Cytokine Secretion from Human Monocytes Potentiated by P-Selectin-Mediated Cell Adhesion

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Key Words

Cell adhesion • Cytokines • Monocytes • P-selectin • Tumor necrosis factor

Abstract

Background/Aim: P-selectin is a carbohydrate-recognizing cell adhesion molecule expressed on activated platelets and endothelial cells. It plays a crucial role in the recruitment of leukocytes to inflammatory and hemorrhagic sites. Cell adhesion mediated by P-selectin induces leukocyte activation, such as the generation of reactive oxygen species and the expression of blood coagulation factors. We assessed how P-selectin-mediated cell adhesion affects cytokine secretion from monocytes. **Methods:** Human peripheral blood monocytes were cultured in a plate that had been coated with P-selectin purified from human platelets, and cytokines released in the culture supernatant from monocytes were determined by ELISA. **Results:** The secretion of tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-8, IL-12 and macrophage inflammatory protein-1 β increased 3- to 10-fold in response to P-selectin compared with unstimulated mono-

cytes. We next examined the effects of cytokine treatment of monocytes on their susceptibility to P-selectin. The secretion of TNF- α from monocytes in response to P-selectin was increased when monocytes were preincubated with granulocyte/macrophage colony-stimulating factor, monocyte chemotactic protein-1 or interferon- γ (IFN- γ); IFN- γ was the most effective in potentiating TNF- α secretion from monocytes. **Conclusion:** These results suggest that the interaction of monocytes with P-selectin plays an important role not only in their trafficking but also in the regulation of cytokine production by these cells.

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Introduction

Selectins are a family of carbohydrate-recognizing adhesion molecules implicated in the initial interactions between leukocytes and platelets or endothelium that lead to inflammatory responses. Three selectin members have been identified and exhibit specific cellular distributions; i.e., activated vascular endothelial cells (E- and P-selec-

tins), activated platelets (P-selectin), and leukocytes (L-selectin) [1–3]. P-selectin is stored in granules of platelets and endothelial cells and is translocated to their surfaces upon cellular activation. This adhesion molecule plays a crucial role in the recruitment of leukocytes to inflammatory and hemorrhagic sites. Leukocyte recruitment into inflamed tissues is a multistep process. P-selectin was shown to mediate leukocyte rolling on activated endothelia, which is thought to be an initial step in the extravasation and recruitment of leukocytes.

A major counter-ligand for P-selectin, called P-selectin glycoprotein ligand-1 (PSGL-1), has been identified on leukocyte cell membranes. This counter-ligand is a mucin-like glycoprotein containing the sialyl Lewis X (sLeX) carbohydrate structure, which P-selectin recognizes [4]. In addition to the physical interaction between P-selectin and PSGL-1, several studies have suggested that the ligation of P-selectin to its ligands induces the activation of leukocytes, including the production of reactive oxygen species (ROS) in neutrophils [5, 6] and the expression of tissue factor on monocytes [7]. It was reported that signals leading to the activation of leukocytes were transduced, at least in part, through PSGL-1. The P-selectin-PSGL-1 interaction triggers the activation of protein tyrosine phosphorylation and mitogen-activated protein kinases in human neutrophils [8, 9]. The intracellular signaling mediated by protein tyrosine kinases may cause the activation of β_2 -integrin including CD11b/18, which promotes the subsequent process of leukocyte transmigration through the vascular endothelium [10, 11]. The P-selectin-mediated adhesion also affects cytokine production by leukocytes. The antibody (Ab) engagement of PSGL-1 upregulates the expression of colony-stimulating factor (CSF)-1 mRNA in Jurkat leukemic cells [12]. Our previous study also demonstrated that P-selectin immobilized on a culture plate effectively induced monocytes to secrete tumor necrosis factor (TNF)- α [13]. Cytokines secreted from monocytes have been shown to regulate various types of immune responses and inflammatory/allergic diseases. In this study, we assessed profiles of cytokine production by monocytes after their interaction with P-selectin, and found that the secretion of various cytokines, including TNF- α , interleukin (IL)-1 β , IL-6, IL-8, IL-12, and macrophage inflammatory protein-1 β (MIP-1 β), was potentiated by P-selectin-mediated cell adhesion. Moreover, we have observed that pretreatment of monocytes with cytokines such as interferon (IFN)- γ raised the susceptibility of the monocyte response to P-selectin. Considering that P-selectin mediates the initial step of monocyte adhesion to activated endothelia and

platelets, the adhesion-induced cytokine production seems to be a critical event for the subsequent progression of inflammation.

