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Research Article

Podocalyxin enhances the adherence of cells to platelets

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Abstract. Podocalyxin (PODXL) is a mucin protein of the CD34 family expressed in kidney glomerular podocytes, vascular endothelium, progenitor bone marrow and tumor cells. It is assumed that PODXL plays an anti-adherent role in kidney podocytes. CHO cells stably expressing human PODXL (CHO-PODXL) or human tumor cells (Tera-1) inherently expressing PODXL showed increased adherence to platelets. The adherence of cells was inhibited (70%) by blockers of platelet P-selectin, prevented by the soluble ectodomain of human PODXL (PODXL-Δ) or by the arginine-glycine-aspartate (RGDS) peptide and partially impeded by inhibition of integrin αVβ3/αVβ5, suggesting a coordinated action of P-selectin and integrins. Colocalization of platelet P-selectin and PODXL expressed on CHO cells was demonstrated by confocal immunofluorescence. No adherence to platelets was observed when PODXL was expressed in glycomutant CHO cells deficient in sialic acid.

Keywords. Podocalyxin, platelets, adhesion, aggregation, Tera-1.

Introduction

Platelets play a central role in preventing blood loss upon disruption of vascular endothelial cells. The contact with extracellular matrix adhesion ligands, hormones, agonists and cytokines induces the activation of platelets, enabling the high-affinity binding of fibrinogen and formation of stable aggregates. The activation of platelets involves conformational changes in the fibrinogen receptor, integrin αIIbβ3, leading to high-affinity ligand binding, surface exposure of P-selectin and secretion of Ca^{2+}, ADP, thrombospondin, fibrinogen and protein C, among other factors. As a result, a plug of fibrin and aggregated platelets prevents the hemorrhage at the point of damage. The participation of platelets in processes other than hemostasis, such as inflammation, tumor metastasis, atherogenesis and reendothelialization, are currently under investigation. It is well known that hemostatic disorders are a common feature in the clinical evolution of tumors. Some tumor cells may induce platelet aggregation that correlates with their metastatic potential [1, 2]. Human carcinomas can also bind recombinant P and E-selectins [3–5]. Small cell lung tumors, which are negative for P-selectin-glycoprotein ligand-1 (PSGL-1), express a mucin-type glycoprotein, CD24, capable of binding P-selectin [6]. Thus, many tumors express mucin-like proteins capable of binding to other cells and forming complexes with leukocytes and platelets. Podocalyxin (PODXL), also known as Myb-Ets transformed progenitor (MEP), may be one of these

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membrane-adhesive proteins. PODXL is found in human testicular adenocarcinomas, leukemias and breast cancer, among other types of tumors [7–9], in which its cellular content seems to correlate with their degree of malignancy. The PODXL is a type I membrane protein with a mucin-like N-terminal domain, strongly sialylated and sulfated conferring a highly negative charge to the native sialoprotein. This protein belongs to the CD34 family, and its major location is on the kidney podocytes, epithelial intraglomerular cells lining the vascular surface of the glomeruli. The estimated mass of the PODXL nascent polypeptide is 59 kDa, but extensive posttranslational processing yields a mature native protein of ~165 kDa [10]. The strongly negative charge of PODXL was the reason why it was originally described as the glomerular epithelial polyanion due to its affinity for cationic stains [11]. The negative charge of PODXL was postulated to be the anti-adhesive force maintaining open the podocyte interdigitations, "urinary slits" that allow the passage of plasma filtrates into the glomerular cavity. PODXL is essential for renal function [12], since null $PODXL^{-/-}$ mice die of anuria a few hours after birth [13]. Based on the latter observations, PODXL was postulated to behave as an anti-adherent molecule. However, recent reports indicate that PODXL-like proteins present on high endothelial venules binds to L-selectin on lymphocytes [14]. Moreover, a parallelism seems to exist between the expression of PODXL and the metastatic ability of tumor cells. On these grounds, we found it interesting to investigate whether PODXL can enable the adhesion of cells to platelets *in vitro*. The results presented here indicate that this is indeed the case, since PODXL-expressing cells (either tumoral or transfected cells) showed binding to platelets mediated by P-selectin and integrins. The use of glycosylation mutants of CHO cells revealed that sialylation of membrane protein glycoconjugates is essential for the interaction of CHO-PODXL and platelets.

Materials and methods

Antibodies and reagents. Murine anti-human αIIbβ3 and anti-human PODXL were produced in our laboratory [15]. Anti-α5 integrin mAb (PB1) was from DSHB (Iowa City, IA), anti-human blocking P-selectin (CD62P) mAb came from R&D systems (Minneapolis, MN), anti-human P-selectin KO.2.3 mAb was a gift from Dr. Pizcueta (Barcelona, Spain), the anti-GPIbα AK2 was from RDI (Flanders, NJ) and secondary goat anti-mouse IgG came from Bio-Rad Laboratories (Hercules, CA). RO0655233–001–001 (RO65), a selective inhibitor of αvβ3 and αvβ5, was a gift of ROCHE (Basel, Switzerland). Thrombin, Arg-Gly-Asp-Ser (RGDS) and Arg-Gly-Glu-Ser (RGES) peptides were from Sigma, and ADP and collagen came from Chrono-Log Corp (Havertown, PA).

