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Staphylococcal SSL5 binding to human leukemia cells inhibits cell adhesion to endothelial cells and platelets

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Abstract. Bacterial proteins provide promising tools for novel anticancer therapies. Staphylococcal superantigen-like 5 (SSL5) was recently described to bind P-selectin glycoprotein ligand-1 (PSGL-1) on leukocytes and to inhibit neutrophil rolling on a P-selectin surface. As leukocytes and tumor cells share many characteristics in migration and dissemination, we explored the potential of SSL5 as an antagonist of malignant cell behavior. Previously, it was demonstrated that rolling of human HL-60 leukemia cells on activated endothelial cells was mediated by P-selectin. In this study, we show that SSL5 targets HL-60 cells. Binding of SSL5 was rapid and without observed toxicity. Competition of SSL5 with the binding of three anti-PSGL-1 antibodies and P-selectin to HL-60 cells identified PSGL-1 as the ligand on HL-60 cells. Presence of sialyl Lewis x epitopes on PSGL-1 was crucial for its interaction with SSL5. Importantly, SSL5 not only inhibited the interaction of HL-60 cells with activated endothelial cells but also with platelets, which both play an important role in growth and metastasis of cancers. These data support the concept that SSL5 could be a lead in the search for novel strategies against hematological malignancies.

Keywords: Leukemia, HL-60, *Staphylococcus aureus*, SSL5, PSGL-1

1. Introduction

Successful metastasis of tumor cells often involves hematogenous dissemination. Thereby, tumor cells need to pass through the blood stream and extravasate from the blood vessels into peripheral organ tissues. While traveling through the bloodstream, interactions with normal host cells like leukocytes, platelets, or endothelial cells are of crucial importance. The ability of tumor cells to bind platelets in the blood stream may be of particular importance as stabilized platelet-enriched thrombi can physically protect tumor cells from destruction or facilitate in tissue adherence and formation of metastatic foci [10,12]. Adhesion to the endothelial lining is also required and involves specific adhesion receptors present on the tumor cells and various adhesive ligands on the endothelium. Initial leukocyte adhesion is mediated by selectins that interact with sialylated, fucosylated lactosaminoglycans such as sialyl Lewis x (sLex) on the cell surface or predominantly when displayed by mucin-like glycoproteins. Receptors important for leukocyte adhesion were shown to play an important role in malignancies as well [22,46]. In this respect, P-selectin was shown to mediate rolling of human HL-60 leukemia cells through P-selectin glycoprotein-1 (PSGL-1) [1]. Immunohistochemical analysis of PSGL-1 in normal prostate tissue and in localized and metastatic prostate tumors revealed that PSGL-1 was detected on the surfaces of bone-metastatic prostate tumor cells. These findings implicated a functional role of PSGL-1 in the bone tropism of prostate tumor cells [14]. In addition, interactions between tumor cells, platelets and endothelial cells are not only important for dissemination and metastasis formation, but also play an important role in tumor-induced angiogenesis. Inhibition of

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these interactions inhibit tumor-induced angiogenesis [27,44].

Conventional cancer therapies traditionally focus on tumor cell death. However, there is a need for new therapies that provide additional value to the already existing therapies. These therapies focus on modulating tumor behavior such as migration and adhesion. Tumor cell migration and metastasizing share many similarities with leukocyte trafficking, which is critically regulated by chemokines and their receptors. Bacteria produce chemokine receptor inhibitors to prevent neutrophil migration and extravasation towards the infection site to escape clearance by innate immune cells [13,34,36]. Recently, staphylococcal superantigen-like (SSL) proteins were shown to play a role in immune evasion [11,28]. SSLs are a family of 14 secreted staphylococcal proteins homologous to superantigens but lacking superantigenic activity [3]. We demonstrated that SSL5 binds to PSGL-1 on leukocytes [6]. Thereby, SSL5 inhibits neutrophil rolling on endothelial cells by disrupting the interaction of PSGL-1 with its natural ligand P-selectin. Sugar moieties were shown to be crucial in this effect. Recently, the crystal structure of SSL5 in complex with sialyl Lewis x (sLex) was determined, and it was shown that SSL5 binds sLex through its C-terminal domain [5]. SSL5 thus inhibits the initial activation and migration of neutrophils to the site of infection.

In this study we describe staphylococcal SSL5 binding to PSGL-1 expressed on human leukemic HL-60 cells. SSL5 inhibited cell adhesion of these leukemia cells to P-selectin expressed on activated human umbilical vein endothelial cells (HUVEC). In addition, SSL5 also inhibited the interaction between the tumor cells and platelets. We describe for the first time inhibition of the interaction between P-selectin and PSGL-1 on leukemic cells by SSL5, thus providing for an attractive new perspective into novel anticancer therapies.

