Published in final edited form as:

Thromb Haemost. 2012 November ; 108(5): 963–972. doi:10.1160/TH12-01-0047.

The Thr715Pro variant impairs terminal glycosylation of P-selectin

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Summary

P-selectin variant 715Pro is associated with lower concentrations of plasma P-selectin and reduced risk for thrombosis. We examined the influence of 715Pro on P-selectin synthesis, post-translational processing, surface expression and function using HEK293 cells, which do not express endogenous P-selectin. Mass spectrometry revealed that HEK293 cells produced recombinant P-selectin which has a glycosylation pattern comparable to platelet P-selectin. Compared to wild-type transfectants, 715Pro transfectants have ~50% less terminally glycosylated P-selectin and accumulate more immature P-selectin in Golgi. Following Brefeldin A treatment, the majority of 715Pro P-selectin is not modified by Golgi enzymes, while wild-type P-selectin undergoes complete modification. Flow cytometry revealed that 715Pro transfectants have ~20% less P-selectin on the cell surface compared to wild-type transfectants. Secretion of P-selectin by 715Pro transfectants was about 38% lower compared to wild-type transfectants. Binding of HL-60 cells to 715Pro transfectants was ~29% lower than to wild-type transfectants. This observation was confirmed by the presence of fewer platelet-monocyte aggregates (PMA) in the blood of healthy individuals and patients with angiographically proven atherosclerosis, carrying 715Pro P-selectin compared to individuals with wild-type P-selectin. We conclude that the 715Pro variant impairs terminal glycosylation of P-selectin in Golgi, leading to reduced amounts of mature P-selectin and subsequently less surface expression and secretion of P-selectin. The reduced surface expression of 715Pro P-selectin contributes to inefficient adhesion to HL-60 cells or monocytes.

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Conflicts of interest

None declared.

Keywords

P-selectin; P-selectin polymorphism; P-selectin glycosylation; soluble P-selectin; platelet-monocyte aggregate

Introduction

P-selectin is an adhesion receptor expressed and stored in the alpha-granules of platelets (1) and the Weibel-Palade bodies of endothelial cells (EC) (2). Following agonist activation, P-selectin is translocated to the surface of platelets (1) and EC (3). Once on the surface, P-selectin can interact with P-selectin glycoprotein ligand 1 (PSGL-1) expressed on leukocytes (4, 5), and initiate the synthesis of pro-coagulatory tissue factor (6) and the subsequent release of tissue factor positive microparticles from leukocytes (7), key events in thrombogenesis. P-selectin mediates leukocyte rolling and extravasations at sites of inflammation (8). P-selectin is also found in soluble form in plasma. Soluble P-selectin is able to bind to PSGL-1 and activate leukocytes (9, 10). Increased levels of soluble P-selectin have been associated with various diseases including coronary artery disease, hypertension, atrial fibrillation, disseminated intravascular coagulation and thrombotic thrombocytopenic purpura (11, 12).

The P-selectin gene was reported to be highly polymorphic (13). The polymorphisms Ser290Asn (rs6131, 1087G>A), Asp562Asn (rs6127, 1902G>A) and Thr715Pro (rs6136, 2361A>C) either individually or in haplotypes were found to be associated with vascular diseases and thrombotic disorders such as myocardial infarction (13–16), coronary artery disease (17, 18), deep venous thrombosis (19) and atherosclerosis (20). Despite the accumulating evidence of an association of P-selectin polymorphisms with thrombotic disorders, it is unclear how the polymorphisms impact on P-selectin synthesis, surface expression, secretion and function.

The P-selectin variant 715Pro is found in 15–20% healthy Caucasian individuals in heterozygous form and only occurs in association with 562Asn (19). 715Pro is located 15 amino acids in front of the transmembrane domain. Volcik et al. reported that individuals carrying 715Pro have less transmembrane P-selectin on platelets (21). Several studies showed an association of 715Pro with low concentrations of secreted/shed P-selectin in plasma (14, 19, 22, 23). The 715Pro variant was proposed to have a protective effect against myocardial infarction in France, Northern Ireland and UK (13, 15, 16). Controversial data was published on patients with coronary heart diseases in Arab/African people (24) and patients from the Leiden thrombophilia study (25). The mechanism leading to reduced surface expression and secretion/shedding of 715Pro P-selectin is still unknown.

