

Cloning and functional characterization of recombinant equine P-selectin

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Abstract

The recent molecular characterization and sequencing of equine P-selectin (ePsel), and its glycoprotein ligand, P-selectin glycoprotein ligand-1 (PSGL-1), have provided the tools for further investigation into their role in leukocyte trafficking. Here, we report the generation of a genetically engineered chimeric protein (ePsel-IgG) in which the equine P-selectin lectin and epithelial growth factor (EGF) domains were covalently linked to the equine IgG1 heavy chain constant region. The soluble ePsel-IgG was observed to bind to equine monocytes by confocal microscopy and flow cytometry. Furthermore, equine monocytes bound to immobilized ePsel-IgG in a time course and dose dependent manner. Not only did ePsel-IgG act as an adhesion molecule, it was also found to activate ERK1/2 kinase and induce IL-8 mRNA expression in equine monocytes. That all of the aforementioned ePsel-IgG-induced cell binding and cell signaling were abolished by the addition of EDTA, suggested that ePsel-IgG chimera mediated events occurred via the P-selectin ligand, PSGL-1. We were able to demonstrate that 78% of equine monocytes cross-reacted with anti-human HECA-452 antibody, which recognizes the sialy-Lewis X (sLex) epitope, a well-known carbohydrate binding site on human PSGL-1. Pre-incubation of equine PBMC with neuraminidase or O-sialoglycoprotein endopeptidase (OSGP) reduced ePsel-IgG monocyte binding to 36% or 60%, respectively. Taken together, these data suggest that there might be two ligand recognition sites on P-selectin, one of which recognizes sLex and another which recognizes P-selectin ligand core protein. The ePsel-IgG chimera can be a useful as a reagent for further studies on the role of equine P-selectin and signal transduction in inflammatory events in horse.

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1. Introduction

The adhesion molecules, which regulate the series of adhesive and signaling events, are of interest because of

their role in a variety of physiological and pathological processes, including leukocyte trafficking and recruitment (Salmi and Jalkanen, 2005; Liu and Kubes, 2003), inflammation (Luster et al., 2005), hemostasis and thrombosis (Shebuski and Kilgore, 2002; Levi et al., 2006) and cancer growth and metastasis (Geng et al., 2004; Varki and Varki, 2001). The initial adhesive step, mediated by selectins, is characterized by leukocytes tethering and rolling on endothelial cells or binding to

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platelets (Furie and Furie, 2004; Furie et al., 2001). P-selectin is expressed on activated platelets and endothelial cells and engages most leukocytes by its major counter-receptor, P-selectin glycoprotein ligand-1 (PSGL-1) (Cummings, 1999; Yang et al., 1999; McEver and Cummings, 1997). Most leukocytes also express a ligand for E-selectin, which is expressed on activated endothelial cells (Martinez et al., 2005; Asa et al., 1995; Lenter et al., 1994). L-selectin is expressed on leukocytes and binds to ligands on endothelial cells and other leukocytes (Green et al., 2004). The expression of the selectins and subsequent PSGL-1 engagement initiates and regulates leukocyte trafficking (Schottelius et al., 2003), during ischemia and reperfusion organ injury (Egami et al., 2006; Lefer, 2000; Garcia-Criado et al., 1995) and associated inflammatory (Lowe, 2003; Hartwell et al., 1998; Ley, 2003), and thrombotic processes (Polgar et al., 2005; Vandendries et al., 2004; André, 2004). Almost two decades ago P-selectin was shown to be a reliable marker of platelet activation and today is regarded as an important pathogenic factor and a disease marker (Kappelmayer et al., 2004; Merten and Thiagarajan, 2004; Coito et al., 2002; Woollard et al., 2006; Cambien and Wagner, 2004). Additionally, its interactions with leukocytes and endothelial cells have been exploited for therapeutic purposes as well (Constantin, 2004; Lowe and Ward, 1997; Van Assche and Rutgeerts, 2002; Bievenu et al., 2001; Hansen et al., 2004; Rijcken et al., 2004; Farmer et al., 2005a; Farmer et al., 2005b).

P-selectin is constitutively expressed in the α -granules of platelets and the Weibel–Palade bodies of endothelial cells and redistributed to the surface of platelets and endothelial cells upon activation by a variety of stimuli such as thrombin, tumor necrosis factor- α and endotoxin (lipopolysaccharide, LPS) (Geng et al., 2004; Furie and Furie, 2004; Polgar et al., 2005). Following platelet activation, P-selectin becomes expressed on the surface where it is rapidly shed which is thought to be main source of the soluble form found in plasma. The translocation of P-selectin to the surface of the endothelial cells is regulated by determinants contained within the P-selectin cytoplasmic tail (Hartwell et al., 1998). P-selectin (CD62P), the largest of the known selectins with a molecular weight of 140 kDa, is a type 1 membrane glycoproteins with an N-terminal C-type Lectin domain, followed by an epidermal growth factor-like motif, a series of consensus repeats, a transmembrane domain and the C-terminal cytoplasmic tail (Whiss and Andersson, 2002; Mehta et al., 1997). Engagement through the lectin domain with specific glycoconjugate ligands is central to selectin mediated cell–cell adhesion. All selectins bind, in

a calcium dependent manner, to the tetrasaccharide sialyl Lewis x (sLex; NeuAc α 2,3Gal β 1,4[Fuc α 1,3]GlcNAc) and its isomer sialyl Lewis a (sLe^a; NeuAc α 2,3Gal β 1,3[Fuc α 1,4]GlcNAc) where AcNeu stands for *N*-acetylneuraminic acid (sialic acid), Gal stands for galactose and GlcNAc stands for *N*-acetylglucosamine (Cummings, 1999; Hirose et al., 1998; Revelle et al., 1996). While P-selectin binds to sulfated carbohydrates, such as heparan sulfate, it binds with highest affinity (or avidity) to only a few molecules including N- and O-glycoproteins (Lowe, 2003). In the immune system, the sLex moiety that is recognized by the three selectins is carried by mucins (O-glycoproteins), i.e., glycoproteins with multiple Ser/Thr-linked oligosaccharides (O-glycans) and repeating peptide motifs, including P-selectin glycoprotein ligand-1 (PSGL-1), the major counter-receptor for P-selectin (Yang et al., 1999; McEver and Cummings, 1997; Martinez et al., 2005).

PSGL-1 is a homodimeric mucin (240 kDa homodimer consisting of two 120 kDa polypeptide chains) constitutively expressed in almost all leucocytes and primarily found in the tips of microvilli (Yang et al., 1999; Tchamitchi et al., 2000; Shodai et al., 2003). The ligand function of PSGL-1 depends notably on two specific post-translational modifications. One consists of the generation of O-linked glycans attached to threonine residues at the PSGL-1 amino terminus includes α (1,3) fucosylation and α (2,3) sialylation. The other involves sulphation of three tyrosine residues in the vicinity of the O-linked glycans. Interestingly, elements of the glycoprotein (GP) Ib α subunit (the GPIb α -IX-V complex) is a platelet receptor for von Willebrand factor (VWF) known to mediate platelet–adhesion, platelet–platelet interactions under conditions of high shear rates share structural homologies with PSGL-1 as it contains a mucin-like domain that separates the NH₂-terminal ligand-binding region from the plasma membrane, and a highly acidic domain with three sulphated tyrosine residues (Romo et al., 1999).

In humans, P-selectin is a key mediator of leukocyte–platelet and leukocyte–vascular endothelium adhesion via interactions with its natural ligand, PSGL-1 (Cummings, 1999; Yang et al., 1999; McEver and Cummings, 1997). The interaction between P-selectin and PSGL-1 plays a central role in inflammatory and thrombotic mechanisms in ischemic conditions by regulating leukocyte trafficking through cell adhesion, platelet–leukocyte aggregate formation (Liu and Kubes, 2003; Egami et al., 2006; Cambien and Wagner, 2004). Specific, P-selectin mediated downstream leukocyte events have been well described in humans including tissue factor expression (Celi et al., 1994), cytokine and

integrin expression (Koike et al., 2000; da Costa Martins et al., 2006), and superoxide anion generation from monocytes (Nagata et al., 1993).

