INVOLVEMENT OF THE SNAKE TOXIN RECEPTOR CLEC-2
IN PODOPLANIN-MEDIATED PLATELET ACTIVATION

BY CANCER CELLS*

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Running Title: Podoplanin Activates Platelets By Binding To CLEC-2.

Podoplanin (aggrus), a transmembrane sialoglycoprotein, is involved in tumor cell-induced platelet aggregation, tumor metastasis, and lymphatic vessel formation. However, the mechanism by which podoplanin induces these cellular processes including its receptor has not been elucidated to date. Podoplanin induced platelet aggregation with a long lag phase, which is dependent upon Src and phospholipase Cγ2 activation. However it does not bind to glycoprotein VI. This mode of platelet activation was reminiscent of the snake toxin rhodocytin, the receptor of which has been identified by us as a novel platelet activation receptor, C-type lectin-like receptor 2 (CLEC-2). Therefore, we sought to evaluate whether CLEC-2 serves as a physiological counterpart for podoplanin. Association between CLEC-2 and podoplanin was confirmed by flow cytometry. Furthermore, their association was dependent on sialic acid on O-glycans of podoplanin. Recombinant CLEC-2 inhibited platelet aggregation induced by podoplanin-expressing tumor cells or lymphatic endothelial cells, suggesting that CLEC-2 is responsible for platelet aggregation induced by endogenously expressed podoplanin on the cell surfaces. These findings suggest that CLEC-2 is a physiological target protein of podoplanin and imply that it is involved in podoplanin-induced platelet aggregation, tumor metastasis, and other cellular responses related to podoplanin.

There has been an increasing body of evidence that platelets are involved in cancer metastasis and/or progression (1, 2). Several studies have reported on tumor cell-induced platelet activation, implicating that aggregation serves for tumor cell nestling while released growth factors serves for angiogenesis or tumor growth. Aggrus, a sialoglycoprotein on the surface of cancer cells, which was later found to be identical to podoplanin, induces platelet aggregation (3-5). Podoplanin/aggrus is a type-I transmembrane sialomucin-like glycoprotein that consists of an extracellular domain with abundant Ser/Thr residues as potential O-glycosylation sites, a single transmembrane portion, and a short cytoplasmic tail with putative sites for protein kinase C and cAMP phosphorylation (3, 6). Increased expression of podoplanin/aggrus was
observed in various tumor cells, including squamous cell carcinomas (7, 8), testicular seminoma (9), and brain tumors (10-12). Recent investigations have indeed reinforced the notion that podoplanin/aggrus expression might be associated with tumor metastasis or malignant progression (11, 13).

Podoplanin is also expressed abundantly on glomerular epithelial cells (podocytes), after which the sialoglycoprotein was named, type I lung alveolar cells, and lymphatic endothelial cells (7, 14). Podoplanin-deficient mice die at birth due to respiratory failure and have defects in lymphatic, but not blood vessel pattern formation, which are associated with diminished lymphatic transport, congenital lymphedema and dilation of lymphatic vessels, suggesting that it is crucially involved in lymphatic vessel formation (15). However, how podoplanin regulates the formation of lymphatic vessels or tumor-platelet interaction has remained totally unknown, and the identification of its pathophysiological target to which podoplanin interacts has been ardently awaited among research workers in a number of scientific fields.

In this study, we sought to identify the target molecule for podoplanin on the platelet membrane, since platelet aggregation can be used as a marker for their interaction. For this end, we investigated the characteristics of podoplanin-induced platelet aggregation, and compared them with those of well-defined platelet stimulators.

In the process of physiologic thrombus formation with platelet aggregation, the first step is platelet interaction with exposed collagen fibers at sites of vessel injury (16). Platelet adhesion and aggregation on collagen fibers and the subsequent stable clot formation is an integrated process that involves several platelet receptors and agonists such as ADP, thromboxane A2 and coagulation factors including thrombin. One of the major and powerful receptors involved herein is a collagen receptor, glycoprotein VI/FcRγ-chain complex (GPVI)1. The signal transduction pathway related to GPVI encompasses a number of intracellular signaling molecules, such as tyrosine kinases Src, spleen tyrosine kinase (Syk), an adapter protein, SH2 domain containing leukocyte protein of 76kDa (SLP-76), and phospholipase Cγ2 (PLCγ2) (16). We have recently identified a novel class of platelet activation receptor, C-type lectin-like receptor 2 (CLEC-2), which belongs to a non-classical C-type lectin, as a receptor on the platelet membrane for a platelet-aggregating snake venom, rhodocytin (17). CLEC-2 generates activation signals depending on protein tyrosine phosphorylation including a tyrosine kinase Src, Syk, an adapter protein SLP-76, and PLCγ2 in platelets in a manner similar to GPVI (17, 18). Although the powerful stimulatory action of CLEC-2 on platelets suggest that it plays an important role in vivo, a physiological ligand of the novel signalling receptor has not been identified to date.

