CD226 Mediates Platelet and Megakaryocytic Cell Adhesion to Vascular Endothelial Cells*

Received for publication, January 21, 2003, and in revised form, May 19, 2003
Published, JBC Papers in Press, July 7, 2003, DOI 10.1074/jbc.M300702200

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Platelet adhesion to vascular endothelial cells is a pathophysiologically relevant cell-to-cell interaction. However, the mechanisms underlying this cellular interaction are incompletely understood. In search of the ligand for CD226 adhesion molecule expressed on platelets, we found that human umbilical vein endothelial cells (HUVEC) express significant amount of putative CD226 ligand. We demonstrated that thrombin-activated, but not resting, platelets bind to intact HUVEC. Anti-CD226 monoclonal antibody specifically inhibited the binding, indicating that CD226 mediates the intercellular binding between thrombin-activated platelet and HUVEC. We also demonstrated that platelet activation with thrombin induces tyrosine phosphorylation of CD226 as well as CD226-mediated platelet adhesion. Moreover, experiments using mutant transfectants suggested that the tyrosine at residue 322 of CD226 plays an important role for its adhesive function. CD226 was also expressed on primary megakaryocytes and megakaryocytic cell lines. Anti-CD226 monoclonal antibody inhibited binding of megakaryocytic cell lines to HUVEC. Taken together, these results reveal a novel mechanism for adhesion of platelets and megakaryocytes to vascular endothelial cells.

Cellular interaction between platelets and vascular endothelial cells plays a crucial role for maintaining the hemostatic system. Platelets roll on activated or inflamed endothelial cells under high shear rate, which is mediated by P-selectin expressed on endothelial cells (1–3). Furthermore, once the endothelial cells are denuded, GPIb (CD42b) on platelet surface binds to immobilized von Willebrand factor (vWF)1 at exposed subendothelium (4, 5). Several other molecules, such as GPIIbIIIa (6, 7), platelet-endothelial cell adhesion molecule-1 (8), fibronogen (9), and b1-integrin (10), have also been reported to be involved in this process, depending on both shear rate and activation status of platelets and endothelial cells. Recently, Harlan and colleagues (7) proposed a model for the interaction between activated platelets and intact endothelial cells, in which platelet-bound adhesive proteins such as fibronogen, fibronecrotin, and vWF mediate bridging between human umbilical vein endothelial cells (HUVEC) and GPIIbIIIa (CD41/CD61) expressed on platelets. However, in their experimental model, adhesion of activated platelets to intact HUVEC was only partially inhibited in the presence of Arg-Gly-Asp-Ser (RGDS) peptide, which blocks ligand binding to GPIIbIIIa (7), suggesting that there might be as yet undetermined adhesion molecules contributing to this interaction.

The hemostatic system is also maintained by the constant production of platelets from megakaryocytes. Platelets are assumed to be cell fragments torn from cytoplasm of mature megakaryocytes that reside in close proximity to bone marrow endothelial cells (11–13). Several lines of evidence suggest that cellular interaction between megakaryocytes and vascular endothelial cells plays a crucial role for megakaryopoiesis (14–18). However, the molecular mechanism underlying this cellular interaction has not been well elucidated before now.

We previously identified a novel adhesion molecule DNAM-1 (CD226), which is a member of the immunoglobulin superfamily containing two Ig-like domains of the V-set and is encoded by a gene on human chromosome 18q22.3 (19). CD226 is a 65-kDa glycoprotein expressed on the majority of NK cells and monocytes and a subset of T-lymphocytes, and involved in cytotoxicity and cytokine secretion mediated by NK cells and T-lymphocytes. It is intriguing that CD226 is also expressed on platelets (20). Cross-linking CD226 with an anti-CD226 monoclonal antibody induces platelet activation and aggregation (21), suggesting that CD226 functions as a signal transducing adhesion molecule in platelets. We demonstrate here that CD226 is involved in adhesion of platelets and megakaryocytes to vascular endothelial cells.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-phosphotyrosine monoclonal antibody (mAb) 4G10 was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Anti-CD226 mAb DX11 was provided by DNAX Research Institute (Palo Alto, CA), and its F(ab’1)2 fragments were prepared as described (19).

