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Interference of neutrophil–platelet interaction by YC-1: A cGMP-dependent manner on heterotypic cell–cell interaction

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Abstract

N-Formyl-Met-Leu-Phe (fMLP) activated neutrophils and then induced neutrophil–platelet complex formation in co-incubation condition. In addition, fMLP induce intracellular calcium mobilization in platelets, only when it is incubated along with neutrophils. This data established that fMLP-stimulated neutrophils activate platelets. 9E1, a monoclonal antibody of P-selectin, significantly blocks the formation of neutrophil–platelet complex induced by fMLP, indicating the involvement of P-selectin in the neutrophil–platelet complex formation. 3-(5′-hydroxymethyl-2′-furyl-1-benzylindazole (YC-1), an unique nitric oxide-independent activator of soluble guanylate cyclase, was evaluated for its effect on neutrophil–platelet complex. YC-1 inhibits fMLP-induced neutrophil–platelet complex formation in a concentration-dependent manner with an IC_{50} value of 15.3 ± 3.5 μM. However, this effect of YC-1 is partially reversed by pre-treatment of 1*H*-(1,2,4)oxadiazolo[4,3-*a*]quinozalin-1-one (ODQ; 10 μM), which is a soluble guanylate cyclase inhibitor. Pre-treatment of either neutrophils or platelets with YC-1 (50 μM) prevent the fMLP-induced neutrophil–platelet complex formation, indicating that YC-1 could potentially exert its effects individually on either neutrophils or platelets alone. Cathepsin G released from fMLP-stimulated neutrophil activates the nearby platelets. YC-1 was also shown to inhibit this release of cathepsin G in a concentration-dependent manner. The IC_{50} value was 6.2 ± 0.2 μM. This inhibitory effect of YC-1 on cathepsin G release is reversed by ODQ (10 μM) and a protein kinase G inhibitor [1-oxo-9.12-epoxy-1*H*-diindolo[1,2,3-fg:3′,2′,1′-kl]pyrrolo[3,4-l][1,6]benzodiazocine-10-carbooxylic acid methyl ester (KT5835); 1 μM]. YC-1 inhibits cathepsin G-induced P-selectin expression on human platelet at the IC_{50} value of 32.5 ± 2.6 μM. A further study showed that YC-1 inhibits fMLP-induced neutrophil–platelet complex formation in whole blood at the IC_{50} value of 35.8 ± 8.1 μM in a concentration-dependent manner. According to these data, it was hypothesized that fMLP stimulates neutrophils to release cathepsin G, which subsequently activates the nearby platelets, creating neutrophil–platelet complexes. YC-1 inhibits fMLP-induced neutrophil from releasing cathepsin G via a cGMP-dependent pathway. This inhibitory effect of YC-1 on cathepsin G release is a major mechanism for affecting fMLP-induced neutrophil–platelet complex. YC-1's inhibition P-selectin expression on platelet may potentiate its effects. These inhibitory effects may contribute to the inhibition of neutrophil–platelet complex formation in whole blood.

Keywords: Neutrophil; Platelet; YC-1; P-selectin; Cathepsin G; fMLP; Whole blood

1. Introduction

Neutrophils and platelets are the predominant cell types found in the microcirculation after an acute inflammatory injury (Brown et al., 1998). Recent studies in patients and in experimental animal models suggest that neutrophil–platelet interactions occur at sites of vascular injury. Furthermore, inflammatory and thrombotic states are associated with circulating neutrophil–platelet aggregation. Neutrophil–platelet aggregation may occur in the circulation and be pathophysiologically significant. Enhanced neutrophil–platelet adhesion has been observed in patients suffering

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from acute myocardial infraction and stroke, as well as those that have undergone coronary angioplasty (Ott et al., 1996). Similarly, increased adhesion of monocyte and neutrophil to platelets has been reported after cardiopulmonary bypass (Rinder et al., 1992; Ogura et al., 2001). Leukocyte–platelet adhesion increases in parallel with the extent of platelet activation. In vitro evidence suggests direct receptor–ligand interactions as being responsible for adhesive interactions between neutrophils and platelets (Hamburger and McEver, 1990). Such heterotypic cell–cell interaction is likely to alter both the neutrophils' and platelets' functional responses, since in vitro evidence suggests that the neutrophils can influence platelet functions and vice versa (Del Maschio et al., 1990; Selak et al., 1988). It has also been suggested that the mediators released from neutrophils and activated platelets may also contribute to allergic asthma (Renesto et al., 1991). Moreover, platelets biologically synthesize active substances such as thromboxane A_2, 12-hydroxy-5,8,10,14-eicosatetraenoic acid or platelet-activating factor (PAF) which are relevant to allergic inflammation (Marcus et al., 1988; Chignard et al., 1979). Thus, such a cell–cell communication may have contributed to various stages of inflammation, and its inhibition may be relevant to anti-inflammatory therapy.

The interaction between a neutrophil and an activated platelet is coordinated by an adhesion cascade, where P-selectin on the platelet binds to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils to promote the initial tethering (Evangelista et al., 1999). Subsequently, integrin $\alpha_M\beta_2$ (CD11b/CD18 or Mac-1) (Sheikh and Nash, 1996) mediates their firm adhesion by binding to platelet GPIb or fibrinogen that is bound to platelet GPIIb/IIIa or $\alpha_V\beta_3$ (Piccardoni et al., 2001). β_2-integrin's ability to bind its ligands, thereby forming a firm complex, is regulated by the cell through an activation process involving intracellular signaling. It is generally accepted that these processes are triggered by G-protein-coupled receptors for chemokines or by lipid chemoattractant coexpressed with the selectins on the cell surface. Moreover, P-selectin's binding to its receptors on neutrophils may promote β_2-integrin's adhesiveness in human and mouse cells (Blanks et al., 1998; Pitchford et al., 2005). The P-selectins expressed on activated platelets or those expressed on P-selectin-transfected Chinese hamster ovary (CHO-P) cells was able to promote a Mac-1-dependent neutrophil–platelet adhesion or CHO-P–cell adhesion. The formation of a β_2-integrin mediated neutrophil aggregation required tyrosine kinase activity and was accompanied by tyrosine phosphorylation of a major protein of approximately 110 Kd (p-110).