As a result of characterizing podoplanin-induced platelet aggregation, we found that the mode of platelet activation induced by podoplanin was reminiscent of the rhodocytin. Therefore, we sought to evaluate whether CLEC-2 serves as a physiological counterpart for podoplanin. Association between CLEC-2 and podoplanin was confirmed by flow cytometry. Moreover, recombinant CLEC-2 inhibited platelet aggregation induced by podoplanin-expressing tumor cells or lymphatic endothelial cells. These findings suggest that podoplanin stimulates platelet aggregation by interacting with CLEC-2 and that the interaction between podoplanin and CLEC-2 regulates various cellular responses related to podoplanin.

Experimental Procedures

Animals and Materials - Genetically modified mice deficient in PLCγ2 were kindly donated by Drs. Masaki Hikida and Tomohiro Kurosaki (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). FcRγ-chain-deficient mice were generous gift
from Drs. Tadashi Yokosuka and Takashi Saito (RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). Rhodocytin purified from the venom of Calloselasma rhodostoma was donated by Dr. Takashi Morita (Meiji Pharmaceutical University, Tokyo, Japan) (19). The dimeric form of soluble GPVI (hGPVI-hFc2) was a generous gift from Dr. Yoshiki Miura and Dr. Masaaki Moroi (Kurume University, Fukuoka, Japan) (20). 293T-REx cell line that expressed CLEC-2 under a tet repressor protein was donated by Dr. Stefan Pöhlmann (University of Erlangen-Nürnberg, Erlangen, Germany) (21). Anti-human podoplanin antibody and anti-mouse podoplanin monoclonal antibody were from AngioBio Co. (Der Mar, CA). Anti-mouse podoplanin antibody 8F11 was obtained from Medical & Biological Laboratories Co., Ltd (Nagoya, Japan). Polyclonal anti–CLEC-2 antibody was from R&D Systems (Minneapolis, MN). Anti-phospho tyrosine antibody (4G10) was purchased from Upstate Biotechnology. Src kinase inhibitor PP2, and its inactive control PP3 were purchased from Calbiochem (San Diego, CA). Horm collagen (predominately Type I; derived from equine tendon) was from Nycomed (Munich, Germany). Thrombin was purchased from Sigma-Aldrich. Gly-Arg-Gly-Asp-Ser (GRGDS) peptide was obtained from Peptide Institute (Osaka, Japan).

**Platelet preparation** - This study was approved by the ethical committees in University of Yamanashi and informed consent was provided according to the Declaration of Helsinki. Venous blood from healthy drug-free volunteers was taken into 10% sodium citrate. Murine blood was drawn from mice, which were terminally anesthetized by diethyl ether, by portal vein puncture and taken into 100 μl of acid citrate dextrose. Washed human and murine platelets were obtained by centrifugation as previously described, using prostacyclin to prevent activation during the isolation procedure (22). Both sets of washed platelets were resuspended in modified Tyrode buffer (22) at a cell density of $2.5 \times 10^8$ and $1 \times 10^9$/ml.

**Transient transfection** - Chinese hamster ovary (CHO) cells were obtained from The Health Science Research Resources Bank (Osaka, Japan). Mouse podoplanin cDNA (AJ297944) incorporated with FLAG tag sequence was subcloned into a vector of pcDNA3 (Invitrogen) as described (mouse pod pcDNA) (4). Cell culture and transient transfection was performed as described previously (23). Briefly, $1 \times 10^7$ CHO cells were placed into an electroporation cuvette with 40 μg of mouse pod pcDNA or pcDNA only (see “Cell lines and stable transfection” below), followed by electroporation. Surface expression of mouse podoplanin was confirmed by flow cytometry using anti-mouse podoplanin monoclonal antibody as shown in Fig.3Ai.

**Cell lines and stable transfection** - CLEC-2 was expressed under a tet repressor protein in 293T-REx cell line (Invitrogen, Carlsbad, CA) and grown as described (21). CLEC-2 expression was induced by addition of 1 μg/mL doxycycline to the medium 24 to 48 hours before experimentation. Vehicle-added cells were used for control. A CHO cell mutant, Lec2 cells was obtained from ATCC. Cell culture and stable transfection of human podoplanin to these cells were performed as described previously (4). Briefly, human podoplanin cDNA (AB127958) incorporated with FLAG tag sequence, were subcloned into a vector of pcDNA3 (Invitrogen) (pcDNA-hpod). CHO and Lec2 cells were transfected with pcDNA-hpod by a procedure using LipofectAMINE reagent (Invitrogen). Stable transfectants were obtained using a selective culture in a medium containing 1 mg/ml Geneticin (G418; Sigma-Aldrich). A glioblastoma cell line LN319 was donated by Dr. Webster K Cavenee (Ludwig Institute for Cancer Research, San Diego, CA) and cultured as described (12). A cell line of murine colon
carcinoma, Colon-26 was obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Colon-26 cells were grown in RPMI 1640 medium containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum. Lymphatic endothelial cells were purchased from Sanko Junyaku Co., Ltd (Tokyo, Japan) and cultured using EGM-2-MV BulletKit (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instruction.

