Pathol. Oncol. Res. (2012) 18:989–996
DOI 10.1007/s12253-012-9531-y

RESEARCH

Activated Platelets Interact with Lung Cancer Cells Through P-Selectin Glycoprotein Ligand-1

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Received: 30 August 2011 / Accepted: 29 March 2012 / Published online: 26 April 2012

Abstract Hematogenous metastasis always leads to the poor prognosis of non-small cell lung cancers (NSCLC). Activated platelets are involved in hematogenous metastasis and may be a potential therapeutic target. P-selectin is an important adhesion molecule and expressed on the surface of activated platelets. P-selectin glycoprotein ligand-1 (PSGL-1) as a transmembrane protein is expressed on the surface of various cell types. P-selectin can bind to PSGL-1, and thereby initiate the platelet-mediated cell adhesion. The aim of the study was to investigate the degree of platelet activation in NSCLC and the roles of PSGL-1 in the activation of platelets. Purified platelets were obtained from NSCLC patients (40 lung adenocarcinomas and 26 lung squamous cell carcinomas), and P-selectin expression was detected by fluorescence-activated cell sorter. The population of peripheral blood platelets with P-selectin expression in lung adenocarcinoma was 63.16±25.44 %, and significantly higher than that in lung squamous cell carcinoma (35.97±17.19 %) and the healthy population (9.12± 7.66 %, n=30). A specific small hairpin RNA (shRNA) for PSGL-1 was transfected into A549 human alveolar cell carcinoma cells**.** The expressions of PSGL-1 mRNA and protein were significantly reduced with the PSGL-1 shRNA (p<0.01). Furthermore, the knockdown of PSGL-1 also resulted in the significantly reduced aggregate formation of activated platelets and A549 cells. Thus, activated platelets may interact with lung cancer cells through PSGL-1. Inhibiting platelet activation and/or down-regulating PSGL-1 expression may be useful for suppression of tumor metastasis.

Keywords Lung cancer · Platelet activation · P-selectin · P-selectin glycoprotein ligand 1 · RNA interference

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Introduction

Lung cancer is the most common cause of cancer-related death in the world. Non-small cell lung cancer (NSCLC) comprises approximately 80 % of lung cancers [1]. Hematogenous and lymphatic metastases are the most common metastatic pathways. In the earlier stages, lung cancer cells metastasizes through the lymphatic system. If hematogenous metastasis occurs, the disease often develops into an advanced stage. Therefore, hematogenous metastasis is often associated with a poor prognosis of lung cancer patients, and can accelerate death of lung cancer patients [1, 2].

The hematogenous metastasis of tumors mainly includes three steps: (i) the tumor cells pass through the vascular endothelial cells of the tumor tissues and move out of their original position; (ii) the tumor cells move via blood, and (iii) the tumor cells become implanted at the metastatic position. Under normal hemodynamic conditions, most tumor cells are destroyed because of the intolerance to the shear forces under flow conditions or because of attack by the immune system in the blood [3, 4]. However, a few tumor cells can induce platelet activation and make the platelets congregate around the tumor cells, thereby leading to the aggregate formation of platelets and tumor cells. Currently, some researchers have demonstrated that platelet activation during circulation occurs in many types of malignant tumors. Since they are involved in

hematogenous metastasis, activated platelets may be a potential therapeutic target [5, 6].

It has previously demonstrated that platelet count and aggregation are significantly increased in patients with highly metastatic lung cancer, especially hematogenously metastatic lung cancer, compared with healthy controls and lung cancer patients without hematogenous metastasis [7]. Studies have indicated that platelet activation was also closely correlated with the hematogenous metastasis of lung cancer [8]. However, it remains unclear about how activated platelets affect the hematogenous metastasis of lung cancer.

The P-selectin on platelets is an important adhesion molecule, which is mainly present in platelet α-particles. After platelet activation, the P-selectin can be rapidly inserted into the membrane and expressed on the platelet surface. Hence, P-selectin is often regarded as a marker for platelet activation [9]. P-selectin glycoprotein ligand 1 (PSGL-1) is a transmembrane glycoprotein with a homologous dimer structure and expressed on the surface of almost all types of leukocytes and cancer cells, such as human colon carcinoma, lymphomas, human metastatic prostate tumor cells and so on [10–12]. Studies have demonstrated that PSGL-1 was a common physiologic ligand used by P-selectin, L-selectin and E-selectin, and could induce physiologic and pathologic processes through adhesion to the selectins [12–14]. Recent studies have shown that PSGL-1 might be closely correlated with the hematogenous metastasis of some tumors. Dimitroff et al. found that PSGL-1 was highly expressed in the bone metastatic focus of prostatic carcinoma [10]. Raes et al. also found that PSGL-1 was involved in the hematogenous metastasis of lymphomas [12]. Recent studies have also indicated that cancer cell-derived microparticles that express PSGL-1 played a key role in thrombus formation in vivo, and these microparticles could be the target for the prevention of thrombosis and for limiting metastasis in cancer patients [15]. All of the studies have indicated that PSGL-1 expression was different in various tumor cells and it might be related to tumor metastasis, although the specific mechanism remains unclear. Further research is required to determine whether the important effects of activated platelets on the hematogenous metastasis of tumors depends on P-selectin or PSGL-1.