3-(5′-hydroxymethyl-2′-furyl-1-benzylindazole (YC-1), an unique nitric oxide (NO)-independent activator of soluble guanylate cyclase (sGC), has been shown to increase the intracellular cyclic GMP concentration in platelets (Wu et al., 1995) and vascular smooth muscle cells (Galle et al., 1999). Further more, the cyclic GMP-increasing effect of YC-1 has been reported to inhibit platelet aggregation (Wu et al., 1995) and mediate vasorelaxation (Galle et al., 1999). Furthermore, it has been demonstrated that YC-1 not only activated sGC but also inhibited cyclic GMP-hydrolyzing phosphodiesterase in human platelets. In our study, YC-1 inhibited *N*-Formyl-Met-Leu-Phe (fMLP)-induced neutrophil–platelet complex formation in both co-incubation and whole blood preparation. Accordingly, this study was designed to investigate the effect of YC-1 on neutrophil and platelets and elucidate the affecting the mechanisms of YC-1's effect on fMLP induced neutrophil–platelet interaction.

2. Material and methods

2.1. Materials

YC-1 was kindly provided by Dr Teng, CM. LDS-751 was purchased from Molecular probe (Eugene, OR), Ficoll was purchased from Amersham Pharmacia Biotech Inc (Piscataway, NJ), HBSS was purchase from Gibco Company (Rockville, MD). Cathepsin G was purchased from ICN, HEPES, substrates of cathepsin G, Suc-AAPF-pNA, dextran, fMLP, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinozalin-1-one (ODQ), NaCl, KCl, $MgCl_2$ and glucose were purchased from Sigma Chemical Co. (St. Louis, MO), CD42b-FITC was purchased from Immunotech Co. (Marseille cedex, France). CD62P-FITC and CD18-FITC were purchased from BD PharMingen Co. (San Diego, CA). 9E1 was purchased from R&D system Inc. (Minneapolis, MN). CD18 (β_2-integrin) blocking mAb YFC118.3 was purchased from Chemicom (Temecula, CA).

2.2. Neutrophil preparation

Venous blood samples were obtained from healthy volunteers of both sexes between the ages of 20 and 40, using syringes that contained heparin (final concentration of 20 unit/ml). All protocols complied with the guidelines set forth by the Chang-Gung Memorial Hospital Ethics Committee. Neutrophil were isolated from the blood samples using the Ficoll gradient centrifugation method and subsequently purified by hypotonic lysis of contamination erythrocytes (Liao et al., 2004).

Briefly, a blood sample was mixed with an equal volume of 3% dextran solution in a 50-ml centrifuge tube and incubated in the upright position for 20 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at 250×*g* for 15 min at 4 °C. Subsequently, the pellet was re-suspended immediately in a volume of phosphate buffer system (PBS) equal to the starting volume of blood. The cell suspension thus obtained was then apportioned into 50-ml centrifuge tubes at 25 ml per tube. Using a pipette, a layer of 10 ml of 1.077 g/ml Ficoll solution was laid beneath the cell suspension. Finally, the sample was centrifuged again (400×*g* for 40 min at 20 °C), at the end of which the upper (PBS) and lower (Ficoll) layers were removed, leaving only the granulocyte/erythrocyte pellets. In order to remove the residual erythrocytes, the pellet was re-suspended in 20 ml cold 0.2% NaCl for 30 s. This is then followed by addition of 20 ml cold 1.6% NaCl to restore tonicity. Finally, the remaining neutrophils were then pelleted, washed twice with ice-cold PBS and re-suspended in an adequate volume of ice-cold Hank's

buffered saline (HBSS). The thus obtained final preparation contained greater than 95% neutrophils, as estimated by differentially counting 200 cells Giemsa staining under the microscope.

2.3. Platelet preparation

The platelet suspension was prepared according to the washing procedure (Wu et al., 1995). Human blood was drawn from healthy, drug-free volunteers. Approximately 60 ml blood was mixed directly with anticoagulant solution (ACD; 25 g trisodium citrate dihydrate, 14 g citric acid, and 20 g glucose per liter) in a 9:1 ratio. Blood was centrifuged at 190×*g* for 15 min to obtain platelet-rich plasma. The plasma was subsequently centrifuged at 1250×*g* for 15 min to obtain the platelet pellets, which was then re-suspended in 10 ml Tyrode's buffer supplemented with 2 units/ml apyrase and 50 units/ml heparin. Next, the cell suspension was incubated at 37 °C for 15 min and centrifuged again at 1000×*g* for 10 min. The platelets were collected and re-suspended gently in 10 ml of Tyrode's buffer and incubated at 37 °C for 10 min. Afterward, the platelets were pelletted at 1000×*g* for 10 min and re-suspended at room temperature in Tyrode's buffer supplemented with 0.3 units/ml apyrase alone. Finally, the platelets in the suspension were counted in an automated cell counter (coulter counter Z1) and suspended at a concentration of 6×10^7/ml. All glassware were siliconized.

2.4. Co-incubation assay

Co-incubation assay was performed according to Tseng et al. (2004). Briefly, purified platelets (6×10^7/ml) were incubated with FITC-conjugated anti-CD42b antibody for 5 min at room temperature for their labeling. Correspondingly, purified neutrophils (2×10^6/ml) were labeled with LDS-751 (1 μg/ml) for 2 min. Mixed preparations were applied to an aggregometer and stirred at 700 rpm with various concentrations of YC-1 (5–100 μM) at 37 °C for 5 min before adding fMLP (1 μM). The reaction was stopped at 5 min after fMLP stimulation with the addition of equal volume of 2% paraformaldehyde. In FACSCcalibur flow cytometeric analysis, a florescence threshold was set to detect only those cells that were labeled with the leukocyte-specific marker LDS-751, thus excluding single platelet from the display. Neutrophil–platelet complexes were considered as those particles that expressed CD42b-FITC fluorescence above background level. In order to identify the effect of YC-1 on neutrophils or platelets, isolated neutrophils or platelets were incubated in the presence or absence of YC-1 (50 μM) at 37 °C for 5 min. Following washing twice, preparations were re-suspended in Hank's buffer containing 0.35% BSA. After mixing neutrophil (2×10^6/ml) with platelets (6×10^7/ml) in tubes, FITC-conjugated anti-CD42b and LDS-751 were added to label platelets and neutrophils, respectively. Following the previous steps, these mixed preparations were analyzed with flow cytometer. A mouse $IgG_1\kappa$ conjugate FITC was a negative control.

