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The CD24/P-selectin Binding Pathway Initiates Lung Arrest of Human A125 Adenocarcinoma Cells[1]

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ABSTRACT

Carbohydrates on tumor cells have been shown to play an important role in tumor metastasis. We demonstrated before that CD24, a M_r 35,000–60,000 mucine-type glycosylphosphatidylinositol-linked cell surface molecule, can function as ligand for P-selectin and that the sialylLe^x carbohydrate is essential for CD24-mediated rolling of tumor cells on P-selectin. To investigate the role of both antigens more closely, we transfected human A125 adenocarcinoma cells with CD24 and/or fucosyltransferase VII (Fuc TVII) cDNAs. Stable transfectants expressed CD24 and/or sialylLe^x. Biochemical analysis confirmed that in A125-CD24/FucTVII double transfectants, CD24 was modified with sialylLe^x. Only double transfectants showed rolling on P-selectin *in vivo*. When injected into mice, double transfectants arrested in the lungs, and this step was P-selectin dependent because it was strongly enhanced in lipopolysaccharide (LPS) pretreated wild-type mice but not in P-selectin knockout mice. CD24 modified by sialylLe^x was required on the tumor cells because the LPS-induced lung arrest was abolished by removal of CD24 from the cell surface by phosphatidylinositol-specific phospholipase C. A125-FucTVII single transfectants expressing sialylLe^x but not CD24 did not show P-selectin-mediated lung arrest. The sialylLe^x epitope is abundantly expressed on human carcinomas, and significant correlations between sialylLe^x expression and clinical prognosis exist. Our data suggest an important role for sialylLe^x-modified CD24 in the lung colonization of human tumors.

INTRODUCTION

During the process of metastasis, tumor cells have to cross the local extracellular matrix, penetrate the vascular endothelium, circulate in the blood or lymph, and extravasate through the vessel wall into the tissue where the formation of secondary tumors eventually occurs (for review, see Refs. 1 and 2). The acquisition of properties that allow the interaction with normal host cells like leukocytes, platelets, or endothelial cells may be of advantage for tumor cells. The ability to bind to platelets in the blood stream may be of particular importance. It has long been known that tumor cells can circulate in the vasculature as stabilized platelet-enriched thrombi that can physically protect tumor cells from destruction (3, 4). The ability to bind to endothelial cells may be also beneficial. After passage through the blood stream, tumor cells have to adhere to the endothelium lining the vessel wall. This step is a prerequisite for tumor extravasation and tissue penetration (5, 6).

Among the endothelial selectins, P- and E-selectin (CD62P and CD62E) play an important role in capturing leukocytes in inflamed tissues (7–9). The initial cell contact with the vessel wall is followed by the selectin-mediated rolling of leukocytes on the endothelial cell surface, leading to integrin-mediated firm adhesion and transmigration (10, 11). P-selectin is found on the surface of activated endothelial cells and platelets. It is stored in intracellular granula and is rapidly mobilized to the cell surface within minutes after stimulation with proinflammatory agents like histamine or thrombin but also with LPS[3] (12, 13). In contrast, E-selectin requires *de novo* synthesis for its expression and is therefore available only at later time points during an inflammatory response (7–9). Selectins mediate the rolling adhesion by interacting with specific carbohydrate ligands on the cell surface that are sialylated, fucosylated lactosaminoglycans such as sialylLe^x and other sialylated or sulfated moieties that are displayed on a limited number of glycoproteins (14, 15). On leukocytes, high-affinity selectin ligands that can support rolling interactions include PSGL-1 (16, 17). PSGL-1 is a mucin-type glycoprotein that contains three unique tyrosine residues near the NH_2 terminus. Sulfatation of one of these tyrosine residues in addition to sialylLe^x expression is essential for P-selectin binding to PSGL-1 (18, 19). The presence of the sialylLe^x oligosaccharide alone is not sufficient for mediating rolling interactions of leukocytes. Studies in knockout mice deficient in FucTVII, which is necessary for the biosynthesis of sialylLe^x (20), or lacking E- and/or P-selectin (21) or lacking PSGL-1 (22) have demonstrated the important role of these structures in rolling and transmigration of blood-borne leukocytes.