Expression and purification of soluble podoplanin
- The cDNA of human CLEC-2 (AF124841), mouse CLEC-2 (AF201457), human podoplanin (AB127958), mouse podoplanin (AJ297944) containing the extracellular domains of these proteins were obtained by PCR using their cDNA as templates. PCR was performed using Pfu turbo DNA polymerase (Stratagene, La Jolla, CA). The following oligonucleotides were used as primers (the EcoRV in the forward primer and the BglII site in the reverse primer were underlined):
  - human CLEC-2 (forward: CCGATTACAGCGCAATTACCT, reverse: GAAGATCTAGGTAGTTGGTCCAC),
  - mouse CLEC-2 (forward: CCGATTACAGCGCAATTACCT, reverse: GAAGATCTAGGTAGTTGGTCCAC),
  - human podoplanin (forward: GGCAAGATCTGGACAGCGCAATCTTCA, reverse: GGCAAGATCTGGACAGCGCAATCTTCA),
  - mouse podoplanin (forward: GTGATATCTGGACAGCGCAATCTTCA, reverse: GTGATATCTGGACAGCGCAATCTTCA).

The PCR products were purified by using a QIAquick gel Extraction Kit (Qiagen K.K., Tokyo, Japan), digested with EcoRV and BglIII, purified again, and ligated to the pFUSE-hFc2 (IL2ss) vector or the pFUSE-rFc2 (IL2ss) vector (hCLEC-2-hFc2, hPod-hFc2, respectively). Those of mouse CLEC-2 and mouse podoplanin were ligated to the pFUSE-rFc2 (IL2ss) vector (mCLEC-2-rFc2, mPod-rFc2, respectively). The ligation mixture was transformed into Escherichia coli DH5α. The obtained construct was verified by restriction enzyme digestion and DNA sequencing. COS-7 cells were transfected with hCLEC-2-hFc, hPod-hFc2, mCLEC-2-rFc, mPod-rFc2 using the electroporation method (23). In following day of the electroporation, the culture medium was replaced by reduced-serum medium, Opti-MEM (Invitrogen). For the purification of the fusion proteins, the medium was centrifuged, and the obtained supernatant was applied to a column of protein A-Sepharose (Amersham Biosciences). After extensive washing with phosphate-buffered saline (PBS), the fusion proteins were eluted by 0.2 M glycine pH 2.0, followed by neutralization using 1M Tris pH 10.0. The proteins were dialyzed against PBS.

Platelet aggregation - Human or murine washed platelets at indicated concentrations were stimulated by 1.5 × 10⁷/ml of indicated cell lines, 20 μg/ml of mPod-rFc2, 10 nM rhodocytin, 2 μg/ml of Horm collagen, 0.5 U/ml of thrombin, or 5 μM U46619. Platelet aggregation was monitored by measuring light transmission with the use of an AG-10 aggregation analyzer (Kowa, Tokyo, Japan) for 15 min under constant stirring at 1000 rpm at 37°C. The instrument was calibrated with buffer for 100% transmission. Where indicated, DMSO, 10 μM PP3, or 10 μM PP2 was incubated with washed platelets for 5 min at 37°C before stimulation. In some experiments, 2.5 × 10⁷/ml of podoplanin expressed cell lines were incubated with 2.5
mg/ml of the indicated recombinant proteins for 10 min at room temperature before addition to washed platelets. The final concentrations of podoplanin expressed cell lines and that of recombinant proteins in platelet suspension were $1.5 \times 10^9$/ml and 10 μg/ml, respectively.

*Western blot analysis* - Western blot was performed as described previously (22). Briefly, human washed platelets ($1 \times 10^9$/ml) were pretreated with 1 mM GRGDS peptide to inhibit platelet aggregation. Then, they were stimulated with $1.5 \times 10^9$/ml of CHO cells transiently-transfected with mouse podoplanin for the indicated duration of times. Reactions were terminated by addition of 2 × ice-cold lysis buffer. Platelet lysates were precleared and detergent-insoluble debris was clarified as described. The supernatant was dissolved with SDS sample buffer, separated by 10% SDS-PAGE, electrotransferred, and Western blotted by anti-phospho tyrosine antibody (4G10). Murine washed platelets ($2.5 \times 10^8$/ml) pretreated with 1 mM GRGDS were stimulated by 20 μg/ml of mPod-rFc2. Reactions were terminated by addition of 4 ×SDS sample buffer, separated by 10% SDS-PAGE, electrotransferred, and Western blotted by anti-phospho tyrosine antibody (4G10).

*Flow Cytometry Studies* - Cells suspended in PBS ($5 \times 10^6$/ml, 100 μl) were incubated with 5 μg/ml of the first antibodies or 100 μg/ml of the recombinant proteins. Where indicated, cells were incubated sequentially with 100 nM rhodocytin and 100 μg/ml of hPod-hFc2 for 20 min at room temperature. After washing with PBS, those cells were resuspended with 100 μl PBS and stained with 3 μl of secondary antibodies conjugated with fluorescein isothiocyanate (FITC) for 15 min. Stained cells were analyzed immediately using a FACS caliber (Becton Dickinson). Data were recorded and analyzed using CellQuest software.