Preparation of platelet-rich plasma. Peripheral blood samples were collected in 3.8% sodium citrate (3.8% w/v) from informed consent patients or normal donors. Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood at 160 × g for 20 min; the upper two-thirds of the supernatant was separated for the experiments. To prevent platelet activation, we added acid-citrate-dextrose (ACD; 1:6) to the PRP. Finally, the PRP was centrifuged at 650 × g for 10 min and platelets resuspended in Tyrode's-Hepes with 1 mM $MgCl_2$.

Cells and culture conditions. Chinese Hamster Ovary (CHO) cells and Tera-1 were obtained from the ATCC repository and grown in DMEM (Gibco) medium supplemented with 10% fetal calf serum (FCS; ICN), 100 U/ml penicillin G and 100 μg/ml streptomycin (Gibco). Parental CHO cells (Pro-5) [16], Lec1 mutants (Pro-Lec1.3C) [17], Lec2 mutants (Pro-Lec2.6A) [18] and Lec8 mutants (Pro-Lec8.3D) [18], obtained from Dr. Stanley (Albert Einstein College of Medicine, NY), were cultured in MEM alpha medium (Gibco).

Stable transfection of CHO cells with the expression vector pEGFP-N1. To generate the fusion protein human PODXL-green fluorescence protein (PODXLGFP), full-length human PODXL-cDNA was subcloned into the BamHI site of the GFP vector pEGFP-N1 (Clontech Labs, Palo Alto, CA). To remove the termination codon of the PODXL gene and fuse it with the 3′ end of GFP (PODXLGFP), a PCR product was generated using the expression vector pcDNA3 (Invitrogen) containing the PODXL cDNA (pcDNA3-PODXL) and the primers 232U19 (5′-GCCCA-GAGGCGACGACACG-3′) and PODXL-AS 1627 (5′-CAGAGGATCCAAGAGGTGTGTGTC-3′) complementary to 1627–1650 of the PODXL-cDNA, followed by restriction of BamHI. The PCR product was cloned into a TA cloning vector, digested with BamHI and ligated to the vector previously digested with the same restriction enzyme. CHO cells were stably transfected using the calcium-phosphate procedure with the expression plasmid pEGFP-N1 containing the cDNA of PODXL or an equimolar amount of the void plasmid, and the positive clones were selected by G418 (400 μg/ml). The expression of PODXL was verified by immunoblotting, binding of cells to anti-PODXL-coated plates or flow cytometry.

Cell adhesion experiments. Cell adhesion experiments were carried out as previously described [19]. Briefly, 7.5×10^6 washed platelets were added to fibrinogen-coated plates (40 μg/ml), incubated for 2 h at 37°C and blocked with 200 ml 1% bovine serum albumin (BSA). CHO cells growing in Dulbecco modified Eagle medium (DMEM) containing 10% FCS were serum-starved for 24 h, harvested by EDTA treatment, collected by centrifugation and resuspended in serum-free DMEM with 0.1% BSA and 0.1% glucose. Then 100 μl cells (5×10^5 cells/ml) were plated onto monolayers of platelets, in duplicate, in the presence or absence of inhibitors and incubated for different periods of time at 37°C. Non-adherent cells were removed by careful washes with PBS, and adherent cells were examined with an Olympus phase-contrast IX-50 microscope. Micrographs were taken with an Olympus DP-70 digital camera. The counting of cells bound to platelets was performed on enlarged photographic images by different observers who had no information about the experiments. The statistical significance of the counting differences was evaluated using the Student's *t*-test.

Cell aggregation. The homotypic aggregation of CHO-PODXL was analyzed by seeding cells (5×10^5 cells/ml) in serum-free DMEM medium in 24-well plates blocked with 1% BSA, followed by incubation at 37°C for 90 min. The formation of aggregates was observed with a phase contrast microscope using a 10× objective. Platelet aggregation was performed by incubating 250 μl whole blood with 500 μl CHO cells (3×10^6 cells/ml) and 250 μl DMEM in an aggregometer cuvette at 1200 rpm, followed by addition of collagen to a final concentration of 2 μg/ml and monitoring the impedance in a two channel Chrono-Log aggregometer.

Confocal immunofluorescence. To study the colocalization of PODXL and P-selectin, glass coverslips in 24-well plates were coated with 40 μg/ml human fibrinogen (Sigma) for 2 h at 37°C, washed with PBS and blocked with 1% PBS-BSA for 1 h. Later on, platelets (2×10^8/ml) in Tyrodes buffer were added, incubated for 2 h at 37°C and activated with thrombin for 10 min. CHO cells expressing a fusion protein of PODXL and green fluorescence protein (CHO-$PODXL_{GFP}$) were then added (1×10^6/ml) and incubated for 45 min, fixed for 15 min in 3.7% PFA in PBS and permeabilized with 0.5% Triton X-100 in PBS-0.5% BSA for 30 min at room temperature. Finally, cells were incubated with an anti-P-selectin mAb (P-sel.KO.2.3), followed by addition of a secondary antibody labeled with Alexa546. Images were taken using a TCS-SP2-AOBS confocal microscope system (Leica, Heidelberg, Germany).

Western blotting. Cell lysates were prepared in Triton buffer (1% Triton X-100, 0.05% Tween 20, 150 mM NaCl, 50 mM Tris pH 7.5) and loaded onto 8% SDS-polyacrylamide gels under reducing conditions. Proteins were transferred to nitrocellulose membranes and visualized by incubation with an anti-PODXL mAb for 2 h at room temperature, followed by incubation with an anti-mouse IgG polyclonal antibody. Blots were visualized using an enhanced chemiluminescence detection system (ECL).