It has been speculated that human tumors may use selectins or their ligands during metastasis (12, 23, 24). In fact, it is known that inflammation and trauma, which favor the expression of selectins, can influence the spread of tumor cells (25, 26). In addition, sialylLe^x or the related sialylLe^x oligosaccharides are commonly expressed on human carcinoma cells and are associated with poor clinical prognosis (27–31). Indeed, many human cancer tissues or established tumor cell lines can bind to E- and P-selectin (32–35). Experimentally, overexpression of sialylLe^x in tumor cells by transfection with fucosyltransferases caused enhanced lung colonization that was correlated with better E-selectin binding (36). Ectopic expression of E-selectin in the liver of transgenic mice leads to a redirectioning of tumor cells that normally colonized the lungs (37). These results indicated that carbohydrates on tumor cell surfaces may be recognized by E-selectin on the endothelium. The potential importance of P-selectin in tumor metastasis was also demonstrated recently (38). Using P-selectin/RAG-2 double knockout mice, a diminished tumor growth and metastasis of human adenocarcinoma cells compared with wild-type P-selectin+/+ mice was observed (38). The reduced tumor dissemination in P-selectin-deficient mice was attributed to the capability of platelets to interact with tumor cells; however, a role for endothelial P-selectin was not excluded (38).

Despite the accumulating evidence that interactions of selectins with sialylLe^x and related structures can play a role in tumor metastasis, the function of particular ligands on the tumor cell surface and their nature have not been addressed. For example, carcinoma cells usually do not express PSGL-1 (35), the major selectin ligand on leukocytes. What could the equivalent ligand molecule(s) on tumor

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[3] The abbreviations used are: LPS, lipopolysaccharide; FucVII, fucosyltransferase VII; mAb, monoclonal antibody; PSGL-1, P-selectin glycoprotein ligand 1; PIPL-C, phosphatidylinositol-specific phospholipase C; FACS, fluorescence-activated cell-sorting; TNF, tumor necrosis factor; RT-PCR, reverse transcription-PCR; GPI, glycosylphosphatidylinositol.

cells be? Indirect evidence suggests that, as on leukocytes, mucin-type glycoproteins account for most of the selectin ligands on carcinoma cells (39). In previous studies, we demonstrated that the GPI-anchored mucin CD24 can act as a P-selectin ligand on human carcinoma cells (35). We also have shown previously that CD24 can support P-selectin-mediated rolling of human tumor cells on the endothelium; however, sialylLe^x had to be expressed concomitantly by the cells (40). The biosynthesis of sialylLe^x requires the addition of a fucosyl residue to the type 2 chain-based structures (Galβ1-4GlcNAc-R) that can be mediated by FucTVII (21). To study the functional role of both antigens in detail, we have now reconstituted human A125 adenocarcinoma cells with CD24 and/or FucTVII cDNAs. We established stable single and double transfected cells and investigated P-selectin-mediated interactions *in vitro* and *in vivo*. Our results indicate that sialylLe^x modified CD24 creates a functional P-selectin ligand at the tumor cell surface that can promote rolling and tumor cell colonization to the lungs. Expression of sialylLe^x or CD24 alone was not sufficient to mediate theses effects. Because CD24 and sialylLe^x are coexpressed by many human carcinoma cells, our data have important implications for the understanding of molecular interactions during the early steps of the process of metastasis.

MATERIALS AND METHODS

Cells

The breast carcinoma cell line KS and the A125 lung adenocarcinoma cells and CD24 transfectants derived from these cells (A125-CD24) have been described previously (35). A125-CD24 cells expressing human FucTVII were established by transfection of a FucTVII cDNA (obtained from Dr. J. Lowe, University of Michigan, Ann Arbor, MI) in pREP4 (Invitrogen, Groningen, the Netherlands) using LipofectAMINE (Life Technologies, Inc., Eggenstein, Germany). After hygromycin selection, sialylLe^x-expressing transfectants were selected by FACS sorting using the sialylLe^x-specific mAb AM-3 (see below). A125 cells were transfected with FucTVII and selected in a similar way. Cells were cultivated in RPMI 1640 supplemented with 10% FCS at 37°C, 5% CO_2, and 100% humidity.