The first events of leukocyte recruitment into the tissues are leukocyte tethering (capture) and rolling along the vessel wall, which are mediated primarily by selectins. The direct role of PSGL-1 in mediating vascular inflammation and thrombosis has been elucidated in several animal models with the ligand specific blocking antibodies or Ig chimeras (Bievenu et al., 2001; Hansen et al., 2004; Rijcken et al., 2004; Farmer et al., 2005a,b). Consistently, these animal models have demonstrated that inhibition of PSGL-1 represents an attractive basis for regulating vascular pathologies in a variety of etiopathogenic mechanisms.

Recent molecular characterization of equine P-selectin (Sayasith et al., 2005; Lalko et al., 2003), alongside our identification and sequencing of equine PSGL-1 (Xu et al., 2005), and bovine PSGL-1 (Xu et al., 2006), have opened the door to further studies that may more precisely identify the functional and cellular events in P-selectin mediated inflammation in the horse. Hence, in this present study, we engineered a soluble recombinant equine P-selectin-IgG chimera protein (ePsel-IgG), containing P-selectin lectin and EGF domains and demonstrated its functionalities *in vitro*. This molecule might prove useful in future studies as an immunogen for the development of anti-equine P-selectin antibody. Such an antibody could facilitate further studies into the precise molecular interactions between P selectin and its natural ligand(s) as well potentially enabling the measurement of soluble P-selectin in equine serum.

2. Materials and methods

2.1. Production of recombinant equine P-selectin-IgG (ePsel-IgG)

The cDNA of equine P-selectin (AY509881), coding the first 198 amino acids, including the signal sequence,

the lectin domain and the EGF (epidermal growth factor) domain (Table 1), was amplified by PCR using primers Psel1 (GCGCTAGCATGGCCAGCTGCCTA-AAAGCCTCT) and Psel2 (GCGCGATATCGTGACG-TATTCACATTCTGGTCCAT), thereby introducing *Nhe I* and *EcoR V* restriction sites at the 5' and 3' end of the PCR fragment, respectively. The PCR fragment was gel-purified and cloned into the vector pcDNA3.1/TOPO (Invitrogen, Carlsbad, CA, USA). After P-selectin sequence verification by nucleotide sequencing, the P-selectin fragment was digested with *Nhe I* and *EcoR V*, and cloned into the mammalian expression vector pcDNA-IGHG1, which originates from the G418-selecting plasmid pcDNA3.1-MycHis₆. The pcDNA-IGHG1 vector contained the equine IGHG1 gene to encode a C-terminal IgG1 "tag". Cloning of the equine P-selectin gene upstream of the IGHG1 gene resulted in the plasmid, pcDNA-ePsel/IGHG1 encoding an equine P-selectin-IgG1 fusion protein (ePsel-IgG).

2.2. Generation of stable transfectants

CHO cells were seeded overnight in 6-well plates at 1×10^5 /well and transfected with 1–2 μ g of the ePsel-IgG plasmid, pcDNA-ePsel/IGHG1/neo, per well using TL1 Transfection Reagent (Mirus, Madison, WI, USA) according to the manufacturer's instructions. Transfected CHO cells were plated into 96-well plates after 24 h at 100 cells/well in DMEM medium containing 10% (v/v) FCS (Hyclone, Logan, UT, USA), 50 μ g/ml gentamycin, and 1.5 mg/ml geneticin (G418, Invitrogen, Carlsbad, CA, USA). After 2 weeks of G418 selection, clones were selected for highest secretion of the ePsel-IgG fusion protein by ELISA, as described previously (Wagner et al., 2005). Cells from high-producing wells were further cloned by limiting dilution. In this manner, several ePsel-IgG high-producing clones were obtained. For recombinant protein production, ePsel-IgG producing clones were

Table 1
Alignment and comparison of the mature P-selectin molecule of human and equine

Molecular structure of mature P-selectin (residues number)	Lectin domain (58–158)	EGF domain (159–195)	CR domain (198–762) (198–763)	TM domain (771/794) (772/795)	Cytoplasmic domain (795–829) (796–830)
Equine	101	47	565	24	35
Human	101	47	566	24	35
Amino Acid homology	80%	91%	79%	83%	82%

The regions for comparison include the regions of the mature P-selectin protein. The signal peptide (1–41 residues) and a short peptide (42–57 residues) between signal peptide and lectin domain of equine P-selectin, which are required for the soluble ePsel-IgG protein processed into the supernatant, are not shown but included in the engineered pcDNA-ePsel/IGHG1/neo plasmid. The P-selectin part of ePsel-IgG contains 198 amino acids (1–198). The human P-selectin amino acid is bolded. EGF, epidermal growth factor; CR, consensus repeats; TM, transmembrane.

then grown in T75 flasks including 0.5 mg/ml of G418 in growth media until they were 70–80% confluent. Cells were washed and maintained in serum-free medium, and the supernatant collected every 24 h and stored at -20°C . The pooled cell supernatants were filtered and concentrated using a YM50 membrane (Millipore, Eschborn, FRG). The concentrated supernatant was applied to a protein G Sepharose column (Amersham Pharmacia, Uppsala, Sweden) and eluted with 0.1 M glycine-HCl, pH 3.0; followed by immediate neutralization with 1 M Tris-HCl, pH 8.0. The elution from the protein G column was pooled, concentrated, dialyzed against PBS, and stored at -20°C . The purity and homogeneity of purified ePsel-IgG chimeras were analyzed by non-reducing SDS-PAGE, followed by silver stain or Coomassie Blue and Western blotting.

2.3. SDS-PAGE and Western blot

The purified ePsel-IgG chimera protein was analyzed by SDS-PAGE on 4–15% Tris-HCl gradient gels (BioRad Laboratories, Hercules, CA, USA) under non-reducing conditions followed by Coomassie Brilliant Blue staining or transfer to a blotting membranes (PVDF, BioRad Laboratories, Hercules, CA, USA) after blocking with 5% (w/v) non-fat dry milk as previously reported (Aga et al., 2002). Blotting membranes were then incubated with peroxidase conjugated goat anti-horse IgG (H + L) Ab or CD62P Ab (BD Biosciences Pharmingen, San Diego, CA, USA).

ePsel-IgG induced ERK1/2 phosphorylation in equine monocytes was studied as follows. Freshly isolated equine PBMC were cultured in a complete medium for 2 h. Non-adherent cells and supernatant was aspirated and the remaining adherent monocytic cells cultured overnight in a serum-free medium before incubating with various concentrations of soluble or immobilized ePsel-IgG and equine IgG (0.3 $\mu\text{g}/\text{ml}$) for various time periods or LPS (1 $\mu\text{g}/\text{ml}$) for 1 h. For experimental conditions involving endotoxin, control and LPS treated samples were treated with and without polymyxin B to evaluate for contamination. In some experiments, the PBMC were pre-treated with U0126 or OSGP, or Neuraminidase before incubated with ePsel-IgG. At the indicated time, monocytes were lysed with M-PER reagent (Pierce, Rockford, IL, USA) supplemented with a protease inhibitor (Roche Applied Science, Indianapolis, IN, USA), and prepared in laemmli buffer (BioRad Laboratories, Hercules, CA, USA). Equal amounts of protein ($\sim 50 \mu\text{g}$) were loaded per lane and separated by SDS-PAGE. Proteins were

transferred to a PVDF membrane and blocked for 2 h in 5% (w/v) BSA at room temperature. Anti-ERK1/2 antibodies (Rabbit anti-human polyclonal antibody) (Cell Signaling, Beverly, MA, USA) that recognize active and inactive forms of both proteins were used at a final dilution of 1:1000 and incubated overnight at 4°C . After thorough washing, membranes were incubated with the peroxidase conjugated anti-rabbit IgG (Cell Signaling, Beverly, MA, USA). All the membranes were washed 3 times with TBS containing 0.05% (v/v) Tween-20, and antibody binding was visualized by the ECL chemiluminescence method (Amersham Bioscience, Piscataway, NJ, USA).