2. Materials and methods

2.1. Antibodies

The monoclonal anti-PSGL-1 antibodies PL1 (function-blocking [31], clone 3E2.25.5) and PL2 (non-blocking [31], clone 5D8.8.12) were obtained from Serotec (Oxford, United Kingdom), while KPL1 (function-blocking [40]) was purchased from BD Biosciences (San Jose, CA, USA). Polyclonal goat anti-mouse Ig-FITC was from Dako (Heverlee, Belgium) and goat anti-human IgG (Fc specific)-FITC from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cloning, expression and purification of SSL5

SSL5 was cloned and expressed as described before [6]. Briefly, the SSL5 gene of the NCTC8325 *S. aureus* strain was cloned into the pRSETB expression vector (Invitrogen, Paisley, United Kingdom) directly downstream of an enterokinase cleavage site and a poly-histidine tag. The vector was transformed in BL21 (DE3) *Escherichia coli* (Novagen, Darmstadt, Germany) for protein expression. The histidine-tagged protein was purified using nickel affinity chromatography (HiTrap chelating HP, GE Healthcare, Piscataway, NJ, USA) and cleaved with enterokinase. Finally, SSL5 was stored in PBS, and its purity was examined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

2.3. Cells

The human promyelocytic HL-60 leukemic cell line was obtained from ATCC (Rockville, MD, USA). Cells were maintained in RPMI 1640 containing L-glutamine (BioWhittaker, Walkersville, MD, USA) with 10% FCS (Biochem AG, Laufelfingen, Switzerland) and 10 μg/ml gentamicin (Gibco, Paisley, United Kingdom).

HUVECs were isolated as described by Jaffe et al. [21]. Freshly-isolated cells were grown to confluent monolayers in endothelial growth medium-2 (Clonetics, Waltersville, MD, USA). Informed consent was obtained from all subjects and was provided in accordance with the Declaration of Helsinki. Approval was obtained from the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

Platelets and red blood cells were isolated from venous blood obtained from healthy volunteers using sodium citrate as anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation (15 min at 200g at room temperature) and acidified by addition of ACD (0.25% trisodium citrate, 0.15% citric acid and 0.2% D-glucose). Platelets were centrifuged (500g, 10 min), and resuspended in HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid)-Tyrode buffer (10 mM HEPES, 137 mM NaCl, 2.68 mM KCl, 0.42 mM NaH_2PO_4, 1.7 mM $MgCl_2$, 5 mM D-glucose, pH 6.5) with prostacyclin (PGI_2, 10 ng/ml) to prevent activation during the following washing step. Platelets were centrifuged and resuspended in HEPES-Tyrode buffer (pH 7.4). Red blood cells were washed twice (2000g, 5 min) with 0.9% NaCl containing 5 mM D-glucose, and finally the cells were packed (2000g, 15 min).

2.4. Binding assays

To determine binding of SSL5 to cells, SSL5 was first labeled with FITC as previously described [6]. HL-60 cells (5×10^6 cells/ml) were incubated with 0–10 μg/ml SSL5-FITC in RPMI 1640 containing 25 mM HEPES and L-glutamine (BioWhittaker), that was supplemented with 0.05% human serum albumin (HSA; Sanquin, Amsterdam, The Netherlands) (RPMI/HSA) for 30 min on ice. After washing, fluorescence was measured with a flow cytometer. For binding of SSL5 over time, HL-60 cells (5×10^6 cells/ml) were incubated with 10 μg/ml SSL5-FITC at 37°C. At several time points, samples were taken and fluorescence was measured. For competition experiments, HL-60 cells were first incubated with 0–10 μg/ml SSL5 for 30 min on ice. After washing, the cells were incubated with 1 μg/ml P-selectin/Fc chimera (R&D Systems) or anti-PSGL-1 mAbs PL1, PL2, or KPL1 for 30 min on ice, washed, and stained with 20 μg/ml goat anti-mouse Ig-FITC or 10 μg/ml goat anti-human IgG (Fc specific)-FITC, respectively. Where indicated, HL-60 cells were first treated with 0.2 U/ml neuraminidase (from *Clostridium perfringens*, Roche Diagnostics, Almere, The Netherlands) for 45 min at 37°C.

2.5. Viability assays

HL-60 cells (5×10^6 cells/ml) were treated with 10 μg/ml SSL5 in RPMI/HSA. At several time points (up to 360 min) samples were taken and immediately stained with 1 mg/ml trypan blue (Merck, Dormstadt, Germany). The number of viable cells was quantified by counting at least 100 cells and calculating the percentage of trypan blue negative cells.