2.5. Calcium measurement in platelets on co-incubation system

Calcium measurement was performed according to Sambrano et al. (2000). Platelets were loaded with 1 μM fura-2/AM for 30 min at 37 °C. Next, neutrophils (2×10^6/ml) and fura-2-loaded-platelets (6×10^7/ml) were suspended together in a cuvette for 3 min before the addition of fMLP (1 μM). In a set of samples, fMLP (1 μM) was added to the fura-2-loaded-platelets to evaluate its direct effect. Conversely, in another set of samples, fMLP was added to fura-2-loaded neutrophils to establish a positive control group. Finally, Hitachi F4500 fluorescencemeter was used to measure the fluorescence signals.

2.6. Whole blood measurement neutrophil–platelet complex

Whole blood assay was performed according to Konstantopoulos et al. (1998). Briefly, venous blood from healthy volunteers (aged 25 to 30 years, equal distribution of male and female subjects) was drawn into tubes containing heparin at a final concentration of 10 U/ml. Specimens were stored at room temperature in capped polypropylene tubes and used within 1 to 1.5 h of collection. Then, anti-coagulated blood (100 μl) was diluted with HEPES buffer (110 mM NaCl, 10 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, and 30 mM HEPES, pH 7.4) in a 1 : 5 ratio. All buffers tested negative for endotoxin. This dilution was determined to be the minimum level that enabled real-time flow cytometric measurements of neutrophil events, eliminating the coincident measurement caused by the presence of >1000-fold excess of red blood cells and platelets in diluted blood. Platelets were labeled by incubating diluted blood with 10 μl of CD42b (GPIb)-FITC for 5 min at room temperature in siliconized glassware. After an additional 2-min incubation with 1.0 μg/ml LDS-751 at 37 °C, platelets were then treated with various concentrations of YC-1 (5–100 μM) for 5 min before being challenged with fMLP (1 μM) in an aggregometer (700 rpm; Model B 600; Payton, Scarborough, Canada). After additional 5 min, samples were aspired into Falcon tube (12 × 75 mm; Becton Dickinson) and analyzed on a FACSCalibur flow cytometer (Becton Dickinson). Neutrophil–platelet complex was considered as those particles that expressed CD42b-FITC fluorescence above a background level as previously described (Konstantopoulos et al., 1998; Rinder et al., 1991). The extent of neutrophil–platelet adhesion was expressed as the percentage of total neutrophils that were bound to platelets. Neutrophil–neutrophil adhesions were defined as those particles that expressed LDS-751 fluorescence levels greater than those of single neutrophils in the presence or absence of platelets.

2.7. Effect of YC-1 on fMLP stimulated cathepsin G release and activity

Neutrophil (2×10^6 cells/ml) was placed in duplicate into tubes containing different concentrations of YC-1 (5–100 μM) at room temperature for 5 min. Neutrophils were stimulated with fMLP (1 μM) for 10 min. At the end of this period, cells were centrifuged for 1 min and 25 μl of the sample were placed in duplicate into wells of flat-bottomed 96-well microplate. Tris-buffer (150 μl) was added to each well in addition to 20 μl of Suc-AAPF-pNA (1 mM) for cathepsin G activity. After 2 h at room temperature, the colored product was measured spectrophotometrically at 405 nm using a Benchmark Microplate reader (Bio-Rad). To determine whether YC-1 affects cathepsin G activity, YC-1 was added to cellular supernatant from fMLP-treated neutrophils and then incubated with Suc-AAPF-pNA (Brown et al., 2003).

2.8. P-selectin measurement with flow cytometer

Samples for the assessment of platelet CD62P expression was prepared as previously described (Endemann et al., 1996). In short,

platelet suspensions (1×10^6/ml; 500 μl) was treated with various concentrations of YC-1 (5–100 μM) containing 5 μl of anti CD62P or control IgG1 antibody for 5 min. Following gentle re-suspension, cathepsin G (500 nM) was added to these cell suspensions at room temperature and allowed to sit for 30 min. Finally, cells were fixed by the addition of equal volume of 2% formaldehyde in phosphate buffer saline at pH 7.4. Flow cytometric analysis was performed within 1 h of fixation.

3. Results

3.1. Effect of YC-1 on fMLP induced platelet–neutrophil interaction

In a consistent stir, neutrophil–platelet complex formation was about 15.2±4.6% of total cells in flow cytometer analysis (Fig. 1D). In addition, it was found that fMLP (1 μM) increased the percentage of such formation when neutrophils were incubated with platelets (Fig. 1A, D; 53.8±5.5%). However, when platelets were incubated in the absence of neutrophils, fMLP was shown to be unable to induce platelet aggregation (data not shown). The monoclonal P-selectin antibody 9E1 was observed to block the formation of neutrophil–platelet complex (Fig. 1B, D; 24.7±4.5%). On the other hand, monoclonal CD18 (β_2-integrin) antibody was not shown to inhibit such formation (Fig. 1C, D;

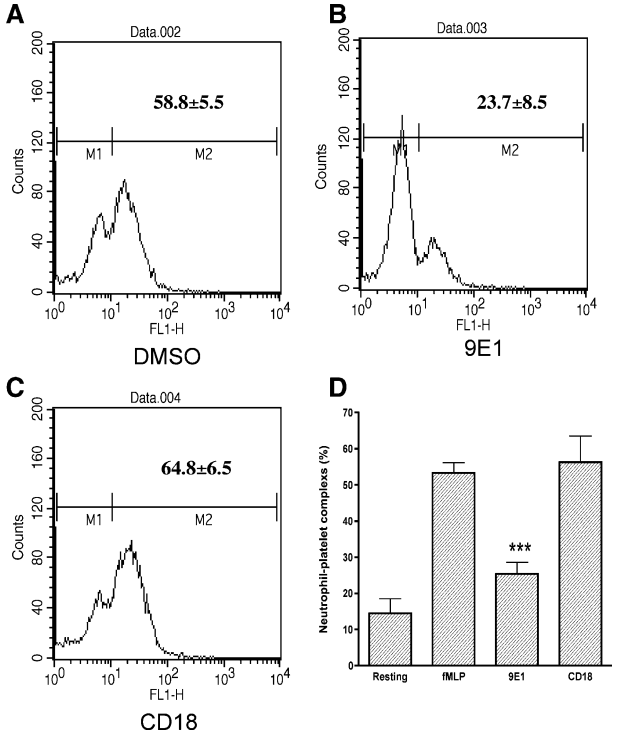

Fig. 1. Fluorescence histograms of single neutrophils associated with platelets with P-selectin or CD18 blocking antibody in co-incubation condition. Purified platelets (6×10^7/ml) were labeled with CD42b-FITC, purified neutrophils (2×10^6/ml) were labeled with LDS-751. Neutrophil–platelet mixture was applied to an aggregometer with (A) DMSO, (B) P-selectin mAb, 9E1, (C) CD18 mAb for 5 min then stimulated with fMLP (1 μM). Two-color flow cytometric analysis was carried 5 min after fMLP was added. In flow cytometric analysis, neutrophil-gated events presenting negative staining of CD42b were defined as singlet neutrophils (M1), positive staining of CD42b represented neutrophil–platelet complexes (M2). The numbers in the plot represent the percentage of neutrophil–platelet complex. (D) Percentage of neutrophil–platelet complex in the presence of 9E1 or CD18 antibody. The basal percentage of neutrophil–platelet complex is 15.2±4.6 (n=4). ***P<0.001 as compared with fMLP.