Access to endothelial cells or megakaryocytes from individuals homozygous for 715Pro is practically impossible as these individuals are rare and biopsy material can only be obtained for medical reasons. Therefore, we chose to investigate the influence of 715Pro (2361C) on P-selectin synthesis, post-translational processing, surface expression, secretion and function in in vitro experiments, using cells that do not express endogenous P-selectin. We selected

HEK293 cells and proofed the suitability of this cell model by comparison of P-selectin synthesised in these cells with platelet P-selectin.

Materials and methods

Materials

Mouse monoclonal P-selectin antibodies, 1E3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and AC1.2 (BD Biosciences, Heidelberg, Germany), Alexa647 labelled P-selectin antibody AK6 (ABDserotec, Oxford, UK), sheep polyclonal P-selectin antibody (R&D Systems, Minneapolis, MN, USA), and mouse monoclonal GFP antibody B-2 (Santa Cruz Biotechnology) were used. All cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal GFP antibody and pEGFP-N1 plasmid were kind gifts of Dr. Werner Sieghart (Center for Brain Research, Medical University of Vienna, Austria). Sar1A (Secretion-associated and Ras-related) dominant negative (Sar1A DN) plasmid and Brefeldin A (BFA) were generously gifted by Dr. Michael Freissmuth (Institute of Pharmacology, Medical University of Vienna). HEK293 cells and HL-60 cells were kindly provided by Dr. Erika Jensen-Jarolim (Institute of Pathophysiology, Medical University of Vienna).

P-selectin plasmids

Human P-selectin cDNA obtained from platelet RNA (genotyped as AA – cDNA positions 1902A/2361A) was amplified by polymerase chain reaction using PfuTurbo DNA polymerase (Stratagene, La Jolla, CA, USA) and cloned into pEGFP-N1 vector. The variants GA coding for 562Asp and 715Thr P-selectin, GC coding for 562Asp and 715Pro P-selectin and AC coding for 562Asn and 715Pro P-selectin were produced by introducing mutations at nucleotides 1902 (A→G) and 2361 (A→C) in the AA (562Asn, 715Thr) plasmid using specific primers and QuikChange site-directed mutagenesis Kit (Stratagene) following the manufacturer's protocol. All plasmids were sequence verified. Primers (Eurofins MWG Operon, Ebersberg, Germany) used for cloning and mutagenesis are listed in Table 1.

Transfection of HEK293 cells

HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% fetal bovine serum and grown at 37°C. Freshly seeded HEK293 cells were transiently transfected with 1.25 µg of plasmid DNA expressing GFP tagged P-selectin (GA, AA, GC and AC) or EGFP using METAFECTENE PRO (Biontex, Martinsried/Planegg, Germany) following the manufacturer's protocol. 24 hours (h) post-transfection, cells from four independent transfections per genotype were used for quantification of cellular and surface P-selectin by FACS, and quantification of P-selectin mRNA by real-time RT-PCR.

Flow cytometry of transfected HEK293 cells

Transfected cells were harvested by scraping and were resuspended in phosphate-buffered saline (PBS) with 0.5% bovine serum albumin and incubated with Alexa647 labelled P-selectin antibody for 30 minutes (min) at room temperature. For each transfectant, cellular (GFP fluorescence) and surface P-selectin (Alexa647 fluorescence) was determined by Becton Dickinson FACS Calibur using CELLQuest software. To eliminate differences in the

number of transfected cells between samples only GFP positive cells were evaluated. 10^4 GFP-positive cells from each transfectant were gated and the median fluorescent intensity (MFI) of GFP and Alexa647 was determined for the gated cells.

P-selectin ELISA

The amount of P-selectin in the cell culture supernatants of transfected cells was determined by human soluble P-selectin/CD62P immunoassay (R&D Systems) following the manufacturer's protocol.

Real-time reverse-transcription polymerase chain reaction

Total RNA was isolated from the transfected cells using RNeasy spin columns (Qiagen, Hilden, Germany). Reverse transcription was performed with an equal amount (1 μ g) of total RNA from each sample using GeneAmp[®] RNA PCR (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. P-selectin and the reference gene GAPDH were quantified by qPCR using a BioRad CFX96 real-time PCR machine and Itaq SYBR Green Supermix with ROX (BioRad, Hercules, CA, USA) following the manufacturer's protocol. The intron-spanning primers used for the real time qPCR are listed in Table I.

Imaging of transfected HEK293 cells

P-selectin transfected cells were grown on glass cover slips for 24 h. Cells were stained with 100 ng/ml 4',6-diamidino-2-phenylindole (DAPI) and 1 μ g/ml CellMask[™] plasma membrane stain (Invitrogen). After washing, the cells were incubated in imaging medium (NaCl 144 mM, KCl 5.4 mM, MgCl₂ 1 mM, CaCl₂ 2 mM, HEPES 10 mM, pH=7.3). Labelled cells were imaged using 63X oil objective lens mounted on a SP5 confocal microscope (Leica). The images were captured and analysed using LAS AF software (Leica).

