and PMNs induced by adenosine (B). Adenosine (1.0 mM) and CPDMX (1.0 and 100 nM) were incubated with PMNs (1 x 10^6 cells/ml) in the presence of FMLP (1 x 10^-6 M) at 37°C for 30 min. TF activity in BAECs was measured as described in “Materials and Methods”. The control (C) was incubated with PMNs without adenosine. Each value represents the mean ± S.E.M. ###P<0.001, compared with control group. *P<0.05, **P<0.01 and ***P<0.001, compared with adenosine-treated group without CPDMX. For other details, refer to the legend of Fig. 2.

Fig. 3. Antagonistic effects of DMPX on the decrease of TF activity induced by adenosine (A) and cell adhesion between BAECs and PMNs induced by adenosine (B). Adenosine (1.0 mM) and DMPX (1.0 and 100 nM) were incubated with PMNs (1 x 10^6 cells/ml) in the presence of FMLP (1 x 10^-6 M) at 37°C for 30 min. Each value represents the mean ± S.E.M. ###P<0.001, compared with control group. *P<0.05, **P<0.01 and ***P<0.001, compared with the adenosine-treated group without DMPX. For other details, refer to the legend of Fig. 2.
Fig. 4. Effects of CHA on the increase of TF activity induced by PMNs (A) and cell adhesion between BAECs and PMNs (B). CHA (10 and 100 nM) was incubated with PMNs (1 \times 10^6 cells/ml) in the presence of FMLP (1 \times 10^{-6} M) at 37°C for 30 min. Each value represents the mean ± S.E.M. For other details, refer to the legend of Fig. 1.

Fig. 5. Effects of CCPA on the increase of TF activity induced by PMNs (A) and cell adhesion between BAECs and PMNs (B). CCPA (10 and 100 nM) was incubated with PMNs (1 \times 10^6 cells/ml) in the presence of FMLP (1 \times 10^{-6} M) at 37°C for 30 min. Each value represents the mean ± S.E.M. For other details, refer to the legend of Fig. 1.

Fig. 6. Effects of CGS-21680 on the increase of TF activity induced by PMNs (A) and cell adhesion between BAECs and PMNs (B). CGS-21680 (5.0, 10 and 50 μM) was incubated with PMNs (1 \times 10^6 cells/ml) in the presence of FMLP (1 \times 10^{-6} M) at 37°C for 30 min. Each value represents the mean ± S.E.M. ***P<0.001 compared with the PMN-treated group without CGS-21680. For other details, refer to the legend of Fig. 1.
CGS-21680 (5.0, 10 and 50 μM) (Fig. 6: A and B), and an adenosine A<sub>2</sub>-receptor agonist, CPCA (1.0, 10 and 100 nM) (Fig. 7: A and B), significantly inhibited the TF activity and the adhesion in a dose-dependent manner. Both basal TF activity and adhesion were not influenced by CGS-21680 and CPCA (data not shown).

DISCUSSION

In this study, we reconfirmed our previous evidence that PMNs could increase TF activity in endothelial cells mediated by adhesion of PMNs to endothelial cells (1). It has been known that endothelial cell injury is caused by inflammatory cell and/or ROS and cytokines released from inflammatory cells (12). Furthermore, the severity of endothelial cell injury has been accepted to be mainly dependent on the inflammatory condition involving the adhesion between PMNs and endothelial cells (13–15). Adenosine, which blocks the adhesion between PMNs and endothelial cells, is reported to inhibit endothelial cell injury during reperfusion following ischemia through adenosine A<sub>2</sub> receptors (16). In the present study, the increase of TF activity and the adhesion was significantly reduced by adenosine, the adenosine A<sub>1</sub>/A<sub>2</sub>-receptor agonist CGS-21680, and the adenosine A<sub>2</sub>-receptor agonist CPCA. Furthermore, the adenosine A<sub>2</sub>-receptor antagonist DMPX antagonized significantly the reduction of the TF activity and the adhesion induced by adenosine, while CPDMX, an adenosine A<sub>1</sub>-receptor antagonist, did not affect it. These results suggest that the inhibitory effect on TF activity stimulated by PMNs is related to the adhesive inhibition of PMNs to endothelial cells through the adenosine A<sub>2</sub>-receptors, but not A<sub>1</sub>-receptors.

Recently, Deguchi et al. (17) have reported that adenosine inhibits through the adenosine A<sub>1</sub>-receptor the TF activity in human umbilical artery endothelial cells (HUVEC) stimulated by tumor necrosis factor-α, thrombin and phorbol 12-myristate 13-acetate. In this study, however, the PMN-stimulated TF activity was not influenced by CHA and CCPA, adenosine A<sub>1</sub>-receptor agonists, suggesting that the TF activity stimulated by PMNs is not regulated by the changes of PMN adhesion to endothelial cells through the adenosine A<sub>1</sub>-receptor. On the other hand, Cronstien et al. (7) have shown that the stimulation of A<sub>1</sub> adenosine-receptors promotes adhesion of neutrophil to endothelial cells. The reasons for these discrepancies of the involvement of adenosine A<sub>1</sub>-receptors on the changes of TF activity are not well documented in the present time. Further experiments to answer these questions must be performed.

In our previous report (1), we showed that the adhesion of PMNs to endothelial cells through intercellular adhesion molecule-1 (ICAM-1, one of adhesion molecules) may play an important role in the changes in TF activity. Interestingly, Mac-1 and LFA-1, counter receptors of ICAM-1, are reported to be decreased by adenosine through adenosine A<sub>2</sub>-receptors (18–21). In addition, Wollner et al. have reported that an adenosine A<sub>2</sub>-receptor agonist produces a dose-dependent inhibition of the increase in Mac-1 expression on neutrophils stimulated with FMLP (22). Hence, these findings including our evidence suggest that adenosine A<sub>2</sub>-receptors play an important role in the changes of PMN-stimulated TF activity in endothelial cells, which is triggered by adhesion to endothelial cells through ICAM-1.

In the present study, effective contribution of adenosine in the changes of PMN-stimulated TF activity in endothelial cells is actually presented. Adenosine, however, exists abundantly in blood of the whole body even in a normal state. Therefore, as it might be difficult to change dramatically the whole blood concentration of adenosine,
it is better to consider the local changes of that in the PMNs and endothelial cells. Indeed, it has been reported that the release of adenosine is increased during myocardial ischemia, suggesting that the accumulation and increase of adenosine are caused by the inhibition of adenosine kinase and/or the increase of adenine nucleotides, such as ATP and ADP, released from platelets, mast cells and endothelial cells (23, 24). Furthermore, vascular ATP diphosphohydrolase/CD39, one of endothelial cell membrane proteins, has been known to possess both ecto-ATPase and ecto-ADPase activities regulating the transformation into adenosine from adenine nucleotides (25, 26). In the inflammatory and ischemic state, it has been also shown that both the release of ATP and ADP are enhanced in the endothelial cells and activated-platelets (27). These evidence suggest that adenosine derived from PMNs and endothelial cells plays an important role in the changes of PMN-stimulated TF activity in endothelial cells.

In conclusion, the reduction of TF activity stimulated by PMNs is suggested to be closely related to the adhesive inhibition between PMNs and endothelial cells through the adenosine-A<sub>2</sub>-receptor-mediated system.

REFERENCES