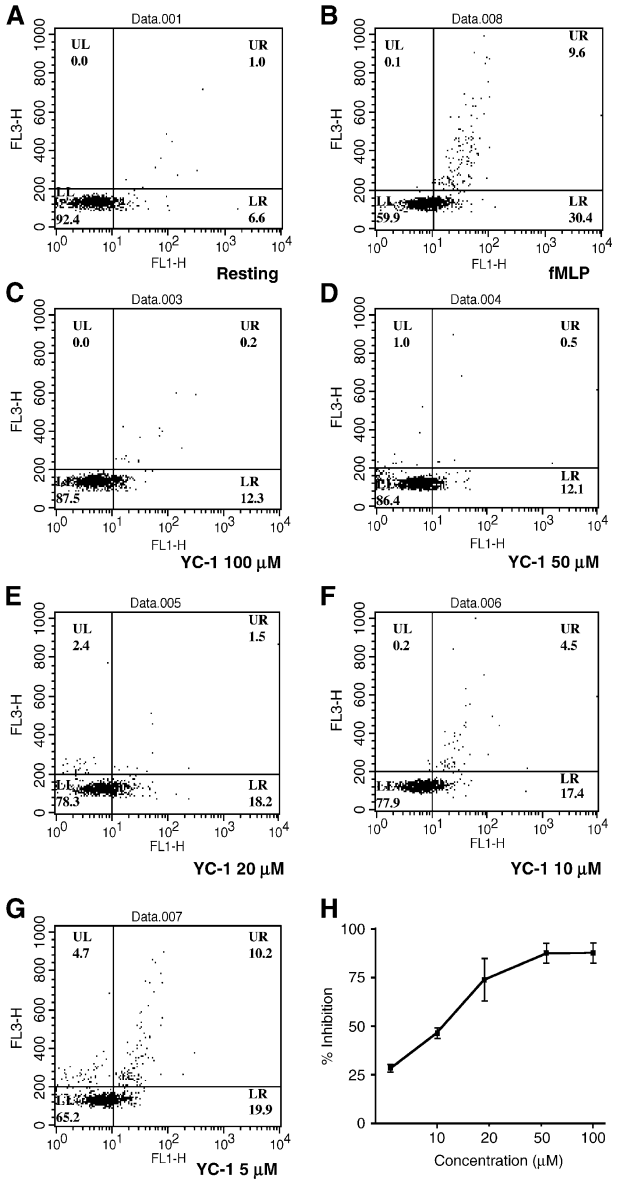

Fig. 2. Flow cytometric analysis of neutrophil–platelet adhesion in co-incubation condition. Purified platelets (6×10^7) were incubated with CD42b-FITC for 5 min at room temperature. Neutrophils (2×10^6) were labeled with LDS-751 for 2 min. Neutrophil–platelet mixture was applied to an aggregometer and stirring at 700 rpm with various concentrations of YC-1 (5–100 μM) at 37 °C for 5 min before fMLP (1 μM) was added. Reaction was stopped 5 min after stimulate with fMLP. Two-color flow cytometric analysis enabled quantification of neutrophil–platelet and neutrophil adhesion. (A) Unstimulated sample showing single neutrophils with (UR+LR) or without (LL) adherent platelets. (B) fMLP increased the cell number of neutrophil–platelet adhesion (UR+LR) and decreased in single neutrophils (LL). Effect of various concentrations of YC-1 on fMLP induced neutrophil–platelet adhesion (C–G). Number in four quadrants representative the percentage of cell number of gating. (H) Percentage of inhibition of YC-1 (5–100 μM) on neutrophil–platelet complex (n=5).

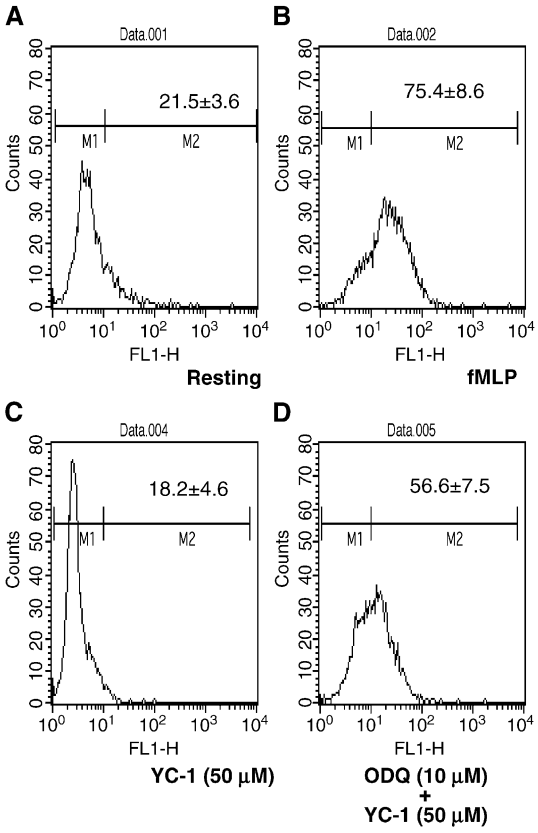

Fig. 3. ODQ reversed inhibitory effect of YC-1 on neutrophil–platelet mixture in co-incubation condition. Isolated platelets (6×10^7/ml) and neutrophils (2×10^6/ml) were labeled with CD42b-FITC and LDS-751, respectively. Mixture are incubated with DMSO (A, resting), fMLP stimulate (B), YC-1 50 μM pre-treat 5 min before fMLP (1 μM) was added (C). ODQ (10 μM) was pre-treated 30 min before incubation with YC-,1 then fMLP was added 5 min after incubation with YC-1 (D). Reaction was stopped 5 min after stimulate with fMLP. Data was present as fluorescence histograms. M1: singlet neutrophil negative CD42b, M2: neutrophil–platelet complex. The numbers in the plot represent the percentage of neutrophil–platelet complexes ($n = 4$).