**RESULTS**
exclude the possibility that Fc portion interacts a stimulatory Fc receptor, FcγRIIA, we used murine platelets, which lack FcγRIIA. mPod-rFc2 also stimulated aggregation (Fig.1E), suggesting that podoplanin, but not other cellular component, is responsible for platelet aggregation.

The long lag phase before the initiation of aggregate formation and Src kinase-dependent platelet activation are reminiscent of platelet activation induced by collagen through GPVI or by the snake venom rhodocytin (18), but not through G protein-coupled receptor (GPCR), platelet aggregation through which is not dependent on Src kinase. Therefore, we first investigated the possibility that podoplanin activates platelets by binding to GPVI, using murine platelets deficient in GPVI/FcγRII complex. Podoplanin or rhodocytin, but not collagen, induced platelet aggregation in mice deficient in GPVI/FcγRII complex (Fig.2A), suggesting that GPVI is not a target protein for podoplanin. We were then prompted to determine whether podoplanin activates platelets by the mechanism similar to rhodocytin. As a receptor on the platelet membrane for rhodocytin, we have recently identified a novel class of platelet activation receptor, C-type lectin-like receptor 2 (CLEC-2), which belongs to a non-classical C-type lectin (17). Since PLCγ2 is a crucial signalling molecule for rhodocytin-induced platelet aggregation through CLEC-2 (17), we first checked whether PLCγ2 is required for podoplanin-induced platelet aggregation. Podoplanin as well as rhodocytin induced platelet aggregation in wild type mice, but not in PLCγ2-deficient mice (Fig.2B). On the other hand, thrombin invariably induced platelet aggregation in both mice (Fig.2B). Since all the data hitherto have suggested that podoplanin-induced platelet aggregation has a profile quite similar to that of rhodocytin, we came to a hypothesis that CLEC-2 is a physiological counterpart for podoplanin.

Podoplanin stimulates platelet aggregation by interacting with CLEC-2. We next investigated the binding of recombinant CLEC-2 to podoplanin-expressing CHO cells. We utilized recombinant extracellular domain of human CLEC-2 expressed as a dimeric human immunoglobulin Fc domain fusion protein (hCLEC-2-hFc2). A fusion protein of human GPVI and human IgG Fc (hGPVI-hFc2) is used as a negative control. CHO cells were transiently-transfected with pcDNA alone or pcDNA encoding mouse podoplanin. Flow cytometric analysis using a specific anti-podoplanin antibody confirmed podoplanin expression on the CHO cells (Fig.3Ai). Mock-(MOCK CHO) or mouse podoplanin-transfected CHO cells (mPod CHO) were incubated with hCLEC-2-hFc2 or hGPVI-hFc2, and then specific binding was detected with FITC-labeled anti-human IgG hCLEC-2-hFc2 (Fig.3Aii), but not hGPVI-hFc2 (Fig.3Aiii), bound to podoplanin-expressing CHO cells, suggesting that CLEC-2 associates with podoplanin. Inversely, specific binding of recombinant podoplanin to CLEC-2-expressing cells was examined. To obtain CLEC-2 expressing cells, we used 293T-REx cells, in which CLEC-2 was expressed under the control of a tet repressor protein. The addition of doxycycline to transfected 293T-REx cells induces surface expression of CLEC-2, as assessed with a specific antibody to the lectin receptor (Fig.3Bi). As shown in Fig.3Bii and 3Biii, mPod-rFc2, but not its negative control mCLEC-2-rFc2 (a fusion protein of mouse CLEC-2 and rabbit IgG Fc), bound to CLEC-2-expressing 293T-REx cell line, further confirming that podoplanin is a physiological ligand of CLEC-2. We also confirmed that extracellular domain of human podoplanin expressed as a dimeric human immunoglobulin Fc domain fusion protein (hPod-hFc2), but not its negative control hGPVI-hFc2, bound to human CLEC-2-expressing 293T-REx cell line (data not shown). Moreover, pretreatment of human podoplanin-expressing CHO cells with hCLEC-2-hFc2, but not with hGPVI-hFc2, completely inhibited podoplanin-induced platelet
aggregation (Fig. 3C). Importantly, hCLEC-2-hFc2 did not inhibit platelet aggregation stimulated by other agonists than podoplanin or rhodocytin (Fig. 3D). These findings confirmed that podoplanin stimulates platelet aggregation by interacting with CLEC-2.

Sialic acid on O-glycans in podoplanin is necessary for its binding to CLEC-2. CLEC-2 belongs to non-classical C-type lectin-like proteins, which contain a C-type lectin-like domain (CTLD) homologous to a carbohydrate recognition domain, but lack the consensus sequence for binding sugars and calcium (25).

