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Binding of human serum amyloid P component to L-selectin

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Serum concentrations of soluble L-selectin by far exceed those of other soluble adhesion molecules, and serum soluble L-selectin concentrations are remarkably stable upon prolonged storage. We present evidence for Ca^{2+}-dependent binding interactions between human serum amyloid P (SAP), a proteolysis-resistant pentraxin glycoprotein, and L-selectin, as shown by surface plasmon resonance measurements, protein band shift assays in a native PAGE system, and after SDS-PAGE and membrane transfer. Monoclonal antibodies to L-selectin strongly reduced binding of biotinylated SAP to L-selectin-IgG chimeras immobilized on microtiter plates. As binding was reduced by prior glycopeptidase F treatment of L-selectin but not of SAP, it appears to be based on SAP lectin domain interactions with N-linked L-selectin carbohydrates. In freshly prepared human lymphocytes, SAP incubation induced expression of a β2 integrin neoepitope associated with high-affinity binding. This was partially blocked by pre-incubation with Fab fragments of two anti-L-selectin antibodies. In flow chamber experiments, SAP inhibited the adherence of human neutrophils to activated endothelium under shear stress. Thus, SAP binds to human L-selectin and affects L-selectin-dependent leukocyte-endothelial interactions.

Key words: Acute-phase reactants · Adhesion molecules · Cell trafficking · Human

Introduction

The extravasation of leukocytes into tissues is a multi-step process initiated by the tethering and subsequent rolling of leukocytes along endothelial surfaces. The three members of the selectin family of cell adhesion molecules (L-selectin, E-selectin and P-selectin) play a pivotal role in these dynamic leukocyte-endothelium interactions. All three selectins are type I transmembrane glycoproteins with N-terminal Ca^{2+}-dependent lectin domains. L-Selectin (CD62L) is expressed constitutively and almost exclusively on leukocytes of both myeloid and lymphoid origin, whereas E- and P-selectin are expressed on activated endothelial cells and platelets. L-Selectin-mediated adhesion is important for the extravasation of predominantly naive lymphocytes into lymph nodes and for the recruitment of various leukocytes into areas of inflammation. Four L-selectin ligands have been identified in vascular endothelium: glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1), CD34, podocalyxin, and en-

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Abbreviations: **CRP:** C-reactive protein · **EDTA:** ethylenediaminetetraacetic acid · **GlyCAM-1:** glycosylation-dependent cell adhesion molecule-1 · **RLU:** relative light unit · **RU:** resonance unit · **SAP:** serum amyloid P · **SLP4:** recombinant human soluble L-selectin lacking the cytoplasmic and transmembrane domain · **SPR:** surface plasmon resonance · **TBS:** Tris-buffered saline

doglycan [1–4]. While CD34, podocalyxin and endoglycan are transmembrane glycoproteins present in both rodents and humans, GlyCAM-1 is a primarily secreted glycoprotein [5]. GlyCAM-1 has been found in rodents and cattle, but not in humans. All L-selectin ligands expressed by vascular endothelium belong to the sialomucin molecule family. Two other sialomucin transmembrane molecules, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is expressed on Peyer's patch endothelium, and the leukocyte surface adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1), have also been shown to bind L-selectin, in addition to binding other adhesion molecules. In all sialomucins identified as potential L-selectin ligands, L-selectin binding requires appropriate post-translational glycosylation and sulfation [6], consistent with the notion that the N-terminal C-type lectin domain of L-selectin is pivotal for its function. In addition, appropriately glycosylated L-selectin from human but not mouse neutrophils or T lymphoblasts serves as a ligand for E-selectin [7, 8]. L-Selectin-mediated adhesion to all of these ligands has an absolute requirement for Ca^{2+} ions.

Following activation of leukocytes by a large variety of stimuli, L-selectin is rapidly cleaved. The shed L-selectin retains functional activity and is present in human plasma at high levels [9]. Such proteolytic release has neither been observed for E- or P-selectin, nor for other leukocyte endothelial cell adhesion molecules belonging to the Ig superfamily, such as ICAM-1, ICAM-3, VCAM-1 or PECAM-1. Soluble isoforms of these molecules have been detected in human serum, although at much lower concentrations than those of soluble L-selectin. On a molar basis, L-selectin serum concentrations exceed those of other soluble adhesion molecules by one to two orders of magnitude. With an average density of 10^4–10^5 surface-expressed L-selectin molecules per leukocyte [10, 11] and L-selectin serum concentrations of 10–12 nmol/L ($6–7 \times 10^{15}$ molecules/L) [12], it can be calculated that the number of soluble L-selectin molecules present in a given volume of blood exceeds that of L-selectin molecules expressed on the surface of the leukocytes in the same sample.

While the generation of soluble L-selectin by proteolytic release has been studied in detail, its mechanism of clearance from the circulation has not been investigated yet. Measurements in pancytopenic patients after bone marrow transplantation have yielded estimated plasma half-lives of human soluble L-selectin in the range of 2–4 days [13]. Importantly, the calculated volume of distribution of soluble L-selectin was found to be 2.5-fold higher than the plasma volume, suggestive of ligands for soluble L-selectin within the circulation [13]. L-Selectin in human serum has been shown to be exceptionally stable upon prolonged storage at 4°C or repeated freeze/thaw cycles [9]. We observed, however, that soluble L-selectin becomes prone to degradation upon partial purification from human serum. Searching for soluble L-selectin ligands in human serum by affinity chromatography, we found that serum amyloid P (SAP), a member of the pentraxin family of proteins, binds L-selectin in a Ca^{2+}-dependent fashion and interferes with L-selectin-dependent leukocyte binding to activated endothelium.

Results

Commercially obtained SAP ran as a single monomer of approximately 26 kD in SDS-PAGE, as visualized by silver stain or immunodetection by the mAb CBL305. A band with the same apparent molecular weight was obtained when CLB305 was substituted for by L-selectin-IgG1 chimera in the presence (but not the absence) of Ca^{2+}, followed by biotinylated Fc-specific mouse anti-human IgG antibodies (Fig. 1). After SDS-PAGE and blotting on polyvinylidene difluoride membranes, the human L-selectin-IgG1 chimera was detected by probing with biotinylated SAP in the presence of 5 mM Ca^{2+} (Fig. 2).

