Lysenin, a Sphingomyelin-Binding Protein: its Role in the Activation of Platelets

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ABSTRACT

Lysenin is a novel protein of 33 kDa isolated from the earthworm, *Eisenia foetida*, and it binds specifically to sphingomyelin (SM) among the phospholipids located in the cell membrane. Since it is known that SM is rich in the cell membrane of platelets, we evaluated the effects of lysenin on platelets. Flow cytometric analysis revealed that lysenin bound to platelets in a concentration dependent manner (1–100 ng/mL). Moreover, lysenin (1 µg/mL) induced irreversible aggregations of platelets with formation of thromboxane B₂. The expressions of P-selectin and platelet endothelial cell adhesion molecule-1 (PECAM-1) on the surface of platelets were increased 5 min after the incubation of platelets with lysenin (10–100 ng/mL). These results indicate that SM in the cell membrane is involved in activation of platelets by lysenin. Lysenin might be a useful tool for studying the mechanisms of the platelet activation.

Lysenin is a novel protein of 33 kDa isolated from the earthworm, *Eisenia foetida* (24) and it binds specifically to sphingomyelin (SM) among the phospholipids located in the cell membrane (33). It has lethal effects on the mammalian red blood cells (RBC) and spermatogonia (12, 14, 33), whose outer leaflets of their cell membranes are rich in SM com-

pared with the inner leaflets (22, 29). It appears that the cell membranes of both RBC and spermatogonia are damaged by either perturbing the lipid layer structure or by forming aqueous pores induced by binding of lysenin to SM in their cell membrane (14, 33). Since the cell membrane of platelets contains SM at higher levels in the outer leaflets than the inner leaflet, as well as in the RBC (5), it is assumed that lysenin binds to SM in the platelet cell membrane and causes some effects on the platelet. In the present study, the effects of lysenin on platelets, aggregation of platelets, formation of thromboxane B₂ and expression of cell adhesion molecules (P-selectin and PECAM-1), which are requisite for the homophilic and heterophilic aggregation (13, 15, 18, 20, 27, 31), were analyzed to determine the mechanism of platelet activation.

MATERIALS AND METHODS

Animals. Female BALB/c mice (8 weeks of age, 18–20 g in weight) were purchased from Charles River

Abbreviations: SM, sphingomyelin; PECAM-1, platelet endothelial cell adhesion molecule-1; RBC, red blood cell; PGE, prostaglandin E; SMase, sphingomyelinase; PE, phycoerythrin; BSA, bovine serum albumin; PFA, paraformaldehyde; PB, phosphate buffer; TXB, thromboxane B₂; ELISA, enzyme-linked immunosorbent assay; CMC, carboxymethylcellulose; MFI, mean fluorescence intensity.

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Japan Inc. (Kanagawa, Japan). They were maintained at around 22°C with 12 h light/dark cycle and given a standard chow and tap water *ad libitum*.

Reagents. Lysenin and polyclonal rabbit anti-lysenin antiserum were purchased from Peptide Institute (Osaka, Japan). Prostaglandin E (PGE) and sphingomyelinase (SMase) from *Bacillus cereus* were purchased from Sigma Chemical (St. Louis, MO, USA). Diclofenac was purchased from Wako Pure Chemical (Osaka, Japan). Multilamellar liposome vesicles (SM-liposome) containing 1 mM SM and 1 mM cholesterol, mixed at a molar ratio of 1 : 1 were kindly prepared by Dr. A. Yamaji (33). The anti-PECAM-1 monoclonal antibodies (BBIG-P1, Seikagaku Corp, Tokyo, Japan), anti-P-selectin monoclonal antibodies (G1/G1-4, Ancell Corp. Bayport, MN, USA), phycoerythrin (PE)-conjugated goat anti-rabbit IgG (Southern Biotech. Assoc. Inc., Birmingham, AL, USA), and PE-conjugated goat anti-mouse IgG (Immunotech, Marseilles, France) were used for flow cytometry evaluation.

Preparation of platelets. Venous blood from healthy subjects was drawn with polypropylene syringe containing 3.8% sodium citrate (one tenth of blood volume) and centrifuged at $100 \times g$ for 15 min to obtain the platelet-rich plasma. Next the platelets were sedimented by centrifugation at $2,000 \times g$ for 10 min and washed twice with 10 mL of Hepes-Tyrode buffer (4 mM Hepes, 1 mM $MgCl_2$, 130 mM NaCl, 3 mM KCl, and 5 mM glucose, 0.35% BSA, pH 6.4) in the presence of PGE (1 $\mu g/mL$). After that, the platelets were resuspended in Hepes-Tyrode buffer (pH 7.4).