Immunoprecipitation and immunoblotting

For immunoprecipitation (IP), transfected HEK293 were lysed in RIPA buffer with protease inhibitors (Pierce, Rockford, IL, USA). Lysates were centrifuged for 20 min at 14,000 x g and 4°C, and the supernatants were incubated with P-selectin antibody (AC1.2) and Protein A/G plus agarose (Santa Cruz Biotechnology) overnight under rotation at 4°C. Then, beads were washed three times with RIPA buffer and twice with PBS before elution with elution buffer (Pierce). The eluted P-selectin was digested with Endoglycosidase H (EndoH) or Peptide: N-Glycosidase F (PNGaseF) at 37°C for 1 h. EndoH specifically cleaves immature glycans attached to proteins located in the endoplasmatic reticulum (ER) and Golgi apparatus, while PNGaseF unspecifically cleaves all sugars attached to the protein.

For Western blotting, cell lysates or IP products were separated on 8–16% Tris-Glycine gels (Invitrogen) and transferred to nitro-cellulose membrane. The membranes were blocked with non-fat dry milk powder, followed by overnight incubation with GFP antibody B2 (for reduced samples) or P-selectin antibody 1E3 (for non-reduced samples). IP products were probed with rabbit polyclonal GFP antibody. For visualisation of the signal, goat anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase was used as secondary

antibody, followed by ECL detection (GE Healthcare, Buckinghamshire, UK). Densitometry analysis of the immunoblots was carried out with ImageJ software.

Intracellular trafficking of P-selectin

In co-transfection experiments, cells were transfected with 0.75 µg of P-selectin plasmids and 0.5 µg of Sar1A DN plasmid. Sar1A DN (Thr39Asn) blocks ER-Golgi transport (26), which leads to the accumulation of newly synthesised proteins in the ER. When indicated, P-selectin transfected cells were incubated with 0.7 µg/ml BFA for 19 h (27). BFA disassembles Golgi and re-distributes cis-Golgi enzymes to the ER (27, 28). After BFA treatment, half of the cells were lysed for Western blotting, while the other half was incubated with Alexa647 coupled P-selectin antibody for FACS analysis.

Mass spectrometric analysis

P-selectin immunoprecipitates were resolved on 8–16% polyacrylamide gels and the gels were stained with coomassie brilliant blue. P-selectin bands from the stained gel were manually excised. In-gel digestion and sample preparation for mass spectrometric analysis was performed as described before (29). The digested peptides were analysed by high capacity ion trap (HCT). A HCT ultra ETDII PTM discovery system (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of m/z 350–1500, and MS/MS spectra in information dependent data acquisition over the mass range of m/z 100–2800. The recording was followed by ten data-dependent CID MS/MS spectra generated from ten highest intensity precursor ions. Peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics) and were searched against latest Swissprot database using MASCOT 2.3.02 (Matrix Science, London, UK). Protein identification information returned from MASCOT were manually inspected and filtered to validate protein identification. Peptides that had been modified due to PNGaseF treatment were verified.

Cell adhesion assay

P-selectin transfected HEK293 cells were grown to confluence in 24-well plates. HL-60 cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and grown at 37°C. In some experiments, transfected HEK293 cells were pre-incubated with P-selectin antibody AK6 (25 µg/ml) for 20 min at room temperature before performing the adhesion assay. For the assay, 10^6 HL-60 cells were resuspended in Hank's Balanced Salt Solution (HBSS) with 0.5% human serum albumin and added to the wells containing transfected HEK293 cells. After incubation at 25°C for 15 min, unbound HL-60 cells were washed away and the amount of adhered HL-60 cells was determined by measuring their myeloperoxidase content as described (30).

Determination of platelet-monocyte aggregates in whole blood

Platelet-monocyte aggregates (PMAs) were determined in 37 non-smoking healthy males between 20 and 60 years of age (median age 34 years). The study participants did not use medications and did not change their eating or physical activity habits during the two weeks prior to blood collection. Furthermore, the number of PMAs was determined in 115 consecutive patients with angiographically proven atherosclerosis [median age 66 years,

range 41 – 78 years; 40 females (34.8%)]]. All individuals gave written informed consent to participate in the study. The study was approved by the ethics committee of the Medical University of Vienna. Blood drawn from every individual was subjected to blood cell counting, DNA isolation followed by P-selectin genotyping performed as described (19). Primers used for P-selectin genotyping are listed in Table 1.