Reagents

Anti-CD24 mAbs ML-5 and SWA11 have been described previously (35). MA CSLEX-1 (anti-sialylLe^x) was a kind gift of E. C. Butcher (Stanford University, Stanford, CA). mAb AM-3 (anti-sialylLe^x) was a gift of Dr. G. Hanski (Benjamin Franklin Clinics, Berlin, Germany). Human P-selectin IgG was a gift from Genetics Institute (Boston, MA). The polyclonal antibody to mouse P-selectin and the mAb to mouse CD41 (clone MWReg30) were obtained from PharMingen (Hamburg, Germany). mAb W6/32 against human MHC class I (biotinylated) was obtained from Dr. Gerd Moldenhauer (German Cancer Research Center, Heidelberg, Germany). mAb 9A9 (rat IgG1) is a blocking mAb against murine E-selectin, and it was used at 50 μg/mouse (41). TNF-α was used at 0.5 μg/mouse and obtained from Genzyme Corp. (Cambridge, MA).

Cytofluorography

The staining of cells with mAbs and phycoerythrin-conjugated goat antibodies to mouse immunoglobulins (SERVA, Heidelberg, Germany) has been described previously (35). Stained cells were analyzed with a FACScan fluorescence-activated cell analyzer (Becton Dickinson, Heidelberg, Germany).

Isolation of RNA and RT-PCR Analysis

The isolation of total RNA from cells and RT-PCR have been described in detail elsewhere (42). Briefly, total RNA was transcribed into cDNA using Moloney murine leukemia virus reverse transcriptase (Promega, Heidelberg, Germany) and oligo(dT)$_{20}$ for priming. After heat inactivation of the enzyme, the RNA/DNA hybrid was treated with RNase H, and the cDNA was used as a template for PCR analysis. The following primers were used: PSGL-1 forward, 5′-GCTATGGAGATACAGACCACTCA-3′; PSLG-1 reverse, 5′-CAGATGGCAGAGTGAGCTAAG-3′ (fragment size, 874 bp); FucTVII forward, 5′-CACCTCCGAGGCATCTTCAACTG-3′; and FucTVII reverse, 5′-CGTTGGTATCGGCTCTCATTCATG-3′ (fragment size, 497 bp).

Enzyme Treatment

Treatment of cells with PIPL-C (500 milliunits/ml) for 2 h was done as described previously (35). To control the successful removal of GPI-anchored proteins, an aliquot of the cells was analyzed by FACS using the mAb to CD24. The remainder of the cells were labeled with ^{51}Cr for 1 h at 37°C, washed twice, adjusted to a concentration 1×10^7 cells/ml, and injected into the tail vein of mice.

Cell Binding Assays

For binding of cells to P-selectin IgG, goat antihuman IgG was coated before the fusion protein to allow directional coating. Vitronectin was obtained from Sigma and coated overnight at 1 μg/ml. Wells were then blocked with BSA, and the binding assay was performed as described previously (42, 43). Cell binding was measured by counting six independent ×10 fields by video microscopy using IMAGE 1.47 software.

Animal Experiments

Rolling experiments were performed on four male 8–10-week-old mice weighing between 22 and 26 grams. Wild-type C57BL/6 mice were obtained from Hilltop (Scottdale, PA) or from Charles River (Sulzbach, Germany). Female P-selectin−/− mice of the same age were obtained from Jackson Laboratory (Ann Arbor, MI). The organ arrest of human tumor cells in mice was studied using ^{51}Cr-labeled cells as outlined by Gosslar *et al.* (44). Briefly, cells were washed and incubated in RPMI 1640 containing 20% FCS in the presence of 500 μCi of ^{51}Cr for 1 h at 37°C. Cells were then washed twice in prewarmed complete medium, counted, and adjusted to a concentration of 10^7 cells/ml. An aliquot of the cell suspension was counted in a gamma counter to determine the specific incorporation per cell. Cells (100 μl) were injected via the tail vein. For LPS treatment, 100 μl of LPS from *Escherichia coli* 026:B6 (Sigma) at 0.1 mg/ml were injected via the tail vein 2 h before application of the tumor cells.