For this purpose, the short interfering RNA (siRNA) interference method was used to study the effects of platelet activation during circulation, as well as the effects of P-selectin and PSGL-1 expression on the hematogenous metastasis of lung cancer.

Materials and Methods

Subjects

A total of 127 cases, including 97 patients (54.12±8.69 years) and 30 healthy controls (43.86±4.72 years) were included in the current study. The patients were diagnosed with primary lung cancer in the Department of Respiratory Diseases, Southwest Hospital, the Third Military Medical University, from March 2009 to May 2010. These patients had no blood diseases, severe malnutrition, infection, cardiovascular diseases, diabetes, and other interfering factors. 97 patients were definitively diagnosed using histopathologic methods: 26 patients with squamous cell carcinoma (53.62±9.41 years, 14 men and 12 women), 40 patients with lung adenocarcinoma (54.02±9.19 years,13men and 27 women), and 31 patients with small cell lung cancer (54.13±8.69 years, 20 men and 11 women). In our study, only NSCLC were included, and 31 cases with small cell lung cancer were excluded. The patients did not take any drugs that would affect platelet function 2 weeks before drawing blood samples. According to the physical examinations performed in our hospital, 30 healthy individuals, including 19 men and 11 women, were assigned as the control group. This study was conducted in accordance with the declaration of Helsinki. This study was conducted with approval from the Ethics Committee of the Third Military Medical University. Written informed consent was obtained from all participants.

Separation of Platelets

Platelets were isolated according to the following steps: (i) blood samples were collected from each of 66 patients and 30 control subjects after arising on an empty stomach in the morning. Then, 7 mL of whole blood with the anticoagulant ethylenediaminetetraacetic acid-K_3 was obtained and centrifuged at 2500 rpm for 12 min at room temperature. (ii) After stratification, the plasma at the upper layer was obtained and placed into another centrifuge tube and then centrifuged at 500 rpm for 6 min to obtain the platelet-rich plasma (PRP) in the upper layer. (iii) The PRP was centrifuged at 3200 rpm for 30 min at 4 °C, and the platelets were collected after discarding the supernatant. (iv) The collected platelets were washed twice with 0.01 M of phosphate-buffered saline (PBS; 3200 rpm, 30 min, 4 °C). At this time, the platelets were ready for testing.

P-Selectin Expression Assay Using Fluorescence-Activated Cell Sorter (FACS)

The platelet activation was reflected by the P-selectin expression on the platelets in peripheral blood from patients with NSCLC. After the platelets were separated and purified, 50 μL of 0.01 M PBS was added in each tube to resuspend the platelets. Then, 5 μL of the P-selectin antibodies (1:50) were added and the samples were incubated for 1 h at 37 °C. The samples were then washed twice with 0.01 M PBS (3000 rpm, 3.5 min). After discarding the

supernatant, 5 μL of the FITC-labeled secondary antibodies (1:50) were added to each tube, and the samples were washed twice with 0.01 M PBS. Samples were analyzed by flow cytometry.

shRNA Vector Construction

The pSilencer GFP 2.0-U6 plasmid was purchased from the GenScript Corporation. According to the PSGL-1 gene sequence, three specific siRNA sequences for PSGL-1 (GI: 68160948) were designed using the siRNA design tool "siRNA sequence selector". The sequences were as follows: A) 5′-CCACGGATTCAGCAGCTAT-3′; B) 5′-GGAGAT ACAGACCACTCAA-3′, and C) 5′-GGAAGCACAGA CCACTCAA-3′. The construction of the pSilenser-PSGL-1-shRNA vector was completed by the Shanghai GeneChem Co., Ltd. (China), and the steps were as follows: the siRNA oligonucleotides were annealed to form the target double-stranded DNA, followed by digestion with *Bam*HI/*Hind*III restriction enzymes and ligated into the linearized pSilenser 2.0-U6 digested with the same enzymes and then transformed into competent *Escherichia coli* DH5α and screened on ampicillin-containing plates to produce the recombinant pSilenser-PSGL-1-shRNA vector, and the positive colonies were picked for sequencing and culture. Subsequently, the plasmids were extracted and digested with *Bam*HI/*Hin*dIII after identification through enzyme digestion and sequencing. After the primary experiment, the silencing effect of the B sequence was the most efficient and used for the following transfection.

Cell Transfection

A549 human alveolar cell carcinoma cells were purchased from the Shanghai Cell Bank (China), and cultured in Dulbecco's modified Eagle's medium supplemented with 10 % fetal calf serum at 37 °C under 95 % air-5 % CO_2. The pSilenser-PSGL-1-shRNA or the pSilencer GFP 2.0-U6 plasmid was introduced into A549 cells according to the instructions for Lipofectamine™ 2000. Briefly, 1 day before transfection, $0.5–2\times10^5$ cells were inoculated into six-well plates. Transfection mixtures containing Solution A (5 μg plasmid+100 μL OptiMEM medium) and Solution B (10 μL Lipofectamine™ 2000+100 μL OptiMEM medium) were prepared in 96-well plates. When A549 cells reached 70 % confluence, cells were transfected with the DNA of the pSilenser-PSGL-1-shRNA and the pSilencer GFP 2.0-U6 plasmid as mock transfection control, respectively. The cells were incubated for 4 h at 37 °C. Subsequently, the transfection medium was replaced with complete medium, and the cells were incubated for 44 h at 37 °C. The transfection efficiency of the cells was observed under an inverted phase contrast microscope and a fluorescence microscope. After transfection for 48 h, the cells were removed from the plates and washed twice with precooled PBS (1500 rpm, 5 min), resuspended and adjusted to a concentration of 20×10^6/L, and selected using the FACS to obtain the mock-transfected or shRNA-transfected GFP-positive cells.