To assess the attribution of glycosylation to podoplanin binding to CLEC-2, recombinant podoplanin was stably expressed on wild type CHO cells (CHO) or CMP-sialic acid transporter-deficient Lec2 CHO cells (Lec2), which lack 90% of common sialic acid decoration in both glycoproteins and glycolipids (4, 5). As we reported previously (4), podoplanin expressed on Lec2 cells (hPod Lec2) failed to induce platelet aggregation, although equal expression of the recombinant molecules in both hPod CHO and hPod Lec2 was confirmed by flow cytometry using a specific antibody (Fig. 4A, B). Consistent with these findings, recombinant hCLEC-2-hFc2 was associated with podoplanin expressed on hPod CHO, but not that expressed on hPod Lec2 (Fig. 4C). These observations lead us to conclude that the presence of sialic acid on O-glycans of podoplanin is critical for interaction between CLEC-2 and podoplanin. Pretreatment of CLEC-2 expressing 293T-REx cells with rhodocytin greatly reduced podoplanin binding to the CLEC-2 expressing cells (Fig. 4D), suggesting that the binding sites of both proteins locate very close to each other. Further identification of CLEC-2 binding site is now under investigation.

Endogenous podoplanin expressed on the surface of tumor cells or lymphatic endothelial cells stimulates platelet aggregation. Since platelet aggregation shown in Fig. 3 and 4 was induced by CHO cells that were forced to express podoplanin by transfection, we next investigated whether tumor cell lines, which express endogenous podoplanin, stimulate platelet aggregation by interacting with CLEC-2. A human glioblastoma cell line, LN319 and a murine colon carcinoma cell line, Colon-26 highly express podoplanin and induce platelet aggregation (12, 26). We confirmed our previous findings; surface expression of podoplanin in LN319 and Colon-26 and platelet aggregation stimulated by the cell lines (Fig. 5A, B). Pretreatment of LN319 or Colon-26 with hCLEC-2-hFc2, but not with hGPVI-hFc2, completely inhibited platelet aggregation induced by the cell lines (Fig. 5A, B). These findings suggest that podoplanin that is endogenously expressed in tumor cells also induces platelet activation through interaction with CLEC-2.

Podoplanin is highly expressed in lymphatic endothelial cells, but not in vascular endothelial cells, and used as a specific marker of lymphatic endothelium (7, 14). We finally investigated whether lymphatic endothelial cells (LECs), which endogenously express podoplanin, stimulate platelets through interacting with CLEC-2. We confirmed our previous report that LECs expressed podoplanin and stimulated platelet aggregation (4) (Fig. 5C). The LEC-induced platelet aggregation was completely inhibited by hCLEC-2-hFc2, but not by hGPVI-hFc2 (Fig. 5C), suggesting that podoplanin expressed on LECs is also able to stimulate platelet aggregation by interacting with CLEC-2, although physiological relevance of the interaction between platelet CLEC-2 and podoplanin expressed on LECs remains to be elucidated.

DISCUSSION

The role of podoplanin has been implicated in tumor metastasis/progression (11) and lymphatic vessel formation (15). Although the identification of the podoplanin receptor should provide a clue to understanding of podoplanin-related cellular responses, search for its physiologic counter has not been successful.
It is well known that platelets are activated by the immunoglobulin superfamily and GPCR. CLEC-2 belongs to a non-classical C-type lectin, which is completely a new class of platelet-activating receptor. CLEC-2 activates platelets through the novel signaling pathway, in which single YxxL motif in its cytoplasmic tail plays a crucial role for platelet activation (17, 27). Moreover, the powerful stimulatory action of CLEC-2 on platelets suggests that it plays an important role in vivo. However, its physiological ligand has not been identified to date.

In this study, we identified the physiological counterparts for the above-mentioned podoplanin and CLEC-2, which turn out to be a receptor and a ligand for each other, and paved the way for new research fields on the role(s) of the CLEC-2-podoplanin interaction. Specific binding of recombinant extracellular domain of CLEC-2 to podoplanin-expressing cells was confirmed by flow cytometry (Fig.3Aii). Inversely, recombinant extracellular domain of podoplanin specifically associated with CLEC-2-expressing cell line (Fig.3Av). Moreover, pretreatment of podoplanin-expressing CHO cells with recombinant CLEC-2 completely inhibited podoplanin-induced platelet aggregation (Fig.3B), suggesting that interaction between CLEC-2 and podoplanin is responsible for podoplanin-induced platelet aggregation.

We next sought to elucidate the mode of podoplanin-CLEC-2 interaction and the mechanism of podoplanin-induced platelet aggregation through CLEC-2. We found that podoplanin expressed in CMP-sialic acid transporter-deficient Lec2 CHO cells (Lec2), which lack 90% of common sialic acid decoration in both glycoproteins and glycolipids, failed to associate with CLEC-2 and was unable to induce platelet aggregation (Fig.4). These results suggest that sialic acid on O-glycans of podoplanin is essential for binding to CLEC-2. A previous report on the structure and mutational binding analysis of CLEC-2 also indicates that an endogenous ligand is likely to be a protein with a predominantly negatively-charged binding surface (28). Our data appear to be consistent with this report, since sialic acid is negatively charged. Binding of podoplanin to CLEC-2 expressing cells was inhibited by rhodocytin (Fig.4D), suggesting that both proteins may target same binding site in CLEC-2. In contrast to podoplanin, glycosylation does not seem to be important for rhodocytin binding to CLEC-2 since rhodocytin has no potential O- or N-glycosylation site deduced from its amino acid sequence (Accession no. AAF79953 and AAF79952). Attempts are now being made to further investigate the precise mechanism of interaction between podoplanin and CLEC-2.