Production of human or mouse PODXL ectodomains. A DNA fragment comprising the entire putative ectodomain (341–1537 bp) of human PODXL (hPODXL-Δ; GeneBank NM-005397) was amplified by PCR using the primers: 5′-GTCGCCGAATTCGTCGCCCTCCCAGAAT-3′ (sense) and 5′-GATGAGAAGCTTGCTGAAGCGGTCCTCG-3′ (antisense) containing EcoRI (sense primer, underlined) and Hind III (antisense primer, underlined) restriction sites to allow inframe subcloning in the same sites of the bacterial expression plasmid pET-24b(+) (Novagen). When expressed in BL21 cells, this construction yields a recombinant PODXL-Δ fragment of 429 aa containing a 6× His-tag at the C-terminus. To generate the mouse ectodomain of PODXL (mPODXL-Δ), a DNA fragment (216–1385 bp; GeneBank AB028048) was amplified by PCR using the primers: 5′-CTGCTGCTGAATTCGTCGCCTGCA-3′ (sense) and 5′-ATGATGAGCTCGAGGCTGAAGCGG-3′ (antisense) containing EcoR I (underlined) and Xho I (underlined) restriction sites. Expression of either human or mouse PODXL (h/mPODXL-Δ) was induced by 1 mM IPTG. Expressed proteins were found only in the soluble fraction and were purified using a Ni++ column.

Flow cytometry. CHO cells were harvested using 0.5 mM EDTA in PBS and suspended at a concentration of 6×10^6 to 10×10^6 cells/ml. Platelets were labeled with anti-αIIb, washed with PBS buffer and exposed to FITC-F(ab′)2 fragment of rabbit anti-mouse IgG (Dako A/S, Denmark) at 4°C for 20 min. Portions of CHO-PODXL cells and platelet suspensions were then incubated for 8 min at room temperature and analyzed in a Coulter flow cytometer (model EPICS XL).

Results

Homotypic interaction in CHO cells expressing PODXL. Identification of cell surface PODXL was made by incubation of CHO-PODXL and/or Tera-1 (inherently expressing PODXL) cells in anti-PODXL-coated plates. Whether recombinant or native PODXL was expressed, both types of cells adhered markedly to anti-PODXL, whereas no adhesion was observed on plates coated with a mouse irrelevant IgG (Fig. 1a). Figure 1b shows a representative experiment in which quiescent CHO-PODXL cells in serum-free medium were plated on culture dishes blocked with BSA. Under these conditions, in agreement with a previous observation made with cells expressing rat PODXL [20], we found no homotypic interactions of CHO-PODXL cells, as indicated by the decreased number of cell aggregates formed (Fig. 1b). The absence of cell-cell interactions of cells expressing PODXL was suggested to result from repulsion forces produced by the dense negative charge of this sulfated sialoglycoprotein [20]. However, the following data indicate that PODXL acts as a pro-adhesive agent when cells are incubated in the presence of serum and platelets.

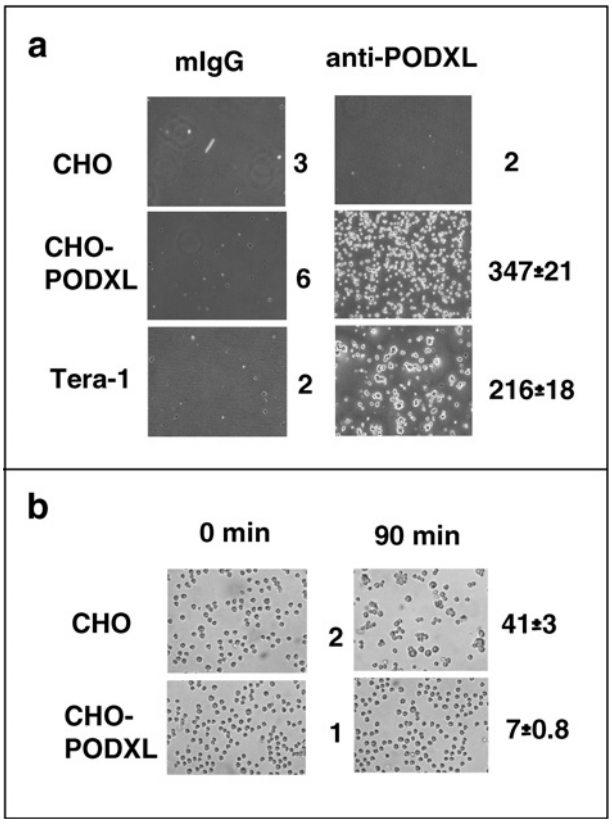

Figure 1. Lack of homotypic aggregation of CHO-PODXL cells. (*a*) CHO, CHO-PODXL and Tera-1 cells (2×10^5 cells/ml) were incubated on plates coated with either anti-PODXL (50 μg/ml) or irrelevant IgG and the number of cells bound counted. (*b*) CHO and CHO-PODXL cells (5×10^5 cells/ml) were seeded in serum-free DMEM medium for 90 min. The formation of aggregates (≥ 3 cells) was recorded with a phase contrast microscope at a magnification of 10×. The number of aggregates per field is indicated to the right of a representative field (showing the formation of aggregates); the results are mean values of three experiments ± SEM.