56.8±6.5%). We know that the CD18 antibody we used was not malfunctioning because it was able to block neutrophils adhesion on immobilized fibrinogen (data not shown). YC-1 inhibited fMLP-induced neutrophil–platelet complex formation in a concentration-dependent manner (Fig. 2C–G). The IC_{50} value was determined to be 15.3±3.5 μM (Fig. 2H). It was further demonstrated that YC-1 exerted its maximal inhibitory effect on the formation of neutrophil–platelet complexes at 50 μM (Fig. 2H; 82.4±3.5% inhibition) and further increases in its concentration to 100 μM did not result in further inhibition. This inhibitory effect of YC-1 was partially reversed when mixtures of neutrophils and platelets were pre-treated with 10 μM of ODQ (Fig. 3D). In the experiment to identify YC-1's effects on neutrophils and platelets individually, isolated neutrophils or platelets were incubated in the presence or absence of YC-1. Under this experimental condition, neutrophil–platelet complex formation was about 10.2±3.6% in a consistent stir condition (Fig. 4A) Similarly, fMLP (1 μM) was also shown to increase such complex formation (56.5±6.5%) in this experiment (Fig. 4B). The percentage of neutrophil–platelet complex formation was decreased in the mixed preparations where neutrophils and platelets were both pre-incubated with YC-1 (50 μM; 20.5±5.6%; Fig. 4C). Moreover, pre-treatments of either neutrophils or platelets alone with YC-1 (50 μM) also resulted in a decreased formation of such complexes (Fig. 4D 34.7±4.5% for YC-1 pre-treat in neutrophils; Fig. 4E, 28.8±2.8% for YC-1 pre-treat in platelets).

3.2. Effect of YC-1 on fMLP trans-activate intracellular calcium mobilization on human platelets

Isolated neutrophils were incubated with fura-2/AM (1 μM)-loaded human isolated platelets. fMLP (1 μM) induced a significant intracellular calcium mobilization in platelet in this mixed preparation (Fig. 5A, B; 310.5±10.5 nM; trace a). In addition, it was observed that this mobilization of calcium was delay for 30 sec with the treatment of fMLP. However, fMLP (1 μM) was not observed to induce human platelet intracellular calcium mobilization in the absence of neutrophil (Fig. 5A, trace C). fMLP increased neutrophils intracellular calcium mobilization

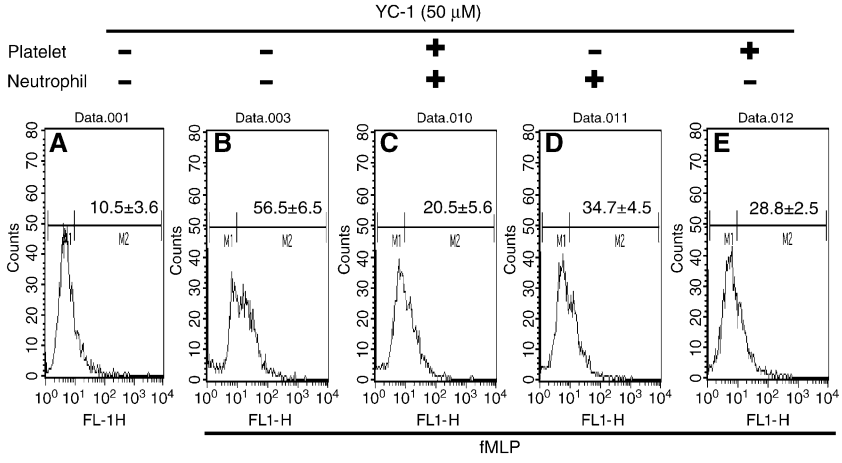

Fig. 4. Effect of YC-1 on neutrophil–platelet complex formation in co-incubation assay. (A–E) Before co-incubation, isolated neutrophils or platelets were pretreated with (+) or without (−) YC-1 (50 μM) at 37 °C for 5 min. YC-1 was washed out from both cells before co-incubation. In flow cytometric analysis, neutrophil-gated events presenting negative staining of CD42b were defined as singlet neutrophils, positive staining of CD42b represented neutrophil–platelet complexes ($n = 4$).

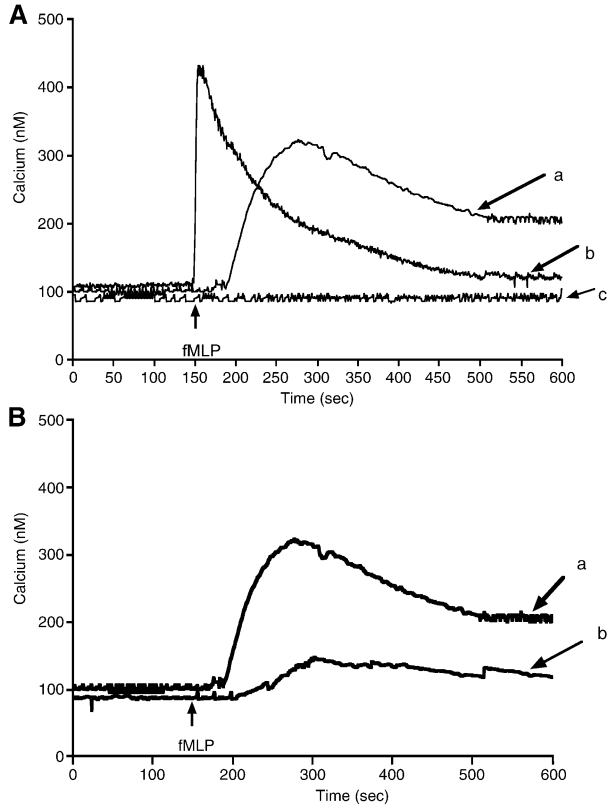

Fig. 5. A typical pattern of neutrophil-dependent calcium mobilization on human platelets in co-incubation assay. Platelets (2×10^6/ml) and neutrophils (6×10^7/ml) were mixed. Calcium mobilization was measured following stimulation with 1 μM fMLP. (A) Trace a: intracellular calcium mobilization in fura-2-loaded platelets caused by fMLP stimulated with fura-2-free neutrophil in mixture; b: fMLP stimulated fura-2-loaded neutrophil; c: fMLP stimulated fura-2-loaded platelet only. (B) Trace a: intracellular calcium mobilization in fura-2-loaded platelets caused by fMLP stimulated with fura-2-free neutrophil; b: fura-2-free neutrophils were incubated with YC-1 50 μM for 5 min. After washing out, neutrophils incubated with fura-2-loaded platelets and stimulated with fMLP as indicated ($n=3$).

as a positive control (Fig. 5A, trace b). fMLP trans-activated human platelet intracellular calcium mobilization was decreased in the mixed preparation where the neutrophils were pre-treated with YC-1 (50 μM; Fig. 5B, trace b).