Binding of lysenin to platelets. Isolated platelets were treated with ice-cold 1% paraformaldehyde (PFA) in PBS to prevent activation of platelets. It has been reported that PFA-treatment does not inhibit the binding of lysenin to SM in the cell membrane (16, 33). After washing with Hepes-Tyrode buffer, the platelets (1×10^6 cells/100 μL) were incubated with various concentrations of lysenin for 30 min at 4°C. After washing with Hepes-Tyrode buffer, platelets were incubated with anti-lysenin antiserum (1 : 1,000) followed by incubation with PE-conjugated goat anti-rabbit IgG (1 : 1,000) for 30 min at 4°C. The fluorescence intensity of the cells (5,000 cells/experimental group) was analyzed with flow cytometry using FACStar (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Specificity of binding of lysenin to SM in platelets cell membrane. To verify specific binding of lysenin to SM in the cell membrane, lysenin (100 ng/mL) preincubated with SM-liposome (200 μM) for 30 min at 37°C was used. Moreover, the binding of lysenin to the platelet cell membrane, in which SM was digested by SMase was evaluated. For this purpose, platelets (1×10^6 cells/100 μL) were suspended in Hepes-Tyrode buffer without BSA, and incubated with SMase (1 mUnit/mL) for 30 min at 37°C. After treatment with 1% PFA, the binding of lysenin (100 ng/mL) to platelets treated with SMase was analyzed with flow cytometry.

Platelets aggregation. Aggregation of platelets was measured by turbidimetric method with the aggregometer (NBS Hema Tracer 601, Niko Bioscience Inc., Tokyo, Japan) after addition of lysenin (1 $\mu g/mL$) to the platelet suspension in the presence of 1 mM $CaCl_2$.

Scanning electron microscopy (SEM). After the incubation of platelets (1×10^8 cells/mL) with 1 $\mu g/mL$ of lysenin in the presence of 1 mM $CaCl_2$ for 3 min at 37°C, the platelets were fixed with 10 mL of 4% PFA in phosphate buffer (PB, pH 6.7) for 30 min at 4°C. After centrifugation at $2,000 \times g$ for 10 min, sedimented platelets were post-fixed with 100 μL of 5% glutaraldehyde in 0.15 M PB (pH 6.7) for 30 min. The platelets were then washed with 0.15 M PB and mounted on cover slides coated with poly-L-lysine (10 $\mu g/mL$). After dehydration in graded ethanol series and replacement of ethanol with isoamyl acetate, the platelets were dried with a critical point dryer (HCP2, Hitachi, Tokyo Japan), sputtered with platinum-palladium, and examined under scanning electron microscopy (S-800, Hitachi).

Formation of thromboxane B_2 . Isolated platelets (1×10^8 cells/mL) were incubated with lysenin in the presence of 1 mM $CaCl_2$. The reaction was terminated by adding indomethacin (10 $\mu g/mL$) at 5 min after incubation of platelets treated with lysenin. The amount of thromboxane B_2 (TXB) in the culture supernatant was determined using TXB immunoassay kit (R&D system Inc., Minneapolis, MN, USA). The enzyme-linked immunosorbent assay (ELISA) procedures were conducted according to the manufacture's instructions.

Expression of P-selectin and PECAM-1 in platelets. Isolated platelets (1×10^6 cells/100 μL) were incubated with lysenin (1, 10, 100 ng/mL) in a CO_2 incu-

bator for 5 min in the presence of 1 mM CaCl_2 or 30 min in the absence of CaCl_2 . The cells were then treated with 1% PFA, and washed twice with Hepes-Tyrode buffer (pH 7.4). The cells were stained for 30 min with a saturated concentration of anti-P-selectin monoclonal antibodies (1 : 100) or the anti-PECAM-1 monoclonal antibodies (1 : 1,000) diluted with Hepes-Tyrode buffer, followed by reaction with PE-conjugated goat anti-mouse IgG (1 : 100) for 30 min. Thereafter, the fluorescence intensity of the cells (10,000 cells/experimental group) was analyzed with flow cytometry.

Effects of lysenin on platelets *in vivo*. We determined the effects of diclofenac on survival time after injection of a lethal dose of lysenin, since diclofenac is known to inhibit the activation of platelets (7). Diclofenac suspended in 1% carboxymethylcellulose (CMC) was administered intraperitoneally 30 min prior to intravenous administration of lysenin (60 $\mu\text{g/kg}$). These experiments were conducted with authorization of the Animal Care and Use Committee at the Research Laboratory of Zen-yaku Kogyo Co. Ltd.