Materials and Methods

Reagents

Heparin from porcine intestine was purchased from Mochida Pharmaceutical (Tokyo, Japan). Dextran 200,000 (molecular weight: 180,000–210,000) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Ficoll-Paque, concanavalin A-Sepharose 4B, heparin-Sepharose 4B, and CNBr-activated Sepharose 4B were products of GE Healthcare (Piscataway, N.J., USA). Nonidet P-40 (NP-40) was purchased from Nacalai Tesque (Kyoto, Japan). PMSF (phenylmethanesulfonyl fluoride), leupeptin, aprotinin, and bovine serum albumin (BSA) were products of Sigma (St. Louis, Mo., USA). Lipopolysaccharide (LPS) from *Escherichia coli* was purchased from Difco (Becton Dickinson, Franklin Lakes, N.J., USA). BCECF-AM (2',7'-bis(carboxyethyl)carboxyfluorescein tetraacetoxymethyl ester) was a product of Dojindo Laboratories (Kumamoto, Japan). Recombinant human cytokines, granulocyte/macrophage (GM)-CSF, macrophage CSF, IFN- γ , and monocyte chemotactic protein (MCP)-1, were purchased from PeproTech (Rocky Hill, N.J., USA) and from Invitrogen (Carlsbad, Calif., USA). BCA protein assay and ELISA kits [IL-1 β , IL-6, IL-8, IL-12 (p70), MIP-1 α , and MIP-1 β] were products of Thermo Fisher Scientific-Pierce-Endogen (Rockford, Ill., USA). A P-selectin antagonistic peptide (Galloyl-N-Gaba-Trp-Val-Asp-Val-OH) [14] was provided by Cosmo Bio (Tokyo, Japan).

Antibodies

Murine monoclonal Ab (mAb) directed against human P-selectin (2T60, IgG1) [15] was a gift from Dr. K. Tanoue (Tokyo Metropolitan Institute of Medical Science). Another anti-P-selectin Ab (2D7, IgG1) [13] was purified from conditioned medium of hybridoma in our laboratory. For purification of P-selectin, mAb 2D7 was conjugated to CNBr-activated Sepharose 4B (GE Healthcare) according to the manufacturer's instructions. Anti-CD14 and anti-CD11c antibodies were products of BioLegend (San Diego, Calif., USA). Anti-sLeX Ab (KM93, IgM) [16] was a generous donation from Dr. Nobuo Hanai (Kyowa Hakko Kirin, Tokyo, Japan). Anti-human PSGL-1 antibodies (PL1 and PL2) were purchased from Serotec (Oxford, UK). Anti-TNF- α Ab was purchased from R&D Systems (Minneapolis, Minn., USA).

Cells

Human monocytes were purified from peripheral blood of healthy volunteers essentially as described previously [13]. Briefly, heparinized whole blood was mixed with an equal volume of 3% dextran 200/0.15 M NaCl and allowed to stand for 30 min. After sedimentation of most erythrocytes at the bottom of the tube, the supernatant was centrifuged at 250 g for 10 min. The pelleted cells were then suspended in 10 mM sodium phosphate buffer (pH 7.3) containing 0.14 M NaCl (PBS) and layered on Ficoll-Paque solution. After centrifugation at 400 g for 30 min, cells at the interface were collected, suspended in PBS, and centrifuged at 250 g for 10 min. Cells were suspended at a density of 1×10^6 cells/ml in

Fig. 1. Flow-cytometric analysis of surface markers of monocytes. Monocytes isolated from human peripheral blood were analyzed by flow cytometry after being stained with mAb against CD14 or CD11c and with FITC-labeled secondary Ab. The profiles without primary Ab staining are indicated by dotted lines.