RT-PCR Assay

According to the PSGL-1 gene sequence (GenBank accession no. 68160948), the primers were designed using the Primer Premier 5.0. The primer sequences were 5′-TGAGTCTA CCACTGTGGAGCC-3′ (sense) and the downstream primer was 5′-GGTTGAGTGGTCTGTATCTCC-3′ (antisense). The primer sequences used for the internal reference β-actin were 5′-ACCCATCACCATCTTCCAGGAG-3′ (sense) and the downstream primer was 5′-GAAGGGGCGGAGATGA TGAC-3′ (antisense).

The RNA was extracted from A549 cells pre- and post-transfected with pSilenser-PSGL-1-shRNA plasmid or the mock control, and subjected to real-time quantitative PCR using SYBR Master Mixture (TakaRa Co., Ltd. Japan) in an iQ5 real-time PCR detection System (Bio-Rad). The PCR amplification procedures were as follows: 94 °C, 2 min, 94 °C, 30 s, 53 °C, 30 s, 72 °C, 60 s, 29 cycles, 72 °C, 10 min. After amplification, 10 μL of the PCR product was detected on 1.5 % agarose gels, observed and photographed under an ultraviolet lamp. The integrated optical density (IOD) ratio of PSGL-1 to the β-actin products was calculated. And the IOD ratio represents the relative expression of PSGL-1.

Western Blot Assay

The A549 cells pre- or post-transfection with pSilenser-PSGL-1-shRNA plasmid or the mock control were collected and dissolved in lysis buffer (7 M of urea, 2 M of thiourea, and 4 % of CHAPS), and centrifuged at 40,000×g for 1 h. The supernatant was then collected. Protein concentrations were determined using Bradford protein assays (Bio-Rad); 25–50 μg of protein was analyzed by 2 % SDS-PAGE, transferred to polyvinylidene fluoride membranes, and blocked overnight with 5 % non-fat milk. The membranes were incubated with rabbit anti-PSGL-1 polyclonal antibodies (1:600; Santa Cruz, USA) at room temperature for 4 h. The membrane was washed three times with Tris-buffered saline (TBS) for 5 min each. After washing, secondary anti-rabbit horseradish peroxidase-IgG (1:2000; Golden Bridge, China) was added, and the membranes were incubated for 2 h at room temperature. The membrane was washed once with TBS for another 5 min. The blots were then developed with chemiluminescent (ECL) reagents (Pierce, Rockford, USA) and imaged on X-ray film by autoradiography. β-actin was used as the control, and Quantity One software

(Bio-Rad, California, USA) was used to measure the band intensity.

Co-immunoprecipitation

The interaction of platelet P-selectin and PSGL-1 of A549 cells was detected by co-immunoprecipitation method, the activated platelets and inactivated platelets were co-cultured with untransfected A549 cells and GFP-positive transfected A549 cells. Inactivated platelets were defined the platelets from the health populations, in which the ratio of p-selectin expression was less than 10 %, while the activated platelets were from the patients with lung cancer, in which the ratio of p-selectin expression was more than 50 %. The activated platelets could bind to PSGL-1 of A549 cells through P-selectin, but the inactivation platelets cannot do through the P-selectin. After co-cultivation, the platelets were washed off gently with PBS for 2–3 times until the platelets were not observed under microscope. Followed, the total proteins in A549 cells and GFP-positive transfected A549 cells were extracted, and the anti-PSGL-1 antibody was used to precipitate the PSGL-1 protein. In the Western blot analysis, the anti-PSGL-1 antibody and anti- P-selectin antibody were used to detect PSGL-1 and P-selectin, respectively. The experiment were divided into five groups: (i) untransfected A549 cells; (ii) co-cultivation of A549 cells and inactivated platelets; (iii) co-cultivation of A549 cells and activated platelets; (iv), co-cultivation of A549 cells transfected with the pSilencer GFP 2.0-U6 plasmid(as mock trasfection control)and activated platelets; (V) co-cultivation of A549 cells transfected with pSilenser-PSGL-1-shRNA plasmid and activated platelets. Only the activated platelets could express P-selectin highly. Antibodies (1–2 μg) were added to the Eppendorf tubes that were slowly shaken overnight (12–16 h) at 4 °C. The prepared "protein A+G" agarose was added to the Eppendorf tubes, and the tubes were slowly shaken at 4 °C for 2–3 h to ensure that the antibodies coupled with the "protein A+G" agarose beads. The "protein A+G" agarose in the Eppendorf tubes was washed with 500 μL cytolytic solution and centrifuged (1000×g, 4 °C for 5 min). Then the wash solution was removed by absorption. The aforementioned steps were repeated 4–5 times. After the last washing, the supernatant was removed and 20–40 μL of PBS was added; the precipitate was resuspended with vortex mixer, and the protein concentration was determined.

