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Activated Platelets Induce Secretion of Interleukin-1β, Monocyte Chemotactic Protein-1, and Macrophage Inflammatory Protein-1α and Surface Expression of Intercellular Adhesion Molecule-1 on Cultured Endothelial Cells

Atherosclerosis is an inflammatory disease. Platelet-endothelium interaction plays an important role in the pathophysiology of atherogenesis. We investigated the role of activated platelets for secretion of interleukin (IL)-1β, monocyte chemotactic protein (MCP)-1 and macrophage inflammatory protein (MIP)-1α and expression of intercellular adhesion molecule (ICAM)-1 on endothelial cells. Human umbilical vein endothelial cells (HUVEC) were incubated with non-stimulated or ADP-activated platelets for 6 hr. Secretion of interleukin (IL)-1β, MCP-1 and MIP-1α and surface expression of ICAM-1 were measured by ELISA and flow cytometry. In the presence of activated platelets, the secretion of IL-1β, MCP-1, and MIP-1α and surface expression of ICAM-1 were significantly increased compared with non-activated platelets. The present study shows that activated platelets may contribute to expression of various inflammatory mediators on endothelial cells.

Key Words: *Blood Platelets; Endothelium; Cell Adhesion Molecules; Atherosclerosis*

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INTRODUCTION

There is increasing evidence that inflammatory processes play a central role in the pathogenesis of atherosclerosis (1). Atherosclerotic plaques exhibit significant infiltration by activated macrophages and T-cells (2, 3). These inflammatory cells in atherosclerotic plaques release matrix-degrading enzymes and thrombogenic substances that may provoke plaque disruption and local thrombosis (4-6). Thus, the local inflammatory process may be critically responsible for plaque destabilization manifesting clinically as acute ischemic stroke (7, 8).

Recent studies have focused on the role of inflammatory mediators such as pro-inflammatory cytokines, chemokines and adhesion molecules in the processes of atherosclerosis (9, 10). Pro-inflammatory cytokines such as IL-1β and TNF-α are released from endothelial cells and macrophages, are modulators of inflammatory responses during atherogenesis (1). Chemokines are increasingly being appreciated as capable of regulating signals that promote recruitment of leukocyte subsets. Chemokines are subgrouped into the α-subfamily (IL-8) acting primarily on neutrophils and the β-subfamily (MCP-1 and MIP-1α etc) attracting mainly lymphocytes and monocytes (11). Among the β-chemokines, MCP-1 seems to be the major chemotactic molecule generated within the vessel wall (12, 13) and is found in macrophage-rich areas of atherosclerotic lesion (1). Macrophage inflammatory protein (MIP)-1α, a kind of β-chemokine, is secreted from endothelial cell, macrophage and fibroblast. It has a function to recruit mainly monocyte (11), but its roles have been not known in the process of atherosclerosis. Intercellular adhesion molecule (ICAM)-1 is a major adhesion receptor expressed on the endothelium (14, 15) and is essential step for initiation, maturation, and destabilization of plaques (9, 10).

Platelets-endothelium interactions play a central role in hemostatic and inflammatory mechanisms within the vessel wall (16). Recently, platelets have been shown to induce pro-inflammatory cytokines and chemokines in leukocytes (17, 18). However, it is exactly unknown whether activated platelets influence the generation of inflammatory mediators on endothelial cells. We also speculate that activated platelets may contribute to

expression of various inflammatory mediators on the endothelial cells.

In the present study, we investigated the effects of activated platelets on IL-1β, MCP-1 and MIP-1α secretion and ICAM-1 expression in cultured endothelial cells.

MATERIALS AND METHODS

Cell culture

Primary human umbilical vein endothelial cells (HUVEC) were harvested by use of 0.25% trypsin (Gibco-BRL, Grand Island, NY, U.S.A.) digestion according to the method of Jeff et al. (19). They were maintained in medium 199 (Gibco-BRL), containing 20% heat-inactivated fetal calf serum, 2 mM glutamine, 3 ng/mL basic fibroblast growth factor (Upstate Biotechnology, Lake Placid, NY, U.S.A.), 5 U/mL heparin, 100 μg/mL streptomycin and 100 U penicillin. HUVEC were serially passaged on gelatin coated flasks. Cells were used within four passages and identified as endothelial cell by their characteristic cobblestone morphology (Fig. 1) and presence of Factor VIII antigen (Sigma, Saint Louis, MO, U.S.A.).

To exclude endotoxin contamination, all cell suspensions at the end of each experiment were evaluated by chromogenic limulus amoebocyte lysate assay and all powdered mediums were reconstituted with endotoxin-free water.