When microwells were coated with L-, P- or E-selectin-IgG1 chimeras, there was negligible binding of biotinylated SAP in the presence of ethylenediaminete-

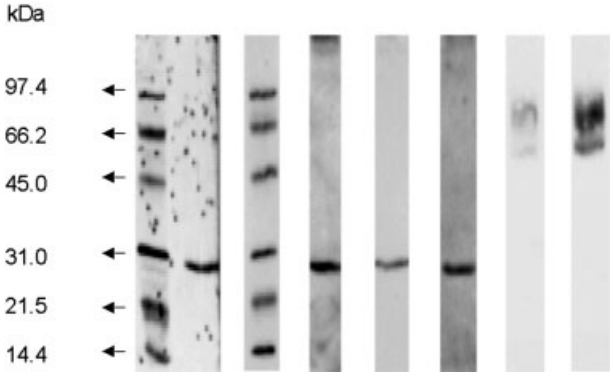

Figure 1. SDS-PAGE (12.5% gel), showing purity of the SAP and soluble L-selectin preparations. In Western blots, SAP was equally detected by anti-SAP (lane 5) or L-selectin (lane 6) antibodies. From left to right: (1) Low-molecular-weight markers, silver stained on blot; (2) SAP (100 ng/lane), silver stained on blot; (3) low-molecular-weight markers, silver stained in gel; (4) SAP (400 ng/lane), silver stained in gel; (5) SAP (800 ng/lane), immunodetection with anti-SAP mAb CBL305; (6) SAP (1000 ng/lane), immunodetection with human L-selectin-IgG1 chimera in the presence of 5 mM Ca^{2+} followed by biotinylated mouse anti-human IgG antibody (Fc-specific); (7) soluble L-selectin (3000 ng/lane), silver stained in gel; (8) soluble L-selectin (1000 ng/lane), immunodetection with anti-human L-selectin mAb Dreg200.

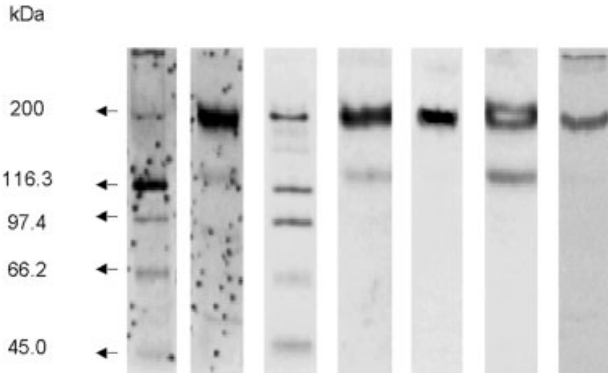

Figure 2. SDS-PAGE (7.5% gel), showing purity of the human L-selectin-IgG1 chimera. In Western blots, the human L-selectin-IgG1 chimera was equally detected by anti-L-selectin antibody (lane 5), anti-human IgG Fc antibody (lane 6) or SAP (lane 7). From left to right: (1) High-molecular-weight markers, silver stained on blot; (2) human L-selectin-IgG1 chimera (100 ng/lane), silver stained on blot; (3) high-molecular-weight markers, silver stained in gel; (4) human L-selectin-IgG1 chimera (400 ng/lane), silver stained in gel; (5) human L-selectin-IgG1 chimera (13 ng/lane), immunodetection with biotinylated anti-human L-selectin mAb Dreg200; (6) human L-selectin-IgG1 chimera (25 ng/lane), immunodetection with biotinylated mouse anti-human IgG antibody (Fc-specific); (7) human L-selectin-IgG1 chimera (200 ng/lane), immunodetection with biotinylated SAP in the presence of 5 mM Ca^{2+}.

traacetic acid (EDTA) (Fig. 3). In the presence of Ca^{2+}, however, a saturable and concentration-dependent binding of SAP to L-selectin chimeras could be detected. The calculated affinity for the binding of SAP to L-selectin chimeras was 0.75×10^{-8} M monomeric SAP, compared to 0.9×10^{-9} M for binding of the mAb Dreg200 to L-selectin chimeras. SAP also bound P- and E-selectin chimeras, although with much lower affinity (2.76×10^{-8} M for SAP binding to P-selectin-IgG, 7.2×10^{-8} M for SAP binding to E-selectin-IgG). Prior treatment of SAP by sialidase or glycopeptidase F did not affect Ca^{2+}-dependent binding to L-selectin chimeras, while enzymatic treatment of L-selectin chimeras with glycopeptidase F reduced subsequent binding of SAP significantly. Binding of SAP to wells coated with human L-selectin chimeras was inhibited by Dreg200 and the two L-selectin-binding sulfated carbohydrate polymers, heparin [half-maximal inhibition at 0.030 μg/mL (1.5×10^{-9} M)] and fucoidan [half-maximal inhibition at 0.075 μg/mL (7.35×10^{-10} M)] (Fig. 4), but not by murine IgG1 controls, asialofetuin, or α2-macroglobulin (no inhibition up to 1000 μg/mL). Binding assays carried out in the presence of purified human serum albumin (2.5 mg/mL) again showed inhibition of SAP binding to L-selectin chimeras that was inhibited by Dreg200 but not murine IgG1 (Fig. 5).

To further study the interaction of human SAP and soluble L-selectin under static conditions in solution, human SAP and soluble L-selectin were allowed to react

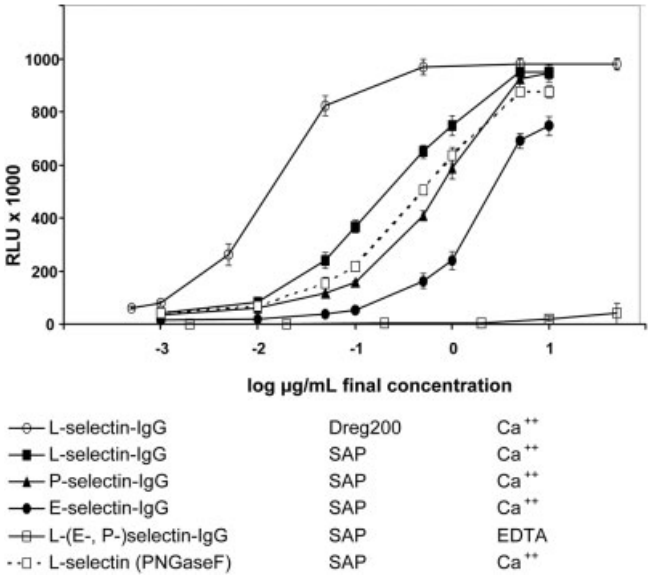

Figure 3. Binding of various concentrations of biotinylated SAP or anti-L-selectin mAb Dreg200 to L-, P- or E-selectin-IgG chimeras coated on microtiter wells. Bound SAP or antibody was detected luminometrically after reaction with ExtrAvidin-alkaline phosphatase (RLU, relative light units). Prior treatment of biotinylated SAP with sialidase or N-glycosidase did not result in altered binding curves. Virtually no binding of biotinylated SAP was observed to uncoated wells or to wells coated with any selectin chimera in the absence of Ca^{2+} (EDTA). Means ± SD of four independent experiments.