2.4. PBMC isolation and enrichment of monocytes

The equine blood donor population was comprised of 4 Quarterhorses, 2 Arabians and 4 Thoroughbred mares, aged between 6 and 14 years and with a bodyweight ranging from 428 to 542 kg (mean 480 kg). All horses were deemed clinically healthy and were maintained on pasture and grass-alfalfa hay throughout the study and blood sampling was conducted in accordance with the Animal Care and Use Committee of University of Wisconsin-Madison. Blood was collected into an EDTA vacutainer tube (BD bioscience, San Jose, CA, USA). Gravity separation of erythrocytes from leucocytes by rouleaux formation was accomplished by allowing whole blood to settle undisturbed at 20°C for 30 min, as described previously (Lalko et al., 2003). The platelet leukocyte-rich plasma (PLRP) was collected in a sterile tube, extended 2:1 with PBS and centrifuged at $250 \times g$ for 7 min. The supernatant was discarded and the pellet resuspended with 15 ml of PBS/2 mM EDTA. In order for the cells to segregate into three layers, the cell suspension was overlaid on 10 ml of Histopaque-1077 (Sigma Chemical Co., St. Louis, Mo, USA) and centrifuged at $400 \times g$ for 30 min. The peripheral blood mononuclear cell (PBMC), containing interphase was collected and washed twice with PBS. To enrich the monocytes population, PBMC were plated in tissue-culture dishes containing medium (RPMI-1640 supplemented with 10% newborn calf serum) and allowed to adhere at 37°C in a humidified atmosphere containing 5% CO_2 for 2 h. Non-adherent cells were removed by replacing the medium. Cell viability was determined by trypan blue exclusion and propidium iodide staining. More than 99% cells were found to be viable, and >95% of the cells isolated were identified as monocytes, as evidenced by nuclear morphology, cytology, and cytochemistry (nonspecific esterase staining).

2.5. Flow cytometry

For HECA-452 antibody staining, freshly isolated PBMC (10^6 cells/ml) treated with or without 0.1 U/ml *Vibrio cholerae* Neuraminidase, or 50 μ g/ml OSGP, as described above, were incubated with fluorescein isothiocyanate (FITC)-labeled rat anti-human HECA-452 polyclonal Ab for 30 min at 4 °C or control FITC-labeled irrelevant antibody of the same isotype (rat anti-human monoclonal IgM). Forward- and side-scatter were used to distinguish the monocytes population from the lymphocyte and polymorphonuclear populations and cell debris. The mean fluorescence intensity (MFI) for each histogram was used to quantify the binding of HECA-452 antibody.

To evaluate ePsel-IgG chimeric protein binding to monocytes the cells were treated similarly as described above. PBMC were resuspended in PBS/1%BSA/1 mM Ca^{2+} and 1 mM Mg^{2+} and incubated with either 5 μ g/ml equine IgG plus FITC-conjugated anti-IgG (H + L), or 5 μ g/ml ePsel-Ig chimera plus FITC-conjugated anti-IgG (H + L) complex at 4 °C for 30 min. Cells bound by ePsel-IgG-Ab complex were detected by fluorescent-activated cell sorting (FACSscan; Becton-Dickinson, San Jose, CA). The specificity of ePsel-IgG binding to cells was verified by the abrogation of ePsel-IgG binding in the presence of 10 mM EDTA. A mouse anti-horse anti-CD11a/18 Ab (clone CVS9), provided by Dr. Paul Lunn and has been previously described (Kydd et al., 1994), was used to identify leukocytes and serve as positive reference control to demonstrate the ePsel-IgG distribution on the leukocytes surface (Lalko et al., 2003).

2.6. Confocal microscopy

Freshly isolated equine PBMCs were incubated in blocking buffer (PBS/1%BSA/5% rabbit serum), on ice for 30 min. After the blocking, the PBMC were resuspended in PBS/1%BSA/1 mM Ca^{2+} and 1 mM Mg^{2+} and incubated with Cy3-conjugated anti-IgG (H + L) Ab (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), immunoglobulin-antibody complex (10 μ g/ml equine IgG1-Ab), chimera-antibody complex (5 μ g/ml ePsel-IgG chimera-Ab), or chimera-antibody complex plus EDTA (10mM EDTA) for 60 min on ice. Protein-antibody complexes were formed by co-incubating Ab with immunoglobulin or chimera on ice for 30 min. In the control group, PBMC were incubated with a mouse anti-horse CD11a/CD18 antibody followed by the Cy3-conjugated anti-mouse IgG1 staining (Southern Biotechnology, Birmingham, AL, USA).

Following washing 3 times, the cell pellets were resuspended in PBS/1%BSA, and dropped onto positively charged slides followed by mounting coverslips. The samples were then sealed with nail polish and observed under a confocal laser scanning microscope (Leica TCS SP2; Heidelberg, Germany) using a HCX PL APO lens at 63×8 magnification, with the numerical aperture set at 1.4, using Leica Confocal Software version 2.

2.7. Cell adhesion assay under static conditions

Static adhesion assays were performed in 96-well flat-bottom plates (Maxisorp, Nunc, Wiesbaden, Germany). Plates were initially coated and incubated with polyclonal anti-equine IgG (H + L) Ab (Jackson ImmunoResearch Lab., West Grove, PA), overnight at 4 °C followed by subsequent incubation with either purified ePsel-IgG (0–10 μ g/ml) chimera or equine IgG1 in PBS overnight at 4 °C. Nonspecific binding sites were blocked by incubating with PBS containing 1% BSA for 3 h. PBMC were labeled with a predetermined optimal concentration of 10 μ M Calcein-AM for 30 min at 37 °C in the dark before the adhesion assays were performed. To allow adhesion, 1×10^5 cells were suspended in PBS/1% BSA supplemented with 1 mM Ca^{2+} and 1 mM Mg^{2+} and incubated for 1 h at 4 °C. Unbound cells were washed gently with cold PBS. Bound cells were lysed with 1% SDS/PBS and the fluorescence measured at an excitation wavelength of 485 nm and an emission wavelength of 520 nm by fluorescence microplate-reader (Bio-Tek Instrument, Winooski, Vermont) with KC4 data reduction software. This protocol served as a basis to investigate the role of protein portion or carbohydrate motif of the P-selectin ligand in regulating ePsel-IgG binding to monocytes. For these inhibition studies, the plates were treated as described above and the PBMC were pre-incubated with 10 μ g/ml (final concentration) of anti-sialyl-Lewis X (sLex) mAb (HECA-452) (BD Biosciences Pharmingen, San Diego, CA) for 20 min on ice; or digested with 0.1 U/ml *Vibrio cholerae* Neuraminidase (Roche Diagnostics, Laval, Quebec, Canada), or 50 μ g/ml *Pasteurella hemolytica* O-sialoglycoprotein endopeptidase (OSGP) (Accurate chemical & Scientific Corp, Westbury, NY, USA) for 1 h at 37 °C. Treated cells were washed twice in PBS/1%BSA before use in the binding assays. Neuraminidase specifically cleaves the sialic acid residues of sLex epitope, and OSGP, an O-sialoglycoprotease, cleaves specific sialylated O-linked glycoprotein backbones (Norgard et al., 1993; Goetz et al., 1997; Moustafa et al., 2004; Steininger et al., 1992). Cell viability after enzyme treatment was >95% as

assessed by trypan blue exclusion. To verify the role for calcium dependent in P-selectin (lectin) binding, the binding assay was performed in PBS containing 10 mM EDTA. All experiments were performed in triplicate from five samples.