Incubation of HUVEC with platelets

Endothelial cells were subcultured into 24-well plates (10^5/well) and were allowed to grow for 48 hr to reach confluent state before experiment.

Platelets were isolated using the methods of Gawaz (20). In brief, human blood was drawn into EDTA tube and was centrifuged (100 *g* for 10 min) to obtain platelets-rich plasma. The platelets rich plasma was recentrifuged (1,300 *g* for 4 min). Washed platelets were suspended in Tyrode's solution-HEPES buffer (mmol/L: HEPES 2.5, NaCl 150, $NaHCO_3$ 12, KCl 2.5, $MgCl_2$ 1, $CaCl_2$ 2, and D-glucose 5.5, and 1 mg/mL BSA, pH 7.4) to obtain a final count of 2×10^8/mL.

Confluent endothelial monolayers were incubated at 37℃ without agitation in culture condition atmosphere for 6 hr with the following treatments: (1) control well without any stimulation, (2) nonstimulated platelets (1×10^8/well)-whole blood was immediately mixed with anti-activation cocktail that contained 1 mmol/L aspirin, 1 mmol/L theophyllin, and 10 nmol/L prostaglandin E_1, (3) activated platelets (1×10^8/well)-adenosine diphosphate (ADP) in final concentration of 50 μmol/L was added to the platelets suspension in the absence of anti-platelets substances, (4) ADP (50 μmol/L) only without platelets, (5) positive control well-100 pg/mL recombinant human IL-1β (rhIL-1β) (Pharmingen, San Diego, CA, U.S.A.).

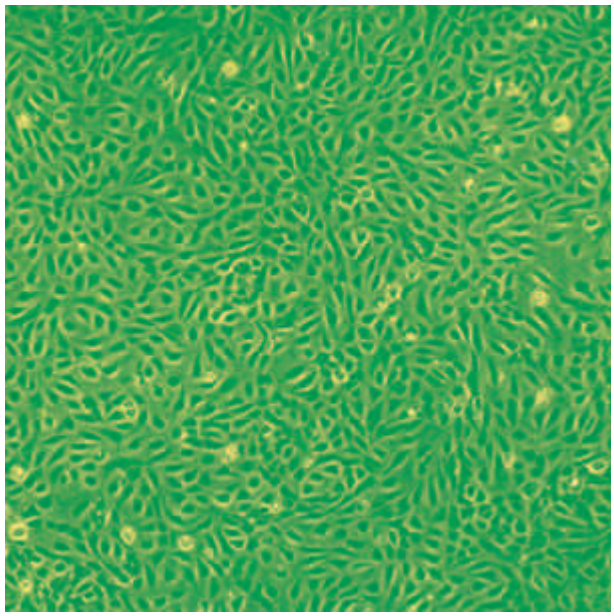

Fig. 1. Human umbilical vein endothelial cells (×100).

Determination of IL-1β, MCP-1 and MIP-1α secretion and surface expression of ICAM-1

The supernatant of cultured endothelial cells treated with platelets or other agents for 6 hr was aspirated and stored at -70℃. Concentration of IL-1β was measured by quantitative "sandwich" ELISA technique. Briefly, standards and test samples were dispensed in duplicate into wells of 96-well microtiter plates, which had been pre-coated with monoclonal anti-human IL-1β antibody (Endogen, Woburn, MA, U.S.A.). Then, horseradish peroxidase (HRP)-conjugated detection antibody (biotinylated anti-human IL-1β, Endogen) was added. HRP-conjugated streptavidin (Endogen) was added to the wells. For color reaction, 100 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added for 15 to 30 min. The absorption at 450 nm was determined using an automated ELISA microplate reader (Bio-Tech, Winooski, VT, U.S.A.). Detection limit for IL-1β was 3 pg/mL. Concentration of MCP-1 and MIP-1α proteins were determined by commercial ELISA kit (R&D, Minneapolis, MN, U.S.A.). Detection limit was 10 and 5 pg/mL.

Surface expression of ICAM-1 was determined by FITC-conjugated anti-CD54 monoclonal antibody (DAKO, Capenteria, CA, U.S.A.) and flow cytometry. After aspi-

ration of cell culture supernatant, endothelial cells were detached by using 0.05% trypsin EDTA and centrifuged at 1,500 rpm (4℃, 5 min). Cell pellets were incubated with FITC-conjugated anti-CD54 monoclonal antibody (10 μL) for 30 min. Thereafter, cell pellets were resuspended with 400 μL staining buffer (2% fetal calf serum in PBS). Cytofluorographic analysis was performed on a FACSort (Becton Dickinson, San Jose, CA, U.S.A.).