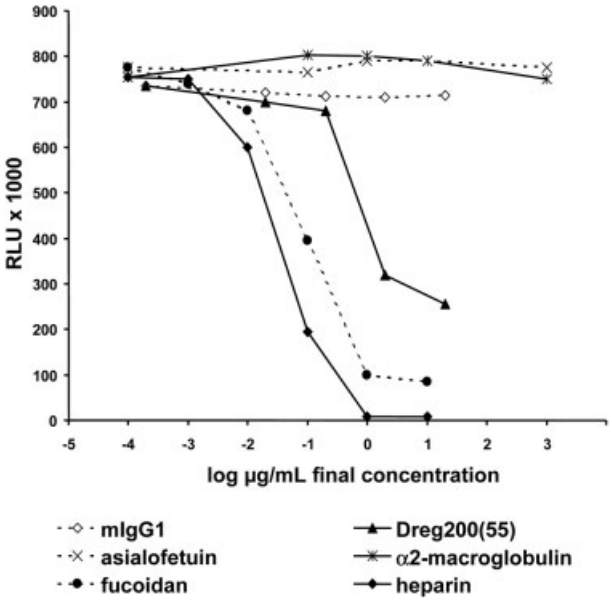

Figure 4. Impact of Dreg200 or Dreg55, murine IgG1 control, heparin, fucoidan, asialofetuin or α2-macroglobulin on the binding of biotinylated SAP (1 μg/mL in TBS containing 5 mM Ca^{2+}) to wells coated with human L-selectin-IgG1 chimera. Means of three independent experiments (SD never exceeded 0.035 RLU).

at a molar (monomeric) ratio of 5 : 1 or 2.5 : 1, followed by native 4–15% gradient PAGE. SAP was found to run as a decamer of approximately 230 kDa, as visualized by silver staining (not shown) or Western blotting (Fig. 6), in the absence of Ca^{2+} or L-selectin. With L-selectin and Ca^{2+} being both present, migration of SAP-positive material was strongly retarded, indicating the formation of higher-molecular-weight complexes. With Ca^{2+} and L-selectin, the 230 kDa SAP decamer band almost completely disappeared when the molar ratio of SAP and L-selectin was decreased to 2.5 : 1.

To investigate the binding of human SAP to L-selectin under flow conditions, we performed surface plasmon resonance (SPR) studies. At flow rates of 20 or 40 μL/min, L-selectin-IgG chimeras coupled to protein G-coated gold particles were found to interact with SAP-coated matrix tracks in a Ca^{2+}-dependent fashion (Fig. 7).

Furthermore, we investigated the potency of SAP to inhibit the adhesion of human neutrophils to TNF-α-activated human umbilical vein endothelial cells under shear conditions. Mock-treated endothelial cells served as reference or negative control and Dreg200 as positive control for inhibition. There was 88% inhibition of neutrophil adhesion to activated endothelial cells by 25 μg/mL SAP and 60% inhibition by 20 μg/mL Dreg200 at a wall shear stress of 2.0–2.6 dyn/cm^2, characteristic for L-selectin-dependent interactions (Fig. 8). At 2.5 μg/mL SAP, the neutrophil adhesion was inhibited to about 42%. BSA and murine IgG1 isotype had no inhibitory activity, and unstimulated endothelial cells did not bind neutrophils at ≥2 dyn/cm^2.

L-Selectin ligation by the murine soluble ligand GlyCAM-1 has been shown to result in β2 integrin

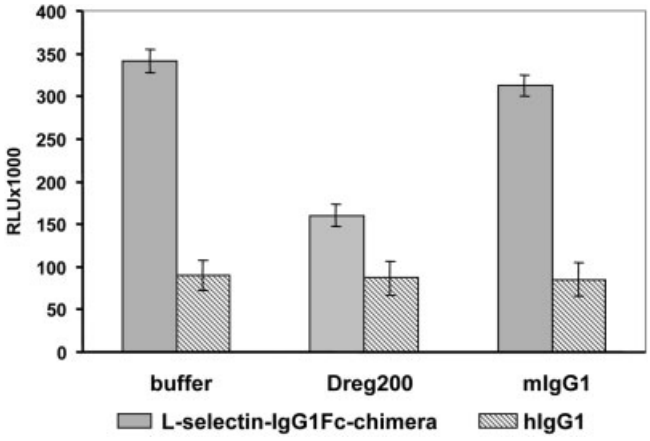

Figure 5. Binding of biotinylated SAP (1 μg/mL in TBS containing 2.5 mg/mL purified human serum albumin and 5 mM Ca^{2+}) to wells coated with human L-selectin-IgG1 chimera or human IgG1, after pre-incubation with the anti-human L-selectin mAb Dreg200 or murine IgG1 control (2 μg/mL). Means ± SD of three independent experiments.

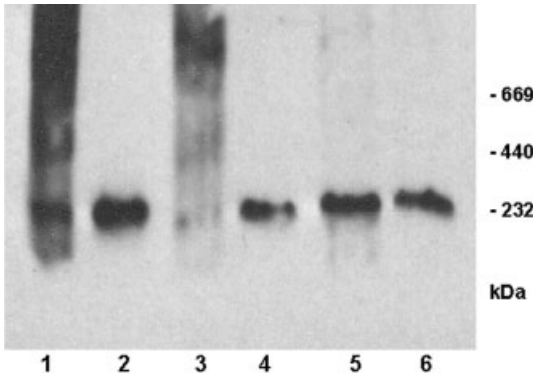

Figure 6. Ca^{2+}-dependent interaction of SAP and soluble L-selectin purified from human plasma. Commercially obtained SAP was subjected to native 4–15% gradient PAGE after reaction with soluble L-selectin, Ca^{2+}, or EDTA. Immunodetection of SAP with anti-SAP mAb CBL305 after blotting on polyvinylidene difluoride membrane. From left to right: (1) SAP (15 pmol monomer/lane) + L-selectin (3 pmol/lane) + 2.5 mM Ca^{2+}; (2) SAP (15 pmol monomer/lane) + L-selectin (3 pmol/lane) + 2.5 mM EDTA; (3) SAP (7.5 pmol monomer/lane) + L-selectin (3 pmol/lane) + 2.5 mM Ca^{2+}; (4) SAP (7.5 pmol monomer/lane) + L-selectin (3 pmol/lane) + 2.5 mM EDTA; (5) SAP (15 pmol monomer/lane) + 2.5 mM Ca^{2+}; (6) SAP (7.5 pmol monomer/lane) + 2.5 mM Ca^{2+}.