In vivo Rolling Analysis

Intravital Microscopy. Mice were anesthesized with an i.p. injection of ketamine hydrochloride (100 mg/kg; Ketalar; Parke-Davis, Morris Plains, NJ) after pretreatment with xylazine (0.05 mg/kg, i.p.) and atropine (0.1 mg/kg, i.p.; Elkins-Sinn, Cherry Hill, NJ). Animals were at 37°C with a thermocontrolled heating pad. Mice were pretreated 2–3 h before surgery with an intrascrotal injection of 0.5 mg of murine TNF-α in 0.3 ml of isotonic saline.

Local catheter. For local injection of the cells into the microcirculation of the cremaster muscle, a heparinized catheter was placed into the proximal part of the right femoral artery and advanced toward the branching section of internal iliac artery from the common iliac artery (45). Transfected cells were labeled with 0.5 μg/ml calcein AM (Molecular Probes, Eugene, OR) for 30 min at 37°C. Cells ($5–10 \times 10^6$ cells/ml) were injected as a 0.1-ml bolus into the cremaster microcirculation via the local catheter. Microscopic observations were made on an intravital microscope (Zeiss Axioskop, Thornwood, NY) with a saline immersion objective (SW 40/0.75, numerical aperture).

Cremaster. The cremaster muscle was prepared for intravital microscopy as described previously (45) and superfused with thermocontrolled 35°C bicarbonate-buffered saline saturated with 95% N_2 and 5% CO_2. The exposed cremaster microcirculation remained well perfused. Time 0 was set at the treatment of cremaster with TNF-α. TNF-α (500 ng) was injected in 0.3 ml of saline. For continuous blood pressure monitoring and blood sampling, the carotic artery was canulated with heparinized PE-10 tubing. All vessels had calculated wall shear rates between 600 and 800 s^{-1}. Microvessel diameters and individual rolling tumor cell velocity were measured using a digital image processing system (46). Freeze frame advancing was used to accurately monitor the movements of the individual rolling transfectants. Each rolling transfectant passing a line perpendicular to the vessel wall was followed for 0.5–1 s. Rolling velocities for individual transfectants were calculated by dividing the traveled distance by the tracking time. Venules with diameters

between 25 and 50 μm were observed, and video recordings were made through a charge-coupled device camera system (model VE-1000CD; Dage-MTI, Michigan City, IN) on a Panasonic S-VHS recorder. Measurements of transfectant rolling were performed using stroboscopic epifluorescence illumination (60 s^{-1}; Strobex; Chadwick Helmuth, Mountain View, CA). For each injection of cells, video scenes of approximately 10 min in duration were recorded (250–300 passing cells). The centerline velocities of the respective vessels were calculated using the distance traveled by free-flowing labeled cells in the center of the vessels in consecutive video frames. After the initial injection of cells, flow was transiently reduced due to lodging of the cells in capillaries. However, in all experiments, flow returned to baseline values within 30 s. For each venule, a critical velocity was determined as the minimal velocity of a freely flowing cell traveling close to the vessel without adhesive interactions (47).

Immunohistological Analysis

Tumor cells were labeled with the fluorescent dye CSFE [5-(and-6)-carboxyfluorescein diacetate, succinimidylester; Molecular Probes, Leiden, the Netherlands] and injected into LPS-pretreated mice as described above. After 6 h, the lung and liver were removed and snap frozen in isopentane. Frozen sections were cut using a microtome and analyzed by histological staining using antibodies to mouse CD41 or P-selectin. Tumor cells were detected in the stained sections by fluorescence microscopy or by the ABC staining procedure (Vector stain; Vector Laboratories, Burlingame, CA) as described previously (48).

Biochemical Analysis

The affinity purification of CD24 has been described in detail previously (35). Sample aliquots were separated by SDS-PAGE on a 10% slab gel under reducing conditions, and proteins were transferred to Immobilon membrane (Millipore). Blots were developed with anti-CD24 mAb SWA11 followed by peroxidase-conjugated goat antimouse IgG and enhanced chemiluminescence detection (Amersham-Pharmacia Biotech, Freiburg, Germany). ELISA analysis of purified CD24 was done as described previously (35).