3.3. Effect of YC-1 on neutrophil cathepsin G release

In this study, we investigated whether YC-1 affected the fMLP-induced cathepsin G release from neutrophils, using Suc-AAPF-pNA as a chromogenic substrate of cathepsin G. fMLP has been observed to increase cathepsin G release by approximately threefold (Fig. 6A; 235.8±15.2 nM). YC-1 inhibited fMLP-induced cathepsin G release in a concentration-dependent manner (Fig. 6A). The IC_{50} value for YC-1 to inhibit the fMLP-induced cathepsin G release was determined to be 6.2±0.6 μM (Fig. 6A) and it was also show that YC-1 did not hydrolyse the Suc-AAPF-pNA (data not shown). Furthermore, ODQ (10 μM), a soluble granulate cyclase inhibitor, or KT5823, a protein kinase G (PKG) inhibitor significantly reversed the inhibitory effect of YC-1 (50 μM) on fMLP induced inhibited cathepsin G release from human neutrophil (Fig. 6B; ODQ: 195.6±20.6 nM; KT5823 271.5±15.6 nM). YC-1 was not shown to inhibit the activity of cathepsin G (Fig. 6B; 266.8±11.2 nM).

3.4. Effect of YC-1 on cathepsin G-induced P-selectin expression on human platelets

The basal mean fluorescence intensity (MFI) value of P-selectin on human platelets was determined to be 10.5±3.5. Cathepsin G (500 nM) was shown to significantly increase the P-selectin expression on human platelets (Fig. 7A; MFI: 110.2±5.5). YC-1

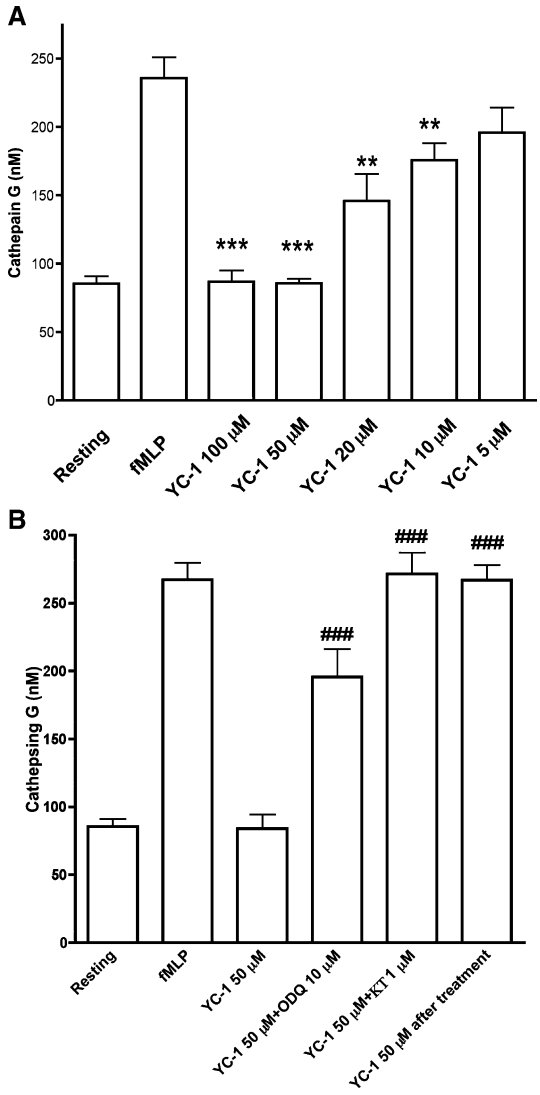

Fig. 6. Effect of YC-1 on fMLP-induced cathepsin G release. (A) Neutrophils were placed into microfuge tubes at a concentration of 2×10^6 cells/ml, in the presence or absence of various concentrations of YC-1 (5–100 μM) for 5 min. fMLP was added to cells for a further 5 min. (B) Pretreatment of ODQ (10 μM, 30 min) or KT5823 (1 μM, 5 min) before YC-1 (50 μM), fMLP was then added for 5 min. Cells were centrifuge and samples of supernatant taken and incubated for 2 h with an cathepsin G substrate, Suc-AAPF-pNA. The color product was measured colorimetrically at 405 nm, using a microplate reader. Results from experiments on cells from six donors, each performed in duplicate, are expressed absorbance mean±standard error of the mean values. $^{**}P<0.01$, $^{***}P<0.001$ as compared with fMLP control. $^{\#\#\#}P<0.001$ as compared with YC-1 50 μM ($n=6$).

(5–50 μM) inhibited cathepsin G induced P-selectin expression on human platelets in a concentration-dependent manner (Fig. 7A–E). The IC_{50} value for YC-1's inhibition of cathepsin G-induced P-selectin expression was found to be 32.5±2.6 μM (Fig. 7F).

3.5. Effect of YC-1 on fMLP-induced neutrophil–platelet complex on whole blood

It was determined that about 17.6% of total cells formed neutrophil–platelet complex in a consistent stir (Fig. 8A; UR+LR). It was observed that fMLP increased this neutrophil–platelet complex formation to 95.4% (Fig. 8B; UR+LR). YC-1 (5–50 μM) inhibited fMLP-induced neutrophil–platelet complex in a concentration-dependent manner (Fig. 8C–F; UR+LR: YC-1 100 μM 32.1±5.8%, YC-1 50 μM 62.4±7.9%, YC-1 20 μM 82.6±5.4%, YC-1 10 μM 84.4±5.5%). The IC_{50} value for YC-1 was determined to be 42.8±2.6 μM. YC-1 100 μM decreased fMLP-induced neutrophil–platelet complex formation to about 32% (about 87% inhibition). In addition, YC-1 (50 μM) demonstrated a less significant inhibitory effect (about 40% inhibition).