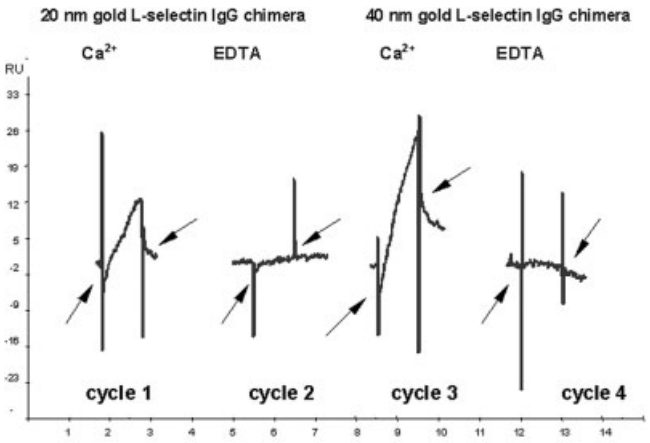

Figure 7. SPR measurements demonstrating Ca^{2+}-dependent binding of mouse L-selectin-IgG ~ protein G ~ gold conjugates to immobilized SAP. Reaction of mouse L-selectin-human IgG ~ protein G ~ gold conjugates (20 nm or 40 nm diameter) to SAP covalently attached to a Biacore CM5 chip (track 2), as opposed to BSA (track 1). The four cycles show the difference in RU between track 2 and track 1 at a flow rate of 40 μL/min. The left arrow of each cycle marks the injection at the start of the cycle. Cycle 1: 20-nm gold conjugate + 1 mM Ca^{2+}; cycle 2: 20-nm gold conjugate + 3 mM EDTA; cycle 3: 40-nm gold conjugate + 1 mM Ca^{2+}; cycle 4: 40-nm gold conjugate + 3 mM EDTA. The right arrows of the four cycles mark the start points of injections of EDTA buffer without conjugate to regenerate the chip. X axis: time (minutes).

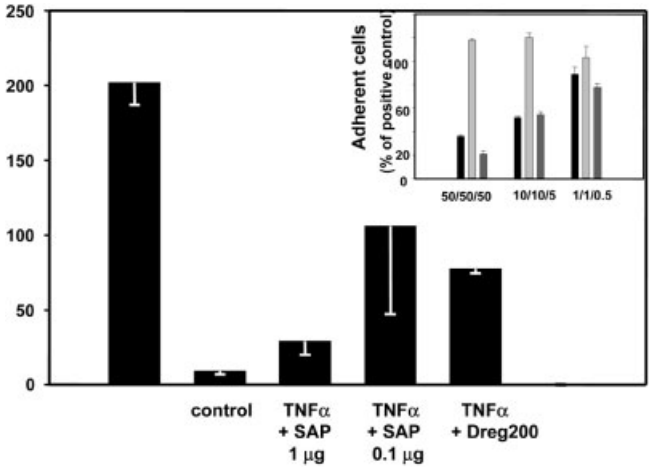

Figure 8. In flow chamber assays with an effective wall shear stress of 2.0–2.6 dyn/cm^2 (representative for L-selectin-dependent adhesion), the number of adherent neutrophils per high power field (HPF) to confluent layers of TNF-α-stimulated (500 U/mL for 4 h at 37°C; TEBU GmbH, Germany) or unstimulated (control) human umbilical cord endothelial cells was counted with or without SAP (0.1 or 1 μmol/L) or the anti-L-selectin antibody Dreg200 (50 μg/mL) being present during the experiment (means ± SD; n = 3–9). The insert shows adhesion of neutrophils to TNF-α-activated endothelial cells in the presence of varying concentrations of Dreg 200 (black; 50, 10, or 1 μg/mL), murine IgG1 isotype (light grey; 50, 10, or 1 μg/mL) and fucoidan (dark grey; 50, 5, or 0.5 μg/mL).

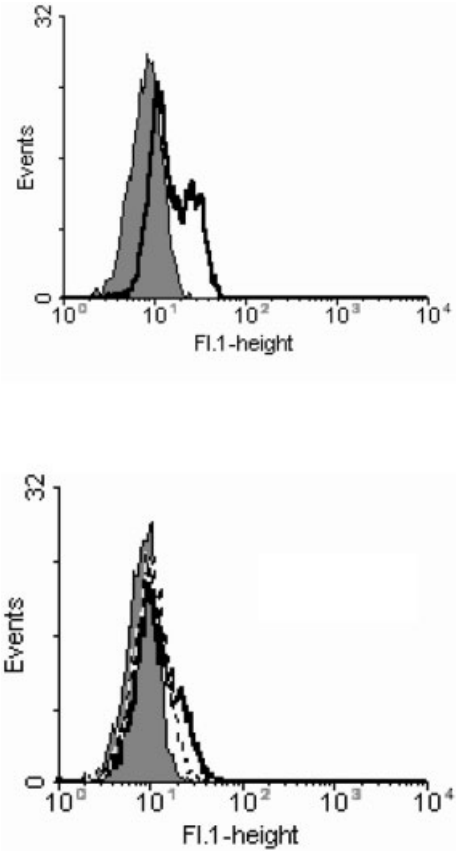

Figure 9. Up-regulation of the lymphocyte activation-dependent integrin neoepitope (mAb 24 epitope) by 10 min of incubation at 37°C with 3 mM Mn^{2+} (positive control, top) or with 5 μg/mL SAP (bottom). Filled histograms: Staining at 0 min (identical to IgG1 negative control histograms at 0 and 10 min). Full-line histograms: Expression at 10 min. Dotted-line histogram: Partial inhibition by Dreg200 Fab.

activation [14], as assessed by expression of the activation-related β2 integrin neoepitope on human PBL recognized by the mAb 24 [15]. When monocyte-depleted, freshly prepared human lymphocytes were incubated with human SAP, expression of the activation-related β2 integrin mAb 24 neoepitope increased to 135.8 ± 11.4%, relative to baseline. SAP-induced mAb 24 neoepitope up-regulation was about half that of the positive control, Mn^{2+} (175.3 ± 7.2%), and was partially (by 38%) but significantly (p = 0.028) blocked by incubation with Fab fragments of the anti-L-selectin mAb Dreg55 and Dreg200 (Fig. 9).

Discussion

The experiments described here were carried out after the provisional purification of putative human L-selectin-binding glycoproteins from human plasma by affinity chromatography involving anti-L-selectin mAb-coupled Sepharose in the presence or absence of Ca^{2+}, and purification by chromatography on L-selectin-bearing Sepharose matrices resulted in the identification of human SAP as a candidate L-selectin-binding glycoprotein. When the final L-selectin-binding glycoprotein preparation was subjected to SDS-PAGE under nonreducing conditions followed by silver staining, there were two major bands of 18–20 and 26–28 kDa and a faint band of 47–54 kDa. All three bands were identified as SAP or SAP split products by mass spectroscopy sequencing. However, the Sepharose matrix itself binds SAP in a Ca^{2+}-dependent fashion [16–18], which readily becomes relevant as SAP concentrations in human plasma [19, 20] exceed those of soluble L-selectin [9, 12]. SAP was also eluted from human plasma-loaded Sepharose control columns upon Ca^{2+} chelation by EDTA, and commercially obtained SAP bound almost equally well to asialofetuin-coupled Sepharose as to Sepharose coupled to recombinant L-selectin. Thus, we went on to characterize the binding of human SAP to L-selectin in detail.