2.8. IL-8 mRNA expression studies

To test the effect of ePsel-IgG on interleukin-8 (IL-8) expression in equine monocytes, we analyzed the mRNA level change by real-time quantitative PCR. Freshly isolated equine PBMCs were cultured overnight in complete medium in suspension in micro-centrifuge tubes, before adding ePsel-IgG into the PBMCs suspension for 1 h incubation. Alternatively, the PBMC were cultured overnight in complete medium in plate adherently and then detached with EDTA. After washed twice, adding PBMCs into the micro-plate coated with immobilized ePsel-IgG for 1 h incubation. Under both condition, 1 μ g/ml LPS stimulation was also performed. To evaluate the role of sLex or sialylated O-linked glycoprotein backbones in regulating ePsel-IgG chimeric-mediated chemokine expression in monocytes, the cells were treated with neuraminidase and OSGP, respectively, as described above. After 1 h incubation at 37 °C, total RNA was isolated from cells as previously described, and treated with DNase (Roche Applied Science, Indianapolis, IN, USA) for 20 min at 37 °C in DNase buffer. The concentration of RNA was determined by measuring the absorbance at 260 nm with a spectrophotometer and the RNA purity was estimated from the ratio of the absorbance at 260 nm and 280 nm. A 1 μ g portion of RNA was used for cDNA synthesis, using oligo (dT) and MLV reverse transcriptase (Promega, Madison, WI, USA). Quantitative RT-PCR analysis was performed by monitoring in real-time the increase in fluorescence of the SYBR-green dye using the iCycler iQ Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA, USA). The equine IL-8 primers for quantitative PCR were designed using the PRIMER express program (PE Biosystem, Foster City, CA, USA). The sequences of the equine IL-8 primers were as follows. Forward: 5'-TTCCAAGCTGGCTGTTGCT-3'; reverse: 5'-TGATACAACCGCAGCTTCACA-3'. The volume of 25 μ l of reaction mix was aliquoted into separate tubes for each cDNA sample. Each sample was performed in duplicate. A no-template control (containing water) was included. Relative mRNA levels for IL-8 were analyzed by using a comparative Ct ($2^{-\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001), with equine 18s rRNA as an internal control to normalize the equivalent

RNA loading for each samples. The IL-8 cDNA amplicon was evaluated for potential DNA contamination and primers dimer formation by gel electrophoresis and dissociation curve analysis, respectively.

3. Results

3.1. Production of ePsel-IgG chimera

Purified ePsel-IgG was analyzed by SDS-polyacrylamide gel electrophoresis under non-reducing conditions and stained by the Coomassie Brilliant Blue methods. A single band with M_r of approximately 158 kDa was observed (Fig. 1). After overnight digestion with enterokinase, a distinct reduction in the relative molecular mass of the fusion protein was found from around 158 kDa–90 kDa. Using the same gel, immunoblotting with a polyclonal goat anti-horse IgG (H + L) Ab demonstrated the same banding pattern as evidenced by Coomassie Brilliant Blue staining. When samples were stained with CD62P antibody, another band with a molecular mass of approximately 34 kDa appeared. The banding patterns in Fig. 1 correspond to the molecular masses of the ePsel-IgG fusion protein (158 kDa), the IgG1 heavy chain constant

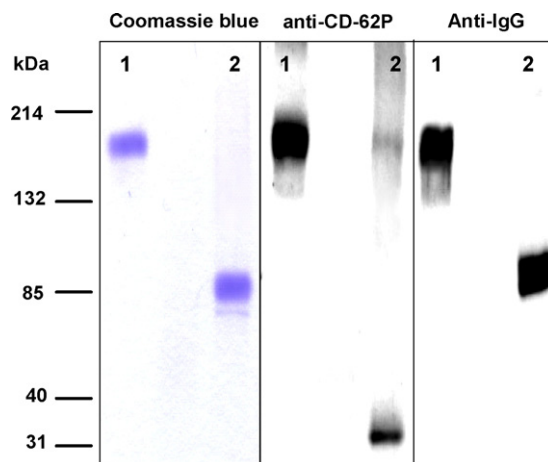


Fig. 1. SDS-PAGE and Western blotting of the purified ePsel-IgG fusion protein. All samples were run in SDS gels under non-reducing conditions. The ePsel-IgG fusion protein was purified by protein G affinity chromatography and digested with or without enterokinase (EK). The samples were separated on a 4–15% SDS gel, and transferred to a PVDF-membrane, and detected with a polyclonal goat anti-horse IgG (H + L) antibody and anti-CD62P (anti-human P-selectin). Lanes 1: ePsel-IgG fusion protein before enterokinase digestion; lanes 2: ePsel-IgG fusion protein after enterokinase digestion. The gel was stained by Coomassie Brilliant Blue (left panel). Samples from an identical gel were transferred for Western blotting by incubating the membranes with the anti-human P-selectin (middle panel) and anti-horse IgG (H + L) antibody (right panel).

region dimer (90 kDa), and one P-selectin monomer (34 kDa) attached on to each Ig heavy chains. The difference between the observed (158 kDa), and theoretical molecular mass of the secreted ePsel-IgG (~111 kDa) according to the predicted amino acid sequences, may be accounted for by glycosylation of the molecule or, in part, by the gel conditions.

3.2. ePsel-IgG binding and distribution on equine monocytes

In order to determine whether this recombinant equine ePsel-IgG chimera retains the capacity to bind equine monocytes, studies were initiated to examine the relative binding avidity of ePsel-IgG to equine PBMC. Incubation of PBMC in suspension with soluble ePsel-IgG provided a means of examining the binding of ePsel-IgG to individual cells by flow cytometry. As shown in Fig. 2a, soluble ePsel-IgG (5 μ g/ml) staining resulted in a strong fluorescence shift and this was completely eliminated by the addition of EDTA, reflecting the calcium requirement for ePsel-IgG

binding. By fluorescence microscopy, the pattern of the ePsel-IgG molecules distribution on the surface of cells showed a “clustered patch” distribution while CD11a/18 staining revealed a diffuse and uniform binding pattern on the cell surface (Fig. 2b). In the presence of EDTA, there was no ePsel-IgG observed on the cell surface.

To mimic the member-anchored P-selectin-mediated leukocytes trafficking *in vivo*, ePsel-IgG was immobilized on anti-IgG pre-coated microplate. We performed ELISA-based static cell adhesion assays to evaluate the dose–response and time kinetics of immobilized ePsel-IgG mediated monocytes binding. As shown in Fig. 3a, the percentage of adhered cells increased in a linear fashion with increasing concentrations of immobilized ePsel-IgG and reached maximal binding at 0.3 μ g/ml. The time kinetic studies demonstrated a linear increase in cell adhesion between monocytes and the immobilized ePsel-IgG from 20 min to 1 h of incubation. Incubation beyond 1 h was not associated with marked further increases in binding (Fig. 3b). Similarly, the presence of EDTA to the monocyte-buffer solution