2.6. Platelet to HL-60 cell binding

Platelets (2×10^8/ml) were labeled with 2 μM calcein-AM for 20 min at room temperature. HL-60 cells (1×10^7/ml) were labeled with 2 μM PKH26 according to manufacturer's protocol (PKH26 RED Fluorescent cell linker kit, Sigma). After washing, HL-60 cells were incubated with 0–10 μg/ml SSL5 or 10 μg/ml KPL1 for 15 min at room temperature. After washing, HL-60 cells were incubated with platelets (1:400) for 1 or 10 min, and platelet binding to HL-60 cells was measured with a flow cytometer. Where indicated platelets were treated with 4 μg/ml collagen for 15 min prior to staining. For binding analysis, HL-60 cells were first identified through side scatter and FL-2 fluorescence (PKH26 RED), eliminating possible selection of platelet aggregates through side scatter analysis. Subsequently, binding of platelets to gated HL-60 cells was examined through FL-1 fluorescence (calcein-AM).

2.7. Adhesion of HL-60 cells on P-selectin, HUVECs, and platelets under flow conditions

To study the effect of SSL5 on the rolling adhesion of HL-60 cells, a flow chamber was used as previously described [6]. Glass coverslips were coated with 10 μg/ml P-selectin/Fc for 1 h at 37°C and blocked with 1% HSA for 1 h. After washing with PBS, the slips were inserted into the flow chamber. HL-60 cells (4×10^6 cells/ml) were kept in perfusion buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$ 1.2 mM KH_2PO_4, with 5 mM glucose, 1 mM $CaCl_2$, and 0.5% HSA) on ice until use. They were put at room temperature for 15 min prior to treatment with 0–30 μg/ml SSL5 or 10 μg/ml anti-PSGL-1 mAb KPL1 for 15 min at 37°C. After dilution to 2×10^6 cells/ml, HL-60 cells were perfused over the P-selectin/Fc surface for 5 min at a shear stress of 0.8 dyn/cm^2 and the cover slips were washed for 1 min. Recording and analysis of data was performed as described earlier [6]. The number of adherent cells was quantified for at least 40 adjacent high power fields recorded along the perfusion chamber (total surface at least 1 mm^2). In other experiments, freshly isolated HUVECs (passage 0) were directly grown to cobblestone confluent monolayers on coverslips precoated with glutaraldehyde-linked gelatin. They were stimulated for 3 min with 100 μM histamine (Sigma) to induce surface expression of P-selectin, and HL-60 cells were immediately perfused at 0.8 dyn/cm^2. To examine HL-60 cell rolling adhesion on platelets, coverslips were first coated with extracellular matrix (ECM) proteins by lysing confluently-grown HUVECs with 0.1 M NH_4OH. Subsequently, freshly isolated platelets were mixed with red blood cells to 2×10^5 platelets/μl and a hematocrit of 40%, and the platelets were perfused over the coverslips to create an activated, P-selectin expressing, platelet-coated surface. After washing with perfusion buffer, HL-60 cells were immediately perfused at 0.8 dyn/cm^2.

2.8. Supplementary data

Supplementary data include videos (available online at: http://www.qub.ac.uk/isco/JCO/).

3. Results

3.1. SSL5 binds to HL-60 leukemia cells

SSL5 binding was investigated to the human promyelocytic HL-60 leukemia cell line. For this purpose, recombinant SSL5 was first labeled with fluorescein (SSL5-FITC). Figure 1(a) shows that HL-60 cells stained positive for SSL5-FITC, and that the binding was dose dependent. Subsequently, binding kinetics of SSL5 were examined. SSL5 quickly bound to maximal levels in 3 min, an effect that was sustained for the full 10 min measured (Fig. 1(b)). Finally, we measured possible toxic effects of SSL5 on HL-60 cells. After 6 h, 97% of SSL5-treated HL-60 cells were identified as viable cells by trypan blue staining, confirming that SSL5 has no direct toxic activity on HL-60 cells.

3.2. SSL5 binds PSGL-1 on HL-60 cells

As SSL5 targets PSGL-1 on leukocytes [6], its ability to block the binding of several monoclonal antibodies directed against PSGL-1 was tested on HL-60 cells. SSL5 dose-dependently inhibited the binding of the two function-blocking antibodies PL1 and KPL1, while binding of the non-blocking antibody PL2 was slightly affected (Fig. 2(a) and (b)). More importantly, binding of P-selectin, the natural ligand for PSGL-1, was also fully blocked when HL-60 cells were treated with SSL5. Thus, SSL5 targets HL-60 cells through PSGL-1.