Binding of commercially obtained human SAP to immobilized L-selectin chimeras was found to be strictly dependent on Ca^{2+} and could be inhibited by the anti-L-selectin mAb Dreg200 and by the L-selectin-binding sulfated carbohydrate polymers, fucoidan and heparin. Binding between SAP and L-selectin chimeras was

observed after SDS-PAGE and transfer onto polyvinylidene difluoride membranes. This argues against the hypothesis that noncovalently bound carbohydrate moieties, *e.g.* glycolipids with terminal 3-sulfated galactose, might mediate the interaction between SAP and L-selectin. It also demonstrates that L-selectin binding does not require pentameric or decameric assembly of SAP. Ca^{2+}-dependent binding of SAP to L-selectin was confirmed by the altered migration pattern of SAP in native SDS-PAGE in the presence of soluble L-selectin. Ca^{2+}-dependent human SAP and L-selectin interaction was also found to occur under shear stress, as demonstrated by real-time SPR studies.

The potential significance of this finding *in vivo* was emphasized by flow chamber assays: SAP inhibited leukocyte adhesion to activated endothelium under shear stress as potently as the L-selectin-specific antibody Dreg200. Morevoer, SAP elicited activation of freshly prepared monocyte-depleted human lymphocytes, as assessed by β2 integrin neoepitope expression, in a fashion similar to the murine soluble L-selectin ligand GlyCAM-1. This activation by SAP was partially inhibited by Fab fragments of antibodies to L-selectin. Both SAP and L-selectin moieties are constituents of human blood, which creates various scenarios for encounters between SAP and L-selectin. This argues for one or more immunologically significant roles of SAP in modulating L-selectin-dependent leukocyte adhesion events, definition of which will have to await a series of well-designed experiments. While circulating SAP may act as a buffer system limiting cellular infiltration by interfering with an early step of leukocyte extravasation, SAP expressed in tissues such as inflamed exocrine glands or degenerating neurons [21, 22] may instigate L-selectin-mediated leukocyte recruitment.

SAP belongs to the pentraxin family of proteins which are endowed with Ca^{2+}-dependent lectin-like binding activity. Native human SAP is arranged as a flat cyclic pentamer consisting of five noncovalently associated identical subunits of 25 462 kDa each [23, 24]. Among the pentraxins, SAP and C-reactive protein (CRP) are secreted proteins with a similar molecular mass, a high degree of conservation throughout evolution, and a comparable genomic organization. Both SAP and CRP are produced and catabolized in the liver of humans and rodents [19, 24], but they show considerable species differences in their regulation. CRP is highly inducible in humans but not in mice during inflammation. In contrast, SAP is the major acute-phase protein in mice while its concentration is only moderately elevated during acute inflammation in humans [25].

Humans unable to synthesize SAP have not been described, while SAP knockout mice show altered responses to gram-negative bacterial infection and spontaneously develop marked anti-chromatin autoimmunity [26, 27]. SAP is a major constituent of amyloid deposits, which is related to its marked resistance to proteolysis [23] apparently conferred to SAP-bound proteins [28]. We assume that the unexpectedly high levels of circulating L-selectin in humans [9, 12] and its long half-life [13] may be linked to protection from degradation conferred to soluble L-selectin by SAP.

Both SAP and L-selectin are C-type lectins that recognize several carbohydrate and other ligands in a Ca^{2+}-dependent fashion. Furthermore, they both contain N-linked but no O-linked glycostructures decorated with terminal sialic acid residues. The requirement for Ca^{2+} strongly suggests the involvement of a lectin domain in mediating the binding between SAP and L-selectin. SAP has a uniquely homogenous glycostructure, with a single N-linked biantennary oligosaccharide [$(Sia\text{-}Gal\text{-}GlcNAc\text{-}Man)_2$-Man-GlcNAc-GlcNAc] [23] which does not contain the L-selectin target structures sialyl Lewis X or the fucosylated lactosamine recognized by the mAb MECA79. Treatment of SAP with either sialidase to remove terminal neuraminic acid or glycopeptidase F to remove N-linked carbohydrates did not affect its L-selectin binding ability, while treatment of L-selectin chimeras with glycopeptidase F reduced SAP binding. This suggests that it is the lectin domain of SAP that interacts with carbohydrates presented by L-selectin to mediate SAP-L-selectin binding. L-Selectin-presented glycostructures are already known to mediate binding to E-selectin [7, 8].

The tendency of SAP to aggregate at supraphysiological concentrations complicates determining the extent to which SAP is complexed with its ligands in solution. While the apparent affinity of SAP to immobilized L-selectin chimeras is high (0.75×10^{-8} M), as compared to its reported plasma concentration of 1.5×10^{-6} M [19, 20], the solution affinity of SAP and soluble L-selectin may be rather low. Unfortunately, the unknown amount of L-selectin molecules bound to the gold particles employed precludes calculations of K_D, k_{on} and k_{off} values in the SPR experiments.

The avidity of most lectins greatly increases with closely spaced repeated recognition motifs [29], and the pentameric assembly of SAP already endows it with multivalency. Immobilization of either SAP [30, 31] or L-selectin [32] may enhance their affinity for respective ligands. SAP has recently been described to be highly absorbed from plasma on biomedical materials such as polystyrene, promoting Ca^{2+}-dependent leukocyte adhesion [31]. We found previously that circulating L-selectin plasma concentrations drop by almost 50% in infants undergoing open heart surgery employing cardiopulmonary bypass [33], which may be due to trapping of soluble L-selectin by SAP absorbed onto the surface of plastic tubings.

L-Selectin molecules are clustered at the tip of leukocyte microvilli [34] and are therefore candidates to explain the binding of SAP observed in leukocytes [35]. Indeed, in a fashion analogous to GlyCAM-1 [14], SAP elicited rapid activation of β2 integrins on human lymphocytes, as assessed by expression of the mAb 24-recognized neoepitope. Thus, by complexing surface-expressed L-selectin, SAP provides signals important for the recruitment of lymphocytes into peripheral lymphoid organs. The extent of β2 integrin neoepitope up-regulation observed with SAP was similar to that published for GlyCAM-1 (136% *vs.* 144%). While almost complete suppression of GlyCAM-1-induced β2 integrin neoepitope up-regulation has been reported by Dreg56 and Dreg200 Fab fragments [14], SAP-induced up-regulation was only partially inhibited by Fab fragments of the anti-L-selectin mAb Dreg55 and Dreg200. Both mAb are directed against other non-overlapping epitopes of the lectin domain and have been reported to inhibit lymphocyte binding to high endothelial venules [36]. Either SAP affects β2 integrin affinity *via* additional pathways, *e.g.* Fcγ receptor binding [18], or the L-selectin binding sites of the two mAb are not close enough to the SAP binding site for complete competition.