Electron Microscopy

On the day before coculturing, 1×10^5 of A549 cells were inoculated on slides in cell-culture plates, and culture plates were placed in an incubator containing 5 % CO_2 at 37 °C. After 24 h, activated platelets were inoculated onto the slides in cell culture plates and cultivated. After 48 h of cocultivation, the cells were collected to conduct subsequent tests. Platelets were isolated from each of the 40 patients with lung cancer, and 29 platelet samples showed the ratio of p-selectin expression more than 50 %.

The specimen-processing steps for scanning under electron microscopy were as follows: (i) The cell specimens fixed with electron microscope fixation solution were extracted, and the fixation solution was removed with a pipette; then, the specimens were washed twice with 0.9 % saline (10 min every time). (ii) Alcohol solutions (30 %, 50 %, 70 %, 80 %, 90 % and 100 %) were used to perform gradient dehydration (once for 30 %, 50 %, 70 %, 80 % and 90 % of alcohol, and twice for 100 % of absolute alcohol, respectively; 4 min every time). (iii) Tert-butyl alcohol solutions (50 %, 70 %, 90 %, 95 % and 100 %) were used to perform gradient substitution (once for 50 %, 70 %, 90 % and 95 % of tert-butyl alcohol, and twice for 100 % tert-butyl alcohol, respectively; 4 min every time;). 100 % tert-butyl alcohol was maintained at 40 °C, otherwise it was easily solidified. Finally, the slides were removed and pasted on the original plate. At this time, they were ready for testing.

Statistics Analysis

All experiments were performed at least three times, and the data are expressed as mean±standard deviation. Data were analyzed using a one-way ANOVA. A p value of less than 0.05 was considered significant.

Results

FACS Assay and Selecting

P-selectin belongs to the family of selectin adhesion molecules, and the P-selectin expression is also considered the major markers of platelet activation [9]. To evaluate the platelet activation in different patients with lung cancer, the P-selectin expression on the surface of activated platelets were compared between patients and healthy subjects by FACS method. The FACS results showed that the P-selectin expression as a platelet activation indicator was significantly higher in the peripheral blood platelets of the patients with lung adenocarcinoma compared with the healthy controls and patients with squamous lung cancer (Fig. 1). The population of peripheral blood platelets with P-selectin expression in lung adenocarcinoma patients (63.16±25.44 %, n=40) was significantly higher than those in squamous cell carcinoma patients (35.97±17.19 %, n=26) and in the healthy controls (9.12±7.66 %, n=30)(Fig. 1).