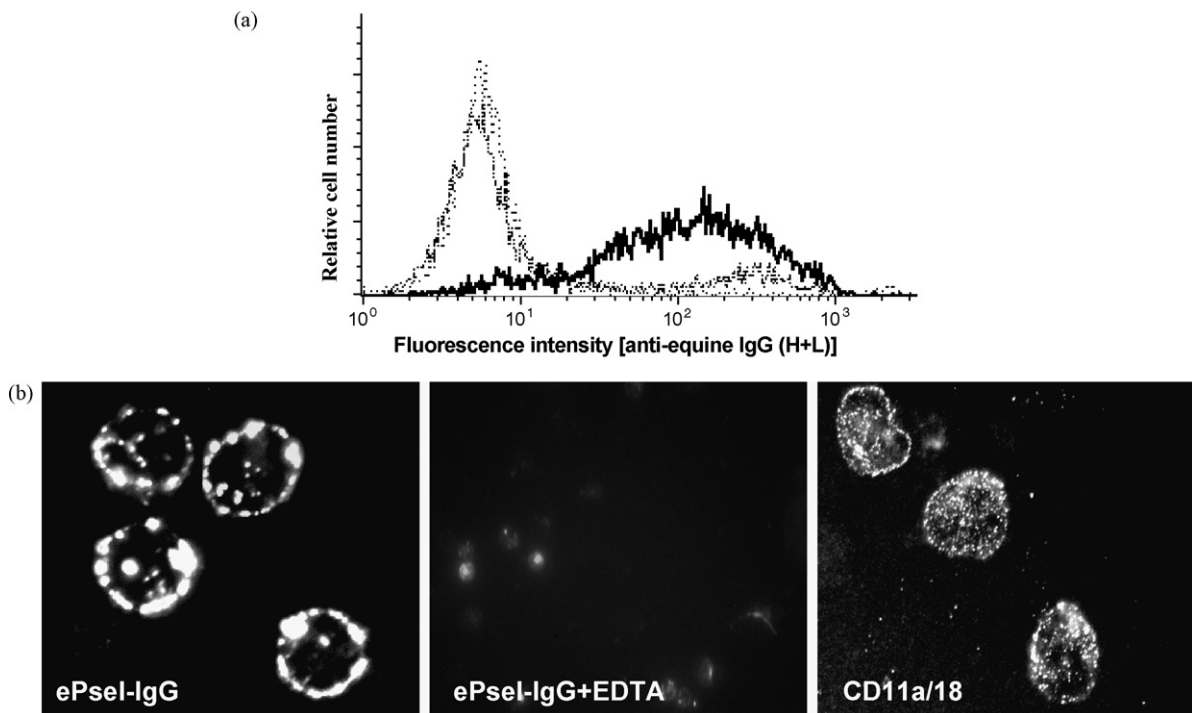


Fig. 2. Binding and distribution of ePsel-IgG to equine monocytes. ePsel-IgG (chimera) was incubated with FITC-conjugated anti-equine IgG (H + L) (Ab) first to form a protein–Ab complex and then co-incubated with equine PBMC. (a) Flow cytometric analysis show three overlapping curves demonstrating the binding of ePsel-IgG to the monocytes. The solid line represents ePsel-IgG–monocyte binding; the dashed line represents ePsel-IgG–monocyte binding in the presence of 10 mM EDTA and the dotted line represents background fluorescence resulting from equine IgG–monocyte binding. (b) Cy3-conjugated anti-equine IgG (H + L)–ePsel-IgG complex distribution on the cells surface without (left) or with EDTA (middle) and CD11a/18 staining (right) under the confocal microscope. No binding was observed for equine IgG or anti-equine IgG (H + L) on monocytes cells surface (data not shown).

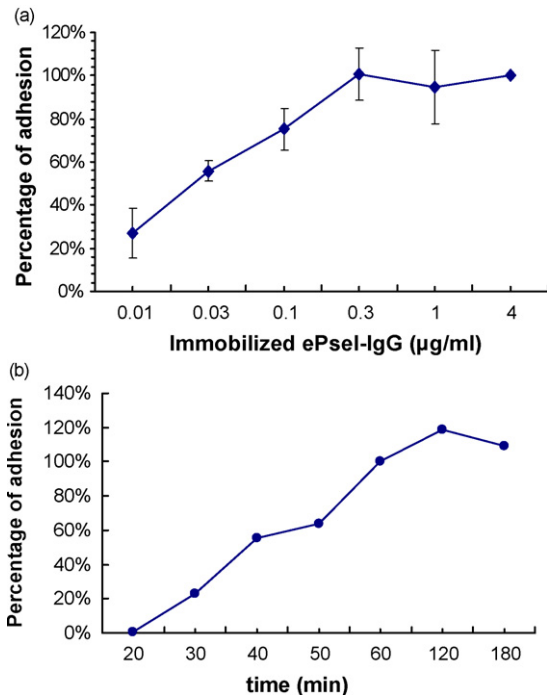


Fig. 3. PBMCs adhesion to immobilized ePsel-IgG under static conditions. Microtiter wells were pre-coated with the anti-equine IgG (H + L) overnight at 4 °C. ePsel-IgG was captured in the indicated wells. Calcein-AM-labeled PBMCs (5×10^4 cells/well) were allowed to settle onto wells coated with various concentrations of ePsel-IgG (0.01–4 µg/ml) for 1 h (a) or 0.3 µg/ml of ePsel-IgG at the indicated time (b). Adhesion under static conditions was measured as described in the Section 2. The final values reported as percentage of peak level (big graph), are means \pm S.E.M., $n = 5$. Specific responses (ePsel-IgG-induced cells binding) were normalized by minus the control level induced by IgG.

completely abolished cell binding to immobilized ePsel-IgG, suggesting a divalent Ca^{2+} -dependent mechanism (data not show).

3.3. Regulation of ePsel-IgG binding to equine monocytes

Subsequent to demonstrating that ePsel-IgG could bind to monocytes, we designed experiments to investigate the potential ligand responsible for in ePsel-IgG-monocyte binding. In humans, the predominant leukocyte P-selectin ligand is PSGL-1, which has appropriate sLex-like carbohydrate determinants for P-selectin recognition (Lenter et al., 1994). Given that the sLex-like carbohydrate determinants displayed on PSGL-1 can react with an oligosaccharide-specific human antibody, HECA-452, we first tested the cross-reactivity of HECA-452 on equine leukocytes and found that 78% of gated equine monocytes demonstrate

positive staining (Fig. 4A(a1)). To further confirm that HECA-452 staining was associated with the sLex epitope expression, equine PBMC were pre-treated with *Vibrio cholerae* neuraminidase (0.1 U/ml) before HECA-452 staining. As expected, neuraminidase treatment reduced HECA-452 positive monocytes to 20% with the mean fluorescence (MF) intensity decreased by 95.6% (no-treatment 399.54 versus neuraminidase treatment 17.62, respectively) (Fig. 4A(a2)). Then, we conducted experiments to study the potential role of sLex in ePsel-IgG-mediated equine monocytes binding by examining the effects of neuraminidase digestion on ePsel-IgG monocytes binding with flow cytometry (Fig. 4B(b1)). As shown in Fig. 4B(b2), neuraminidase (0.1 U/ml) digestion inhibited about 60% of ePsel-IgG cell binding. In the static adhesion assay, neuraminidase treatment also reduced monocytes binding to immobilized ePsel-IgG to a similar degree (data not shown).

This partial inhibition by neuraminidase digestion suggested the existence of another non-sLex P-selectin recognition site on monocytes, such as the protein portion of P-selectin ligand. We treated PBMC with OSGP and evaluated ePsel-IgG-monocyte binding. In Fig. 5A, the histogram of flow cytometry analysis (Fig. 5A(a1)) shows that OSGP treatment of PBMC decreased ePsel-IgG-monocyte binding by about 36% (Fig. 5A(a2), bar graph). However, in the static adhesion assay, OSGP treatment abolished immobilized ePsel-IgG-monocyte binding (data not shown). The presence of EDTA abolished ePsel-IgG-monocyte binding when monocytes were incubated with ePsel-IgG in suspension (Figs. 4 and 5, bar graph) or incubated with immobilized ePsel-IgG on a plate (data not shown).

Considering the potential physical relationship between sLex epitope and the glycoprotein backbone, we investigated HECA-452 staining to OSGP treated PBMC. As shown in Fig. 5B, HECA-452 staining was reduced only 8% with the mean fluorescence intensity decreased by 14%, which supports the notion that the sLex domain is not appreciably affected by sialylated O-linked glycoprotein digestion.

3.4. ePsel-IgG 1 activated ERK1/2 phosphorylation

Recent studies suggest that P-selectin engagement of PSGL-1 induces tyrosine phosphorylation, activates mitogen-activated protein (MAP) kinases (ERK-1 and ERK-2, ERK1/2) through MEK (MAP kinase kinase) (Ba et al., 2005; Cerletti et al., 1999). To test the effect of ePsel-IgG chimera on the cell signaling activation, we evaluated ERK phosphorylation state subsequent to