RPMI 1640 medium (GIBCO/Invitrogen, Carlsbad, Calif., USA) containing 10% FBS, and the cell suspension was incubated at 37°C for 30 min in a plastic culture dish (Falcon 353004, BD Biosciences). After nonadherent cells were washed twice with warm PBS, adherent cells were recovered with a cell scraper in PBS containing 0.02% EDTA. The cells were washed with RPMI 1640 medium containing 10% FBS. All procedures were performed under sterile conditions. The purity of the monocyte preparations was >90%, as evaluated by May-Grünwald-Giemsa staining. The expression of CD14 and CD11c was measured by flow cytometry, and ~85% of the monocyte population were positive for both markers (fig. 1). A trypan blue exclusion assay showed that 99% of the cells were viable.

HL60, a human myelocytic leukemia cell line, was supplied by Riken Cell Bank (Tsukuba, Japan). The cells were cultured in RPMI 1640/10% FBS medium in a 5% CO₂ atmosphere.

Flow Cytometry

The expression of CD14 and CD11c was measured with a flow cytometer (FACS Verse™, BD Biosciences, San Diego, Calif., USA) using specific mAb and FITC-labeled anti-mouse IgG Ab, as described previously [17].

Purification of P-Selectin from Human Platelets

P-selectin was purified from human platelets essentially by the method of Johnston et al. [18], with a modification described previously [15]. Human platelets were lysed by sonication in PBS containing 1% NP-40 and protease inhibitors (1 mM PMSE, 0.2 mM leupeptin, 0.1 μM aprotinin, 4 mM EDTA) at 4°C, and the lysate was centrifuged at 100,000 g for 30 min. The supernatant thus obtained was applied to a column of concanavalin A-Sepharose 4B. After the column was washed with PBS containing 0.1% NP-40, bound glycoproteins were eluted with PBS containing 0.1% NP-40 and 0.5 M methyl-α-D-mannoside. The concanavalin A-Sepharose-bound fraction was diluted with an equal volume of deionized water and then applied to a column of heparin-Sepharose 4B that had been equilibrated with 0.1 M NaCl/0.1% NP-40/

10 mM sodium phosphate buffer (PB; pH 7.3). The elution was sequentially conducted with 0.1 M NaCl/0.1% NP-40/PB, 0.3 M NaCl/0.1% NP-40/PB, and 0.5 M NaCl/0.1% NP-40/PB. The eluate with 0.3 M NaCl/0.1% NP-40/PB was subjected to affinity chromatography on a column of anti-P-selectin Ab (2D7)-Sepharose 4B. After the column was washed with 0.1% NP-40/PBS and then with 1.5 M KSCN/0.1% NP-40, the P-selectin bound to the column was eluted with 3 M KSCN/0.1% NP-40 and immediately dialyzed against PBS at 4°C.

Cell Adhesion Assay

The adhesion of HL60 cells to P-selectin was assayed according to the method described previously [19]. A 96-well culture plate was coated with purified P-selectin (0.1–4 μg/ml) in 0.2 M NaHCO₃ at 4°C for 16 h. The well was then washed with PBS and blocked with 3% BSA/PBS. HL60 cells were labeled with a fluorescent dye, BCECF-AM (3 μM) at 37°C for 30 min, and the labeled cell suspension (1 × 10⁶ cells/ml, 0.1 ml) in RPMI 1640/1% BSA was placed in a 96-well culture plate that had been coated with P-selectin. After the plate was incubated at 4°C for 1 h, non-adherent cells were removed by washing with RPMI 1640/1% BSA, and adherent cells were lysed with 0.2 ml of 1% NP-40. The fluorescence intensity was measured with a fluorescence spectrophotometer (excitation = 490 nm/emission = 520 nm).

Monocyte Culture and Cytokine Assay

A 96-well culture plate was coated with purified P-selectin as described above. A monocyte suspension (0.75–2.5 × 10⁶ cells/ml, 0.2 ml) in RPMI 1640/10% FBS was placed in a P-selectin-coated plate and cultured at 37°C for 1–24 h. Cytokines [TNF-α, IL-1β, IL-6, IL-8, IL-12 (p70), MIP-1α, and MIP-1β] secreted into the culture supernatants were measured by ELISA.