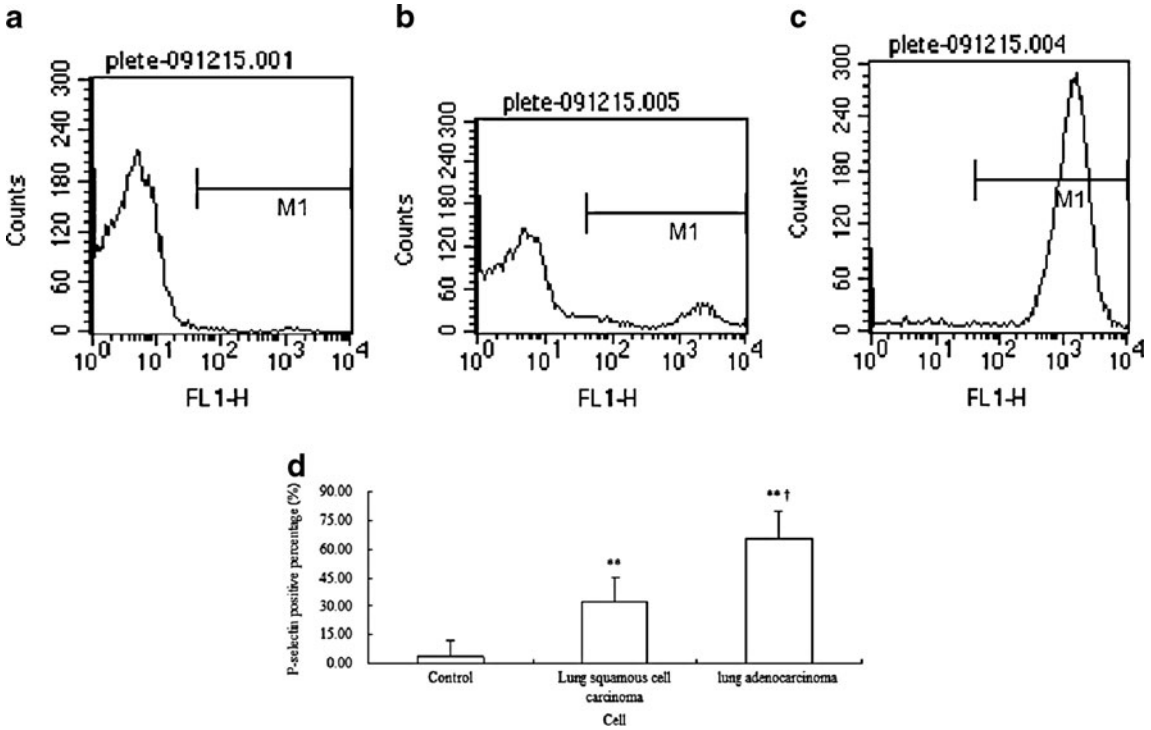

Fig. 1 P-selectin activation on platelets in the peripheral blood of patients with NSCLC. (A) Healthy control. (B) Lung squamous cell carcinoma. (C) Lung adenocarcinoma. M1 represents P-selectin-positive platelets. (D) The percentage of P-selectin-positive platelets. Healthy control subjects (n=30, 9.12±7.66 %, each sample repeated three times), patients with squamous cell carcinoma (n=26, 35.97±17.19 %, each sample repeated three times), and patients with Lung adenocarcinoma (n=40, 63.16±25.44 %, each sample repeated three times). The value was obtained by the calculation of the mean of samples of each group. $^{**}p<0.01$ versus healthy control; $^{+}p<0.05$ versus lung squamous cell carcinoma

Isolation of GFP-Positive A549 Cells

The double-stranded PSGL-1 shRNA or the empty plasmid was transiently expressed in A549 cells. After transfection, A549 cells were selected with FACS to obtain the GFP-positive cells. The GFP-positive A549 cells that were transfected with shRNA were 32.4 % (Fig. 2).

PSGL-1 Gene Expression

To observe whether the PSGL-1 gene expression was knocked down by shRNA, we measured the expression

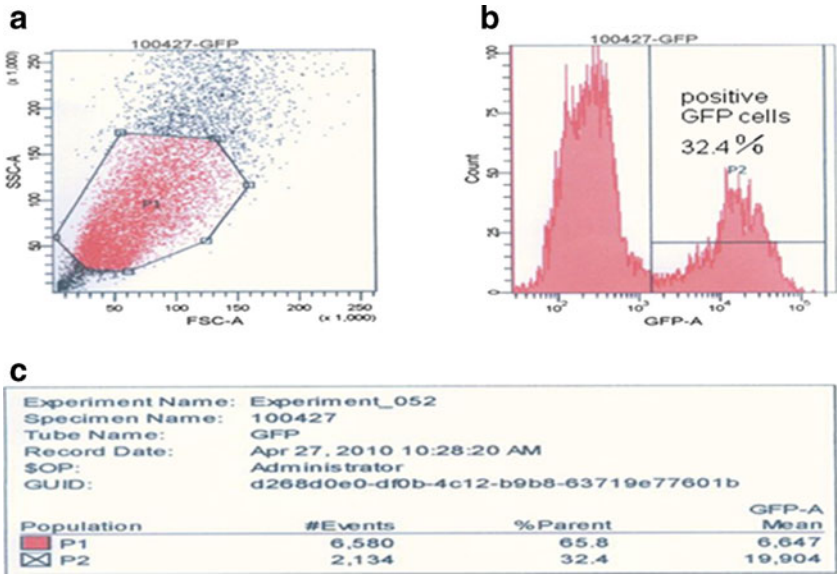

Fig. 2 Section of the GFP-positive cells transfected with the pSilenser-PSGL-1-shRNA plasmid by FACS. After transfection for 48 h, the cells were removed from the plates and washed twice with precooled PBS, resuspended, adjusted the concentration, and selected using the FACS to obtain the GFP-positive cells. **a** The distribution of main cell populations (P1) under FACS screening. **b** The count of the GFP-positive cells (P2). (C) The ratio of P1 and P2 in the all screened cell populations

levels of PSGL-1 mRNA and protein using RT-PCR and Western blot analysis. Among three designed siRNA sequences, B sequence could effectively silence the PSGL-1 gene expression (data not shown).

The relative expression level of PSGL-1 mRNA was significantly lower in the PSGL-1 shRNA transfected A549 cells than that in the untransfected A549 cells ($p<0.05$; Fig. 3). Consistent with the above result, the PSGL-1 protein expression was reduced by 80 % after transfection with the pSilenser-PSGL-1-shRNA plasmid ($p<0.01$; Fig. 4).

Co-immunoprecipitation Assay

The interaction of platelet P-selectin and PSGL-1 of A549 cells was detected by co-immunoprecipitation method using anti-PSGL-1 antibody and anti- P-selectin antibody. The activated platelets could bind to PSGL-1 of A549 cells through P-selectin, but the inactivated platelets cannot bind through the P-selectin. In the immunoprecipitation analysis, the anti-PSGL-1 antibody was used to precipitate the PSGL-1 protein. In the Western blot analysis, the anti-PSGL-1 antibody and anti-P-selectin antibody were used to detect PSGL-1 and P-selectin, respectively.