Cell Lines and Cell Preparation—HEL, BW5147, COS-7, and 293T

* This work was supported in part by Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant 12670972 (to T. N.) and Grants 12051201 and 12470111 (to A. S.) and by special coordination funds from the Science and Technology Agency of the Japanese Government (to A. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: vWF, von Willebrand factor; HUVEC, human umbilical vein endothelial cells; NK, natural killer; mAb, monoclonal antibody; PRP, platelet-rich plasma; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; FP, fusion protein; RIPA, radiimmunoprecipitation assay.

2 H. Kojima, H. Kanada, S. Shimizu, E. Kasama, K. Shibuya, H. Nakauchi, T. Nagasawa, and A. Shibuya, unpublished observations.
cells were purchased from American Type Culture Collection (Manassas, VA). UT7/TPO and CMK are megakaryocyte cell lines provided by Dr. Norio Komatsu (Jichi Medical School, Tochigi, Japan) (22) and Dr. Takeyuki Sato (Chiba University, Chiba, Japan) (25), respectively. NF-κB is an erythroleukemic cell line established in our laboratory. Platelet-rich plasma (PRP) and gel-filtraited platelets were prepared as described elsewhere (24, 25).

HUEVC were prepared by the standard procedure. Briefly, inner lumen of the umbilical vein was gently washed with sterile PBS and filled with trypsin-EDTA solution (Sigma). After a 30-min incubation at 37 °C, detached cells were harvested and washed once with RPMI 1640 containing 10% fetal calf serum. Cells were resuspended in RPMI 1640 containing 15% horse serum, 6 units/ml heparin, 2 mm γ-glutamine, 1 mM sodium pyruvate, 1 mM Hepes, and 1% sodium deoxycholate.

Expression of the CD226-Ig Fusion Protein (FP) and Flow Cytometry—293T cells were transiently transfected with the plasmid of the CD226-Ig FP, which was constructed in pcDNA expression vector containing a human CD8 leader sequence followed by the Fc segment of human IgG at the COOH terminus (provided by Dr. Gerald Zurawski, DNAX Research Institute), and the CD226-Ig FP was purified by affinity chromatography using immobilized protein A (Bio-Rad). Cells (2 × 10^6) were stimulated for 1 h on ice with 1 μg/ml CD226-Ig FP or control FP. FITC-labeled anti-human IgG (Dako, Glostrup, Denmark) was used as a second step reagent. Flow cytometry was performed to detect the binding of CD226-Ig FP to HUEVC.

Adhesion Assay of Calcine-loaded Platelets—Platelet adhesion to HUEVC was determined by the method, as described (7), with modification. Briefly, PRP was treated with 1 μM aspirin for 20 min, and after adding 0.1 volume of ACD solution (2.2% (w/v) sodium citrate, 0.8% (w/v) citric acid, and 2.2% (w/v) dextrose), the PRP was centrifuged at 200 × g for 20 min at room temperature. Platelets were resuspended in 200 μl of Hepes-Tyrode buffer (137 mM NaCl, 2 mM KCl, 0.4 mM NaH2PO4, 1 mM MgCl2, 1 mM CaCl2, 5.6 mM glucose, 0.1% (w/v) BSA, 1% sucrose, and containing 10% ACCD), and 0.5 μl of 5 mM calcein AM (Molecular Probes, Eugene, OR) was added to the platelet suspension. After a 30-min incubation at room temperature in the dark with gentle shaking, platelets were washed twice with Hepes-Tyrode buffer containing 10% ACCD and were resuspended in Hepes-Tyrode buffer (1 × 10^7/ml). Platelets were stimulated or not with thrombin (1 unit/ml, for 10 min at room temperature) under non-stirring condition, and then hirudin (2 units/ml, Sigma) was added to inactivate thrombin. After additional treatment with F(ab′)2 fragments of control Ig (Jackson Co., West Grove, PA) or anti-CD226 mAb alone or in combination with Arg-Gly-Asp-Ser (RGDS) peptide (500 μM, Sigma) at room temperature for 10 min, 100 μl of calcine-loaded platelets were transferred on HUVEC monolayers well of 12-well microtiter plates (Iwaki, Tokyo, Japan) containing 900 μl of RPMI 1640 with 1 mM CaCl2. Platelets were co-cultured with HUEVC for 1 h at 37 °C. After washing out the non-adherent platelets, HUEVC were detached by cell scraper, vigorously pipetted, and washed once. The platelet binding to HUEVC was detected by flow cytometry in triplicate experiments.