Statistical analysis. Statistical analyses were conducted utilizing the Mann-Whitney's U test for non-parametrical data in the experiments for effects of diclofenac on the survival time after injection of lysenin. P values of less than 0.05 were considered statistically significant.

RESULTS

Binding of lysenin to platelets

Flow cytometric analysis revealed that lysenin bound to the surface membrane of the platelet in a concentration dependent manner (1–100 ng/mL, Fig. 1). Lysenin (100 ng/mL) bound to the PFA-treated platelet and the mean fluorescence intensity (MFI) was 647.9. However, it virtually diminished to MFI: 21.2 with the use of lysenin (100 ng/mL) preincubated with SM-liposomes. Next, we determined the binding of lysenin (100 ng/mL) to platelets, which had been treated with SMase. There was no shift in fluorescence intensity (MFI: 8.7), when the effects of lysenin on the platelets treated with SMase were evaluated, indicating a lack of lysenin bound to the platelets. These findings indicate that SM located in the platelet cell membrane is a requisite for binding of lysenin to the platelets.

Fig. 1 Flow cytometric analysis of lysenin binding to platelets. Binding of lysenin to platelets after incubation of lysenin with platelets for 30 min at 4°C. Lysenin bound to platelets in a concentration dependent manner (1, 10, 100 ng/mL).

Induction of aggregation of platelets by lysenin

Addition of lysenin (100 ng/mL or 1,000 ng/mL) to platelet suspension resulted in an increase in light transmission. The level of light transmission reached a plateau 1 min after incubation of platelets with lysenin (1,000 ng/mL, Fig. 2 *left*). Morphologically, the platelets (1×10^8 cells/mL) incubated with lysenin (1,000 ng/mL) for 3 min were swollen, projected many long pseudopodia and aggregated (Fig. 2 *right*). Thus, lysenin (1,000 ng/mL) induced irreversible aggregation within 1 min after incubation.

Effects of lysenin on the formation of TXB₂, P-selectin and PECAM-1 expression in platelets.

The formation of TXB₂ by platelets increased in a concentration dependent manner (10–1,000 ng/mL, Fig. 3). Moreover, flow cytometric analysis revealed that the expression of P-selectin on the surface of platelets was increased by incubation of platelets with 10 or 100 ng/mL of lysenin in the presence of CaCl_2 , but not in the absence of CaCl_2 (Fig. 4a). On

Fig. 2 Aggregation of platelets induced by lysenin. Light transmission reached its maximal level at 3 min after incubation of platelets with lysenin (1,000 ng/mL) in the presence of 1 mM CaCl_2 (left). Cells treated with lysenin (1,000 ng/mL) were swollen and projection of numerous long pseudopodia was confirmed (right). Scale: 5 μm .

Fig. 3 Formation of TXB₂ in platelet induced by lysenin. Platelets were incubated with lysenin for 5 min in the presence of 1 mM CaCl_2 . The formation of TXB₂ was determined by ELISA. Each value is expressed as the mean $\pm$ SD of data from three independent experiments.

the other hand, the expression of PECAM-1 on platelets increased in a concentration dependent manner by incubation of platelets with lysenin (10, 100 ng/mL) for 5 min. This up-regulation of PECAM-1 expression induced by lysenin (100 ng/mL) was also revealed after incubation in the absence of 1 mM CaCl_2 for 30 min (Fig. 4b). These results indicated that lysenin induced the degranulation in platelets which occurs along with the activation of platelets.

Effects of lysenin on platelets in vivo

In rats injected with lysenin (15, 30 or 60 $\mu\text{g/kg}$) into the tail vein, the doses above 30 $\mu\text{g/kg}$ is re-

ported to cause respiratory distress and death within 10 min after injection (24). In our study, lysenin (15, 30 or 60 $\mu\text{g/kg}$) was intravenously injected into five mice. Mice injected intravenously with lysenin at 15 or 30 $\mu\text{g/kg}$ did not die within one week after the injection. However, the injection of lysenin (60 $\mu\text{g/kg}$) induced respiratory distress and death within 10 min after injection. Then, we evaluated the effects of diclofenac on the survival time after injection of lysenin (60 $\mu\text{g/kg}$). The survival time (949.4 ± 98.5 s; $n=5$) of the mice treated with diclofenac (150 mg/kg, *ip*) and lysenin (60 $\mu\text{g/kg}$) was significantly ($P<0.05$) greater than that (165.6 ± 33.3 s; $n=5$) of mice receiving 1% CMC and lysenin (60 $\mu\text{g/kg}$, Fig. 5). These findings indicate that lysenin induces the activation of platelets resulting in death.