3.3. SSL5 binding to PSGL-1 is glycan dependent

SSL5 binds sLex [5] and its interaction with leukocytes is indeed described to be glycan-dependent [6]. Therefore, the importance of sugar moieties in the SSL5 binding to HL-60 cells was investigated. For this purpose, HL-60 cells were treated with neuraminidase, which removes sialic acids and thereby disturbs the sLex epitope important for SSL5 binding. While SSL5 binds to untreated HL-60 cells, binding was completely disrupted after treatment with neuraminidase (Fig. 2(c)). Subsequently, the competition experiment with SSL5 and anti-PSGL-1 antibodies was examined on neuraminidase-treated cells as well. Upon treatment, SSL5 did not inhibit the binding of anti-PSGL-1 to HL-60 cells (Fig. 2(d)), confirming the importance of sLex epitopes in binding of SSL5 to PSGL-1.

3.4. SSL5 inhibits rolling adhesion of HL-60 cells on endothelial cells

Cancer metastasis and leukemic organ infiltration require initial adhesive interactions through selectins. Therefore, we first investigated whether HL-60 cells can interact with a P-selectin surface under shear conditions. Figure 3(a) and (b) shows that HL-60 cells were indeed capable of rolling on P-selectin (Suppl. videos S1 and S2: http://www.qub.ac.uk/isco/JCO/). SSL5 dose-responsively blocked the rolling adhesion, reaching full inhibition at a concentration of 30 μg/ml. Subsequently, rolling adhesion assays were performed with HL-60 cells and histamine-stimulated HUVECs under shear conditions, mimicking the interaction between leukemic cells and the P-selectin expressing endothelial vessel walls. SSL5 strongly and dose-dependently inhibited the interaction between HL-60 cells and HUVECs (Fig. 3(c) and (d), Suppl. videos S3 and S4: http://www.qub.ac.uk/isco/JCO/). KPL1 also effectively blocked HL-60 cell rolling on P-selectin and HUVECs (Fig. 3(a) and (c), Suppl.

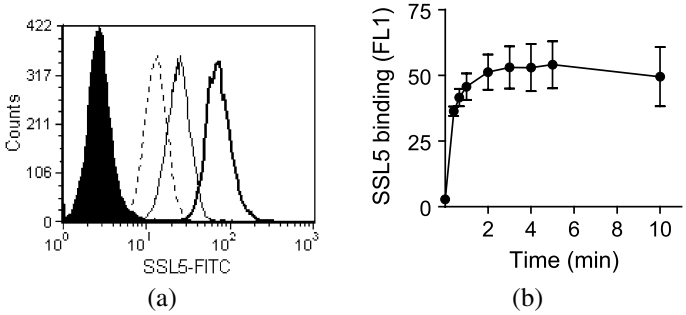

Fig. 1. SSL5 binding characteristics to HL-60 leukemia cells. (a) HL-60 cells were incubated with 0 (black histogram), 1 (dashed line), 3 (thin continuous line) or 10 (thick continuous line) μg/ml SSL5-FITC. Binding was assayed 30 min after incubation at 4°C. Results are representative of three independent experiments. (b) For binding kinetics, HL-60 cells were treated with 10 μg/ml SSL5-FITC at 37°C. At several time points fluorescence was measured. Data are mean values ± SEM of three independent experiments.