PMAs were identified in anti-coagulated blood within 20 min to 1 h after blood withdrawal as previously published (31). In brief, 5 μ l of whole blood was resuspended in HEPES-buffered saline and incubated with monoclonal antibodies, anti-CD45-peridinin chlorophyll protein (clone 2D1, Becton Dickinson), anti-CD41-phycoerythrin, (clone P2, Immunotech, Beckman Coulter, Fullerton, CA, USA), and anti-CD14-allophycocyanin (clone M ϕ P9, Becton Dickinson), or isotype-matched controls. After 15 min, samples were diluted with FACS lysing solution and at least 10,000 CD45+ events were acquired immediately in flow cytometer at a rate of 200–600 events per second. Monocytes were identified as CD14+ and the percentage of CD14+CD41+ events was recorded.

Statistical analysis

Data are reported as mean values $\pm$ SEM for cell culture experiments, and median and range for PMA analysis in the control and patients groups. Four independent transfections were performed for every genotype and all biochemical assays were done in triplicates. Differences were evaluated for statistical significance by two-tailed Student's t-test.

Results

The GA genotype (1902G, 2361A) coding for 562Asp and 715Thr is referred to as wild-type.

715Pro impairs terminal glycosylation of P-selectin

Western blot results showed that HEK293 cells transfected with GFP-tagged P-selectin synthesise two forms of P-selectin – one migrating with an approximate molecular weight of 168 kDa and the other migrating at 148 kDa. The 168 kDa product was EndoH resistant, indicating terminal glycosylation of the protein while the 148 kDa product was susceptible to EndoH digestion. The digested product exhibited similar migration behaviour as P-selectin treated with PNGaseF, an enzyme which unspecifically cleaves all carbohydrates attached to the protein (Fig. 1A). This result indicates that the 148 kDa product represents an immature not yet terminally glycosylated P-selectin.

Cells transfected with plasmids coding for 715Pro P-selectin (GC and AC) had only about half the amount of terminally glycosylated 168 kDa P-selectin and almost twice as much 148 kDa immature P-selectin compared to wild-type transfectants (Fig. 1B). We obtained similar results in Chinese hamster ovary (CHO) cells transfected with 715Pro constructs (see Suppl. Fig. 1, available online at www.thrombosis-online.com), indicating that the influence of 715Pro on terminal glycosylation is not only observed in HEK293 cells.

To find out whether the EndoH sensitive 148 kDa immature P-selectin was retained in the endoplasmic reticulum (ER), we performed Sar1A DN co-transfection experiments. Sar1A

DN blocks ER-Golgi transport (26), which leads to the accumulation of newly synthesised proteins in the ER. In Sar1A DN co-transfected cells, we observed that wild-type and 715Pro P-selectin migrate at 130 kDa (Fig. 2A). This finding suggests that the 148 kDa form of P-selectin does not represent the ER-retained high mannose form.

715Pro seems to impair post-translational modification of P-selectin by Golgi enzymes

Following BFA treatment, newly synthesised proteins are exposed to Golgi enzymes for protein modification. BFA treatment of wild-type P-selectin leads to modification resulting in an electrophoretic migration at 140 kDa under non-reducing conditions. In contrast, a large part of 715Pro (GC) remained un-modified and migrated at 130 kDa. Under reducing conditions, both wild-type and 715Pro P-selectin migrated at 130 kDa indicating that the post-translational modification of P-selectin conferred by Golgi enzymes was affected by reduction (Fig. 2A). Replacement of 715Thr by 715Ala also leads to impaired modification of P-selectin by Golgi enzymes following BFA treatment. However, the 715Ala exchange did not seem to affect terminal glycosylation of P-selectin (Fig. 2B).

Glycosylation of recombinant P-selectin

P-selectin has 12 potential Asn-linked glycosylation sites in the extracellular domain of the protein. By mass spectrometry, we could show that in P-selectin of human platelets all potential sites were glycosylated. We could demonstrate that recombinant P-selectin synthesized in HEK293 cells has a glycosylation pattern comparable to P-selectin present in blood platelets. The identified glycosylation sites are listed in Table 2.