Depletion of TNF-α from Conditioned Medium by Anti-TNF-α Ab

Conditioned medium (0.18 ml) of monocytes cultured in a P-selectin-coated plate was mixed with anti-TNF-α Ab-conjugated

Fig. 2. Purification of P-selectin from human platelets. **a** Purified P-selectin was analyzed by SDS-polyacrylamide gel electrophoresis followed by silver staining. **b** Activity of purified P-selectin was assayed by cell adhesion of HL60 leukemic cells. A 96-well culture plate was coated with purified P-selectin (4 μ g/ml) and fluorescently labeled cells (1×10^5 cells) were added. After the plate was incubated at 4°C for 1 h, non-adherent cells were removed by washing with RPMI 1640/1% BSA, and adherent cells were lysed with 1% NP-40. The fluorescence intensity was measured with a fluorescence spectrophotometer. 2T60 = Anti-P-selectin Ab; PL1 = anti-PSGL-1 blocking Ab; PL2 = anti-PSGL-1 non-blocking Ab; KM93 = anti-sLeX Ab. The assays were carried out in triplicate, and the data are presented as means $\pm$ SD. *** $p < 0.005$ vs. positive control without Ab.

protein G-Sepharose (0.05 ml, 20% suspension) and incubated at 4°C for 16 h. Measurement by ELISA revealed that >98% of TNF- α present in the conditioned medium was gone after sedimentation of protein G-Sepharose. A monocyte suspension (6×10^6 cells/ml, 0.1 μ l) was added to the TNF- α -depleted conditioned medium thus obtained (0.1 μ l), and the mixture was incubated at 37°C for 20 h. The culture supernatant was then assayed for IL-8 by ELISA.

Results

Purification of P-Selectin from Human Platelets

When purified P-selectin was analyzed by SDS-polyacrylamide gel electrophoresis, a single band with 130–140 kDa was observed after silver staining (fig. 2a). The activity of purified P-selectin was assayed by the adhesion of HL60 cells to immobilized P-selectin. The plate coating with P-selectin greatly increased HL60 cell adhesion, and the increased cell adhesion was inhibited by the addition of anti-P-selectin (2T60), anti-PSGL-1 blocking (PL1), or anti-sLeX (KM93) antibodies but not by anti-PSGL-1 non-blocking Ab (PL2; fig. 2b). These results indicated that purified P-selectin possessed binding activity specific for P-selectin ligands and promoted cell adhesion.

Potentiation of Cytokine Secretion from Monocytes by P-Selectin

Secretion of TNF- α from monocytes was potentiated ~10-fold when these cells were cultured in a P-selectin-

coated plate compared to the culture in a BSA-coated plate (fig. 3a). Heat treatment (100°C, 10 min) of P-selectin before plate coating suppressed TNF- α secretion to almost the control level, whereas TNF- α secretion induced by LPS was not affected by heat treatment under the same conditions (fig. 3a). In addition, TNF- α secretion was significantly decreased when P-selectin was depleted by the absorption with anti-P-selectin Ab (2D7) plus protein G-Sepharose prior to the plate coating (fig. 3b). Thus, it is unlikely that possible contamination of LPS in the P-selectin specimen used in this experiment contributed to monocyte stimulation. TNF- α secretion was also inhibited by 75% in the presence of a P-selectin antagonistic peptide at 3 μ M [14] (data not shown), suggesting that specific interaction mediated by P-selectin and its counter-ligands induced monocytes to produce TNF- α .

Because monocytes are known to produce various inflammation-related cytokines, we examined the changes in the production of IL-1 β , IL-6, IL-8, IL-12, and MIP-1 β by monocytes when the cells were cultured in P-selectin-coated plates for 4 or 20 h. The secretion of IL-1 β , IL-8, and IL-12 was not substantially affected after 4 h of culture, whereas the secretion of these cytokines was potentiated 4.5- to 10-fold after 20 h of culture (fig. 4). In contrast, MIP-1 β secretion was increased 4.6-fold after 4 h and 12-fold after 20 h. IL-6 secretion was also induced by P-selectin, and was 4.3- and 6.8-fold increased after 4 and

Fig. 3. TNF- α secretion from human monocytes induced by P-selectin. Human monocytes (1.5×10^5 cells) were incubated at 37°C for 4 h in a culture plate that had been coated with P-selectin (0.4 μ g/ml) or in the presence of LPS (100 ng/ml). The culture supernatant was assayed for TNF- α by ELISA. The effects of heat treatment (HT; 100°C, 10 min) of P-selectin or LPS (**a**) and absorption of P-selectin with anti-P-selectin Ab plus protein G-Sepharose (**b**) were also examined. The assays were carried out in triplicate, and the data are presented as means $\pm$ SD. *** $p < 0.005$ vs. control in the absence of P-selectin; ** $p < 0.01$ vs. positive control without absorption by Ab-protein G conjugate.