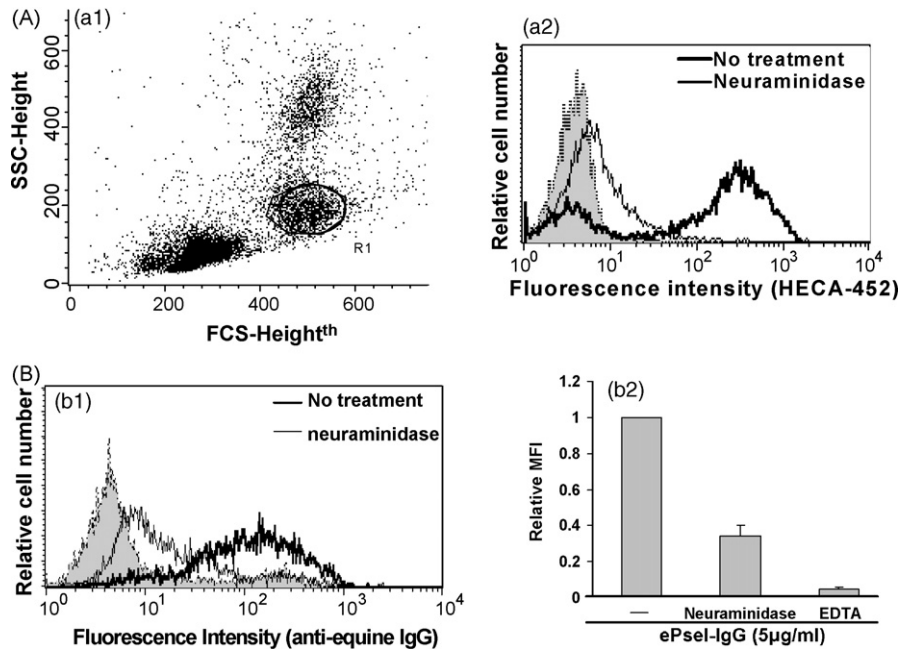


Fig. 4. Effect of neuraminidase treatment on sLex expression and ePsel-IgG binding to equine PBMCs. PBMCs were incubated with or without 0.1 U/mL of *Vibrio cholerae* neuraminidase for 1 h at 37 °C. (A) Representative dot plot (left) and histogram (right) of PBMC incubated with FITC conjugated anti-sLex (HECA-452) mAb. The mean fluorescence (of the gated population) was determined by flow cytometry. Flow cytometric analysis show three overlapping curves demonstrating HECA-452 staining to untreated monocytes (bold line), neuraminidase treated monocytes (thin line) and isotype control staining (grey shadowed dashed line). (B) Binding of ePsel-IgG to neuraminidase-treated and -untreated PBMC was determined as described in Materials and Methods. Representative flow cytometric analysis of three different experiments (left) show three overlapping curves demonstrating ePsel-IgG binding to untreated monocytes (bold line), neuraminidase treated monocytes (thin line) and equine IgG binding as control (grey shadowed dashed line). The bar graph (right) shows the average relative MFI of ePsel-IgG binding to untreated, neuraminidase and EDTA treated monocytes, respectively, from three independent experiments.

monocyte binding to soluble or immobilized ePsel-IgG. The phosphorylation of the ERK1/2 (p44/p42) isoforms of MAPK was assessed by immunoblot using phosphorylation state-specific antibodies following the incubation of various concentrations of ePsel-IgG (0.1–5 µg/ml) to equine monocytes. As shown in Fig. 6a, immobilized ePsel-IgG induced detectable ERK1/ERK2 phosphorylation at 0.3 µg/ml and reached a maximal level of phosphorylation at 2.7 µg/ml. ERK1/2 phosphorylation could be further increased by extending the incubation time to 60 min (Fig. 6b). Pretreatment of monocytes with U0126 (a specific ERK1/2 inhibitor) or OSGP, or in the presence of EDTA, completely abolished the ERK1/2 activation (Fig. 6c). However, experiments with soluble ePsel-IgG and monocyte incubation did not appreciably induce ERK1/2 phosphorylation (data not show).

3.5. Induction of IL-8 mRNA expression by ePsel-IgG

To investigate the down-stream event of ePsel-IgG-mediated cell signaling in monocytes we assayed for IL-8

mRNA expression in monocytes subsequent to the incubation with soluble ePsel-IgG in suspension or immobilized ePsel-IgG coated on microplate. Equine IgG was used as a control for non-specific Ig induction of IL-8. As shown in Fig. 7, both soluble and immobilized ePsel-IgG induced significantly higher IL-8 mRNA expression level than the control, while the response was most pronounced in monocytes bound to immobilized ePsel-IgG. Neuraminidase pretreatment of monocytes attenuated ePsel-IgG induced-IL-8 expression to a similar degree under both conditions. However, OSGP digestion resulted in contrasting results; it abolished immobilized ePsel-IgG induced-IL-8 expression but had no appreciable effect on soluble ePsel-IgG induction of IL-8.

4. Discussion

While the P-selectin system has been shown to play an important role in the pathomorphology of cancer and ischemia/reperfusion injury associated vascular inflammation and thrombosis in humans and various animal models (Levi et al., 2006; Geng et al., 2004; Varki and Varki, 2001; Furie and Furie, 2004; Furie et al., 2001;

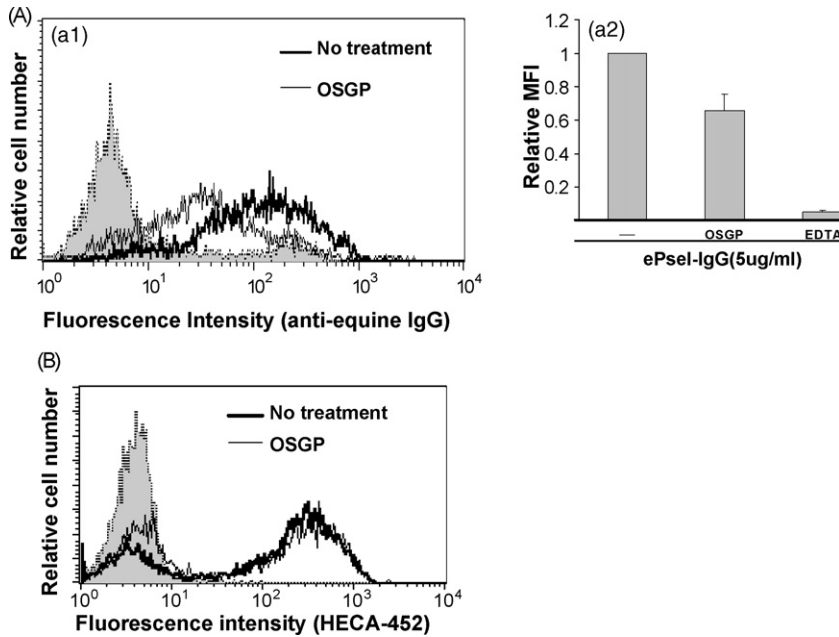


Fig. 5. Effect of OSGP treatment on sLex expression and ePsel-IgG binding to PBMCs. PBMCs were incubated with or without 50 $\mu\text{g/ml}$ OSGP for 1 h at 37 $^{\circ}\text{C}$. (a) Binding of ePsel-IgG to OSGP-treated and -untreated PBMC was determined as described in Section 2. Representative flow cytometric analysis of three different experiments (left) show three overlapping curves demonstrating ePsel-IgG binding to untreated monocytes (bold line), OSGP treated monocytes (thin line) and equine IgG binding as control (grey shadowed dashed line). The bar graph (right) shows the average relative MFI of ePsel-IgG binding to untreated, OSGP and EDTA treated monocytes, respectively, from three different experiments. (b) Representative histogram of PBMC incubated with FITC conjugated anti-sLex (HECA-452) mAb. The mean fluorescence (of the gated population) was determined by flow cytometry. Flow cytometric analysis show three overlapping curves demonstrating HECA-452 staining to untreated monocytes (bold line), OSGP treated monocytes (thin line) and isotype control staining (grey shadowed dashed line).

Celi et al., 1994) there is a paucity of information about equine P-selectin (Sayasith et al., 2005; Lalko et al., 2003). Given the importance of the P-selectin system in other species and the high incidence of life threatening vasculitis and thrombosis that accompanies neonatal septicemia and acute gastrointestinal disease of the adult horse (Ouellette et al., 2004; Dallap, 2004; Roy, 2004), it is logical to investigate its role in the equine. Here, we report for the first time, an engineered recombinant form of equine P-selectin-IgG chimera protein and demonstrate its functionalities being as an adhesion molecule and coordinated signaling molecule to induce cytokine expression.

Human and equine P-selectin share a relatively high level of amino acid homology throughout the entire peptide sequence, ranging between 91% for the EGF domain to 79% for the CR, with the proximal extracellular lectin domain being 80%. Previous reports have suggested that the lectin and EGF domains of P-selectin are sufficient to mediate PSGL-1 binding (Mehta et al., 1997). Taken together, we hypothesized that an engineered lectin and EGF domain of equine P-selectin fused into a vector containing equine heavy chain IgG could serve as a tool to investigate cellular

and molecular modeling of equine leukocyte trafficking. The IgG tag has been reported as a useful tool for the protein detection, purification and orientation when there is lack of specific antibody, such as is the case for equine P-selectin (Wagner et al., 2005). Single band recognized by Coomassie Brilliant Blue staining proved the purity or homogeneity of the purified ePsel-IgG protein. A different migrating pattern of ePsel-IgG under reduced and non-reduced condition on SDS-PAGE gel suggested its dimerization structure (data not shown). The difference between the theoretical molecular weight and the relative molecular masses observed in SDS-PAGE of the ePsel-IgG fusion protein and the fragment after enterokinase digestion may be due to glycosylation. Evidence for this is based on prediction that equine P-selectin and the IgG1 heavy chain constant region have three and two *N*-glycosylation sites, respectively (ExpASY Proteomics Server, in press; Wagner et al., 2002).