The Thr715Pro variation leads to reduced surface expression and secretion of P-selectin

P-selectin on the surface of 10^4 GFP positive cells was quantified by flow cytometry. We found that cells transfected with the 715Pro variants, GC and AC, had $21.2 \pm 4\%$ and $22.8 \pm 3.2\%$, ($n=4$, $p<0.01$) less P-selectin on the surface, respectively, compared to cells transfected with wild-type P-selectin (Fig. 3B). ELISA assays revealed that cell culture supernatants of GC and AC transfectants contained $52.8 \pm 4.8\%$ and $38.8 \pm 5.7\%$ ($n=4$, $p<0.01$) less P-selectin, respectively, compared to supernatants of wild-type P-selectin transfectants (Fig. 3C).

The concentration of cellular P-selectin protein (represented by GFP fluorescence) in 10^4 transfected cells was $20.2 \pm 3.3\%$ ($n=4$, $p<0.01$) and $12.3 \pm 3.5\%$ ($n=4$, $p>0.01$) lower in GC and AC transfectants respectively, compared to wild-type transfectants (Fig. 3D). Interestingly, the P-selectin mRNA levels did not differ between the different transfectants (Table 3).

Confocal imaging of cells transfected with wild-type or 715Pro variant showed that only a small amount of P-selectin was expressed on the plasma membrane, while most of the P-selectin was found intracellularly (Fig. 4).

The Thr715Pro variant influences binding of P-selectin transfected HEK293 cells to HL-60 cells

Surface expression of P-selectin allows interaction with PSGL-1 and CD24 ligands constitutively expressed on HL-60 cells. As expected, P-selectin transfected HEK293 cells bound HL-60 cells. Cells expressing P-selectin 715Pro variants, GC and AC, bound $29.2 \pm 2.8\%$ and $28.2 \pm 6.2\%$ ($n=4$, $p<0.01$) less HL-60 cells, respectively, compared to wild-type transfectants. Mock transfected cells bound less than 10% of total HL-60 cells added (Fig. 5). Pre-treatment of transfected HEK293 cells with P-selectin antibody reduced the binding to HL-60 cells by approximately 70% (data not shown).

Association of P-selectin genotype with PMAs in whole blood

Determination of PMAs in the anti-coagulated whole blood of 14 healthy heterozygous Thr715Pro and 23 healthy homozygous Thr715Thr carriers indicated slightly lower levels of circulating PMAs in Thr715Pro carriers compared to Thr715Thr carriers, but the differences were not statistically significant. The analysis of 115 consecutive patients with proven atherosclerosis – 29 heterozygous Thr715Pro and 86 homozygous Thr715Thr carriers – revealed significantly lower levels of PMAs in Thr715Pro patients compared to Thr715Thr patients (16% vs. 24%; $p<0.05$, Table 4).

Discussion

P-selectin is a glycoprotein expressed on the surface of activated platelets and EC. Similar to other transmembrane proteins, nascent P-selectin is translated and transferred through the ER membrane. For N-linked glycosylation, an oligosaccharide containing several mannose sugars is attached to Asn residues on the nascent protein. Then, the precursor protein transits through the Golgi, where the immature glycans attached to the protein are edited by Golgi-resident enzymes and subsequently, the proteins are sorted to respective sub-cellular locations.

Corresponding to data reported for endothelial cells (2) and transfected AtT20 cells (32), we identified two differentially glycosylated species of P-selectin in HEK293 cells transfected with wildtype P-selectin. The two forms corresponded to terminally glycosylated (168 kDa) and immature (148 kDa) P-selectin. We could show that recombinant P-selectin has a glycosylation pattern comparable to P-selectin present in blood platelets with glycosylation of all potential glycosylation sites. Analysis of recombinant 715Pro P-selectin indicated that the Thr>Pro variation lead to reduced terminal glycosylation of P-selectin. Three potential mechanisms could explain how 715Pro impairs terminal glycosylation of P-selectin (i) 715Pro P-selectin is preferably retained in the ER and poorly exposed to Golgi for terminal glycosylation. (ii) 715Pro P-selectin is not processed properly leading to P-selectin accumulation in Golgi (iii) 715Pro P-selectin has poor sensitivity to glycan-modifying Golgi enzymes.

AtT20 cells transfected with wild-type P-selectin retained some immature P-selectin in ER, but the majority of the protein was completely processed and localised in late Golgi compartments, secretory granules and plasma membrane (32). Also in HEK293 cells, the

majority of wild-type P-selectin was processed and terminally glycosylated. But, in cells transfected with 715Pro variants a high amount of P-selectin was not terminally glycosylated.