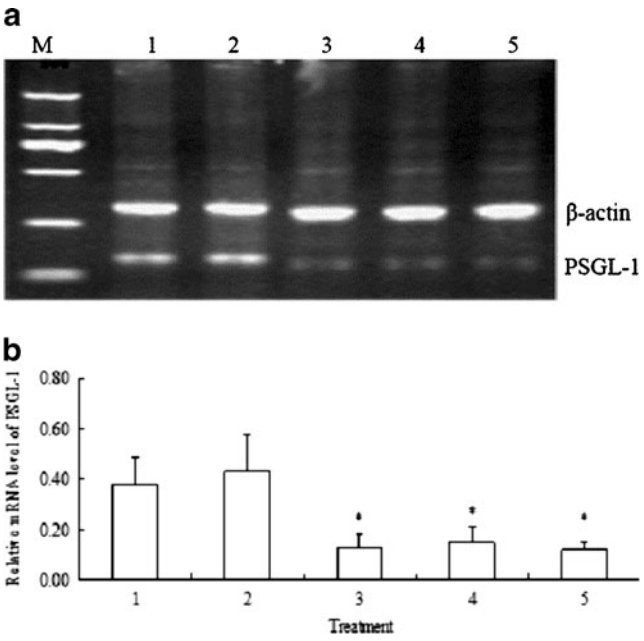

Fig. 3 The change of PSGL-1 mRNA level before and after transfection. **a** RT-PCR electrophoresis results. **b** The relative expression of PSGL-1 mRNA pre- and post-transfection by pSilenser-PSGL-1-shRNA. The amount of PSGL-1 mRNA was normalized to the amount of β-actin mRNA. The mean values were derived from three independent experiments. M, marker; Lane 1, A549 cells; lane 2, the screened positive cells transfected by pSilencer GFP 2.0-U6 plasmid as mock transfection control; lanes 3–5, the screened positive cells transfected by pSilenser-PSGL-1-shRNA

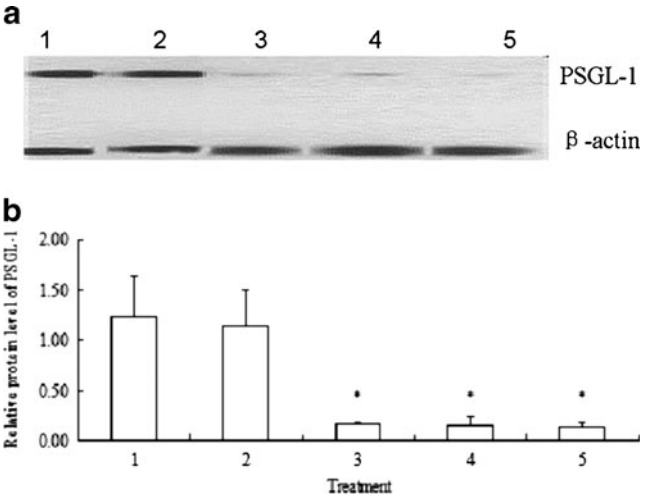

Fig. 4 PSGL-1 protein expression in the A549 cells pre- and post-transfected with pSilenser-PSGL-1-shRNA. **a** Western Blot analysis. **b** The relative level of PSGL-1 proteins before and after transfection. After transfection of the pSilenser-PSGL-1-shRNA, the PSGL-1 protein expression was significantly reduced. The mean values were obtained from three independent experiments. Lane 1, A549 cells; lane 2, the screened positive cells transfected by pSilencer GFP 2.0-U6 plasmid as mock transfection control; lanes 3–5, the screened positive cells transfected by pSilenser-PSGL-1-shRNA

As shown in Fig. 5, only PSGL-1 could be detected in the A549 cells alone (lane 1). When A549 cells were co-cultured with inactivated platelets, the P-selectin was not found because the P-selectin expression level was too low in inactivated platelets (lane 2). However, when A549 cells (untransfected or transfected with mock plasmid) were co-cultured with activated platelets, the P-selectin interacted with the corresponding PSGL-1 could be observed (lane 3, 4). After the PSGL-1 gene expression was silenced by the pSilenser-PSGL-1-shRNA, the interaction of P-selectin and PSGL-1 of A549 cells could be significantly decreased by silenced PSGL-1 (lane 5, 6).

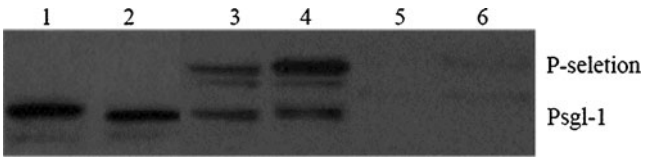

Fig. 5 Co-immunoprecipitation assay. The interaction of P-selectin and PSGL-1 was analyzed by co-immunoprecipitation assay using anti- PSGL-1 antibody and anti- P-selectin antibody. The activated platelets and in activated platelets were co-cultured with untransfected A549 cells and GFP-positive transfected A549. Lane 1, A549 cells; lane 2, co-cultivation of A549 cells and inactivated platelets; lane 3, co-cultivation of A549 cells and activated platelets; lane 4, co-cultivation of A549 cells transfected with the pSilencer GFP 2.0-U6 plasmid(as mock trasfection control)and activated platelets; lanes 5 and 6, co-cultivation of A549 cells transfected with pSilenser-PSGL-1-shRNA plasmid and activated platelets