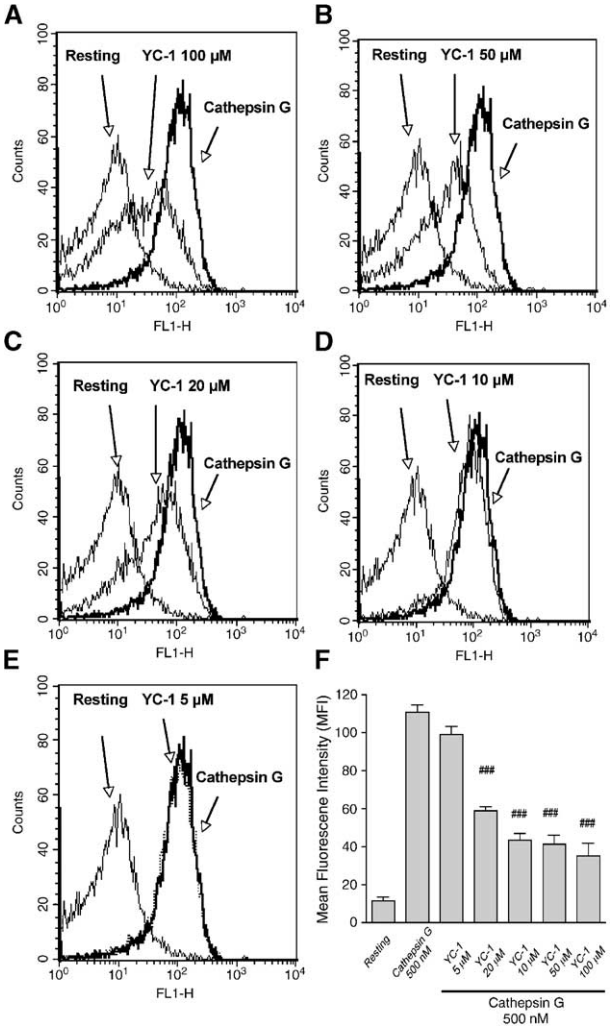

Fig. 7. Effect of YC-1 on P-selectin expression caused by cathepsin G on human platelets. Fluorescence histogram of P-selectin on human platelets was recorded. Various concentrations of (A) YC-1 100 μM, (B) YC-1 50 μM, (C) YC-1 20 μM, (D) YC-1 10 μM and (E) YC-1 5 μM on cathepsin G (500 nM)-induced P-selectin expression on human platelets. (F) Quantify the mean fluorescence intensity. $^{###}P<0.001$ as compared with cathepsin G ($n=5$).

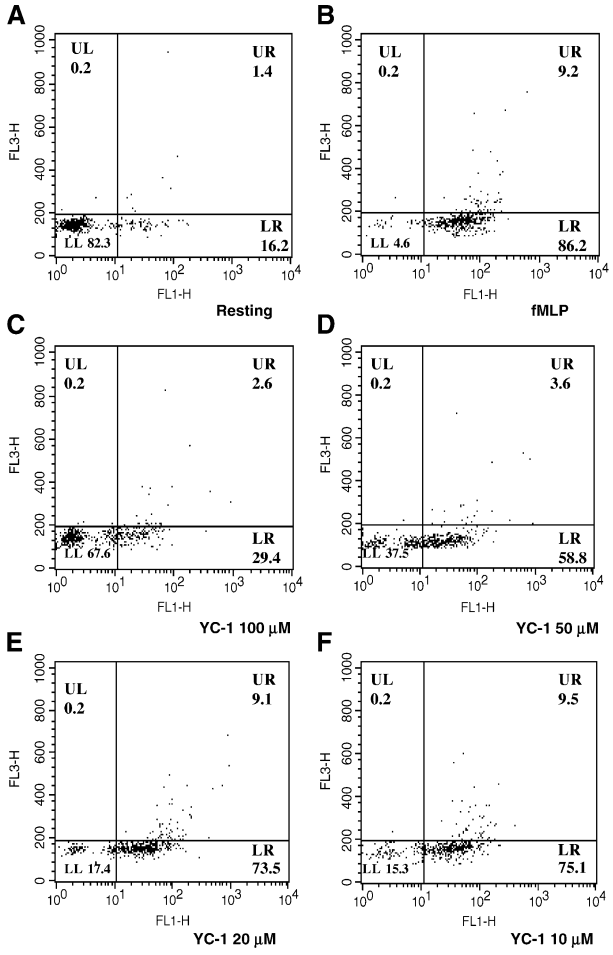

Fig. 8. Flow cytometric analysis of neutrophil–platelet adhesion on whole blood. Diluted whole blood was incubated with CD42b-FITC (for label platelets) at room temperature. Leukocytes were labeled with LDS-751. The sample was applied to an aggregometer and stirring at 700 rpm with various concentrations of YC-1 (5–100 μM) at 37 °C for 5 min before fMLP (1 μM) was added. Two-color flow cytometric analysis enabled quantification of neutrophil–platelet adhesion. (A) Resting, (B) fMLP (1 μM), (C) YC-1 100 μM, (D) YC-1 50 μM, (E) YC-1 20 μM, (F) YC-1 10 μM on fMLP induced neutrophil–platelet complex.

4. Discussion

The basic mechanisms of YC-1's effect on neutrophils (Wang et al., 2002) and platelets have been well studied. YC-1 increased cGMP through activation of soluble guanylate cyclase (Ko et al., 1994). In a further study, YC-1 was also found to increase the level of cGMP via the inhibition of a phosphodiesterase (Mullershausen et al., 2004). However, the application of YC-1 has not been well reported. We incubated neutrophils with platelets in our experiment to explore the effect of YC-1 on neutrophil–platelet interaction in this study. The mechanisms of YC-1's individual effects on either neutrophils or platelets were also investigated this

study. In addition, we also evaluated the effect of YC-1 on a whole blood system, which was suitable for drug evaluation due its proximity to the in vivo environment. YC-1 has multiple effects on the formation of neutrophil–platelet complexes induced by fMLP. First of all, its dominant effect lies in the inhibition of cathepsin G release from fMLP-stimulated human neutrophil, thereby preventing the formation of neutrophil–platelet complexes. This effect of YC-1 is produced via a cGMP-dependent pathway, which is parallel with YC-1's effect on fMLP-induced neutrophil–platelet complex in co-incubation condition. Secondly, YC-1 also inhibits cathepsin G from activating platelets, which further potentiates its original effect on fMLP-induced neutrophil–platelet interaction. The effects of YC-1 on fMLP-induced cathepsin G release and P-selectin expression on platelets could also be the mechanisms behind its inhibitory effects in a whole blood system.

fMLP induces neutrophil–platelet adhesion in whole blood and co-incubation experiments. According to previous studies, fMLP does not directly activate human platelet. Consequently, it can be deduced that fMLP induces neutrophil–platelet complex formation via activated neutrophils. This theory was confirmed in our study, which indicated that fMLP was unable to induce platelet aggregation (data not shown) in the absence of neutrophils. Moreover, fMLP increased platelet intracellular calcium mobilization only in the presence of neutrophils. YC-1 may have affected either or both neutrophils and platelets in this experimental condition. The actual interaction of YC-1 with either neutrophils or platelets alone remains to be elucidated. A further study showed that pre-treatments of either human neutrophils or platelets with YC-1 inhibited the formation of fMLP-induced neutrophil–platelet complexes. It was also shown that YC-1's effects on neutrophils and platelets were irreversible.