Binding of CHO-PODXL to platelets immobilized on fibrinogen-coated plates. PODXL-expressing cells showed enhanced adhesion to platelets immobilized on fibrinogen-coated plates (Fig. 2a, b). CHO-PODXL cells showed an increased rate of adherence to platelets, further enhanced by activation of platelets by agonists (Fig. 2c), suggesting additional surface exposure of PODXL-binding sites. The enhancing effect of agonists suggested that platelet P-selectin could be a binding site for PODXL. Under our experimental conditions, we found increased surface expression of P-selectin on activated platelets (results not shown). Blockade of platelet P-selectin inhibited the binding of CHO-PODXL to platelets by 70% (Fig. 3a), whereas no effect was observed using mAb against another surface protein of platelets such as GPIbα. Moreover, colocalization of PODXL and P-selectin at the contact points of CHO-PODXL$_{GFP}$ (fusion protein with GFP) and platelets (labeled with Alexa546) was demonstrated by confocal microscopy. The merging yellow color tracing indicates the sites of coincidence of both proteins along the contact line of cells (Fig. 3b).

Tera-1 cells, isolated from a human testicular carcinoma, express PODXL abundantly [7]. Tera-1 and CHO-PODXL both exhibited pronounced binding to platelets (Fig. 4a) that was abolished by the presence of the human soluble ectodomain of PODXL (hPODXL-Δ), whereas the mouse ectodomain mPODXL-Δ, whose sequence shows very low similarity with that of human [15], was ineffective (Fig. 4a). The role played by integrins in the PODXL-platelet interaction was investigated using several inhibitors. The interaction of CHO-PODXL and platelets was virtually abolished by the RGDS peptide, a competitive inhibitor of many integrins (Fig. 4b). Since the progression of several tumors depends on integrin α5β1 function [21], we also studied the effect of blocking α5β1 (mAb PB1) on adhesion of CHO-PODXL cells to platelets; the blockade of integrin α5β1 failed to prevent the PODXL-induced binding to platelets (Fig. 4c). Integrins αvβ3 and αvβ5 were inhibited by preincubation of PODXL-expressing cells with the specific inhibitor RO65, obtaining a decrease of 54% in their binding to platelets (Fig. 4d).

Coincubation of platelet suspensions with PODXL-expressing cells increased the agonist-induced aggre-

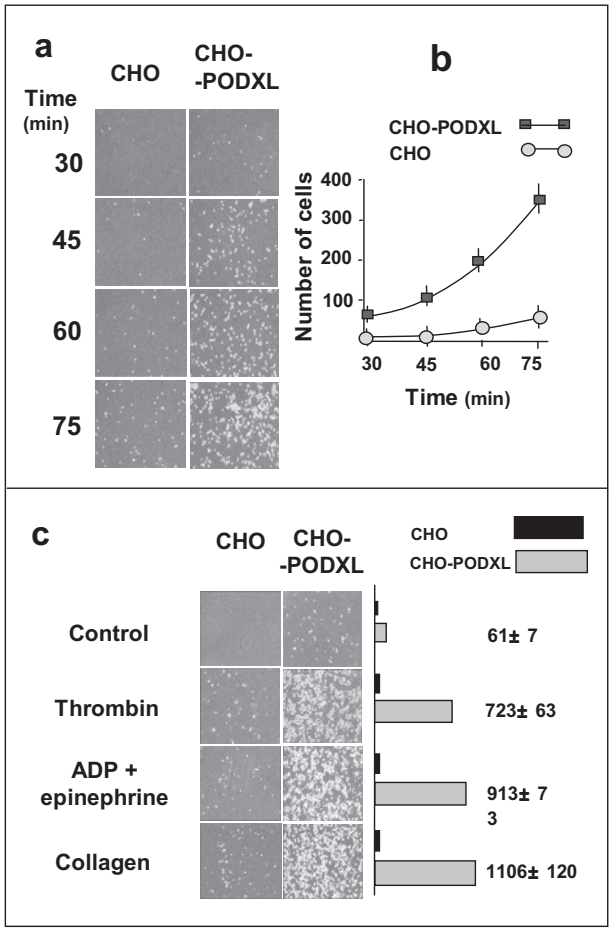

Figure 2. Time course of adhesion of CHO-PODXL cells to immobilized platelets. CHO and CHO-PODXL cells (5×10^5 cells/ml) were seeded onto a monolayer of fibrinogen-immobilized platelets and incubated for the indicated times; representative fields (*a*) and the number of cells bound to platelets (*b*) are shown. (*c*) The platelets were activated with thrombin (1 U/ml), ADP (10 μM) + epinephrine (20 μM) or collagen (10 μg/ml) for 10 min before adding CHO or CHO-PODXL cells; the number of adhered cells was counted (bars on the right) 30 min thereafter. The results are mean values ± SEM of three experiments performed in duplicate.