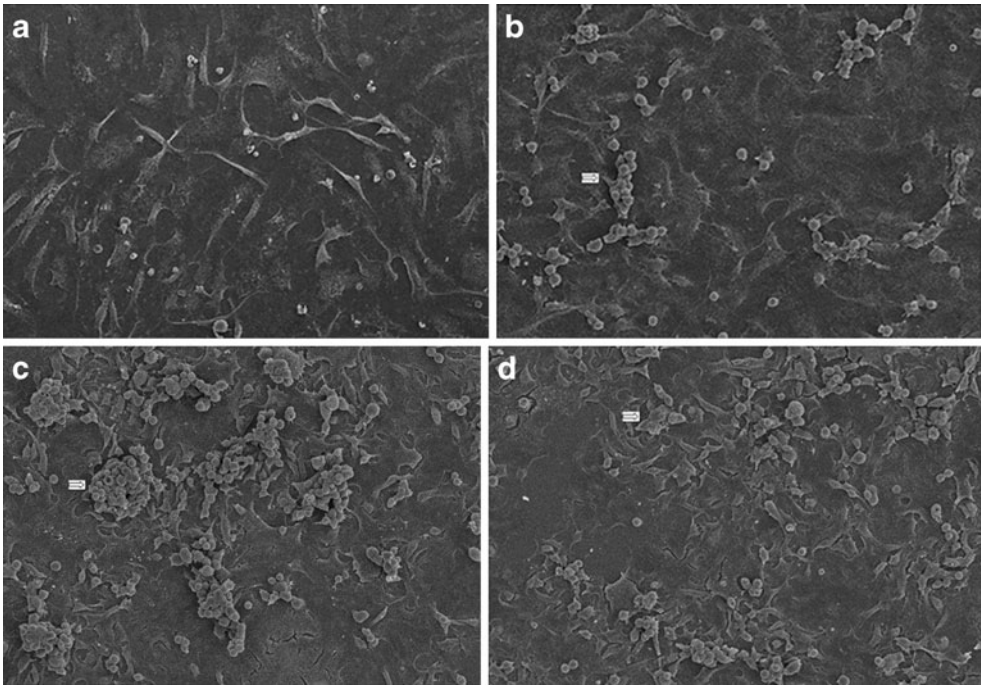

Fig. 6 The electron microscopy assay. The platelet and A549 cell complexes were scanned under the electron microscopy. The white arrow indicates the aggregation of platelets and lung cancer cells. **a** A549 cells; **b** co-cultivation of A549 cells and inactivated platelets; **c** co-cultivation of A549 cells and activated platelets; and **d** co-cultivation of A549 cells with pSilenser-PSGL-1-shRNA plasmid and activated platelets. Each of samples was repeated three times

Electron Microscopy

Compared with the normal cultured A549 cells, only a small number of platelets congregated around the tumor cells in the A549 cells co-cultured with inactivated platelets (Fig. 6A and B). After the A549 cells were cocultured with activated platelets (with higher P-selectin expression), obvious aggregations of tumor cells and platelets were found (Fig. 6C). However, the interactions between platelets and lung cancer cells were significantly reduced after the pSilenser-PSGL-1-shRNA transfected into the A549 cells (Fig. 6D). Platelets from three patients were analyzed in each group, and each of samples was repeated three times.

Discussion

During circulation, lung cancer cells interacted with many cellular factors and cytokines to influence the hematogenous metastasis of cancerous cells [16, 17]. Recent studies have confirmed that the activation and aggregation of platelet in circulation were relevant to cancer-associated thrombosis and tumor metastasis in a variety of malignancies, and the increased expression of active molecules was the main characteristics of platelet activation [5, 18, 19].

Our studies indicated that P-selectin expression on platelets in the peripheral blood of patients with lung cancer was significantly higher than that of the healthy control group. And the significant difference was also found between the lung adenocarcinoma and the squamous lung cancer groups. Activated platelets ccould combine with cultured human alveolar cell carcinoma A549 cells to form the aggregation of platelets and lung cancer cells. PSGL-1 expression was detected in the cultured A549 cell strain. After the PSGL-1 expression of the A549 cells was silenced by shRNA, the aggregation of activated platelets and A549 also significantly decreased ($p<0.01$).

Activated platelets reacted with their ligand PSGL-1 on lung cancer cells via P-selectin, and the aggregated formation of platelets and lung cancer cells might be mediated by the binding of the P-selectin/PSGL-1 pairs. Inhibiting the expression of endogenous PSGL-1 could effectively prevent the A549 carcinoma cells from interacting with activated platelets, thereby probably leading to the distant hematogenous metastasis of the lung cancer cells. This is a great potential for further research on the correlations of platelets and its adhesion molecules to the hematogenous metastasis of tumor cells. Importantly, antiplatelet activation and anti-selectins and selectin ligands could play a potential role in therapy of suppression of tumor metastasis [5, 20, 21].

The platelet-lung cancer cell complex protected cancer cells from mechanical injury in the bloodstream. In addition, the complex might influence the interaction between the tumor cells and the vascular endothelial cells, which might be one of the main mechanisms involved in platelet activation and hematogenous metastasis of tumors [22–24]. In future studies, we would investigate the formation of platelet-lung cancer cell complexes and their effects on the rolling and adhesion of lung cancer cells onto the surface of the vascular endothelium under simulated flow conditions, and the mechanisms of how P-selectin/PSGL-1 pairs affect the interaction of lung cancer cells and the vascular endothelium.