A recent study on the crystal structure and mutational binding analysis of CLEC-2 revealed that ligand binding to CLEC-2 is unlikely to transmit signals by inducing a major conformational change of CLEC-2 upon ligand binding (28). Alternatively, podoplanin binding to CLEC-2 may bring the cytoplasmic signaling domains of several CLEC-2 molecules into closer proximity. Recombinant podoplanin expressed as a dimeric IgG Fc fusion protein (mPod-rFc2) induced more powerful platelet aggregation and protein tyrosine phosphorylation than podoplanin expressed on the surface of CHO cells (Fig.1). Conceivably, a small dimeric recombinant protein, mPod-rFc2 more effectively clusters CLEC-2 than podoplanin expressed on the surfaces of CHO cells, which may not be located close enough to form dimers. Alternatively, the difference may be due to podoplanin concentration. Since the difference in the amount of podoplanin molecules and/or sialic acid on them may contribute to this phenomenon, further studies are required to get conclusions on this issue.

The findings in this study clearly demonstrate that podoplanin on the surface of tumor cells induces platelet aggregation by interacting with CLEC-2. A study on the metastatic abilities of several clones from a mouse
colon adenocarcinoma 26 cell line revealed that a highly metastatic clone expressed more podoplanin and induces platelet aggregation to a greater extent (26). Moreover, anti-podoplanin antibody that inhibits podoplanin-induced platelet aggregation suppressed lung colonization of colon adenocarcinoma (24). These findings, taken together, suggest that podoplanin-induced platelet activation through CLEC-2 is one of the mechanisms of tumor metastasis. The role of podoplanin-CLEC-2 interaction may not be confined to tumor metastasis. Activated platelets release a number of angiogenic factors that are stored in platelets, including vascular endothelial growth factor, platelet-derived growth factor, and sphingosine-1-phosphate (29). Angiogenic factors released from tumor-activated platelets along with adhesive molecules on the platelet surfaces may contribute to the process of tumor angiogenesis, thereby facilitating tumor growth or metastasis (30). Thus, inhibition of the interaction between CLEC-2 and podoplanin may be a good therapeutic target to prevent tumor growth and metastasis.

Whether the interaction between podoplanin and platelet CLEC-2 plays any role in physiological hemostasis remains to be elucidated, since podoplanin is not expressed in endothelial cells in blood vessels. However, it may be possible that podoplanin is expressed in atherosclerotic lesions and upon plaque rupture, it may contribute to pathological thrombus formation by activating platelets through CLEC-2 binding. If this is the case, blocking the interaction between CLEC-2 and podoplanin is an ideal therapeutic target, as this would inhibit only pathological thrombus formation without affecting physiological hemostasis. This hypothesis is now under investigation.

We found that podoplanin on the surface of lymphatic endothelial cells also induced platelet aggregation. Physiological significance of this finding remains to be elucidated, since lymphatic vessels under the physiologic conditions are not filled with blood. However, it may be of great importance during organ development or under pathologic conditions. Podoplanin-deficient mice have defects in lymphatic vessel pattern formation (15). Intracellular signaling molecules, Syk and SLP-76, regulate blood and lymphatic vascular separation (31), although they are not detected in endothelium, which suggest that Syk and SLP work by way of blood cells. Syk and SLP-76 in platelets are requisites for podoplanin-induced platelet activation mediated by CLEC-2 (17). Thus, although we have no direct evidence up to date, it is tempting to speculate that podoplanin-induced platelet activation through CLEC-2 regulates proper formation of lymphatic vessels.

In conclusion, we propose that a novel platelet activation receptor, CLEC-2, is the physiological counterpart for podoplanin, and their interaction induces platelet aggregation. The sialic acid residue on O-glycans of podoplanin appears to be important for its recognition by CLEC-2. The interaction between CLEC-2 and podoplanin may regulate tumor growth/metastasis, and furthermore, it may be related to the formation of lymphatic vessels.

REFERENCES


FOOTNOTES

*We are very grateful to Drs. Masaki Hikida, Tomohiro Kurosaki, Tadashi Yokosuka, Takashi Saito, Takashi Morita, Yoshiki Miura, Masaaki Moroi, Webster K Cavenee, Stefan Pöhlmann, Cell Resource Center for Biomedical Research, and Tohoku University for their generous donation of animals, cell lines, and proteins. Gratitude is expressed to Ms. Chiaki Komatsu and Ms. Haruka Nakagomi for their excellent technical assistance. We thank Dr. Yasuo Yamazaki for his helpful suggestion for the study. This study is supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan (grant no. 18591052) (K. S.-I.), from Mitsubishi Pharma Research Foundation (K. S.-I.), from the Japan Society for the Promotion of Science for Young Scientists, Japan (Y. K.), from Kanae Foundation for Life and Socio-medical Science (Y. K.), and from the Osaka Cancer Research Foundation (Y. K.).