CD226 Mediates Thrombin-activated Platelet Binding to HUEVC—To explore the involvement of CD226 in platelet binding to vascular endothelial cells, we first examined whether vascular endothelial cells express CD226L. We generated a soluble protein consisting of the extracellular domain of CD226 fused to the Fc portion of human IgG. The fusion protein of CD226 specifically bound to HUEVC, indicating that HUEVC express the putative CD226L (Fig. 1A). To examine whether the CD226L expressed on HUEVC mediates intercellular binding, we co-cultured 51Cr-labeled BW5147 transfectants expressing CD226 with HUEVC monolayers and determined specific intercellular binding. As shown in Fig. 1B, the BW5147 transfectants expressing CD226 bound HUEVC at significantly higher levels than the parental BW5147 cells. Moreover, the binding of the transfectants to HUEVC was specifically inhibited in the presence of F(ab′)2 fragments of anti-CD226 mAb. These results indicate that HUEVC express the functional CD226L that mediates intercellular binding.

RESULTS

HUEVC Express the Putative Ligand for CD226 (CD226L)—To determine the role of CD226 in platelet adhesion to vascular endothelial cells, we first examined whether vascular endothelial cells express CD226L. We generated a soluble protein consisting of the extracellular domain of CD226 fused to the Fc portion of human IgG. The fusion protein of CD226 specifically bound to HUEVC, indicating that HUEVC express the putative CD226L (Fig. 1A). To examine whether the CD226L expressed on HUEVC mediates intercellular binding, we co-cultured 51Cr-labeled BW5147 transfectants expressing CD226 with HUEVC monolayers and determined specific intercellular binding. As shown in Fig. 1B, the BW5147 transfectants expressing CD226 bound HUEVC at significantly higher levels than the parental BW5147 cells. Moreover, the binding of the transfectants to HUEVC was specifically inhibited in the presence of F(ab′)2 fragments of anti-CD226 mAb. These results indicate that HUEVC express the functional CD226L that mediates intercellular binding.