As a adhesion molecule, this recombinant ePsel-IgG chimera protein showed strong binding avidity to equine monocytes and neutrophils (data not shown) in a Ca^{2+} dependent sialidase-sensitive manner, which is characteristic of lectin-mediated binding (Whiss and

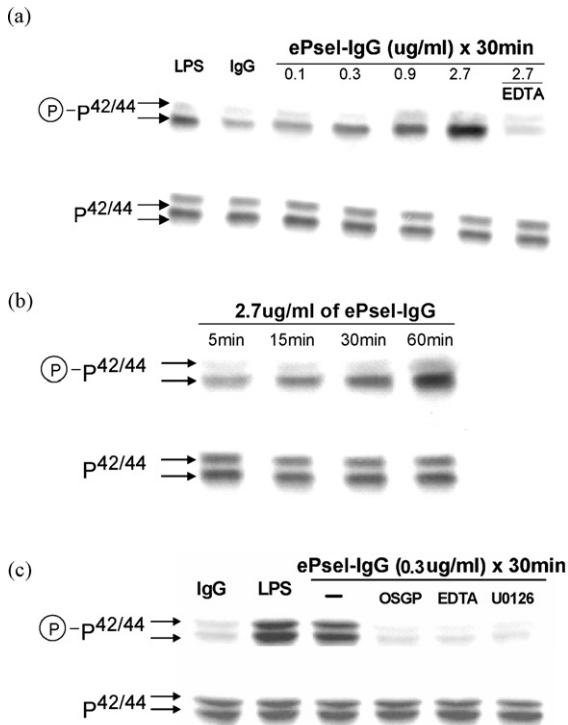


Fig. 6. ePsel-IgG triggered ERK1/2 MAP kinase phosphorylation. Equine monocytes were stimulated by various concentrations of immobilized ePsel-IgG (a), or 2.7 ug/ml of immobilized ePsel-IgG at the indicated time (b), or 0.3 ug/ml of immobilized ePsel-IgG following U0126 or OSGP pretreatments (c). Cells were lysated and performed the immunoblotting with Abs specific for phosphorylated (the top p42/44 bands) and nonphosphorylated ERK (the lower p42/44 bands), which confirms equivalent protein loading of each sample. This figure is representative of three separate experiments. LPS and equine IgG stimulation were used a positive and background control, respectively.

Andersson, 2002). P-sel-IgG, in Fig. 2, was used to represent soluble P-selectin (as in circulating platelet microparticles *in vivo*), which has been shown to bind and activate circulating monocytes (Woollard et al., 2006). The authors recognize the limitations of the technique reported here to mimic flow *in vivo*, however a flow chamber was not available. Under the fluorescent microscope, we observed that the immunofluorescence labeled ePsel-IgG molecules were not evenly distributed on the surface of equine monocytes. Instead, the ePsel-IgG molecules were clustered in sparse, but discrete patches, similar to the human PSGL-1 patches observed on human neutrophils (Norgard et al., 1993). The unique “clustered saccharide patch” characterized large numbers of closely spaced oligosaccharides carried on the P-selectin ligand. Thus, the ligand for equine P-selectin may also present clusters of oligosaccharides for high avidity recognition. Studies are

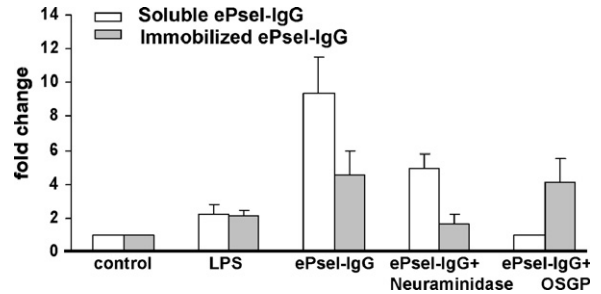


Fig. 7. ePsel-IgG binding mediates IL-8 mRNA expression in equine monocytes. Equine monocytes (5×10^5 cells) were stimulated by soluble ePsel-IgG (0.3 $\mu\text{g/ml}$) in micro-centrifuge tubes or by immobilized ePsel-IgG (0.3 $\mu\text{g/ml}$) coated on a microplate. After 1 h incubation, total RNA was isolated and subjected to RT-QPCR under the conditions described above. QPCR using primers for 18s as an internal control were also performed. The final values reported as a fold change of control level induced by IgG, are means \pm S.E.M., $n = 5$.

underway to determine the structural basis for high avidity binding of equine P-selectin to monocytes as well as the physiologic significance of this binding for leukocyte adhesion.

The saturating concentration of immobilized ePsel-IgG for monocyte binding was 0.3 ug/ml, while that reported for immobilized human P-selectin is 10 $\mu\text{g/ml}$ (Koike et al., 2000). Differences in methodology, such as plate-coating, likely contributed to the 30-fold differences in saturating concentrations. In our studies, the plates were initially coated with a specific anti-equine IgG antibody followed by ePsel-IgG, which orientated ePsel-IgG molecule to expose all P-selectin portions to the monocytes. In the human adhesion studies, P-selectin protein was plated directly, which can result in some P-selectin molecules being oriented up-side down and consequently unable to bind monocytes. As such, this technique may require higher concentrations of P-selectin to achieve binding saturation. After 20 min incubation at 0.3 ug/ml ePsel-IgG, cell binding was detected and lasted up to 3 h. The fact that we demonstrated stable monocyte binding to immobilized ePsel-IgG as quickly as 20 min, which included the time it took for the cells to settle-out by gravity, suggests that this interaction was relatively quick and stable. Future studies are underway using shear force, which will enable us study ePsel-IgG-ligand interaction kinetics.

While we have demonstrated ePsel-IgG functionality as a leukocyte adhesion protein, the question of possible recognition sites for P-selectin on equine monocytes has never been described. A large body of prior evidence indicates that binding by human P-selectin involves either sLex or sle^a as a minimal recognition structure (Phillips et al., 1990; Hiraiwa et al., 1995; Easton et al.,

1993). In our studies, ePsel-IgG bound 98% of the cells within the monocyte population and 78% of gated monocytes were positive for HECA-452 staining, which is similar to that reported in human monocytes. With neuraminidase pre-treatment, the percentage of HECA-452 positive staining monocytes was substantially decreased. These data suggested that most of gated equine monocytes carry the sLex epitope. The difference between total ePsel-IgG positive (98%) and HECA-452 positive (78%) cells may result from conformational differences between natural and ePsel-IgG chimera or possibly not all the cells within the gated population express the sLex epitope but are ePsel-IgG-positive. This could happen if large lymphocytes, such as NK cells or activated T cells, were included in the monocyte gate that expresses selectin ligands (e.g., ePsel-IgG positive), but not necessarily stain positive for HECA-452 (Kieffer et al., 2001; Fuhlbrigge et al., 1996). Alternatively, there may be two kinds of functional epitopes in equine P-selectin ligand recognition. Support for this notion is based on the results where removal of majority of sLex from equine monocytes by neuraminidase failed to inhibit ePsel-IgG-monocyte binding to the same degree as that of the sLex-mediated ePsel-IgG-monocyte binding. Taken together, the data suggest that some of ePsel-IgG-positive cells may not express the sLex epitope, but carry another kind of functional non-sLex epitope, such as the core protein portion of equine P-selectin ligand. Hirose et al. (1998) demonstrated that the anti-rat P-selectin mAb C215 and synthetic peptides containing the C215 epitope block the binding of rat and human P-selectins to PSGL-1, but failed to inhibit the binding of rat and human P-selectin-IgG to sLex oligosaccharides (Hirose et al., 1998). The mAb C215 epitope spans amino acid residues of P-selectin that are completely conserved in the equine P-selectin portion of ePsel-IgG. This finding provides direct evidence that another structural feature beyond sLex may contribute to P-selectin-PSGL-1 binding. Taken together, our results suggest that sialic acid is an essential component of the equine P-selectin ligand structure, but not sufficient for equine P-selectin recognition. The availability of a specific antibody to define equine monocytes, such as CD14, will greatly facilitate identifying the population of CD14/ePsel-IgG positive monocytes, respectively.