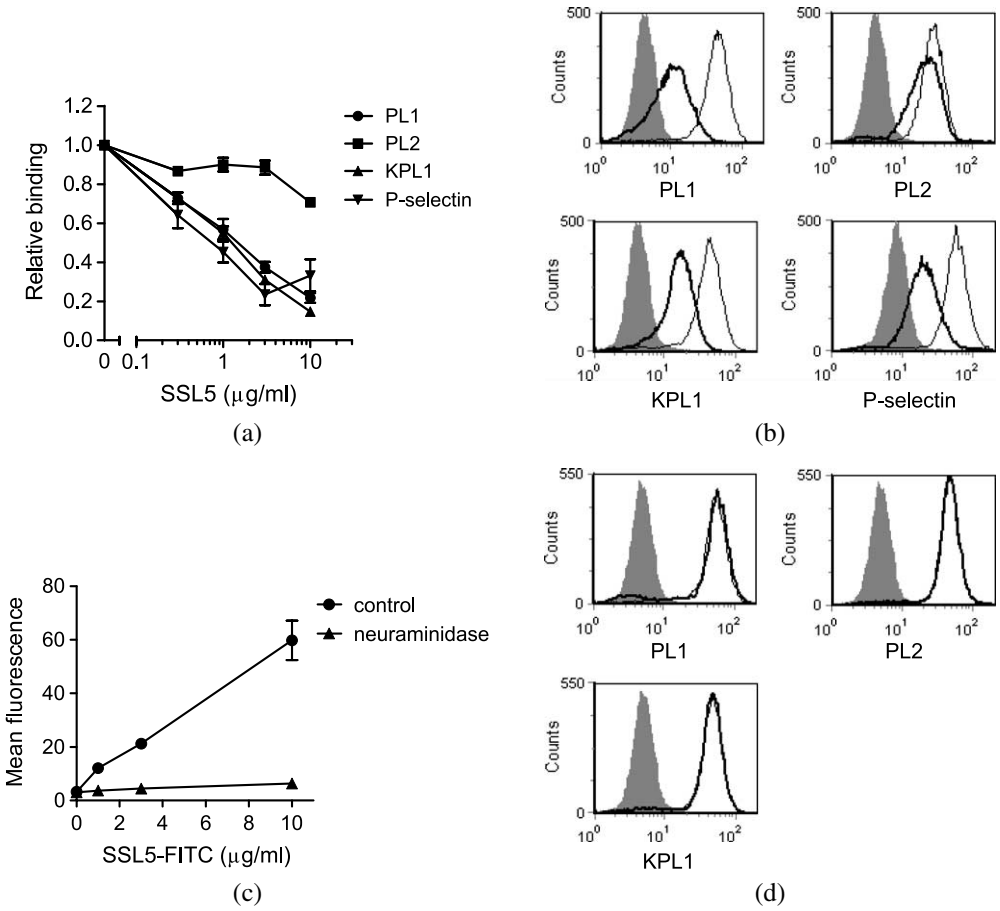

Fig. 2. SSL5 binds PSGL-1 on HL-60 cells in a glycan-dependent manner. (a) HL-60 cells were treated with 0–10 μg/ml SSL5 for 30 min at 4°C. Subsequently, binding of 1 μg/ml P-selectin or anti-PSGL-1 mAbs PL1, PL2, or KPL1 was examined. Data represent relative ligand binding to cells treated with SSL5 compared to control-treated cells and are mean values ± SEM of four independent experiments. (b) Histograms depict binding of PL1, PL2, KPL1, or P-selectin to HL-60 cells with (thick line) or without (thin line) pretreatment with 10 μg/ml SSL5. Grey histograms represent cells only stained with the secondary antibody. Results are representative of three independent experiments. (c) HL-60 cells were treated with 0.2 U/ml neuraminidase for 45 min at 37°C and examined for binding of 0–10 μg/ml SSL5-FITC. Data are mean values ± SEM of three independent experiments. (d) Binding of 1 μg/ml anti-PSGL-1 mAbs to neuraminidase-treated HL-60 cells preincubated with (thick line) or without (thin line) 10 μg/ml SSL5. Grey histograms represent cells only stained with the secondary antibody. Representative of three experiments.

video S5: http://www.qub.ac.uk/isco/JCO/), indicating that the rolling adhesion was PSGL-1-dependent. Thus, SSL5 functionally inhibits HL-60 cells rolling adhesion through PSGL-1 binding.

3.5. *SSL5 inhibits binding of HL-60 cells and platelets*

Blood platelets are also involved in the process of cancer metastasis. Not only was P-selectin shown to be important for the cancer cell–platelet interaction [20,24,30,41], but an increased mucin expression and glycosylation on cancer cells also contributed to cancer metastasis [22,25,32]. As SSL5 binds to PSGL-1, its role in tumor cell–platelet interaction was examined. First, SSL5 effects on platelet aggregation were tested in a standard platelet aggregometer. In platelet-rich plasma, SSL5 did not induce platelet activation (data not shown). Subsequently, HL-60 cell interaction with an activated platelet surface was examined. For this purpose, HL-60 cells in the presence or absence of SSL5 were allowed to roll on a surface coated with activated platelets that express P-selectin. Figure 4(a) and (b) shows that SSL5 blocked HL-60 cell rolling adhesion to activated platelets (Suppl. videos S6 and S7: http://www.qub.ac.uk/isco/JCO/). KPL1 also inhibited HL-60 cell adhesion to platelets, confirming PSGL-1-