Statistical analysis

Results are presented as mean±SD of three independent experiments. Differences between groups were tested by Student's t-test. A value of $p<0.05$ was considered statistically significant.

RESULTS

Activated platelets enhance secretion of IL-1β, MCP-1 and MIP-1α on HUVECs

HUVECs were little released IL-1β under basal condition (1.8±1.3 pg/mL, Fig. 2). There was a small increase in IL-1β production in the experiment where the endothelial cells were incubated with nonstimulated platelets (8.3±4.1 pg/mL, Fig. 2). Direct addition of ADP in the absence of platelets did not significantly induce IL-1β secretion. In contrast, platelets activated by ADP induced significant release of IL-1β (36.7±3.1 pg/mL) compared with levels of basal condition or other conditions ($p<0.05$, Fig. 2).

Activated platelets significantly increased secretion of MCP-1 (13.6±3.1 ng/mL, approximately 80% of maximal rhIL-1β 100 pg/mL-induced secretion). In nonstimulated platelets, MCP-1 secretion was a little increased (Fig. 3).

Secretion of MIP-1α was significantly enhanced on endothelial cells incubated with activated platelets (162.7 ±23.7 pg/mL). However, there was little secretion of MIP-1 (21.3±2.5 pg/mL) when endothelial cells were incubated with rhIL-1β (Fig. 4).

Surface expression of ICAM-1 on endothelial cells

Fig. 5 delineates the endothelial cells expression of ICAM-1, demonstrated by FACS analysis. Nonstimulated platelets or ADP without platelets induced no significant changes of surface expression of ICAM-1 on endothelial cells, compared with resting state. However, surface ex-

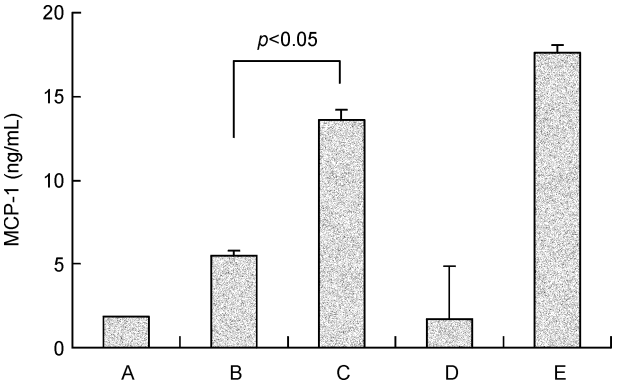

Fig. 3. Activated platelets induce the secretion of MCP-1 on cultured endothelial cells. Results (mean±SD) of 3 independent experiments are shown. A: cultured endothelial cells, B: nonstimulated platelets+cultured endothelial cells, C: ADP-activated platelets+cultured endothelial cells, D: ADP only+cultured endothelial cells, E: IL-1β+cultured endothelial cells.

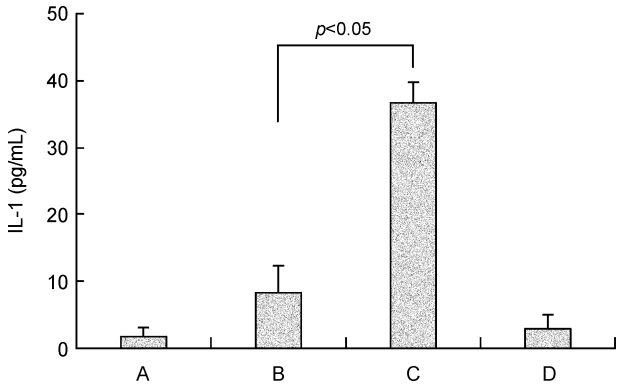

Fig. 2. Activated platelets induce the secretion of IL-1β on cultured endothelial cells. Results (mean±SD) of 3 independent experiments are shown. A: cultured endothelial cells, B: nonstimulated platelets+cultured endothelial cells, C: ADP-activated platelets+cultured endothelial cells, D: ADP only+cultured endothelial cells.

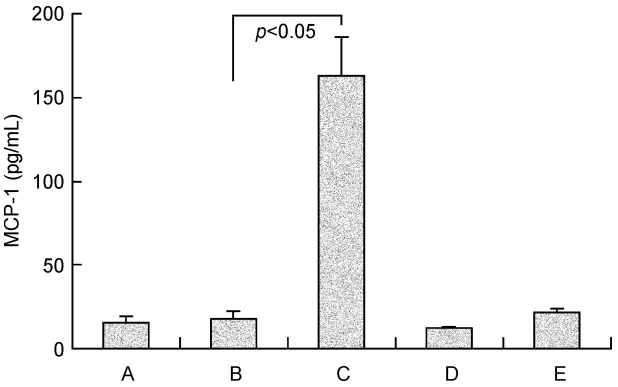

Fig. 4. Activated platelets induce the secretion of MIP-1α on cultured endothelial cells. Results (mean±SD) of 3 independent experiments are shown. A: cultured endothelial cells, B: nonstimulated platelets+cultured endothelial cells, C: ADP-activated platelets+cultured endothelial cells, D: ADP only+cultured endothelial cells, E: IL-1β+cultured endothelial cells.