1The abbreviations used are: GPVI, glycoprotein VI; Syk, spleen tyrosine kinase; SLP-76, SH2 domain containing leukocyte protein of 76kDa; PLCγ2, phospholipase Cγ2; CLEC-2, C-type lectin-like receptor 2.

FIGURE LEGENDS

Fig. 1. Src kinase dependent platelet aggregation induced by Podoplanin. A, i) CHO cells transiently-transfected with MOCK (MOCK CHO) or mouse podoplanin (mPod CHO) and their surface expression was confirmed by flow cytometer. Human washed platelets (1 × 10⁹/ml) were stimulated by 1.5 × 10⁶/ml of MOCK CHO or mPod CHO and platelet aggregation was monitored, using an aggregometer for 15 min. B, CHO cells stably-transfected with human podoplanin (hPod CHO) were incubated with 50 μg/ml of control rat IgG (anti-mouse podoplanin; 8F11) or human podoplanin antibody (NZ-1) for 15 min at room temperature. Then they were added to human washed platelets (1 × 10⁹/ml) (final concentration of hPod CHO: 1.5 × 10⁶/ml). Platelet aggregation was monitored, using an AG-10 aggregometer for 15 min. C, Human washed platelets (1 × 10⁹/ml) were preincubated with DMSO, 10 μM PP3, or 10 μM PP2 for 5 min at 37°C. They were stimulated by 1.5 × 10⁶/ml of mPod CHO and platelet aggregation was monitored, using an aggregometer for 15 min. D, Human washed platelets (1 × 10⁹/ml) were pretreated with 1 mM GRGDS peptide to inhibit platelet aggregation. Then, they were stimulated with 1.5 × 10⁹/ml of mPod CHO for the indicated duration of times. Reactions were terminated by addition of 2× ice-cold lysis buffer. Platelet lysates was dissolved with SDS sample buffer, separated by 10% SDS-PAGE, and Western blotted by anti-phospho tyrosine antibody (4G10). E, Murine washed platelets (2.5 × 10⁹/ml) were stimulated by 20 μg/ml of recombinant extracellular domain of mouse podoplanin expressed as a rabbit IgG Fc domain fusion protein. Platelet aggregation was monitored using an AG-10 aggregation analyzer for 15 min. The data are representative of at least two experiments.

Fig. 2. PLCγ2-dependent, GPVI-independent platelet aggregation induced by Podoplanin. A, Washed murine platelets (2.5 × 10⁹/ml) from wild type (WT) or GPVI/FcRγ-deficient (GPVI/FcRγ KO) mice were
stimulated by $1.5 \times 10^6$/ml of mPod CHO (pod), 10 nM rhodocytin (rhod), 2 μg/ml of collagen (col), and platelet aggregation was monitored, using an aggregometer for 15 min.  B, Washed murine platelets (2.5 $\times 10^8$/ml) from wild type (WT) or PLCγ2-deficient (PLCγ2 KO) mice were stimulated by $1.5 \times 10^6$/ml of mPod CHO (pod), 10 nM rhodocytin (rhod), 0.5 U/ml of thrombin (thr) and platelet aggregation was monitored, using an aggregometer for 15 min.  The data are representative of at least two experiments.

Fig. 3.  Platelet aggregation stimulated by association between CLEC-2 and podoplanin.  A, CHO cells transiently-transfected with MOCK (MOCK CHO) or mouse podoplanin (mPod CHO) were incubated with anti-mouse podoplanin antibody (i), hCLEC-2-hFc2 (ii), or hGPVI-hFc2 (iii) for 20 min at room temperature.  After unbound antibodies or proteins were removed by centrifugation, cells were stained with FITC-conjugated anti-hamster IgG (i) or anti-human IgG (ii, iii) for 15 min and analyzed by a FACScalibur.  B, 293T-REx cells, which express CLEC-2 under a tet repressor, were incubated with vehicle (CLEC-2 (-) 293T-REx) or 1 μg/ml of doxycycline for 48 hours (CLEC-2 (+) 293T-REx).  Cells were incubated with anti-human CLEC-2 antibody (i), mPod-rFc2 (ii), or mCLEC-2-rFc2 (iii) for 20 min at room temperature.  After unbound antibodies or proteins were removed by centrifugation, cells were stained with FITC-conjugated anti-goat IgG (i) or anti-rabbit IgG antibody (ii, iii) for 15 min and analyzed by a FACScalibur.  C, mPod CHO were incubated with PBS, hCLEC-2-hFc2, or hGPVI-hFc2 for 10 min at room temperature.  The cell-recombinant protein mixture was added to human washed platelets (1 $\times 10^9$/ml) (final concentration of mPod CHO: $1.5 \times 10^6$/ml).  Platelet aggregation was monitored, using an AG-10 aggregometer for 15 min.  D, A thromboxane A2 mimetic, U46619 (1 mM, 1.5 μl) was incubated with PBS (9 μl) or hCLEC-2-hFc2 (0.5 mg/ml, 9 μl) for 10 min at room temperature.  The agonist-recombinant protein mixture was added to human washed platelets (1 $\times 10^9$/ml, 300 μl) (final concentration of U46619: 5 μM).  Platelet aggregation was monitored using an AG-10 aggregometer for 15 min.  The data are representative of at least two experiments.