Theoretically, 715Pro P-selectin could be preferably retained in the ER and be poorly exposed to Golgi for terminal glycosylation as suggested by the high amount of EndoH sensitive P-selectin. However, EndoH sensitivity is not a direct indicator for ER retention. The enzyme can cleave high mannose glycans from proteins retained in ER as well as hybrid glycans from proteins transiting Golgi. Sar1A DN co-transfection experiments showed that ER retained P-selectin migrates at 130 kDa. Thus, we conclude that the 148 kDa P-selectin does not represent the ER retained form but presumably is the Golgi transiting P-selectin with hybrid glycans.

When exposed to Golgi enzymes by BFA treatment, wild-type P-selectin was modified as indicated by a change of the electrophoretic mobility from 130 kDa to 140 kDa under non-reducing condition. In contrast, 715Pro P-selectin was poorly modified by Golgi enzymes. Interestingly, under reducing conditions, the modification brought about by Golgi enzymes was neither detectable in wild-type nor 715Pro P-selectin. We speculate that the modification of P-selectin that occurred after BFA treatment was S-palmitoylation. It is known that (i) palmitic acid is linked to Cys766 in the cytoplasmic tail of P-selectin through thioester linkage and can be removed by reducing agents (33), (ii) Golgi is the hot spot for palmitoylation and can retain proteins that cannot be palmitoylated (reviewed in reference [34]), (iii) Palmitoylation leads to slower gel migration as reported for cysteine-string protein (35), (iv) Palmitoylation of transmembrane proteins is critical for proper targeting to the cell surface (34), and (v) Palmitoylation is required for the secretion of alpha-granule proteins like P-selectin in platelets (36).

The observation that 715Ala also affected P-selectin modification underlines the importance of Thr at 715 for proper processing of the protein in Golgi. However, 715Ala did not impair the terminal glycosylation of P-selectin. This indicates that the non-conservative amino acid exchange of Thr to Pro at 715 plays a critical role in impaired terminal glycosylation.

The concentration of P-selectin on the cell surface and in the cell culture supernatant of 715Pro transfected cells was lower than of wild-type transfectants. This corresponds well with several reports showing that 715Pro is associated with low surface P-selectin on platelets (21) and low soluble P-selectin in plasma (14, 19, 22, 23). Our results suggest that the impaired terminal glycosylation and the possible lack of palmitoylation of 715Pro P-selectin in Golgi affect the export of P-selectin from Golgi and are responsible for the reduced amount of P-selectin on the cell surface and in the cell culture supernatant. In type 2 diabetes mellitus patients, the concentration of soluble P-selectin was increased compared to healthy controls. 715Pro had no influence on the amount of soluble P-selectin in those patients. After stratification of the patients according to body weight, an association between 715Pro and lower concentrations of soluble P-selectin was found in non-obese (body mass index [BMI] <22.4 kg/m²) but not in obese (BMI >22.4 kg/m²) patients (37). The relationship between platelet activation, obesity and soluble P-selectin was not studied in detail.

Surprisingly, we found less cellular P-selectin in 715Pro transfectants, even though the presence of 715Pro did not prevent de novo synthesis of P-selectin, as fairly large amounts of the protein were present in the cells. We can rule out any influence of different transfection efficiencies between transfections as well as an effect of the variation on transcription as mRNA concentrations were comparable in all transfectants tested. Degradation of the protein can also be excluded since no evidence of degraded products was found in cell lysates and culture supernatants by Western blot. Possibly, the reduced amount of cellular P-selectin in 715Pro transfectants was due to the retention of immature P-selectin in Golgi.

We found that 715Pro transfectants bound about 29% less HL-60 cells than wild-type transfectants. As 715Pro is located relatively far from the ligand binding domain (amino acids 17–117), we propose that the variation does not directly influence ligand binding. Rather, it seems that the reduced amount of P-selectin expressed on the surface of the 715Pro transfectants is responsible for the reduced adhesion to HL-60 cells.

Apparently, the lower number of P-selectin molecules on the surface of 715Pro platelets leads to the generation of fewer platelet-monocyte aggregates. This could be observed in healthy individuals, and more pronounced in patients with atherosclerosis. In the small group of healthy individuals the effect of the 715Pro polymorphism on the amount of PMAs formed was not statistically significant. In addition to the small group size the fact that a variety of receptor-ligand interactions are involved in the firm adhesion of platelets to monocytes could explain the lack of the statistical significance. In atherosclerosis patients, the P-selectin-PSGL-1 axis may have a stronger influence on PMA formation and therefore the Thr715Pro P-selectin polymorphism has a statistically significant impact.