Several studies have indicated that mucins (O-linked glycoproteins) or mucin-like domains of glycoproteins including PSGL-1, CD34, CD44 or CD45 serve an important role in presentation of carbohydrate ligands to human P-selectin (Yang et al., 1999; McEver and Cummings, 1997; Martinez et al., 2005). These mucins,

which are sensitive to cleavage by OSGP, abolished human P-selectin and its ligand binding without significantly decreasing overall cell surface sLex expression (Norgard et al., 1993). Consistently, OSGP treatment completely eliminated equine monocytes binding to immobilized ePsel-IgG without significantly decreasing overall cell surface sLex expression, supporting the notion that OSGP-sensitive sites are required for optimal binding of equine monocytes to immobilize equine P-selectin. Whereas simple expression of sLex on monocytes is not sufficient to support cell adhesion to immobilized ePsel-IgG. In performing the immobilized ePsel-IgG-monocyte binding study, the cells were exposed to modest degree of force during gentle washing procedures. Consequently, under this condition, high avidity may be required to be stably bound by immobilized ePsel-IgG and expression of sLex on intact monocytes may not be sufficient to support optimal cell adhesion to P-selectin. When the same OSGP-treated PBMC were incubated with soluble ePsel-IgG in suspension, the binding of soluble ePsel-IgG to monocytes was only modestly decreased and the inhibitory effect was lower than that caused by neuraminidase digestion. That soluble ePsel-IgG binding in suspension assay did not involve washing “forces”, and sLex removal inhibited ePsel-IgG-monocyte binding to a greater degree than OSGP digestion infers the degree of ePsel-IgG binding to sLex and OSGP-sensitive sites may be related directly to their expression level that there are fewer OSGP-sensitive sites than that of sLex epitope, respectively. Since the high avidity recognition of this P-selectin ligand may be derived from a “clustered saccharide patch” of sialylated fucosylated O-linked oligosaccharide sequences (Norgard et al., 1993), it is quite likely that the cleavage of O-linked oligosaccharide sequences by OSGP digestion abolished the ePsel-IgG-monocyte binding. This concept is consistent with other reports demonstrating that higher avidity binding (nanomolar range) is achieved either by multiple binding sites within a single lectin molecule or by molecular aggregation of the monovalent lectin on the cell surface (Varki, 1997). In our study, when ePsel-IgG was fixed on the solid surface, high avidity binding may be achieved through multiple binding sites within a single lectin molecule. Since with the selectins, the binding affinities for sLex are very weak, being in the mid micromolar to millimolar range, we hypothesize that with equine monocytes, OSGP-sensitive sites are critical to form high avidity binding. However, the issue of how selectins generate functional high avidity binding remains unresolved.

PSGL-1-induced signal transduction in human neutrophils is characterized by tyrosine and ERK phosphorylation and chemokine synthesis (Ba et al., 2005; Ma et al., 2004; Ma et al., 1994; Weyrich et al., 1996). P-selectin has been shown to induce cytokine mRNA expression in human monocytes (Koike et al., 2000). In our study soluble ePsel-IgG was used to mimic plasmatic or platelet microparticle P-selectin in contact with monocytes while immobilized chimeric protein mimicked P-selectin expressed on endothelial cells *in vivo*. We demonstrated that immobilized ePsel-IgG induced detectable ERK1/2 phosphorylation in a dose and time dependent manner. In addition, the presence of EDTA, OSGP, and the ERK1/2 inhibitor U0126 completely eliminated ePsel-IgG-induced ERK1/2 phosphorylation. These results are consistent with those of the binding studies in which EDTA or OSGP abolished PBMC binding to immobilized ePsel-IgG. However, soluble ePsel-IgG did not induce detectable ERK1/2 phosphorylation and induced lower level of IL-8 mRNA expression than did immobilized ePsel-IgG. Similarly, Mahoney et al. (2001) reported greater protein synthesis of urokinase plasminogen activator receptor in monocytes adherent to immobilized P-selectin versus monocytes were incubated in suspension with P-selectin (Mahoney et al., 2001). These differences may reflect the different role of soluble P-selectin (plasmatic or platelet-associated P-selectin) and immobilized P-selectin (membrane-anchored or endothelial-associated P-selectin) under different pathophysiological states. Soluble P-selectin may induce tissue factor expression and platelets–leukocytes aggregation in thrombophilic conditions without affecting cell signaling, while immobilized P-selectin may regulate leukocyte trafficking and inflammation by activating cell-signaling and inducing chemokine expression and firm adhesion. Activation of other kinases and phosphorylation of their targets may also be important for signal integration. The use of specific MAP kinase inhibitors may elucidate cooperative signal activation pathways in ePsel-IgG stimulated monocytes. To decrease the interference of serum protein activating ERK1/2, the PBMCs were serum-starved overnight before incubating with ePsel-IgG. However, the lack of serum leads to the absence of LBP (lipopolysaccharide binding protein), which is critical for LBP/CD14-dependent activation by LPS at concentrations. We have previously shown in human monocytes (Aga et al., 2002), that incubation for less than 24 h in serum-free medium does not prevent LPS-induced signal transduction at 1 $\mu\text{g/ml}$. Our results show that under similar conditions, there is still ERK1/2

phosphorylation induced by LPS stimulation. This response might be due to some residual serum LBP remaining bound to monocytes since there is no thorough PBS washing after culturing in complete medium. Another possible reason might be LBP/CD14-independent pathway that mediates LPS induction of ERK1/2 phosphorylation. This notion is supported by Steinemann et al. (1994), who reported that LPS induction of TF expression in human monocytes is mediated by both LBP/CD14-dependent and independent pathways, depending on the LPS dosage used to stimulate the cells. Low concentration of LPS (0.1–1 ng/ml) induced TF expression via LBP/CD14-dependent pathway, whereas high concentration of LPS (100 ng/ml) induced TF expression via LBP/CD14-independent pathway. In our study, LPS concentration is 1 $\mu\text{g/ml}$. Thus, the LPS-induced ERK1/2 phosphorylation in the present study was likely influenced by the presence of previously bound LBP and/or an LBP/CD14-independent mechanism.

The effects of neuraminidase and OSGP digestion on ePsel-IgG-mediated cell signaling and IL-8 expression are constant with their effects on binding. Neuraminidase treatment partially inhibited soluble and immobilized ePsel-IgG-monocyte binding, and consequently inhibited soluble and immobilized ePsel-IgG-monocyte binding induced IL-8 expression. OSGP-digestion abolished immobilized ePsel-IgG-monocyte binding, and consequently abolished immobilized ePsel-IgG-monocyte binding induced IL-8 expression. OSGP-digestion partially inhibited soluble ePsel-IgG-monocyte binding, and consequently changes little soluble ePsel-IgG-monocyte binding induced IL-8 expression. Consequently, the binding state of ePsel-IgG to monocytes appears to greatly influence the subsequent cellular events.

In summary, our studies indicate that the chimeric protein ePsel-IgG binds to equine monocytes and induces signaling events that are associated with mRNA expression of IL-8. Most significantly, both soluble and immobilized ePsel-IgG bound to and activated monocytes, indicating that both are physiologically active. However, soluble ePsel-IgG (mimicking P-selectin positive platelets or platelet microparticles), and immobilized ePsel-IgG (mimicking endothelial-associated P-selectin), demonstrated different binding and cellular activation properties. It is tempting to suggest that the role of equine P-selectin in hemostatic and inflammatory processes may differ between soluble and immobilized state. Our results suggest that there are at least two types of recognition motifs, sLeX and OSGP-sensitive sites, which are involved in equine P-selectin

binding and may play different role in high avidity binding. Further molecular probes and immunologic tools are required to investigate the role of P-selectin and its ligand(s) in regulating vascular pathophysiological processes.

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