Fig. 4.  Sialic acid-dependent binding of podoplanin to CLEC-2.  A, CHO cells (hPod CHO) or a CHO cell mutant, Lec2 (hPod Lec2), which are stably-transfected with human podoplanin were incubated with anti-human podoplanin antibody or control rat IgG for 20 min at room temperature.  After unbound antibodies were removed by centrifugation, cells were stained with FITC-conjugated anti-rat IgG antibody and analyzed by a FACScalibur.  B, Human washed platelets (1 $\times 10^9$/ml) were stimulated with 1.5 $\times 10^6$/ml of hPod CHO or hPod Lec2 and aggregation was monitored by an AG-10 aggregometer for 15 min.  C, hPod CHO or hPod Lec2 was incubated with PBS or hCLEC-2-hFc2 for 20 min at room temperature.  After unbound proteins were removed by centrifugation, these cells were incubated with hPod-hFc2 for 20 min at room temperature.  A thromboxane A2 mimetic, U46619 (1 mM, 1.5 μl) was incubated with PBS (9 μl) or hCLEC-2-hFc2 (0.5 mg/ml, 9 μl) for 10 min at room temperature.  The agonist-recombinant protein mixture was added to human washed platelets (1 $\times 10^9$/ml, 300 μl) (final concentration of U46619: 5 μM).  Platelet aggregation was monitored using an AG-10 aggregometer for 15 min.  The data are representative of at least two experiments.

Fig. 5.  Platelet aggregation induced by podoplanin-expressing tumor cell lines or lymphatic endothelial cells through association between CLEC-2 and podoplanin.  A, i) Human glioblastoma cell line, LN319 was incubated with anti-human podoplanin antibody (line) or control rat IgG (fill) for 20 min at room temperature.  After unbound antibodies were removed by centrifugation, cells were stained with FITC-conjugated anti-rat antibody and analyzed by a FACScalibur.  ii) LN319 was incubated with PBS,
hCLEC-2-hFc2, hGPVI-hFc2 for 10 min at room temperature. The cell-recombinant protein mixture was added to human washed platelets (1 × 10^9/ml) (final cell concentration of LN319: 1.5 × 10^6/ml). Platelet aggregation was monitored, using an AG-10 aggregometer for 15 min. B, i) Colon-26 (a mouse colon carcinoma cell line) was incubated with anti-mouse podoplanin antibody (line) or control hamster IgG (fill) for 20 min at room temperature. After unbound antibodies were removed by centrifugation, cells were stained with FITC-conjugated anti-hamster IgG antibody and analyzed by a FACSscalibur. ii) Colon-26 were incubated with PBS, hCLEC-2-hFc2, hGPVI-hFc2 for 10 min at room temperature. The following procedure is the same as described in A, ii). C, i) Human lymphatic endothelial cells were incubated with anti-human podoplanin antibody (line) or control rat IgG (fill) for 20 min at room temperature. After unbound antibodies were removed by centrifugation, cells were stained with FITC-conjugated anti-rat antibody and analyzed by a FACSscalibur. ii) LECs were incubated with PBS, hCLEC-2-hFc2, hGPVI-hFc2 for 10 min at room temperature. The following procedure is the same as described in A, ii). The data are representative of at least two experiments.
Figure 1
Figure 2
(A) CHO cells

i) anti-mouse podoplanin

ii) hCLEC-2-hFc2 & anti-human IgG FITC

iii) hGPVI-hFc2 & anti-human IgG FITC

Fill: MOCK CHO
Line: mPod CHO

(B) 293T-REx cells

i) anti-human CLEC2

ii) mPod-rFc2 & anti-rabbit IgG FITC

iii) mCLEC-2-rFc2 & anti-rabbit IgG FITC

Fill: human CLEC-2 (-) 293T-REx
Line: human CLEC-2 (+) 293T-REx

(C) mPod CHO + PBS
mPod CHO + hCLEC-2-hFc2
mPod CHO + hGPVI-hFc2

(D) U46619 + PBS
U46619 + hCLEC-2-hFc2

Figure 3
Figure 4
Figure 5
Involvement of the snake toxin receptor CLEC-2 in podoplanin-mediated platelet activation by cancer cells
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J. Biol. Chem. published online July 6, 2007

Access the most updated version of this article at doi: 10.1074/jbc.M702327200

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