In summary, our in vitro experiments demonstrate for the first time that the Thr715Pro missense mutation has a profound effect on post-translational processing of the protein in Golgi, leading to reduced transport of P-selectin to the cell surface and an impaired binding to HL60 cells and monocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Dr. Viacheslav Nikolaev, Gajalakshmi Ramanathan, Brinda Subbarayal and Birgit Horvath for valuable help and technical assistance.

Financial support:

This study is a part of the PhD program, Cell Communication in Health and Disease (CCHD) funded by Austrian Science Fund (FWF) and the Medical University of Vienna. Experimental work in Wuerzburg was supported by the Deutsche Forschungsgemeinschaft (DFG, SFB688).

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What is known about this topic?

- P-selectin triggers the adhesion of platelets to leukocytes, and promotes thrombogenesis.
- The P-selectin gene is highly polymorphic.
- The polymorphic variant 715Pro was shown to be associated with low concentrations of plasma P-selectin (representing a marker for platelet activation) and reduced risk for thrombotic disorders.

What does this paper add?

- We could show that HEK293 cells transfected with wild-type P-selectin produce recombinant P-selectin, which has a glycosylation pattern comparable to P-selectin present in blood platelets.
- The P-selectin variant 715Pro impairs terminal glycosylation of the protein, which then leads to less surface P-selectin and lower concentration of soluble P-selectin.
- The reduced surface P-selectin in cells expressing the 715Pro form leads to inefficient adhesion to HL-60 cells or monocytes.

Figure 1.

Immunoblots of different forms of P-selectin. A) Wild-type P-selectin immuno-purified from transfected HEK293 cells (lane 1) following digestion with EndoH (lane 2) and PNGase F (lane 3). Samples were resolved in 8–16% polyacrylamide gels followed by immuno-blotting with GFP antibodies. B) HEK293 cells were transfected with P-selectin wild-type and variants and lysed 24 hours post-transfection. Aliquots of the lysates (containing 50 ng/ml P-selectin) were subjected to immuno-blotting as above. Immunoblots were scanned and the intensity of the bands was quantified by the ImageJ program. A

representative blot of three independent experiments is shown. The graph represents the mean $\pm$ SEM of three independent experiments. Significant differences compared to wild-type P-selectin are indicated (** $p < 0.01$).

Figure 2.

Effect of Sar1A DN co-transfection and BFA treatment on P-selectin. A) HEK293 cells transfected with P-selectin alone (lanes 1 and 4), P-selectin together with Sar1A DN (lanes 2 and 5), and P-selectin transfected HEK293 cells after BFA treatment (lanes 3 and 6) were lysed and subjected to electrophoresis in 8–16% polyacrylamide gels under non-reducing and reducing conditions. Of BFA treated samples four-fold more protein was applied. For detection of the recombinant protein, P-selectin antibodies and GFP antibodies were used for non-reduced and reduced samples respectively. B) HEK293 cells transfected with 715Ala P-

selectin alone (lane 1), 715Ala together with Sar1A DN (lane 2) and 715Ala P-selectin transfected HEK293 cells after BFA treatment (lane 3) were lysed and subjected to immunoblotting as described above. Representative blots of three independent experiments are shown.

Figure 3.

Synthesis, surface expression and secretion of P-selectin wild-type and genetic variants. P-selectin transfected HEK293 cells were analysed 24 hours after transfection. A) Cell sizes of different transfectants were compared and are represented in the histogram. B) Surface P-selectin was quantified in 10^4 GFP positive cells by flow cytometry using Alexa647 labelled P-selectin antibodies. C) Soluble P-selectin in the cell culture supernatant of transfected cells was quantified by ELISA. Data is normalised to cell number. D) Cellular P-selectin (GFP fluorescence) in 10^4 transfected cells was quantified by flow cytometry. The data represent the mean $\pm$ SEM of four independent experiments. Significant differences compared to wild-type P-selectin are indicated (** $p < 0.01$). ND – not detectable.

Figure 4.

Localisation of GFP-tagged P-selectin in transfected HEK293 cells. Analysis was performed by confocal microscopy after staining DNA and plasma membrane with DAPI and Cell Mask™ Plasma Membrane, respectively. A small amount of P-selectin can be detected on the plasma membrane (arrowhead). Scale bar – 10 μ M.

Figure 5.