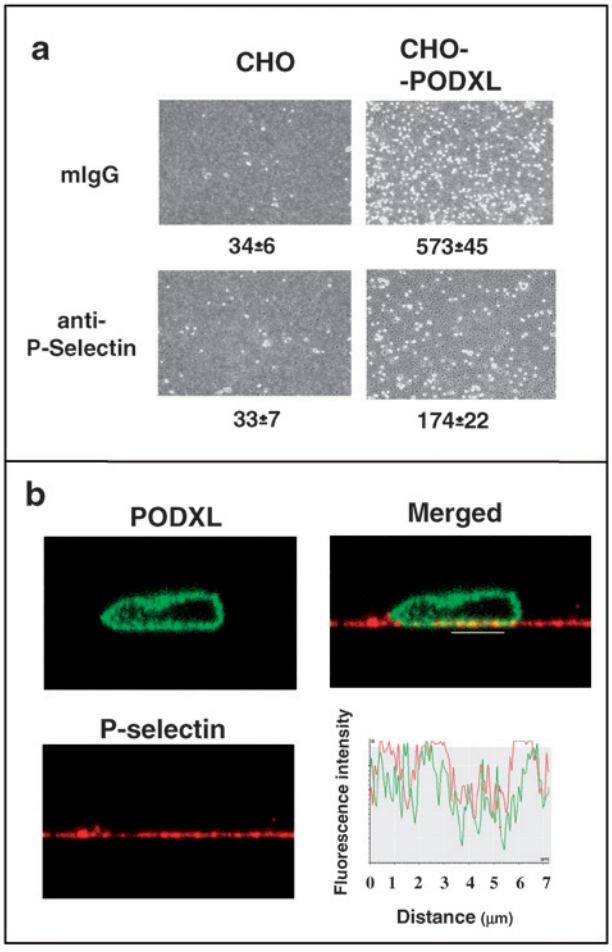

Figure 3. Platelet P-selectin interaction with CHO-PODXL cells. (*a*) Platelets were incubated for 45 min with anti-P-selectin mAbor irrelevant IgG (10 mg/ml) before addition of CHO or CHO-PODXL cells. The number of adherent cells is indicated below a representative field. Results indicate means±SEM of six different experiments. (*b*) Expression of $PODXL_{GFP}$ (green) by CHO cells and platelet P-$selectin_{Alexa546}$ (red); the figure shows a vertical section analyzed by confocal microscopy, and the yellowish points in the merged image indicate colocalization of the two molecules. The colocalization was further verified by densitometric analysis of the section underlined with a white line. The figure is representative from three experiments in which at least eight cells were analyzed.

gation of platelets by ~58% (Fig. 5a). This aggregating effect was prevented by addition of the PODXL ectodomain (PODXL-Δ) to the aggregation cuvette (Fig. 5a, b). To elucidate whether the enhancing action of PODXL on platelet aggregation was the result of binding of CHO-PODXL to platelets, we studied the interaction of CHO or $CHO\text{-}PODXL_{GFP}$ with platelets prelabeled with mouse anti-human αIIb and rabbit anti-mouse IgG-Alexa546, showing that CHO-PODXL but not the parental CHO cells formed aggregates with platelets (Fig. 6a). By flow cytometric analysis of cell suspensions, the ratio of gated CHO-PODXL cells to gated platelets was 50–150% higher than the ratio of CHO cells to platelets. An identical observation was made when the ratios were established between the CHO-PODXL cells and aggregated platelets, suggesting that a significant number of platelets were trapped in the pool of CHO-PODXL cells (Fig. 6b). We also tested whether Tera-1 cells behaved as the CHO-PODXL cells. The platelets were prelabeled with mouse anti-human αIIb mAb and a secondary rabbit anti-mouse FITC-labeled IgG. The redistribution of platelet labeling between platelets and Tera-1 gated windows once again indicated the binding of platelets to Tera-1 cells (Fig. 6b).

PODXL expression in glycosylation mutants of CHO cells. Approximately two-thirds of the PODXL mass

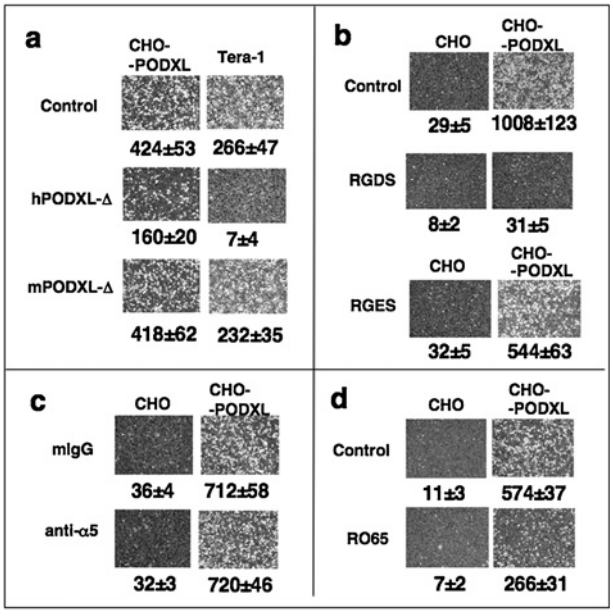

Figure 4. Effects of human or mouse PODXL ectodomains or integrin inhibitors on adhesion. (*a*) Either CHO-PODXL or Tera-1 cells ($5x10^5$ cells/ml) were seeded on platelet monolayers and incubated for 30 min in the presence or absence of the ectodomain of human or mouse PODXL (20 μg/100 ml). CHO or CHO-PODXL cells were preincubated with 1 mM RGDS or RGES peptides (*b*), treated with 50 μg/ml anti-α5 blocking mAb or irrelevant IgG (*c*) or treated with 10 mM of the αvβ3 inhibitor RO65 (*d*). Each experiment was performed at least four times in duplicate and eight different fields were analyzed. Numerical values represent means ±SEM of the number of adherent cells.