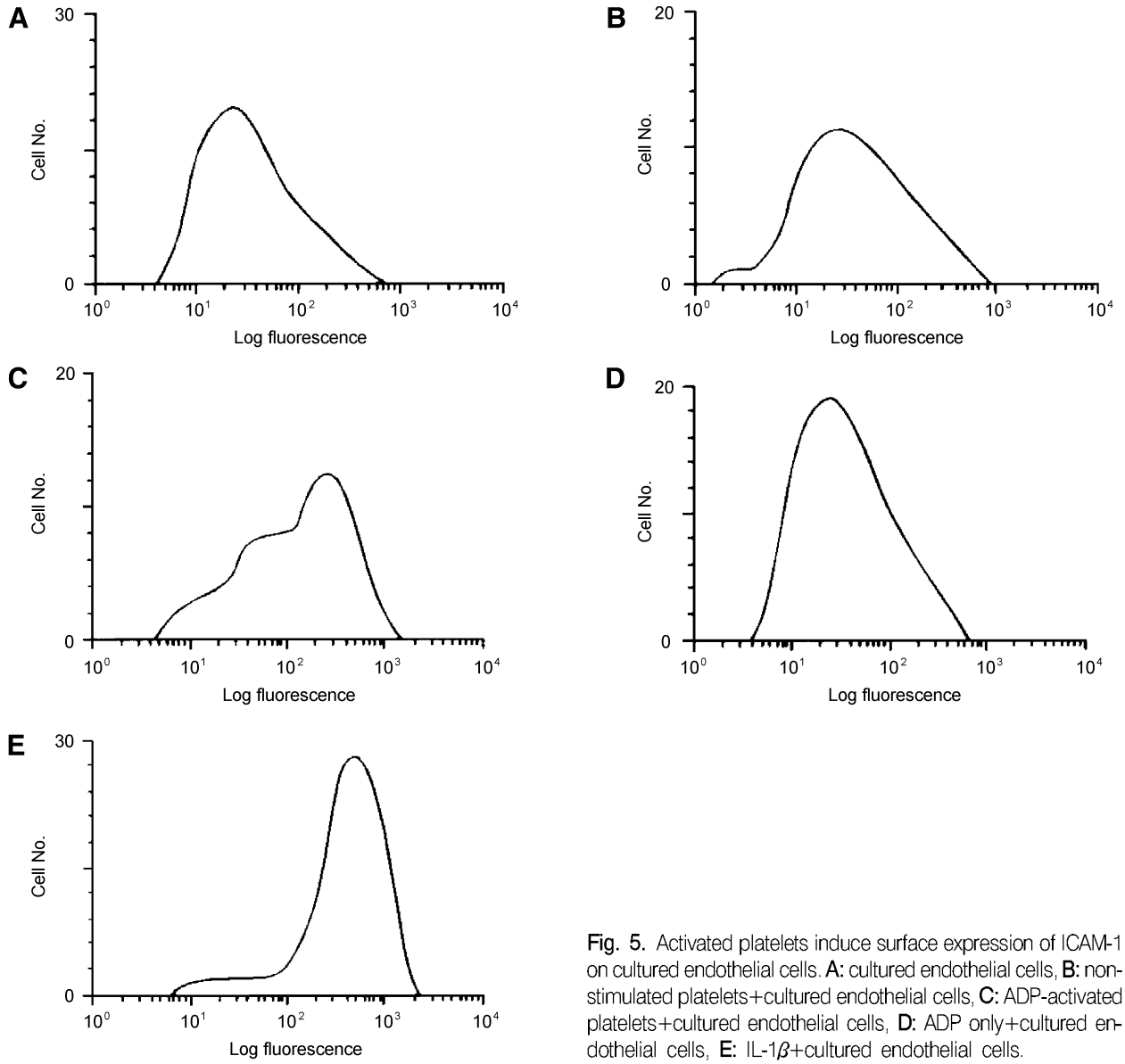

Fig. 5. Activated platelets induce surface expression of ICAM-1 on cultured endothelial cells. **A:** cultured endothelial cells, **B:** non-stimulated platelets+cultured endothelial cells, **C:** ADP-activated platelets+cultured endothelial cells, **D:** ADP only+cultured endothelial cells, **E:** IL-1β+cultured endothelial cells.

pression of ICAM-1 in the presence of ADP-activated platelets was significantly enhanced compared with experiments with resting state and with nonstimulated platelets, Reaching 50% of maximal inducible ICAM-1 surface expression (100 pg/mL of rhIL-1β).