Statistical Analysis

Statistical analysis of the data was done using the unpaired Student's *t* test or Wilcoxon's rank-sum test.

RESULTS

Characterization of A125 Cells Transfected with CD24 and FucTVII. The lung adenocarcinoma line A125 and a subline stably transduced with CD24 (A125-CD24) have been characterized previously (35). We report here that both cell lines do not express FucTVII mRNA as shown by RT-PCR analysis (data not shown) and are negative for the sialylLe^x carbohydrate epitope at the cell surface (see Fig. 1). To alter the cellular glycosylation, A125-CD24 cells were transfected with FucTVII cDNA, and stable transfectants were selected in the presence of hygromycin. Transfected cells expressed sialylLe^x and were further enriched for homogenous expression of the epitope by FACS sorting using the sialylLe^x-specific mAb AM-3. Fig. 1 shows the cytofluorographic analysis of all transfectants using the breast carcinoma cell line KS as a positive control. The levels of CD24 expression at the cell surface of A125-CD24 cells and the CD24/FucTVII double- or mock-transfected cells did not differ significantly. Double-transfected cells were stained with the sialylLe^x-specific mAbs AM-3 and CSLEX-1, respectively.

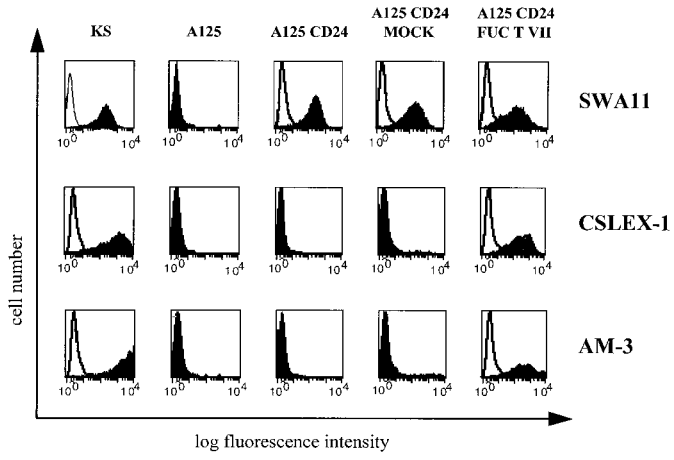

Fig. 1. Characterization of A125 adenocarcinoma cells transfected with CD24 and Fuc TVII. Cytofluorographic analysis of transfected cells using mAbs specific for CD24 (*SWA11*) or the sialylLe^x epitope (*AM-3* and *CSLEX-1*) followed by FITC-conjugated goat antimouse IgG.

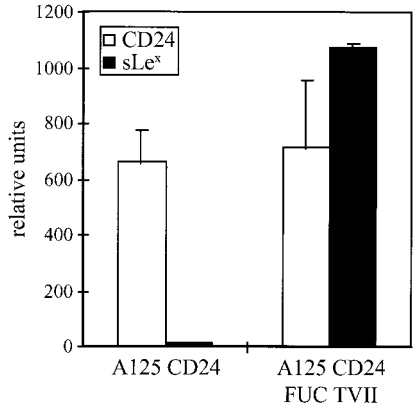

Fig. 2. Analysis of affinity-purified CD24 for sialylLe^x modification. ELISA analysis of affinity-purified CD24 from A125-CD24 or A125-CD24/FucTVII double-transfected cells using mAbs to CD24 or sialylLe^x.

PSGL-1 has been shown to be a major ligand for P- and E-selectin on leukocytes (17, 18). To rule out the possibility that PSGL-1 was expressed by A125 cells or induced on transfection, we investigated the PSGL-1 expression by RT-PCR analysis. The PSGL-1-specific primers amplified an expected band of 874 bp from control HL60 cells; however, all transfected carcinoma cell lines did not express detectable levels of PSGL-1 (data not shown).

FucTVII Expression Alters CD24 Glycosylation. To establish that FucTVII expression affected the glycosylation of CD24, the antigen was affinity purified from A125-CD24 or A125-CD24/FucTVII double-transfected cells using a mAb ML-5 column as described previously (35). SDS-PAGE and Western blot analysis revealed a single band at approximately M_r, 40,000–50,000, in agreement with our previous studies (35). Staining of the gel with colloidal Coomassie revealed no contaminating protein bands in the CD24 preparations (data not shown).