DISCUSSION

Lysenin appears to bind to cell membrane of the platelets, as well as RBC, spermatogonia, fibroblast and oligodendrocytes. The binding of lysenin to platelets diminished, when lysenin was preincubated with SM-liposomes. Furthermore, lysenin did not bind to the platelets pretreated with SMase. These results indicate that lysenin binds specifically to SM located in the cell membrane of platelets. The binding may be related to the morphological changes of the platelet cell surface as revealed by scanning electron microscopy. These results coincide with the findings that lysenin increased the formation of TXB₂ in platelets and the expression of both P-selectin and PECAM-1 on the surface of the platelets. Thus, it is revealed that lysenin induced the activation of platelets. It is scientific merit to clarify the

Fig. 4 The up-regulation of P-selectin and PECAM-1 expression on the platelets induced by lysenin. Platelets were incubated with lysenin for 5 min in the presence (closed circle) or 30 min in the absence (open circle) of 1 mM CaCl₂. The expressions of P-selectin (a) and PECAM-1 (b) were analyzed with flow cytometry. Each value is expressed as the mean $\pm$ SD of data from three independent experiments. MFI: Mean fluorescence intensity.

Fig. 5 Effect of diclofenac on the lethal effects of lysenin in mice. Survival time of mice injected with lysenin (60 μ g/kg, *iv*) was greater in the diclofenac (150 mg/kg, *ip*)-treated mice than control mice. Time of each group is expressed as the mean $\pm$ SEM (n=5). The number in each column indicates the number of mice. * P <0.05 (Mann-Whitney's U test).

mechanisms involved in the activation of platelets by lysenin. It is known that platelets are activated *via* receptors for thrombin or collagen, however, the amino acid sequence of lysenin has no homology to those of known proteins (25). Moreover, lysenin does not have the RGD sequence which induces the activation of platelets (32). Accordingly, lysenin appears to activate the platelets through a different

mechanism from those of known activators. In other words, lysenin appears to activate the platelets in specific way: through its binding to SM located in the cell membrane.

Since SM inhibits activities of phosphatidylinositol phosphodiesterase and 12-lipoxygenase in platelets (8, 11), SM is postulated to inhibit the activation of platelets. However, the involvement of SM in the activation of platelets remains obscure. There is a possibility that lysenin binds to SM resulting in the disruption of the lipid layer structure or forming aqueous pores in the cell membrane of platelets, and may induce colloid osmotic changes resulting in influx of Ca²⁺ into the platelets. The increase of intracellular Ca²⁺ induces the activation of platelets (1). Another possibility is that lysenin may affect the "raft" which is SM and cholesterol-rich microdomain located in the cell membrane. Specific proteins assemble in the rafts and play a role in signal transduction (3, 21, 26). It has been reported that Src family kinase is associated with SM in rafts of platelets (30). Src family kinase is involved in the up-regulation of PECAM-1 expression on platelets and phosphorylation of PECAM-1, which is induced by thrombin or convulxin in the presence of EGTA, indomethacine and apyrase (6). In the present studies, the up-regulation of PECAM-1 expression on platelets was revealed after incubation with lysenin in the absence of Ca²⁺. Thus, it was suggested that the binding of lysenin to SM in the rafts may lead to the activation of the Src family kinase.

Activation of platelets is accompanied by an al-

teration in the distribution of lipids located in the cell membrane (2). Negatively charged lipids like phosphatidylserine become exposed at the outer leaflets of cell membrane and facilitate the conversion of factor X to factor Xa by the factor IX-factor VIIIa and Ca^{2+} complex as well as conversion of prothrombin to thrombin by a complex factor Xa-Factor Va and Ca^{2+} (23, 28). Lysenin induced the up-regulation of P-selectin expression on the platelets in this study. The binding of P-selectin to monocyte induces the up-regulation of tissue factor expression on the cell surface of monocyte (4). Tissue factor binds to factor VII and factor VIIa and activates factor IX and factor X (19). Thus, it was suggested that the activation of platelets by lysenin influences the blood coagulation factors as well as heterophilic and homophilic aggregation of platelets (13, 15, 18, 20, 27, 31) resulting in the formation of thrombosis *in vivo*. This suggestion was supported by the results that the lethal effects of lysenin were attenuated by diclofenac, which inhibited the activation of platelets.

Recently, it has been revealed that C type lectins from snake venom affect the function of collagen receptor on platelets (9, 17). However, the mechanism of platelets activation has not been fully clarified, therefore, research and development of a new reagent is required to solve the mechanism of platelets activation (10). In this study, we indicated that lysenin activated the platelets *via* binding to SM located in the cell membrane of platelets. Thus, lysenin may be an useful tool for determining the mechanism involved in platelet activation in relation to the cell membrane.

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