DISCUSSION

Adhesion of monocyte to endothelial cell is an early event in vascular inflammatory syndromes, and together with subsequent endothelial transmigration, is a feature of developing atherosclerosis (1). During these pathologic processes, many inflammatory mediators such as pro-inflammatory cytokines, chemokines and adhesion molecules are involved (9, 10). Many risk factors for atherosclerosis can increase production of IL-1β and TNF-α, transforming the vessel endothelium from anticoagulant state to a procoagulant state (21). These pro-inflammatory cytokines induce the secretion of MCP-1, a β-chemokine, that may be responsible for the chemotaxis and accumulation of mainly monocytes and T-cells (1).

In this study, we showed that activated platelets induced endothelial secretion of IL-1β and MCP-1. Our results are similar with previous report (18). These findings suggest that activated platelets can evoke the local inflammatory process in endothelial cells. Less significantly endothelial IL-1β and MCP-1 secretion were shown in the presence of nonstimulated platelets in our study. These small increases may have been due to spontaneous activation of the platelets with longer ex vivo incubation period (22).

MIP-1α is a β-chemokine that attributes to recruitment of monocyte and activate macrophage in inflammatory process (11). However, their roles are not exactly known in the atherogenesis. We presumed that MIP-1α would be induced in endothelial cells by variety stimulation (for example pro-inflammatory cytokines and activated platelets, etc) before this study. Our results show that activated platelets induce the secretion of MIP-1α on endothelial cells. However, IL-1β did not increase the release of MIP-1α on endothelial cells. These findings suggest that activated platelets may be significantly correlated with secretion of MIP-1α on endothelial cells. To the best of our knowledge, we show for the first time that MIP-1α is released from endothelial cells under activated platelets. According to previous report (23), IL-1β and TNF-α could not secrete the MIP-1α on HUVECs. However, it has been demonstrated that MIP-1α increased its secretion on endothelial cells in the presence of many monocytes (24). This raises the possibility that initial adhesion events between endothelium and cells such as platelets or monocytes can upregulate the secretion of MIP-1α on endothelial cells. But we do not know exactly why MIP-1α could be induced by activated platelets, instead of IL-1β. We predict that MIP-1α may be involved in the chemotactic process in endothelium by activating platelets. Further studies will be needed.

As the counterpart of leukocytes, ICAM-1 presence on the endothelium is critical for monocytes binding to the endothelium and extravasation to inflammation site within the vessel (14, 15). Our results demonstrate that surface expression of ICAM-1 on endothelial cells was markedly increased in the presence of ADP-activated platelets. DeGraba et al. (7) reported that elevation of ICAM-1 expression in atherosclerotic plaque is involved in the conversion of atherosclerotic plaque to a symptomatic state. Therefore, above findings suggest that activated platelets may correlate with instability of atherosclerotic plaque.

NF-κB regulates a variety gene coding for pro-inflammatory cytokines (IL-1β and TNF-α), chemokines (IL-8 and MCP-1), and adhesion molecules (ICAM-1 and E-selectin). Recently, activated NF-κB was identified in endothelial cells, smooth muscles, macrophages of human atherosclerotic plaques, suggesting a pathophysiological role of NF-κB in inflammatory and proliferative processes in atherosclerosis (25, 26). Particularly, Gawaz et al. have demonstrated that activated platelets induce activation of transcription factor NF-κB in endothelial cells (27). Above findings suggest that activated platelets may induce the secretion of IL-1β, MCP-1, and MIP-1α and the expression of ICAM-1 on the endothelial cells by activation of NF-κB.

Our study has a limitation that does not explain the nature of mediators released from activated platelets, triggering induction of inflammatory mediators on endothelial cells. Activated platelets may release compounds from their granules that might be potential activator of inflammatory mediators in endothelial cells (28). Moreover, activated platelets has been shown to be IL-1-like activity (29). Therefore, we speculate that these biological active mediators of activated platelets may change the property of endothelium.

The present study shows that activated platelets can introduce the secretion of inflammatory mediators on endothelial cells. Thus, modulation of platelet activation and accumulation of at the vessel may be an effective strategy in downregulating atherosclerotic mechanisms.

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