Conflict of Interest All authors have no conflict of interest regarding this paper.

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ (2007) Cancer statistics. CA Cancer J Clin 57:43–66
2. Inamura K, Ishikawa Y (2010) Lung cancer progression and metastasis from the prognostic point of view. Clin Exp Metastasis 27:389–397
3. Geiger TR, Peeper DS (2009) Metastasis mechanisms. Biochim Biophys Acta 1796:293–308
4. Joyce JA, Pollard JW (2009) Microenvironmental regulation of metastasis. Nat Rev Cancer 9:239–252
5. Borsig L (2008) The role of platelet activation in tumor metastasis. Expert Rev Anticancer Ther 8:1247–1255
6. Sierko E, Wojtukiewicz MZ (2007) Inhibition of platelet function, does it offer a chance of better cancer progression control? Semin Thromb Hemost 33:712–721
7. Kunita A, Kashima TG, Morishita Y, Fukayama M, Kato Y, Tsuruo T, Fujita N (2007) The platelet aggregation-inducing factor aggrus/podoplanin promotes pulmonary metastasis. Am J Pathol 170:1337–1347
8. Gonzalez Barcala FJ, Garcia Prim JM, Moldes Rodriguez M, AlvarezFernandez J, Rey Rey MJ, Pose Reino A, Valdes Cuadrado L (2010) Platelet count: association with prognosis in lung cancer. Med Oncol 27:357–362
9. Ludwig RJ, Schön MP, Boehncke WH (2007) P-selectin: a common therapeutic target for cardiovascular disorders, inflammation and tumour metastasis. Expert Opin Ther Targets 11:1103–1117
10. Dimitroff CJ, Descheny L, Trujillo N, Kim R, Nguyen V, Huang W, Pienta KJ, Kutok JL, Rubin MA (2005) Identification of leukocyte E-selectin ligands, P-selectin glycoprotein ligand-1 and E-selectin ligand-1, on human metastatic prostate tumor cells. Cancer Res 65:5750–5760
11. Baïsse B, Galisson F, Giraud S, Schapira M, Spertini O (2007) Evolutionary conservation of P-selectin glycoprotein ligand-1 primary structure and function. BMC Evol Biol 14:166
12. Raes G, Ghassabeh GH, Brys L, Mpofu N, Verschueren H, Vanhecke D, De Baetselier P (2007) The metastatic T-cell hybridoma antigen/P-selectin glycoprotein ligand 1 is required for hematogenous metastasis of lymphomas. Int J Cancer 121:2646–2652
13. Baumann K, Kowalczyk D, Gutjahr T, Pieczyk M, Jones C, Wild MK, Vestweber D, Kunz H (2009) Sulfated and non-sulfated glycopeptide recognition domains of P-selectin glycoprotein ligand 1 and their binding to P- and E-selectin. Angew Chem Int Ed Engl 48:3174–3178
14. Marathe DD, Buffone A Jr, Chandrasekaran EV, Xue J, Locke RD, Nasirikenari M, Lau JT, Matta KL, Neelamegham S (2010) Fluorinated per-acetylated GalNAc metabolically alters glycan structures on leukocyte PSGL-1 and reduces cell binding to selectins. Blood 115:1303–1312
15. Thomas GM, Panicot-Dubois L, Lacroix R et al (2009) Cancer cell-derived microparticles bearing P-selectin glycoprotein ligand 1 accelerate thrombus formation in vivo. JEM 206(9):1913–1927
16. Chambers AF, Naumov GN, Varghese HJ, Nadkarni KV, MacDonald IC, Groom AC (2001) Critical steps in hematogenous metastasis, an overview. Surg Oncol Clin N Am 10:243–255
17. Sleeman J, Steeg PS (2010) Cancer metastasis as a therapeutic target. Eur J Cancer 46:1177–1180
18. Tsuruo T, Fujita N (2008) Platelet aggregation in the formation of tumor metastasis. Proc Jpn Acad Ser B Phys Biol Sci 84:189–198
19. Noble S, Pasi J (2010) Epidemiology and pathophysiology of cancer-associated thrombosis. Br J Cancer 102:S2–S9
20. Barthel SR, Gavino JD, Descheny L, Dimitroff CJ (2007) Targeting selectins and selectin ligands in inflammation and cancer. Expert Opin Ther Targets 11:1473–1491
21. Läubli H, Borsig L (2010) Selectins promote tumor metastasis. Semin Cancer Biol 20:169–177
22. Iiizumi M, Mohinta S, Bandyopadhyay S, Watabe K (2007) Tumor-endothelial cell interactions: Therapeutic potential. Microvasc Res 74:114–120
23. Ley K (2009) Cell adhesion under flow. Microcirculation 16:1–2
24. Fuchs B, Budde U, Schulz A, Kessler CM, Fisseau C, Kannicht C (2010) Flow-based measurements of von Willebrand factor (VWF) function: Binding to collagen and platelet adhesion under physiological shear rate. Thromb Res 125:239–245