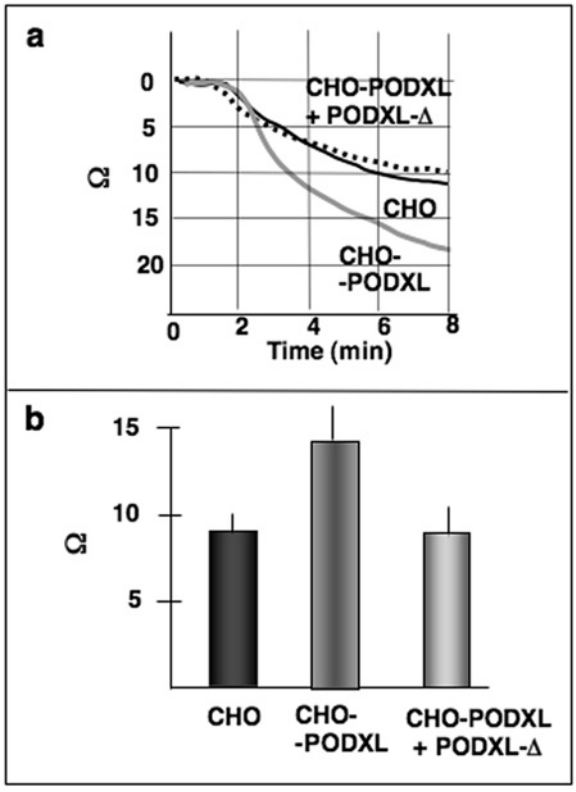

Figure 5. Aggregation of platelets in the presence of CHO-PODXL. (*a*) 250 μl citrated blood and CHO-PODXL cells (3×10^6/ml) were incubated with or without the ectodomain of human PODXL (30 μg/ml) and the platelet aggregation monitored amperometrically after collagen stimulation. CHO cells incubated without the ectodomain served as a control. (*b*) The results were quantified, and the vertical bars represent mean values ± SEM of three duplicate experiments.

can be accounted for by glycosylation, suggesting that quantitative or qualitative glycosylation changes could have a functional significance. The role of protein glycosylation in the pro-adhesive action of PODXL was investigated by stable expression of $PODXL_{GFP}$ in parental CHO cells (W5) or in the glycosylation mutants of CHO cells (CHO-Lec1, CHO-Lec2 or CHO-Lec8). The Lec1 cells lack N-acetylglucosaminyltransferase I activity (GlcNAc-TI), producing incomplete intermediates of N-linked carbohydrates; the Lec2 cells are CMP-sialic acid transporter-deficient; and the Lec8 cells showed a reduced capacity to transport UDP-galactose into the Golgi compartment. Both Lec 2 and Lec8 glycomutant cells produced a marked reduction in the sialylation of membrane glycoproteins. The western blot analysis of glycomutant CHO-PODXL cells showed changes in the apparent mobility of PODXL, most likely due to quantitative differences in glycosylation (Fig. 7a). The surface expression of PODXL was further investigated by incubating cells on plates coated with anti-human PODXL mAb. As shown in Fig. 7b, all glycomutant cells exhibited marked binding to plates – but not on plates coated with an irrelevant mouse IgG. Parental or glycomutant cells were seeded onto monolayers of human platelets. The parental, non-glycomutant cells expressing PODXL showed enhanced binding to platelets. Adhesion of Lec1-PODXL cells to platelets was not impeded, but adherence of Lec2-PODXL or Lec8-PODXL cells to platelets was virtually abolished (Fig. 7c).

Discussion

The herein reported experiments indicate that expression of PODXL enhances the attachment of cells to platelets. The lack of adherence of CHO cells not expressing PODXL suggests that PODXL itself or changes induced by its presence were responsible for the cell-adherent effect. Moreover, the ectodomain of PODXL (PODXL-Δ) prevented the adherent effect of PODXL-expressing cells, most likely by competing for binding sites on platelets. The present data agree with previous work indicating that mucin-like tumor proteins may induce platelet aggregation [1, 22, 23]. It is