CD226 Mediates Thrombin-activated Platelet Binding to HUEVC—To explore the involvement of CD226 in platelet binding to vascular endothelial cells, HUEVC monolayers were incubated with calcine-loaded platelets. HUEVC were then harvested, and platelet binding was determined by flow cytometry. Whereas the binding of resting platelets to HUEVC was hardly detected (data not shown), thrombin-activated platelets effectively bound to HUEVC (Fig. 2). Pretreatment of thrombin-activated platelets with RGDS peptide, which blocks ligand binding to GPIIIb/IIIa, inhibited the platelet binding to HUEVC, consistent with a previous report (7). However, we observed that blockade of CD226 by F(ab′)2 fragments of anti-CD226 mAb alone also significantly inhibited the thrombin-activated
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Thrombin Induces Tyrosine Phosphorylation of CD226 in Platelets—Because platelet activation with thrombin induces CD226-mediated adhesion of platelets to HUVEC, we considered that thrombin may either up-regulate CD226 expression or modulate CD226 function on platelets. As the amount of platelet-surface CD226 did not change, as determined by flow cytometry, after stimulation with thrombin (data not shown), we supposed that platelet activation with thrombin modulates the avidity or affinity of CD226 molecule. We therefore investigated whether platelet activation by natural agonist such as thrombin or collagen induces signaling events in CD226. Because CD226 cytoplasmic portion contains the tyrosine at residue 322, which can be phosphorylated by the Fyn tyrosine kinase (29), we examined whether thrombin or collagen induces tyrosine phosphorylation of CD226. As demonstrated in Fig. 3 (A and B), stimulation of platelets with collagen or thrombin under stirring condition induced tyrosine phosphorylation of CD226. In a sharp contrast to the rapid phosphorylation by collagen that peaked at 1 min, tyrosine phosphorylation of CD226 by thrombin was achieved at the maximum level after 10 min. Judging from the slower time course of the phosphorylation in thrombin-stimulated platelets, we assumed that the phosphorylation is mediated mainly by fibrinogen binding to GPIIbIIIa and/or the resultant platelet aggregation. To explore this possibility, we examined whether thrombin-induced tyrosine phosphorylation of CD226 is observed under non-stirring condition, in which fibrinogen binding to GPIIbIIIa is reduced. As demonstrated in Fig. 3C, although platelet stimulation with thrombin under non-stirring condition induced significant level of tyrosine phosphorylation of CD226, the phosphorylation level was considerably lower than that induced under stirring condition. Moreover, addition of RGDS peptide, which blocks fibrinogen binding to GPIIbIIIa, significantly inhibited thrombin-induced tyrosine phosphorylation of CD226. These results suggest that fibrinogen binding to GPIIbIIIa is critical for achieving the maximal level of tyrosine phosphorylation of CD226 in thrombin-activated platelets. In fact, platelet adhesion to immobilized fibrinogen via GPIIbIIIa resulted in significant levels of tyrosine phosphorylation of CD226 (Fig. 3D).

The Tyrosine (Tyr322) Is Involved in CD226-mediated Adhesion—We previously reported that phosphorylation of the serine at residue 329 by PKC plays an important role for CD226-mediated adhesion (26). However, we also observed that the site-specific mutant CD226 at residue 329 (Ser329 \to Phe) is

![Fig. 1. HUVEC express functional ligand for CD226. A, HUVEC were stained with CD226-Ig fusion protein (filled histogram) or control (Cont.) fusion protein (open histogram), followed by an FITC-labeled anti-human Ig. Histograms displaying the fluorescence of live cells are presented. The data are representative of several independent experiments. B, \[^{31}C\]-labeled BW5147 transfectants expressing CD226 or parental BW5147 cells were treated or not with various concentrations of F(ab\(^\prime\))\(_2\) fragments of control Ig or anti-CD226 mAb at room temperature for 10 min, and incubated on HUVEC prepared in 48-well microtiter plates at 37 °C for 1 h. The adherent cells were lysed, and the radioactivities in the lysates were assayed by a \(\gamma\)-counter. Total radioactivities after \[^{31}C\] labeling were confirmed to be statistically identical between BW5147 transfectants expressing CD226 and parental BW5147 cells. Results are presented as the mean radioactivities in triplicate experiments. Error bars denote standard deviations (\(*, p < 0.05; **, p < 0.01\). The data are representative of several independent experiments.](image1)