Several types of interactions between human neutrophils and platelets have been documented under in vitro condition (Gardiner et al., 2001). One of the most striking effects is a direct activation of platelets by a factor released from the stimulated neutrophil. It is well established that fMLP induces the release of cathepsin G from neutrophil, which in turn, activates the nearby platelets (Gardiner et al., 2001; Renesto and Chignard, 1993). Our studies generated similar results and further showed that fMLP induced platelet intracellular calcium mobilization through cathepsin G released from neutrophils. Cathepsin G, a serine protease stored in the azurophilic granules of neutrophil was first suspected and finally established as the responsible mediator of activation of platelets. Purified cathepsin G activates platelets in terms of causing their aggregation, serotonin release, calcium movements and thromboxane A_2 formation (Kinlough-Rathbone et al., 1999). Previous study proved that cathepsin G triggered platelet activation via protease-activated receptor (PAR)-4 (Renesto and Chignard, 1993; Sambrano et al., 2000). Stimulation with fMLP (1 μM) resulted in highly reproducible neutrophil–platelet complex in our experiments, and this effect was found to be mediated by neutrophil released cathepsin G. For this reason, we evaluated the effect of YC-1 on fMLP-induced cathepsin G activity. In our study, YC-1 inhibits fMLP-induced cathepsin G release in a concentration-dependent manner. A comparison of the inhibitory potency of YC-1 on fMLP-induced cathepsin G release and fMLP-induced neutrophil–platelet complex formation was performed. ODQ and KT5823 were able to reverse YC-1's inhibition of cathepsin G release, indicating that this effect is through a cGMP-dependent pathway. In addition, the possibility of YC-1's inhibitory effect on cathepsin G activity was excluded because its treatment did not result in the altered activity of cathepsin G. Furthermore, elastase, another serine proteinase in azurophilic granules of human neutrophil, has also been reported to enhance the activity of cathepsin G on human platelets. YC-1 also inhibited elastase release from neutrophil (data not shown), which further supported the theory that YC-1 inhibited cathepsin G release from neutrophils. In previous studies, YC-1 was reported to increase in cGMP levels in human and rat neutrophil (Wang et al., 2002). These studies support our results that a cGMP-dependent pathway is involved in effect of YC-1 on neutrophil–platelet complex. YC-1 was also reported to elevate cAMP levels and activated PKA (Hwang et al., 2003); however, *N*-[2-(*p*-bromocinnamyl-amino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H89; a PKA inhibitor) was unable to reverse the inhibitory effect of YC-1 in our study (data not shown). According to these data, we excluded the possibility of YC-1 inhibited neutrophil–platelet interaction via a cAMP-dependent mechanism. In our further study, forskolin, an adenylate cyclase activator, also inhibited neutrophil–platelet interaction via a cAMP dependent manner which can be reversed by H89.

9E1 inhibited the fMLP-induced neutrophil–platelet complex formation, indicating P-selectin's possible involvement in this cell–cell interaction. In addition, pre-treatment of isolated human platelets with YC-1 also prevented such complexes from forming, indicating that YC-1 may also inhibit platelets' P-selectin expressions. In order to observe the effect of YC-1 on P-selectin expression, exogenous cathepsin G was used to induces P-selectin expression on human platelets. We did not use mixed suspension here because P-selectin expression was not significant under such condition. In our experiment, YC-1 inhibited cathepsin G induced P-selectin in a concentration-dependent manner. Our data showed that YC-1 inhibits P-selectin expression on platelets, thereby contributing to the inhibition of neutrophil–platelet complex formation. Inhibition of cathepsin G-induced platelets P-selectin expression in platelets required a higher concentration of YC-1 than that which was required to inhibit fMLP-induced neutrophil–platelet complex formation. This could have been due to the high concentration of cathepsin G used in our studies. P-selectins expressed

on the surface of activated platelets aid in the tethering and rolling of neutrophils from the free stream (de Gaetano et al., 1999). The binding ligand of P-selectin on human neutrophil is PSGL-1. In our study, fMLP did not modify the PSGL-1 expression on human neutrophils. Furthermore, YC-1 was not found to modify the expression of PSGL-1 on human neutrophils either (data not shown). In addition, firm adhesion and transmigration across adherent platelets in a chemotactic gradient is mediated through the β_2-integrin (Zhelev and Alteraifi, 2002). CD18 mAb did not affect the interaction between neutrophils and platelets in our study, this result was supported by Peters et al.'s (1997) study which also showed CD18 mAb did not block neutrophils–platelet interaction. Although CD18 mAb did not affect the interaction between neutrophils and platelets, we evaluated the effect of YC-1 on fMLP-induced CD18 expression on human neutrophils. According to our study, YC-1's inhibition on fMLP-induced CD18 expression could have further effect on neutrophil–platelet complex. The mechanism of YC-1 on fMLP-induced CD18 expression is under investigation. This inhibition is in a cGMP-independent manner (ODQ did not reverse YC-1's effect). Moreover, YC-1 also inhibited the formation of fMLP-induced neutrophil–platelet complex in whole blood. This data demonstrate the potential clinical application of YC-1 in the future.

In conclusion, fMLP-induced cathepsin G release is crucial in the formation of neutrophil–platelet complex. The inhibition of cathepsin G release from neutrophils via a cGMP-dependent pathway is the major mechanism through which YC-1 inhibits fMLP-induced neutrophil–platelet complex formation. Furthermore, YC-'s inhibitory effect on cathepsin G-activated platelet could also be one of the mechanisms of its inhibitory effect on neutrophil–platelet complex formation.

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