Adhesion of HL-60 cells to P-selectin transfected HEK293 cells. HEK293 cells transfected with P-selectin wild-type or P-selectin variant or mock plasmid were grown in 24-well plate. The transfected cells were incubated with HL-60 cells. After washing off unbound cells, the number of bound HL-60 cells was determined by myeloperoxidase assay. The number of bound cells was calculated from a standard curve of myeloperoxidase activity versus number of HL-60 cells. Data represent the mean $\pm$ SEM of four independent experiments. Significant differences compared to wild-type P-selectin are indicated (* * $p < 0.01$).

Table 1

List of primers used (5'→3').

Cloning	
forward	GTAAGCGCTAGCGACCATGGCCAACTGCCAAATA
reverse	TACGGTACCGGTGCAGGACTCGGGTCAAATG
Real-time PCR	
forward	CCATGGACTGTGTTTCATCCGCTCACT
reverse	TGTGCAGGGAATGGCTTGGC
Site-directed mutagenesis	
562Asp forward	CTCCACCTGTCATTTCTTGTGACAATGGCTTTAAG
562Asp reverse	CTTAAAGCCATTGTCACAAGAGAAATGACAGGTGGAG
715Pro forward	CCACTGGTCACCTACCGTGCCAACCTGC
715Pro reverse	GCAGGTTGGCACGGTAGGTGACCAGTGG
P-selectin genotyping	
715 forward	CTGTGAAATGCTCAGAACTACATG
715Thr reverse	GCAGGTTGGCACGGTTGT
715Pro reverse	CCTGCTTGATAGGTTGCCACGGAAGG

Table 2

Asn-linked glycosylation sites in recombinant and platelet P-selectin. The identified peptides presented in the table correspond to P-selectin precursor (Uniprot accession number: P16109).

Glycosylation site	Peptide (Ion score/mass error)	Enzyme
N54	50 K.AYSWN* ISR.K Deamidation(N) 57 (37/-0.0398)	Trypsin
N98	92 W.IGIRKNN* KTW.T Deamidation(N) 101 (35/0.0436)	Chymotrypsin
N180	171 K.QGECLETIGN* YTCSCYPGFYGPCEYVR.E Deamidation(N) 198 (70/-0.1773)	Trypsin
N212 N219	212 M.N* CSHPLGN* FSFNSQCSF.H 2 Deamidation(N) 228 (43/0.0722)	Chymotrypsin
N272	259 L.AAQCPPLKIPERGN* MTCL.H Deamidation(N) 276 (42/-0.0267)	Chymotrypsin
N411	405 R.AFYDTN* CSFR.C Deamidation(N) 415 (79/-0.0451)	Trypsin
N460	459 R.VN* CSHPFGAFR.Y Deamidation(N) 469 (47/-0.0845)	Trypsin
N518	514 L.LSPQN* GTMTCVQPLGSSSY.K Deamidation(N) 532 (109/0.0138)	Chymotrypsin
N665	663 F.GFN* TTCY.F Deamidation(N) 669 (33/-0.0151)	Chymotrypsin
N716	709 H.VNKPIAMN* CSNLW.G Deamidation(N) 721 (53/-0.0023)	Chymotrypsin
N723	722 W.GN* FSYGSICSF.H Deamidation(N) 732 (61/0.0651)	Chymotrypsin
N741	733 F.HCLEGQLLN* GSAQ.T Deamidation(N) 745 (63/0.0506)	Chymotrypsin

Table 3
Relative quantification of P-selectin using comparative C_T method.

Genotype	P-selectin Average C _T	GAPDH Average C _T	C _T = P-selectin C _T -GAPDH C _T	$\frac{C_T}{C_{TGA}}$	P-selectin Relative to GA (=2 ^{-C_T})
GA	13.3 ± 0.22	17.3 ± 0.12	-4.0 ± 0.25	0.0 ± 0.25	1 (-0.26–0.26)
AA	13.5 ± 0.16	17.3 ± 0.15	-3.7 ± 0.22	0.3 ± 0.22	0.8 (0.97–0.71)
GC	13.0 ± 0.12	17.4 ± 0.07	-4.4 ± 0.14	-0.4 ± 0.14	1.3 (1.49–1.22)
AC	13.6 ± 0.62	17.4 ± 0.15	-3.8 ± 0.64	0.2 ± 0.64	1 (1.37–0.56)

Table 4
Platelet-monocyte aggregates (PMA) in relation to P-selectin genotype.

P-selectin genotype	Samples (n)	Platelet-monocyte aggregates (%)		P-value
		Median	Range	
Healthy individuals				
Thr715Thr	23	25	5–48	P=0.19
Thr715Pro	14	20	2–35	
Patients with atherosclerosis				
Thr715Thr	86	24	4–74	P=0.03
Thr715Pro	29	16	5–45	