The purified CD24 was analyzed for sialylLe^x modification by ELISA. Only CD24 isolated from A125-CD24/FucTVII-transfected cells was reactive with mAb AM-3 to sialylLe^x (Fig. 2). These results indicated that CD24 in double but not single transfectants was decorated with sialylLe^x.

FucTVII Expression Augments P-selectin-mediated Platelet Binding. The binding of cells to P-selectin IgG immobilized to a solid support is shown in Fig. 3. Robust cell binding to P-selectin was only observed with A125-CD24/FucTVII double transfectants. CD24 single transfectants, mock-transfected cells, or A125-FucTVII-transfected cells showed slightly enhanced binding compared with A125 cells. The adhesion of the cells to vitronectin was quite similar for all cell lines.

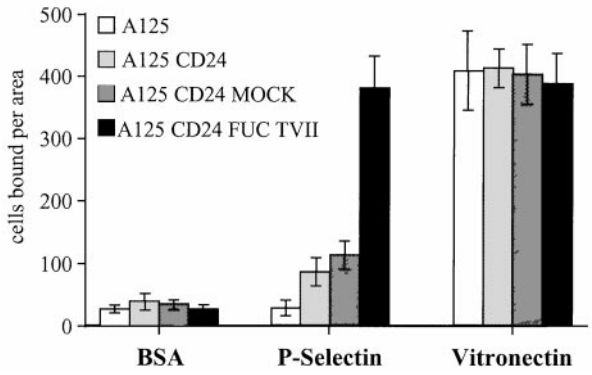

Fig. 3. Binding of transfected cells to P-selectin. Binding of cells to immobilized P-selectin IgG. Tumor cells were allowed to bind to P-selectin IgG (coated at 1 μg/ml) or vitronectin (coated at 10 μg/ml). Bound and nonbound cells were separated by buoyant density and counted.

FucTVII and CD24 Expression Allows Rolling of Tumor Cells. It has been shown previously that P-selectin can support the CD24-mediated rolling of tumor cells *in vitro* and *in vivo* (40). To determine whether transfection had changed P-selectin-mediated rolling on vascular endothelium, we injected cells into the femoral artery of TNF-α-treated mice and observed their transit through post-capillary venules of the exteriorized cremaster muscle. The results from these experiments are summarized in Fig. 4, showing that only A125-CD24/FucTVII double transfectants were able to roll on endothelium. CD24 expression alone was not sufficient to support rolling.

P-selectin-dependent Colonization of Tumor Cells in the Lungs. Having established that FucTVII transfection modified CD24 glycosylation and augmented P-selectin-mediated binding and rolling, we examined the *in vivo* behavior of transfected cells. In a short-term homing assay, ^{51}Cr-labeled cells were injected into the tail vein of C57/B6 mice, and the accumulation of tumor cells in individual organs was determined by whole-organ counting. In initial kinetic experiments, the A125-CD24 and the double-transfected A125-CD24/FucTVII cells were compared at 2, 6, and 24 h. It was found that arrest of tumor cells in the lungs peaked after 2 h (between 5–10% of the total) and in the liver after 6 h (between 20–30% of the total). Accumulation of radioactive cells in the spleen and kidney was highest after 6 h (between 2–5%). Fig. 5*A* (*left panel*) gives a representative distribution pattern of label after 6 h, showing that the initial values for the lung had already declined to approximately 2–3% of injected cells. Tumor cells reach the lung as the first organ. To allow the initial flush of cells to pass by, the animals were sacrificed after 6 h to specifically detect retained cells. At this time point, no radioactivity was detectable in the blood, making the perfusion of organs to remove blood-borne cells unnecessary for the analysis.

To induce P-selectin expression on endothelial cells, mice were injected i.v. with LPS 2 h before application of labeled tumor cells. As shown in Fig. 5*A* (*right panel*), the treatment significantly increased the percentage of double-transfected A125-CD24/FucTVII cells in the lungs but not in the other organs investigated. LPS pretreatment only weakly augmented the arrest of A125-CD24 cells in the lungs (Fig. 5*A*, *right panel*), and A125-CD24/mock-transfected cells behaved in the same way (data not shown).