20 h of culture, respectively. The secretion of these monocyte-derived cytokines was stimulated when cultured in P-selectin-coated plates, but the kinetics appear to be different among cytokines. Therefore, we analyzed the kinetics of secretion of TNF- α , IL-1 β , and IL-12. As shown in figure 5, the secretion of TNF- α was elevated after 2 h of culture and peaked at 4 h. On the other hand, the secretion of IL-1 β and IL-12 exhibited delayed responses; it began to increase at 8 h and gradually increased until 16–24 h of culture. We then assumed the possibility that TNF- α secreted in an early phase secondarily stimulates monocytes to produce other cytokines, and assessed the effect of depletion of TNF- α from a conditioned medium of monocytes cultured for 4 h in a P-selectin-coated plate. The absorption of TNF- α with a complex of anti-TNF- α Ab and protein G-Sepharose resulted in the reduction of IL-8 production to 38% (table 1), indicating that increased IL-8 secretion in the late phase is caused, at least in part, by stimulation with TNF- α secreted in the early phase.

Priming of Monocytes with Cytokines for Stimulation by P-Selectin

We next assessed the effect of cytokine pretreatment on monocyte susceptibility to P-selectin. When monocytes were treated with GM-CSF (50 ng/ml), MCP-1 (50 ng/ml), or IFN- γ (5 ng/ml) at 37°C for 20 min followed by culture in P-selectin-coated plates for 4 h, TNF- α secretion from the cells was significantly increased (fig. 6a–

c). However, the increases in TNF- α secretion from cytokine-treated monocytes were limited (1.2- to 1.5-fold) compared to those from untreated monocytes. We then examined the effect of monocyte priming with IFN- γ on TNF- α response to a low dose of P-selectin. When monocytes treated with or without IFN- γ were cultured in plates coated with a low concentration (0.1 μ g/ml) of P-selectin, TNF- α secretion from untreated monocytes was comparable to the control level, but that from IFN- γ (5 ng/ml)-treated monocytes was markedly increased (3.3-fold; fig. 6d). These results indicate that pretreatment of monocytes with IFN- γ increased their susceptibility to P-selectin-dependent cellular activation.

Discussion

Cell adhesion mediated by P-selectin and its ligands is considered to play a crucial role in leukocyte trafficking through their interaction with activated endothelium and platelets. In this study, we demonstrated that cell adhesion of monocytes to immobilized P-selectin induced the secretion of cytokines, including TNF- α , IL-1 β , IL-6, IL-8, IL-12, and MIP-1 β . These cytokines are involved in cascade reactions of inflammation processes; for example, IL-8 (CXCL8) and MIP-1 β (CCL4) exert chemotaxis of neutrophils and T cells to recruit these cells to inflammatory tissues [20–22], and IL-1 β and IL-12 regulate var-

Fig. 4. Secretion of various cytokines from monocytes induced by immobilized P-selectin. Human monocytes (1.5×10^5 cells) were incubated at 37°C for 4 or 20 h in a culture plate coated with P-selectin (0.4 μ g/ml) or in the presence of LPS (100 ng/ml). The

culture supernatant was assayed for IL-1 β , IL-12 (p70), IL-8, MIP-1 β , and IL-6 by ELISA. The assays were carried out in triplicate, and the data are presented as means $\pm$ SD. *** p < 0.005 vs. control in the absence of P-selectin.

Fig. 5. Time course of cytokine secretion from monocytes induced by P-selectin. Human monocytes (1.5×10^5 cells) were incubated at 37°C for 1–24 h in a culture plate coated with P-selectin (0.4 μ g/ml). The culture supernatant was assayed for TNF- α , IL-1 β , and IL-12 (p70) by ELISA.