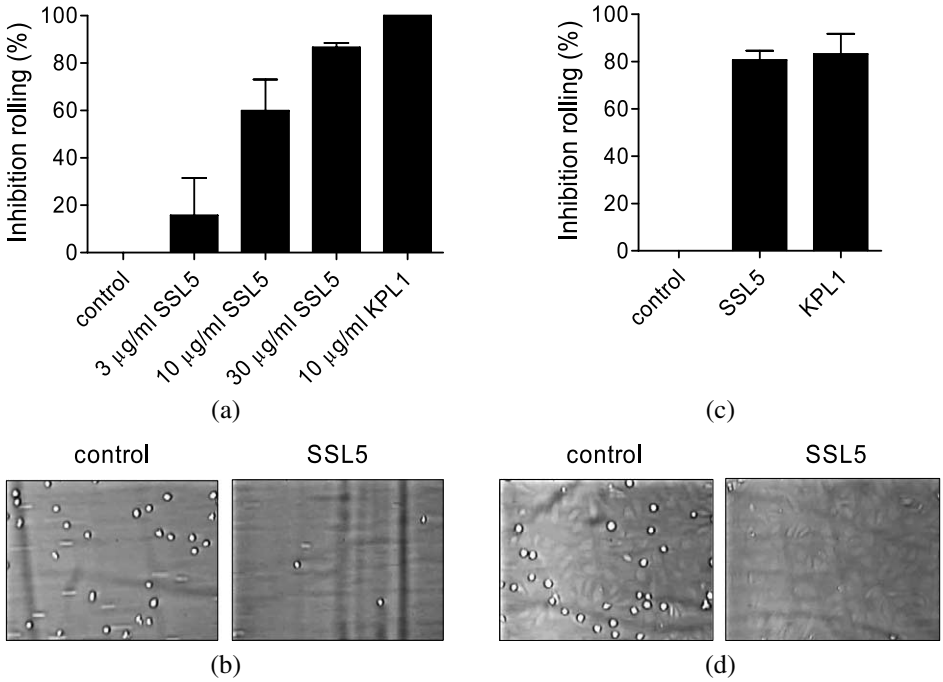

Fig. 3. SSL5 inhibits HL-60 cell rolling on P-selectin and endothelial cells. (a and b) Coverslips were coated with P-selectin and HL-60 cells pretreated with 0–30 µg/ml SSL5 or 10 µg/ml KPL1 for 15 min at 37°C were perfused for 5 min. After washing, accumulated HL-60 cells were quantified to assess rolling adhesion of the cells. (c and d) Freshly isolated HUVECs were cultured on glass coverslips. After stimulation of HUVECs with 100 µM histamine for 3 min for P-selectin surface expression, HL-60 cells pretreated with 30 µg/ml SSL5 or 10 µg/ml KPL1 for 15 min at 37°C were perfused for 5 min. The data of the antagonists were calculated relative to control-treated cells and are mean values ± SEMs of 3 independent experiments. Screen shots represent control- and SSL5-treated HL-60 cell rolling after 5 min of perfusion over a P-selectin (b) and endothelial cell surface (d).