While SAP and GlyCAM-1, the first L-selectin ligand to be identified on a molecular level [1], bear no structural homologies, we propose that human SAP could play part of the role GlyCAM-1 has in murine blood. In addition to being the only L-selectin ligand not found in humans, GlyCAM-1 is also unique among L-selectin ligands in that it is a secretory product [5]. GlyCAM-1 present in mouse serum interferes with L-selectin-mediated cell attachment [37] and appears to trap soluble L-selectin present in murine plasma, although its solution affinity has been found to be low [32, 37]. On a molar scale, GlyCAM-1 concentrations in normal adult mouse serum (~1.3–1.6 μg/mL, or 26–32 nmol/L) [38] slightly exceed those of soluble L-selectin (~1.7 μg/mL, equivalent to 23–26 nmol/L) [39]. As does GlyCAM-1, soluble L-selectin inhibits lymphocyte attachment to activated endothelium *in vitro* [9], and injection of soluble L-selectin into mice inhibits neutrophil migration into inflammatory sites [40] or lymphocyte homing to peripheral lymph nodes [39]. Thus, while both GlyCAM-1 and soluble L-selectin have the capability to negatively affect leukocyte migration, they appear to mutually neutralize their inhibitory activity. As SAP concentrations in human plasma [19, 20] exceed those of soluble L-selectin [9, 12], SAP may act as a sink for soluble L-selectin. So far, none of the numerous reports on soluble L-selectin plasma concentrations in various clinical settings has discriminated between free and bound soluble L-selectin, and SAP may modulate the functional consequences of altered circulating L-selectin concentrations.

Material and methods

Antibodies and affinity chromatography matrices

The monoclonal anti-SAP antibodies CBL304 and CBL305 were from Cymbus Biotech (Chandlers Ford-Hants, UK). The mAb Dreg55 and Dreg200 (murine IgG1 isotype), directed against the lectin domain of human L-selectin [41], were isolated from culture supernatants of hybridoma cell lines, kindly provided by Dr. E. C. Butcher (Stanford University, Palo Alto, CA) using protein G-Sepharose 4FF (AmershamBiosciences) affinity chromatography. CBL305, Dreg55 and Dreg200 were biotinylated with biotin-ε-amidocaproate-N-hydroxysuccinimide ester (Sigma) according to standard procedures, to calculated substitution rates of 30 biotin moieties per mAb. Recombinant human L-, E- and P-selectin-IgG1 Fc chimeras were commercially obtained from R&D Systems (Wiesbaden, Germany), and recombinant human soluble L-selectin lacking the cytoplasmic and transmembrane domain (SLP4) and a similar cDNA construct bearing a 6His-tag at its C terminus (6His-SLP4) were expressed in baby hamster kidney cells as described [42]. Purity of soluble L-selectin and L-selectin chimeras was checked by SDS-PAGE followed by silver staining or Dreg200-mediated immunodetection (Fig. 1, 2). Antibodies (2.5 mg/mL gel), SLP4 (1.5 mg/mL gel), or asialofetuin (Sigma; 2.5 mg/mL) were coupled to BrCN-activated Sepharose 4B, and 6His-SLP4 was coupled to HiTrap NHS-activated Sepharose HP according to the manufacturer's instructions (AmershamBiosciences). 6His-SLP4 ~ anti-6His-tag mAb ~ protein G-Sepharose matrix was made by sequential reaction of anti-6His-tag mAb (Serotec, Düsseldorf, Germany) and 6His-SLP4 with ProteinG-Sepharose4 Fast Flow (AmershamBiosciences).

Isolation of L-selectin-binding proteins and human L-selectin from human plasma

For each batch, 1–1.5 L of fresh-frozen human plasma (Charité Virchow Blood Bank, Berlin) were thawed, adjusted to 5 mM Ca^{2+} (final concentration) and allowed to stand at room temperature for 2 h. NaN_3 was added to this and all subsequent steps to a final concentration of 0.05%. After stirring, the resulting fibrin clot was precipitated by 1-h centrifugation at 4°C and 2000 × g. The supernatant was diluted with an equal volume of 5 mM $CaCl_2$ adjusted to pH 7.5, stored overnight at 4°C, and then cleared by another round of centrifugation (2000 × g) and filtration (0.45 μm).

For isolation of the supposed L-selectin-Ca^{2+}-ligand complexes, the serum solution was loaded at 4°C and 1 mL/min onto a 15-mL Dreg55-Sepharose 4B column, previously equilibrated with 20 mM Tris adjusted to pH 7.5 containing 5 mM Ca^{2+}. Unbound material was washed out with equilibration buffer until absorbance at 280 nm reached baseline. Elution of bound L-selectin ligands was performed with 20 mM Tris (pH 7.5) containing 10 mM EDTA. The protein-containing fractions were pooled, centrifuged for 1 h

at 4°C and 2000 × g, and the clear supernatant was concentrated by ultrafiltration (Centriplus YM-10; Millipore, Bedford, MA), yielding approximately 10–30 mg protein per 1000 mL of starting plasma.

To remove residual soluble L-selectin, the crude ligand preparation was run repeatedly over a 5-mL Dreg55-Sepharose 4B column containing 10 mM EDTA as running buffer, followed by elution with 0.1 M glycine (pH 2.6) until no more protein was bound by the column. This was followed by similar runs over a combination of two columns containing murine IgG1-Sepharose 4B (2.5 mg/mL gel) and ProteinG-Sepharose4 Fast Flow in the presence of 5 mM Ca^{2+}, to remove unspecifically bound material and contaminating human IgG.

The final ligand purification was achieved by repeated affinity chromatography runs over 5 mL SLP4-Sepharose 4B, 1 mL 6His-SLP4-Sepharose HP or 1 mL 6His-SLP4 ~ anti-6His-SLP4 mAb ~ protein G-Sepharose 4B at 1 mL/min in 20 mM Tris (pH 7.3) or 20 mM Tris-buffered saline (TBS), respectively, each containing 5 mM Ca^{2+}, as running buffers, and 20 mM Tris or TBS containing 10 mM EDTA as elution buffers. Pooled elution peaks were transferred to 20 mM Tris (pH 7.3) by ultrafiltration.