Table 1. Effect of TNF- α secreted from P-selectin-stimulated monocytes on IL-8 production

Stimulator	Absorption with	IL-8, ng/ml ^b
Control	none	20 $\pm$ 4
Conditioned medium ^a	protein G-Sepharose	117 $\pm$ 20
	protein G-Sepharose plus anti-TNF- α	45 $\pm$ 3
LPS, 0.1 μ g/ml	none	288 $\pm$ 40

^a Supernatant of monocyte culture for 4 h in a P-selectin-coated plate.

^b Concentration in the supernatant of monocyte culture for 20 h. The assays were carried out in triplicate, and the data are presented as means $\pm$ SD.

Fig. 6. Priming of monocytes with cytokines for TNF- α response to P-selectin. Human monocytes (1.5×10^5 cells) were treated with GM-CSF (50 ng/ml; **a**), MCP-1 (50 ng/ml; **b**), or IFN- γ (5 ng/ml; **c**, **d**) and incubated at 37°C for 4 h in culture plates coated with 0.4 (**a–c**) or 0.1 μ g/ml (**d**) P-selectin. The culture supernatant was assayed for TNF- α by ELISA. The assays were carried out in triplicate, and the data are presented as means $\pm$ SD. * p < 0.05, ** p < 0.01, vs. control.

ious functions of T cells and natural killer cells [23–25]. Thus, cytokine production induced by the interaction of monocytes with P-selectin seems to be of considerable interest in the pathogenesis of various inflammatory diseases. A number of studies indicated the significance of P-selectin expression and inflammatory/allergic diseases, such as ischemia/reperfusion injury [26–28], allergic asthma [29, 30], and atopic dermatitis [31]. It seems likely that these inflammatory cytokines secreted from P-selectin-activated leukocytes play a crucial role in the initiation and progression of inflammation. Previous studies demonstrated that P-selectin-mediated cell adhesion induced leukocyte activation, e.g., superoxide anion production by neutrophils [5, 6, 32] and expression of tissue factor on monocytes [7]. Endothelial dysfunction caused by reactive oxygen species and activation of the blood coagulation system as well as production of various cytokines may be synergistically involved in the development of inflammatory and allergic diseases.

Intracellular signaling of leukocyte activation was reported to involve protein tyrosine kinases and mitogen-activated protein kinases [8, 9]. For instance, Nef-associated factor-1 formed a stable complex with the cytoplasmic domain of a major P-selectin ligand (PSGL-1) and was phosphorylated by Src kinase, leading to the recruitment of phosphoinositide 3-kinase and activation of leukocyte β_2 -integrins [11]. The activation of β_2 -integrins is thought to be an important step for leukocyte extravasation because they must adhere firmly to inflamed endothelia in order for them to migrate to inflammatory sites. The P-selectin-dependent cytokine secretion shown in this study may be involved in the process of leukocyte transmigration. However, we have so far no evidence that the signals for cytokine secretion were transduced via PSGL-1. Although the secretion from monocytes of various cytokines, including TNF- α , IL-1 β , IL-6, IL-8, IL-12, and MIP-1 β , was potentiated by their interaction with P-

selectin, the kinetics of their secretion to culture medium were somewhat different (fig. 5). Because the absorption of TNF- α secreted in the early phase abrogated the successive production of IL-8 (table 1), it is likely that the potentiation of cytokines was, at least in part, of secondary response through TNF- α [33]. However, the production of IL-12 by monocyte-derived macrophages was reported to be suppressed by TNF- α [34], and enhanced secretion of those cytokines may not necessarily be ascribed to TNF- α .

Finally, we found a synergistic effect of pretreatment of monocytes with cytokines such as IFN- γ on P-selectin-induced TNF- α secretion. Monocytes pretreated with IFN- γ were effectively stimulated on a plate coated with a low concentration of P-selectin where unprimed monocytes were not activated (fig. 6). Thus, IFN- γ pretreatment seems to make monocytes more susceptible to producing TNF- α . The P-selectin-mediated monocyte activation of cytokine production is likely to be readily induced in the microenvironment of inflammatory sites.

In conclusion, the interaction of P-selectin with the counter-ligands on monocytes plays an important role not only in their translocation into inflammatory tissues and damaged endothelia but also in the regulation of cytokine production by these cells.

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