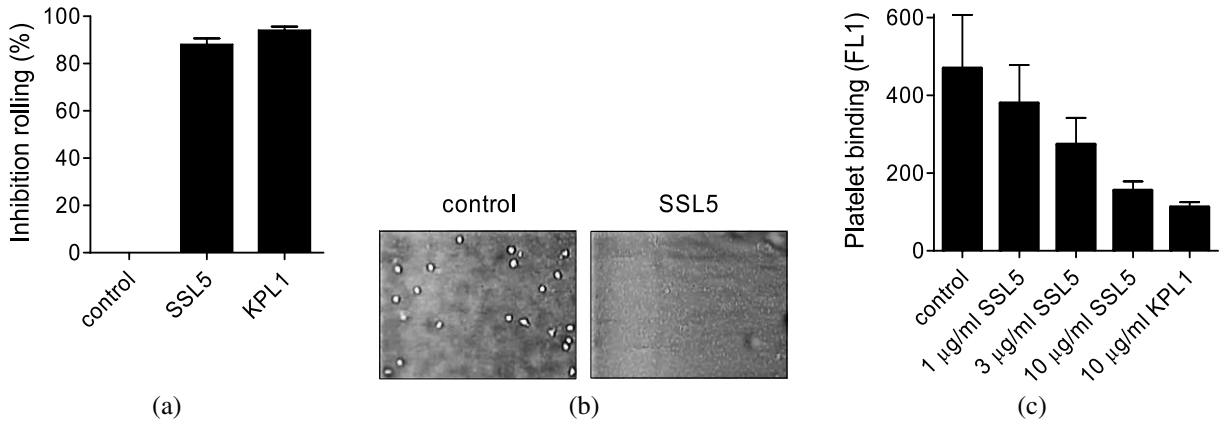

Fig. 4. SSL5 inhibits the interaction between platelets and HL-60 cells. (a and b) Effect of SSL5 on HL-60 cell rolling on activated platelets. An activated platelet-coated surface was generated by perfusing platelets with erythrocytes over a HUVEC-derived ECM surface. After washing, HL-60 cells treated with 30 µg/ml SSL5 or 10 µg/ml KPL1 were immediately perfused for 5 min. The data of the antagonists were calculated relative to control-treated cells and are mean values ± SEMs of 3 independent experiments. (b) Screenshots represent control- and SSL5-treated HL-60 cell rolling after 5 min of perfusion over an activated platelet surface. (c) HL-60 cells were incubated with 0–10 µg/ml SSL5 or 10 µg/ml KPL1 for 15 min. After washing, binding of calcein-labeled platelets to HL-60 cells was examined after a 10 min incubation by flow cytometry. Data represent mean fluorescence values ± SEM of four independent experiments.

dependent effects. As it is described that under *in vitro* and *in vivo* settings malignant cells also bind platelets in suspension, we also tested the effect of SSL5 on the interaction of HL-60 cells and platelets under these settings. Inhibition of the interaction between the two cell types by KPL1 confirmed PSGL-1 dependency of the assay (Fig. 4(c)). Under these conditions, SSL5 blocked binding of platelets to HL-60 cells in a concentration-dependent manner; 10 μg/ml SSL5 was comparable to the effect observed for KPL1. Time kinetics show that the platelet to HL-60 cell binding was already observed within 1 min and was affected by SSL5 (data not shown). The platelet–HL-60 interaction was also abrogated by SSL5 when collagen-activated platelets with higher P-selectin expression were used (data not shown). In conclusion, SSL5 is effective in inhibiting the interaction between tumor cells and platelets.

4. Discussion

In this study, the staphylococcal protein SSL5 is put forward that modifies tumor cell behavior through a novel mechanism. SSL5 targets PSGL-1 on HL-60 leukemia cells and thereby modifies their interactions with platelets and endothelial cells; interactions which are highly relevant during hematological dissemination. For more than a century, bacteria and bacterial products have been used for the treatment of cancer [39]. Starting from the practical observation of tumor regression in individuals with concomitant bacterial infection, the field has evolved into some standard clinical practices, such as the use of BCG for the treatment of superficial bladder cancer. However, in the last few years, new applications have started to emerge that may profoundly change the perspective of the field. Not only whole bacteria but also single bacterial products have been implicated in anti-cancer therapies [9]. Staphylococcal SSL5 is a potential candidate for such a therapy by interfering with malignant cell dissemination.

The understanding that receptors important for leukocyte adhesion also play an important role in malignancies and are thus important candidates to target, led to an increasing literature on cell adhesion molecules and cancer metastasis. Several carcinomas and cancer cell lines are described to bind P-selectin [4], and positive staining for P-selectin on intratumoral vessels was found to be negatively associated with overall survival in melanoma patients [38]. Studies with P-selectin-deficient mice demonstrated significantly slower growth of subcutaneously implanted human colon carcinoma cells and generated fewer lung metastases from intravenously injected cells [24]. In addition to binding P-selectin on endothelial cells, platelets provide a significant source of P-selectin for carcinoma binding [41]. Platelets promote survival of tumor cells as they directly protect them from lysis by natural killer cells *in vitro* as well as *in vivo* [33]. In a mouse model of experimental metastasis, tumor seeding of three different tumor cell lines in the target organs was reduced when the host was platelet depleted, but only if the tumor cells were NK sensitive. Additionally, P-selectin on platelets was shown to allow the formation of platelet-tumor aggregates as P-selectin-deficient mice show less coating of platelets on tumor cells [24]. The platelet-tumor aggregates seemed beneficial in metastasis formation. SSL5 interferes with HL-60 binding to platelets. This inhibition could be detrimental for tumor cells upon entering the bloodflow. Borsig et al. [8] demonstrated that sialomucin and P-selectin interaction is important for the tumor–platelet interaction *in vivo*. Removal of mucins on tumor cells attenuated metastasis by disruption of the interaction between the tumor cells and platelets.