After elution of L-selectin ligands from Dreg55 columns by EDTA, soluble L-selectin was eluted by 0.1 M glycine (pH 2.6), followed by immediate neutralization with 1 M Tris (pH 9.0). Protein-containing fractions were pooled, cleared by centrifugation (1 h, 2000 × g, 4°C) and concentrated by ultrafiltration. Unspecifically bound material and contaminating human IgG was removed by three runs over two columns containing mouse IgG1-Sepharose 4B (2.5 mg/mL gel) and ProteinG-Sepharose4 Fast Flow (20 mM Tris, pH 7.5/5 mM Ca^{2+}) until no more protein was eluted from the columns with 0.1 M glycine, pH 2.6. Final purification was performed by Dreg200-Sepharose 4B affinity chromatography with 20 mM Tris pH 7.5 containing 10 mM EDTA as running buffer, followed by elution with 0.1 M glycine (pH 2.6) into 1 M Tris pH 9.0-filled vials. The purity of soluble L-selectin was checked by 12.5% SDS-PAGE under nonreducing conditions followed by silver staining and immunodetection of L-selectin by Dreg55.

Protein sequencing by mass spectroscopy

After preparative SDS-PAGE (NuPAGE System; Invitrogen, Karlsruhe, Germany), proteins (7.5–10 μg) of the purified ligand preparation were separated by SDS-PAGE followed by mass spectroscopy-compatible silver staining. Bands were excised from the gels, destained with 50 mM $Na_2S_2O_3$/15 mM $K_3Fe(CN)_6$, sequentially washed with water and digestion buffer (25 mM ammonium bicarbonate), dehydrated with acetonitrile and vacuum-dried in a SpeedVac concentrator. Digestion was performed with sequencing-grade trypsin (Promega, Madison, WI) by adding 2–3 μL of a solution containing 20 ng/μL trypsin in digestion buffer to each of the gel pieces. After 30 min of proteolysis at 37°C, a further 10 μL of digestion buffer was added and digestion was continued overnight. The supernatants were concentrated and desalted on ZipTips (Millipore).

For matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) analysis, the peptides were eluted with 1 μL 50% acetonitrile/0.1% trifluoroacetic acid (TFA) onto the MALDI target. Of a saturated α-cyano-4-hydroxycinnamic acid solution in 70% acetonitrile/0.1% TFA, 1 μL was added to each sample. Spectra were acquired on a Voyager-DESTR (Perseptive Biosystems, Framingham, MA). The peptide mass fingerprints obtained were analyzed searching the NCBI non-redundant (NCBI nr) protein database with the ProFound (http://prowl.rockefeller.edu/cgi-bin/ProFound) or Mascot software (http://www.matrixscience.com).

Native and SDS-PAGE

PAGE was carried out in a native fashion with 90 mM Tris/80 mM boric acid buffer (pH 8.4) containing 2.5 mM Ca^{2+} or EDTA, respectively, or in the presence of SDS using precast gels (12.5%, 7.5%, and 4–15% gradient gels), buffer strips, ECL reagents and the PhastSystem including the semidry blotting system from AmershamBiosciences. Proteins were detected by silver staining in gels or after blotting [43] after specific reaction with mAb directed against human L-selectin (Dreg55) or SAP (CBL305), or after reaction with human L-selectin-IgG1 chimera or SAP in the presence of 5 mM Ca^{2+}, followed by biotinylated mouse anti-human IgG (Fc specific) (JacksonImmunoResearch). The immunoblots were blocked with 50% horse serum (Biochrom) or 5% membrane blocking agent (Amersham Biosciences) in TBS containing 0.05% Tween-20, with 5 mM Ca^{2+} for experiments when SAP was detected by L-selectin-IgG1 chimera or L-selectin-IgG1 chimera by SAP.

Biotinylation, desialylation, and de-N-glycosylation of SAP or L-selectin chimera

Human SAP was biotinylated with biotin-ε-amidocaproate-N-hydroxysuccinimide ester (Sigma) according to standard procedures to calculated substitution rates of ten biotin moieties per SAP decamer, and tested for quality by 12.5% SDS-PAGE or 4–15% native PAGE, followed by silver staining or biotin detection on polyvinylidene difluoride blots using ExtrAvidin-peroxidase conjugates (Sigma) and the ECL system (Amersham Biosciences). The biotinylation had no influence on the apparent molecular weights of SAP monomers or decamers.

For desialylation, 0.7 mg biotinylated SAP was transfered into 70 μL 100 mM acetate buffer pH 5.0 by ultrafiltration using YM-10 Centricon units (Millipore) and incubated at 37°C for 24 h with 10 μL sialidase (100 mU; Arthrobacter ureafaciens sialidase; Roche). Thereafter, the product was transferred into PBS (0.02% NaN_3) by ultrafiltration.

To remove N-linked carbohydrates, 0.7 mg biotinylated SAP was transferred into 70 μL 250 mM sodium phosphate buffer pH 8.6 by ultrafiltration using YM-10 Centricon units (Millipore) and incubated at 37°C for 18 h with 10 μL glycopeptidase F (25 U, PNGase F; Sigma). Thereafter, the product was transferred into PBS (0.02% NaN_3) by ultrafiltration. The same reaction was carried out with 80 μg L-selectin-IgG chimera and 3 μL glycopeptidase F.

SAP binding assays

In a first series of experiments, 96-well microtiter plates (F16 black maxisorp; Nunc, Wiesbaden, Germany) were coated

with 50 μL/well 10 μg/mL human selectin-IgG Fc chimeras (R&D Systems) in 50 mM carbonate buffer (pH 9.65; 0.02% NaN_3) for 18 h at 5°C, washed three times with 150 μL/well TBS pH 7.4, containing 0.05% Tween-20, 0.02% NaN_3, and 5 mM Ca^{2+} or EDTA, respectively, and then incubated for 1.5 h at 37°C with 50 μL/well of Dreg200-biotin or SAP-biotin in TBS (pH 7.4; 0.05% Tween-20, 0.02% NaN_3, 5 mM Ca^{2+} or EDTA). After washing the wells as above, they were incubated for 1 h at 37°C with 60 μL/well 1 : 1000 dilution of ExtrAvidin-alkaline phosphatase (Sigma) in the same buffers. Finally, after three times washing as above, the wells were incubated at room temperature with 70 μL/well 0.5 mM CSPD (Roche Diagnostics, Mannheim, Germany) in 0.1 M Tris buffer (pH 9.5; 0.1 M NaCl, 50 mM Mg^{2+}, 5 mM Ca^{2+}) and the emitted light, given in relative light units (RLU), was measured after 10–15 min using a luminometer (ML1000; Dynatech, Denkendorf, Germany).