![Fig. 2. CD226 mediates platelet binding to HUVEC. Calcein-loaded platelets stimulated or not with thrombin under non-stirring condition were further treated with F(ab\(^\prime\))\(_2\) fragments of anti-CD226 mAb and RGDS peptide further inhibited platelet binding to HUVEC. These results indicate that CD226 is involved in thrombin-activated platelet adhesion to HUVEC.](image2)
still capable of binding to the putative CD226L (data not shown and Ref. 26), suggesting that CD226-mediated adhesion depends in part on undetermined molecular events other than the phosphorylation of Ser329. Here we have demonstrated that thrombin induces both CD226-mediated platelet adhesion to HUVEC and tyrosine phosphorylation of CD226. These results suggest that tyrosine phosphorylation of CD226 may also be involved in CD226-mediated adhesion. To examine this possibility, we established BW5147 transfectants expressing either wild type or site-directed mutant CD226 at residues 322, 325, or both (Tyr322 → Phe, Tyr325 → Phe, or Tyr322 → Phe/Tyr325 → Phe, respectively). As shown in Fig. 4B, the introduction of the site-directed mutations at residues 322 (Tyr322 → Phe) and both 322 and 325 (Tyr322 → Phe/Tyr325 → Phe), but not at 325 alone (Tyr325 → Phe), completely abolished tyrosine phosphorylation of CD226 after stimulation with pervanadate, suggesting that Tyr322 as well as Ser329 may play an important role for CD226-mediated adhesion. For binding assay, we used COS-7 cells, instead of HUVEC, which also express the putative CD226L, as confirmed by flow cytometry using the CD226-Ig fusion protein (data not shown), because COS-7 cells are hardly detached from plates by vigorous washing to extremely reduce nonspecific binding of 51Cr-labeled BW5147 transfectants. Whereas these transfectants express comparable amount of CD226 (Fig. 4A), the transfectants expressing mutated CD226 at residue(s) 322 (Tyr322 → Phe) or both 322 and 325 (Tyr322 → Phe/Tyr325 → Phe), but not 325 alone (Tyr325 → Phe), bound to COS-7 cells at a significantly lower level, as compared with that expressing wild type CD226 (Fig. 4C). Taken together, these results suggest that tyrosine phosphorylation of CD226...
at residue 322 plays an important role for CD226-mediated adhesion of the transfectant to COS-7.

**CD226 Mediates Binding of Megakaryocytic Cells to HUVEC**—We examined the expression of CD226 on megakaryocytes or megakaryocytic cell lines. Fig. 5A shows that human primary megakaryocytes express significant amount of CD226, as determined by immunohistochemistry. Analyses by flow cytometry and immunoblotting showed that CD226 is also expressed on various megakaryocytic cell lines, including UT7/TPO, HEL, and CMK, but not on an erythroleukemia cell line NH, and platelets were stained with control Ig (open histogram) or anti-CD226 mAb (filled histogram), followed by an FITC-labeled anti-mouse Ig. Histograms displaying the fluorescence of live cells are presented. The data are representative of several independent experiments. C, lysates prepared from each indicated cells were analyzed by immunoblotting with anti-CD226 mAb (1 μg/ml). CD226 proteins were detected by using an horseradish peroxidase-conjugated goat anti-mouse Ig, and the blot was developed with the ECL-immunoblotting detection kit.

**Fig. 6.** CD226 mediates adhesion of megakaryocytic cells to HUVEC. 

**A**—Labeled HEL cells were treated or not with F(ab')2 fragments of control Ig (Cont.) or anti-CD226 mAb at room temperature for 10 min, and then incubated on HUVEC prepared in 48-well microtiter plates at 37 °C for 1 h. The adherent HEL cells were lysed, and the radioactivities in the lysates were assayed by a γ-counter. Results are presented as the mean radioactivities in triplicate experiments. Error bars denote standard deviations (*, p < 0.05; **, p < 0.01). The data are representative of several independent experiments.

**B**—A bone marrow smear obtained from a healthy volunteer was stained with anti-CD226 mAb, followed by staining with Alexa 488 (green fluorescence)-conjugated horse anti-mouse Ig (center panel). The smear was then fixed with Vectashield mounting medium including 4',6-diamidino-2-phenylindole (blue fluorescence) to stain cell nuclei and analyzed by using fluorescence microscopy under dark-field illumination (left panel). The left and center panels are photographs obtained from the same field. The white arrowheads in the left panel indicate megakaryocytes in the bone marrow smear. A megakaryocyte expressing green fluorescence was shown at higher magnification in the right panel. The megakaryocytes stained with control Ig instead of anti-CD226 mAb did not show any green fluorescence (data not shown). The data are representative of several independent experiments.

**C**—A, megakaryocytic cell lines UT7/TPO, HEL, and CMK, an erythroleukemia cell line NH, and platelets were stained with control Ig (open histogram) or anti-CD226 mAb (filled histogram), followed by an FITC-labeled anti-mouse Ig. Histograms displaying the fluorescence of live cells are presented. The data are representative of several independent experiments.