An increasing literature documents that *in vivo* binding of tumor cells to platelets at least involves expression of P-selectin on platelets. However, it is uncertain whether naive platelets possibly express low levels of P-selectin that enable tumor cell binding, or whether the P-selectin expression on platelets is induced by the tumor cells themselves [23]. In the latter case, tumor cells are described to produce agonists such as tissue factor that lead to platelet activation and P-selectin expression [47]. In our assay examining platelet–tumor cell interactions in suspension, we clearly observed binding of platelets to HL-60 cells. This interaction was also enhanced by preactivating platelets with low amounts of collagen, which induces P-selectin expression. In both the conditions using naive and collagen-preactivated platelets, the platelet to HL-60 cell binding was inhibited by SSL5. The naive platelet preparation thus most likely also displayed some P-selectin. This minor expression could probably have been caused by prior platelet isolation and/or calcein-labeling procedure. Tumor-induced P-selectin expression on the platelets was less likely to have occurred, as maximal platelet–HL-60 interactions were already achieved within a minute. Nevertheless, low platelet activation may be representative of the thrombotic status often associated with cancer

patients. Individuals diagnosed with cancer experience a 4.1-fold increased risk of venous thromboembolism when compared to the general population. That risk increased an additional two-fold following the administration of chemotherapy [19,47]. Importantly, SSL5 itself does not induce platelet activation in platelet-rich plasma.

P-selectin requires the carbohydrate ligand sLex presented on PSGL-1 for binding. PSGL-1 mediates rolling of human HL-60 leukemia cells on activated endothelial cells through P-selectin [1]. Clinical relevance of PSGL-1 was demonstrated by the enhanced expression of PSGL-1 on the surfaces of prostate cancer cells and the implication of PSGL-1 in the tropism of these tumor cells for bone tissues [14]. Recently, the significance of PSGL-1 in metastasis was supported in an invasive and metastatic T-cell lymphoma model. Raes et al. [35] demonstrated that, although overexpression of PSGL-1 may not be sufficient for successful dissemination, PSGL-1 played a crucial role in the hematogenous, disseminative properties in organ colonization of the lymphoid cancer cells investigated. Malignant cell transformation is commonly associated with increased and altered expression of glycans at the cell surface and the aberrant glycosylation is a prominent feature of carcinoma progression [16]. Among the glycans associated with cancer cells, sLex expression on cancer cells is known to be correlated with a poor prognosis and an increased rate of metastasis [32]. Many anti-cancer therapies have therefore focused on targeting sLex epitopes. Compounds mimicking sLex, compounds and antisense-DNA strategies directed against enzymes responsible for sLex synthesis [45] and antibodies against sLex [26] have all proven to be beneficial in mouse models. SSL5 binds sLex [5], and we demonstrated here that SSL5 binding to sLex on PSGL1 inhibits PSGL-1-dependent leukemic cell adhesion.

PSGL-1 requires sialylated, fucosylated O-linked glycans and tyrosine sulfate to bind P-selectin [29, 37]. SSL5 also targets PSGL-1 through the same ligand determinants. Here we show that SSL5 binds PSGL-1-bearing sLex on HL-60 cells. Our previous experiments using CHO-PSGL-1 cells demonstrated that SSL5 also requires sulfation for optimal binding of PSGL-1 [6]. It is therefore likely that sulfation is also necessary for binding of PSGL-1 on HL-60 cells. Another point of interest is that PSGL-1 is not only a P-selectin ligand but is also described to bind L-selectin and E-selectin. The L-selectin ligand determinants of PSGL-1 overlap that of P-selectin, but PSGL-1 does not require tyrosine sulfate to bind E-selectin [17,29,43]. It is possible that through targeting of PSGL-1, SSL5 not only inhibits the interaction with P-selectin, but also with its other ligands L-selectin and E-selectin. This will be the subject for future research. Finally, as SSL5 targets sLex, it might also bind other sLex-bearing glycoconjugates. CD44 is a widely expressed cellular adhesion molecule that is a well-characterised receptor for hyaluronan but also functions as a selectin ligand when properly glycosylated. CD44 variants with P-selectin-, L-selectin- and/or E-selectin-binding capacity carry sLex-epitopes [15,18]. Carcinoembryonic antigen (CEA or CD66e) is another sialofucosylated glycoprotein that mediates selectin binding. CEA possesses E- and L-selectin but not P-selectin ligand activity, and it also carries the sLex epitope [2,42]. Both glycoproteins are implicated in cancer metastasis. Due to the expression of sLex, SSL5 may target these glycoconjugates. Our previous experiments with leukocytes, however, demonstrate that sLex is presumably not the only binding determinant of SSL5 to sLex-carrying glycoproteins [6,7]. Other determinants such as sulfates and specific amino acid residues also seem to play a role.

In conclusion, this study demonstrates that SSL5 can alter malignant cell behavior by targeting PSGL-1 and interfere with its binding to P-selectin. SSL5 not only blocks adhesion of HL-60 malignant leukemia cells to endothelial cells but also to platelets. Not unimportantly, SSL5 binding to HL-60 cells and PSGL-1 is glycan dependent. Therefore, SSL5 is a promising, potential bacterial-derived lead for novel cancer therapies as it inhibits the adhesive properties of leukemia cells.

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