In a second series of experiments, wells were coated as above with human L-selectin-IgG1 Fc chimera or human IgG1 (both from R&D Systems). After three times washing with 150 μL/well TBS (pH 7.4; 0.05% Tween-20, 0.02% NaN_3, 5 mM Ca^{2+}), the wells were incubated for 1.5 h at 37°C with 50 μL/well 1 μg/mL biotinylated SAP, together with different concentrations of one of the following agents: Dreg200 (or Dreg55), mouse IgG1 (R&D Systems), asialofetuin, α2-macroglobulin, fucoidan, heparin (all from Sigma), purified human serum albumin (DRK-Blutspendedienst, Springe, Germany; depleted of soluble L-selectin and SAP by three runs over a Dreg55-Sepharose column in the presence of 5 mM Ca^{2+}; residual content of soluble L-selectin <5 fMol/mL, SAP 0.56 ng/mL) in TBS (pH 7.4; 0.05% Tween-20, 0.02% NaN_3, 5 mM Ca^{2+}). The amount of bound SAP was again determined by reaction with ExtrAvidin-alkaline phosphatase and CSPD.

SPR studies

Binding to L-selectin under shear stress was studied in real time by SPR on a BIAcore 2000 instrument (BIAcore AB, Uppsala, Sweden). SPR arises when light is reflected from a conducting film at the interface between the sample and the sensor chip, causing a reduction in the intensity of reflected light at a specific angle of reflection when molecules in the sample bind to the sensor surface. SPR response values are being expressed in resonance units (RU), with 1 RU representing a change of 0.0001° in the angle of the intensity minimum. Equal amounts of commercially obtained SAP or BSA (control) were covalently bound at pH 4.0 to the carboxymethylated dextran matrix on separate tracks of CM5 chips using the EDC-NHS method (BIAcore). As analytes, L-selectin-IgG chimera was bound to 20-nm (Sigma) or 40-nm (RDI, Flanders, NJ) gold particles coated with protein G as follows: 10 mL gold particles was incubated for 1 h at ambient temperature under rotation with 300 μL protein G (2 mg/mL in purified water). After centrifugation for 20 min at 36 000 × g (20 nm Au) or 15 000 × *g* (40 nm Au), the supernatant was carefully removed and the pellet was resuspended in 10 mL reaction buffer [10 mM Hepes pH 7.4, 150 mM NaCl, 0.005% polyoxyethylenesorbitan (P20)]. This was repeated twice, and the final pellet was resuspended to 240 μL. Of the protein G ~ gold conjugate, 50 μL was added to 5 μL chimera [7 μg/μL in HBSS (0.6 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.125% human serum albumin)] in a siliconized microtube, immediately mixed by slow vortexing and allowed to react overnight at 4°C. L-Selectin-chimera ~ protein G ~ gold conjugates were diluted with reaction buffer (1 : 50 to 1 : 100) just prior to SPR, performed at flow rates of 40 μL/min.

Flow chamber assay

Human umbilical vein endothelial cells were prepared after dispase digestion as described [44] and used within the second or third passage. Peripheral blood neutrophils were obtained from healthy volunteers by Ficoll-Hypaque (Amersham biosciences) centrifugation and hypotonic lysis of contaminating red blood cells as described [45]. For shear flow experiments, endothelial cells were exposed to near physiologic flow conditions using a flow chamber with parallel-plate geometry as described [46]. The effective wall shear stress at constant flow rate was a function of the width of the flow chamber, which widens hyperbolically along the flow direction. Therefore, wall shear stress decreases linearly with increasing distance from the flow chamber entrance. At a flow rate of 117 μL/min, the wall shear stress varied between 0.45 and 2.6 dyn/cm^2. The assembled flow chamber was placed on the stage of an inverted Zeiss microscope (Zeiss, Oberkochen, Germany) and its temperature was maintained throughout the experiment at 37°C by bypassing water. An injection port at the entrance permitted the addition of cell suspensions to the main stream of the perfusion medium (medium 199, containing 20 mmol/L Hepes and 0.1% BSA). The perfusion medium was prewarmed to 37°C and the flow rate was maintained by a perfusion pump (Fresenius, Bad Homburg, Germany). Usually, 100 μL of a freshly prepared neutrophil suspension (10^6 cells/mL) was added and adhesion was allowed to take place during a 5-min interval. The number of firmly attached cells was counted after 5 min, with the perfusion medium still flowing. Firm adhesion represents a composite measure of attachment rate, detachment rate and rolling velocity, referred to as rolling adhesion.

Up-regulation of the mAb 24-reactive activation-related β2 integrin neoepitope

β2 integrin activation in response to L-selectin ligation was examined by assessing expression of the activation-related β2 integrin neoepitope on human PBL recognized by the mAb 24 [15], as previously employed for the murine soluble L-selectin ligand GlyCAM-1 [14]. Human PBMC were isolated from freshly drawn blood by centrifugation over a Ficoll cushion (AmershamBiosciences) and transferred into PBS (w/o Ca^{2+}, Mg^{2+}; 0.5% BSA, 2 mM EDTA) to 5 × 10^7 cells/mL. Monocytes were removed by two rounds of negative depletion using CD14 microbeads (100 μL/mL cell suspension; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. More than 98% of monocytes could be removed as detected by size/granularity parameters and CD14 staining (Diatec, Oslo, Norway) by flow cytometry.

For measurement of mAb 24 binding, 5 × 10^5 lymphocytes in 200 μL HBSS (0.6 mM Ca^{2+}, 0.2 mM Mg^{2+}, 0.125% human serum albumin) were pre-incubated at 22°C for 10 min with

15 μg/mL mAb 24 (kindly provided by Dr. Nancy Hogg and Dr. Alison McDowall, Cancer Research UK, London, UK) or murine IgG1 control (R&D Systems) and 15 μg/mL FITC-conjugated $F(ab')_2$ goat anti-mouse IgG (H+L, Fc-directed) (Dianova, Hamburg, Germany) and then for a further 1 min with or without 11.4 μg/mL Fab fragments of the anti-L-selectin mAb Dreg55 and Dreg200. These Fab fragments had been prepared using the ImmunoPure Fab Preparation Kit according to the manufacturer's instructions (Pierce, Perbio Science, Bonn, Germany) and found to block the adhesion of freshly prepared neutrophils to TNF-α-activated endothelial cells under stress as described below. Cells were warmed to 37°C and the addition of stimulants (2.5 or 5 μg/mL SAP or 3 mM Mn^{2+}) was counted as time point zero. For measurement of fluorescence, 3000 events were collected within 10 s at time point zero and after 10 min of incubation at 37°C under stirring. The up-regulation of the mAb 24-recognized β2 integrin neoepitope observed with Mn^{2+} (positive control) was similar to published data (175% *vs.* 180%).

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