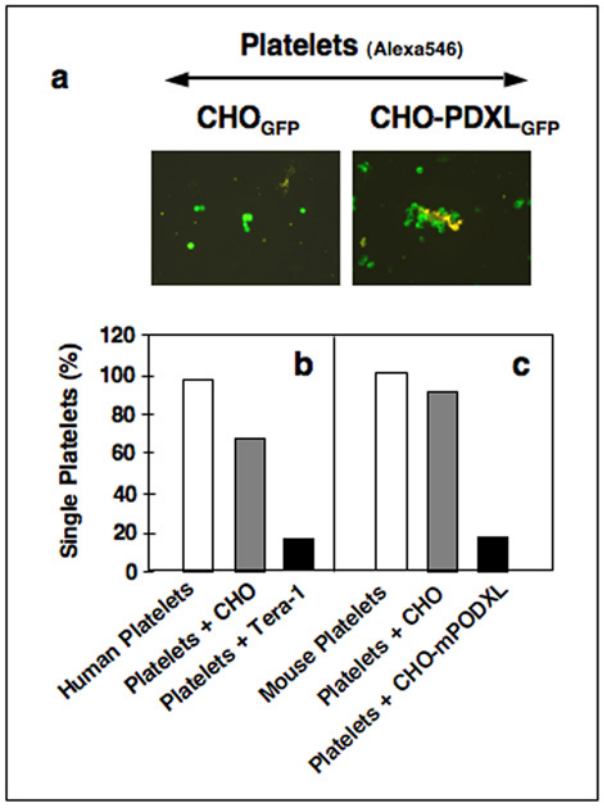

Figure 6. Morphological and cytometric analysis of binding of PODXL-expressing cells to platelets. (*a*) Either CHO cells expressing green fluorescence protein (CHO_{GFP}) or CHO cells expressing PODXL fused to green fluorescence protein ($CHO\text{-}PODXL_{GFP}$) and human platelets labeled with Alexa546 were incubated as described for the platelet aggregometry. Aggregates were only observed in the incubations of CHO-PODXL and platelets. For the sake of clarity (to avoid confusion with $CHO\text{-}PODXL_{GFP}$ cells) the color of platelets was digitally changed to yellow. At least 30 fields were analyzed and a representative figure is shown. (*b*) Flow cytometric analysis of cell suspensions. Tera-1 cells (inherently expressing PODXL) or CHO cells ($PODXL^-$) were incubated with platelets prelabeled with mouse anti-human αIIb mAb and a secondary rabbit anti-mouse FITC IgG. At least 2×10^4 cells were counted in each experiment. The bar indicates the percentage of unbound platelets. (*c*) Unlabeled mouse platelets were incubated with either $CHO\text{-}mPODXL_{GFP}$ or CHO_{GFP}. At least 10 000 cells per sample were analyzed by flow cytometry. The bar indicates the percentage of unbound platelets. The values shown are representative of at least four experiments in duplicate.

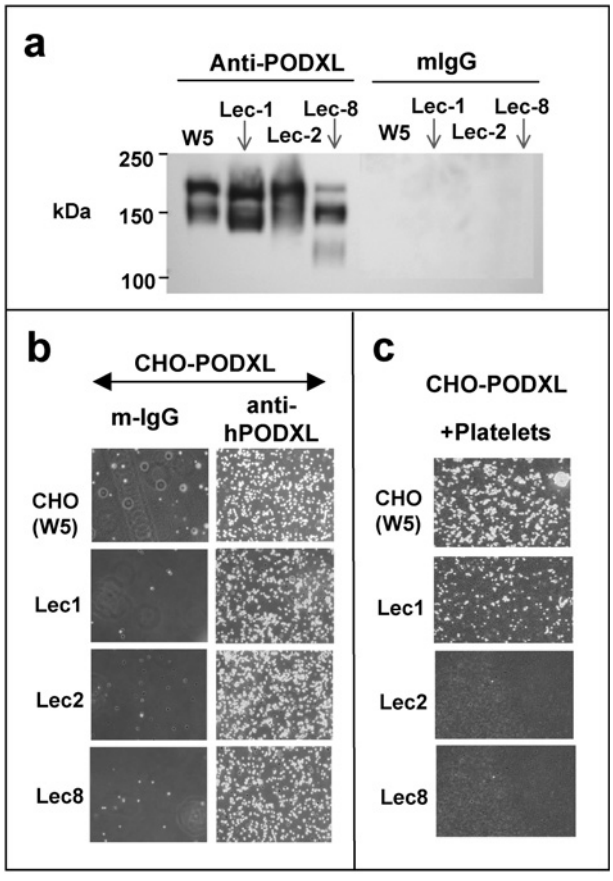

Figure 7. Immunoblotting and adhesion of glycomutant CHO cells to platelets. (*a*) Cell lysates were loaded into 8% SDS-polyacrylamide gels under reducing conditions. The proteins were blotted with anti-PODXL mAb or irrelevant IgG. (*b*) Adhesion of glycomutant CHO cells stably expressing PODXL to immobilized anti-PODXL. (*c*) Glycosylation mutant CHO-PODXL cells were seeded on platelet monolayers and the number of adherent cells counted. Experiments were performed at least four times in duplicate.

also consistent with the observation that anti-platelet agents inhibit the spreading of tumor metastasis [24]. The enhancing effect of platelet activation on the binding of CHO-PODXL cells strongly suggested further surface exposure [25] of a potential PODXL ligand such as P-selectin [14]. Although selectins seem to interact weakly with strongly sialylated moieties [26] such as PODXL, our experiments indicate an important role for platelet P-selectin in the recognition of PODXL expressed on CHO cells and most likely for the initiation of all the subsequent cellular events. Our data also agree with reports indicating that certain tumor cells may bind platelets via P-selectin [3, 4]. Nevertheless, the binding of CHO-PODXL to non-activated platelets and the effect of thrombin in enhancing the binding in the presence of anti-P-selectin suggest that P-selectin may not be the exclusive platelet ligand for PODXL.

Full activity of integrins is needed for PODXL binding to platelets. Integrins are essential for cell attachment, migration, proliferation and the formation of new blood vessels [27, 28]. Thus, the attachment of PODXL-expressing cells to platelets must take place in a coordinated fashion with integrins. The inhibition observed with RGDS peptide and RO65 supports this assertion. A lateral association of PODXL with integrins, as reported for other membrane proteins [29], may also be part of the regulatory mechanism of integrin function. The αvβ3 integrin

localizes in a Rac-dependent process of lamellipodia, where the cell adheres to the extracellular matrix [30]. The 54% reduction in the binding of PODXL-expressing cells to platelets by inhibitors of αvβ3 agrees with both the reported inhibition of binding of tumor cells to platelets by blockade of αv integrins [31] and the observation that αvβ3 integrins localize in the lamellipodia, where cell-matrix adhesions are formed, and their signaling may help to reinforce integrin-cytoskeleton linkages. Blocking α5 integrins did not alter the capacity of PODXL-expressing cells to bind platelets, indicating that this process operates through an α5 integrin-independent mechanism.