**DISCUSSION**

In the present study we have found that the putative ligand for CD226 is expressed on HUVEC (Fig. 1). This observation led us to examine whether CD226 mediates intercellular binding between platelets and HUVEC. We have demonstrated that thrombin-activated, but not resting, platelets bind to HUVEC, and CD226 is in part responsible for this binding (Fig. 2). We have also shown that platelet activation with thrombin induces tyrosine phosphorylation of CD226 (Fig. 3B) as well as CD226-mediated adhesion. Significance of tyrosine phosphorylation of CD226 for its adhesive function was demonstrated at least in CD226-expressing BW5147 transfectants (Fig. 4). Taken together with these and our previous observations (26, 29), it may be feasible to consider that thrombin modulates CD226 adhesive function possibly via CD226 tyrosine phosphorylation, which enables CD226-mediated platelet adhesion to HUVEC.
Although we have demonstrated that the tyrosine phosphorylation at residue 322 of CD226 is in part responsible for CD226-mediated adhesion in BWS5147 transfectant system (Fig. 4), it remains undetermined whether the tyrosine phosphorylation is involved also in thrombin-induced platelet adhesion to HUVEC. Indeed, thrombin gave rise to CD226-mediated platelet adhesion to HUVEC under non-stirring condition (Fig. 2), in which CD226 was only weakly tyrosine-phosphorylated (Fig. 3C). One possible explanation is that, besides tyrosine phosphorylation of CD226, other signaling events induced by thrombin co-operatively modulate CD226 adhesive function. In fact, in our previous experiment, we demonstrated that serine phosphorylation of CD226 may be sufficient to exert its adhesive function.

Fibrinogen binding to GPIIbIIIa, which should be induced by platelet activation by thrombin, seems to play an important role for thrombin-induced tyrosine phosphorylation of CD226 (Fig. 3, C and D). We previously demonstrated that cross-linking CD226 on NK cells derived from a patient with leukocyte adhesion molecule deficiency, whose leukocytes lack LFA-1 expression, fails to induce NK cell activation (29). Furthermore, CD226 is associated with LFA-1 in NK cells and activated T-lymphocytes (29), indicating physical and functional relationship between CD226 and LFA-1. Presently, the signaling pathway leading to CD226 tyrosine phosphorylation in thrombin-activated platelets is uncertain. However, the requirement of ligand binding to GPIIIbIIIa for the tyrosine phosphorylation in thrombin-activated platelets may indicate functional association between GPIIIbIIIa and CD226, corresponding with the association of LFA-1 and CD226 in lymphocytes. On the other hand, the rapid time course of the tyrosine phosphorylation in collagen-stimulated platelets suggests a possibility that an Src family tyrosine kinase Fyn, which is rapidly activated by collagen in platelets (30), may phosphorylate the tyrosine at residue 322 of CD226, as was demonstrated in lymphocytes (29).

Cellular interaction between megakaryocytes and vascular endothelial cells is crucial not only for maturation and differentiation of megakaryocytes (14–18) but also for migration into the inner lumen of vascular endothelial cells, whereby platelets are generated (31). We have described here that human primary megakaryocytes and several megakaryocytic cell lines express significant amount of CD226, which is involved in intercellular binding between megakaryocytic cell lines and HUVEC. Because cross-linking CD226 induces platelet activation and aggregation (20),2 CD226 may mediate signals for cytoplasmic processing in megakaryocytes, possibly contributing to megakaryocyte maturation and/or platelet production.

In summary, we have revealed a novel mechanism for adhesion of platelets and megakaryocytic cells to vascular endothelial cells. The present studies suggest that CD226 may play an important role for thrombosis and hemostasis. Further studies are under way to identify the CD226L and determine the structural requirements involved in CD226 binding. Activation mechanisms of CD226 during platelet activation by natural agonists should also be clarified in future experiments.

Acknowledgments—We thank Dr. Lewis Lanier for critical reading of this manuscript. We also thank Satoshi Yamazaki and Yukinori Kozuma for technical assistance.

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J. Biol. Chem. 2003, 278:36748-36753.
doi: 10.1074/jbc.M300702200 originally published online July 7, 2003

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