The ectodomain of PODXL (PODXL-Δ) failed to enhance the adherence of CHO cells to platelets, suggesting that signaling through transmembrane and cytosolic C-terminal domains of the protein are needed for full action of PODXL. The cytosolic C-terminus of PODXL binds NHERF-2 (Na^+/H^+ exchange regulatory factor 2) and SLC9A3R2 (Solute carrier family 9 isoform A3 regulatory factor 2) [32]. NHERF-2 is a scaffolding signaling protein containing two tandem PDZ (PSD-95, Dlg, ZO-1) domains and a carboxyl terminal ERM (ezrin, radixin, moesin) binding domain. In hematopoietic cells, the intracellular domain of PODXL interacts with NHERF-1 *via* the second PDZ domain of NHERF-1 and the last four residues at the C-terminus (DTHL) of PODXL [33]. It is reasonable to interpret the effect of PODXL-Δ in inhibiting the binding of CHO-PODXL to platelets as a competition for binding sites. A conflict seems to arise upon observing that O-glycosylation mutant CHO cells (Lec-2-PODXL and Lec8-PODXL) failed to bind platelets, whereas the recombinant non-glycosylated ectodomain of PODXL (PODXL-Δ) prevented the binding of CHO-PODXL cells to platelets. However, this conflict is only apparent, since all cell macromolecules – not only PODXL – will be affected by the glycosylation mutation. On the other hand, there is not a sound reason to believe that unglycosylated PODXL-Δ may not adopt a functional conformation capable of competing for ligands with the native protein. Moreover, a significant number of recombinant *E. coli*-expressed unglycosylated proteins have been found to preserve functional properties similar to those of the native protein [34–36]. In our case, the enhancing effect of human PODXL on the binding of CHO cells to platelets was prevented by the ectodomain of human PODXL-Δ, whereas the mouse ectodomain, which shows very low amino acid similarity, or an irrelevant peptide were ineffective.

Influence of membrane protein glycosylation on PODXL action. We also analyzed the functional features of PODXL expressed in glycosylation mutants of CHO cells. All the mutant cells showed a stably robust expression of PODXL. Expression of PODXL on N-acetyl-D-glucosamine (GlcNAc) transferase I-deficient (Lec1) cells did not prevent the association with platelets. Since the synthesis of N-glycan complexes is impeded in the CHO Lec1 mutant [17], this result indicates that cells carrying N-glycosylation mutations retained the capacity to interact with platelets. Lec1 cells incorporate ~10-fold less [^{3}H]-fucose into macromolecules than parental cells (Pro-5). Structural analysis of fucosylated oligosaccharides found an α(1,6) linkage to the core GlcNAc of relatively small oligomannose N-glycans in CHO-Lec1 cells [37, 38]. PSGL-1, a counter-receptor for P-selectin, must undergo two post-translational modifications in order for it to function: α(1,3)fucosylation and α(2,3)-sialylation [39, 40]. Despite the expected quantitative and qualitative changes in the fucosylation of PODXL in CHO-Lec1 cells, no significant changes were detected in the PODXL-mediated binding of CHO-Lec1-PODXL cells to platelets. Thus, the PODXL differs from other counter-receptors for P-selectin in that it may not need to be extensively fucosylated. A reason for the difference may be found in that bonds between P-selectin and PSGL-1 mediate the rolling phase of leucocytes on the endothelial surface, that is, cell-cell interactions under flow conditions.

Unlike Lec1 cells, CHO-Lec2-PODXL and CHO-Lec8-PODXL cells failed to bind to platelets. Since CHO-Lec2 or CHO-Lec8 mutations produce sialic acid-deficient glycoproteins [18, 41], it seems plausible to conclude that mutated O-glycan conjugates are required for the pro-adherent effects of PODXL. O-glycosylation mutant CHO cells expressing PODXL reacted normally with immobilized anti-PODXL mAbs, implying normal or near-normal intracellular trafficking and surface exposure of PODXL. Thus, as mentioned above, surface exposed unsialylated PODXL reacted normally with a specific mAb, suggesting that the lack of CHO-Lec2-PODXL or CHO-Lec8-PODXL adherence to platelets is most likely due to inadequate sialylation of membrane proteins other than PODXL involved in the process.

Physiopathological significance of the binding of PODXL to platelets. The interaction of tumor cells expressing PODXL and platelets may be important in metastasis. A vascular injury is immediately followed by adherence and activation of platelets to the exposed subendothelium. Once activated, the platelets secrete the chemokine SDF-1α, which supports recruitment of bone marrow progenitor cells [$CD34^+$ and c-$Kit^+Sca\text{-}1^+Lin^-$ (KSL)] to support vascular growth [42]. CD34 and PODXL show coincident

expression on hematopoietic precursor cells in adulthood. Moreover, PODXL from the high endothelial venules binds L-selectin [14] from leukocytes. Therefore, PODXL may also be involved in reendothelialization through binding of bone marrow precursor cells to platelets.

To conclude, PODXL-expressing cells exhibited an enhanced capacity to bind platelets. The PODXL action seems to be the result of a coordinated action between platelet P-selectin and integrins that requires normal sialylation of membrane proteins. The ability of PODXL-expressing cells to bind platelets may provide an explanation for the bleeding disorders accompanying many tumors, the formation of metastasis and, likely, the recruitment of bone marrow progenitor cells for vessel reendothelialization.

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