To study a putative role for P-selectin in the lung arrest, similar experiments were carried out in P-selectin$-/-$ mice. As shown in Fig. 5*B*, in contrast to P-selectin$+/+$ wild-type mice, in P-selectin-deficient animals, the LPS injection did not increase lung arrest of the double-transfected tumor cells. Instead, a minor increase of similar size as seen for A125-CD24 cells in wild-type animals occurred in double transfected cells. These results suggested that P-selectin in the lung and sialylLe^x-bearing ligands on the tumor cells were important in mediating arrest of human A125 tumor cells.

Histological examination of lungs from LPS-treated and nontreated animals indicated that P-selectin expression was induced on lung vessels (Fig. 6*A*). This observation supports the notion that endothelial P-selectin might initiate the rolling of tumor cells in the lung vascu-

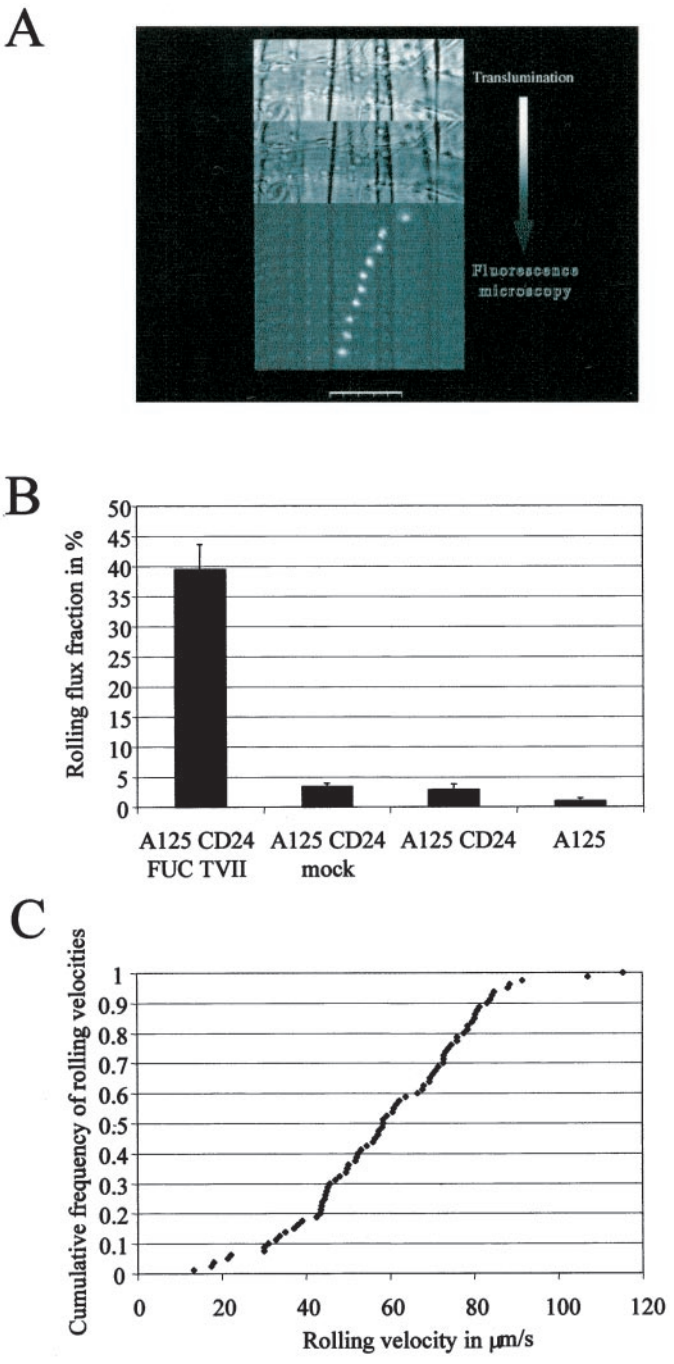

Fig. 4. Analysis of cell rolling *in vivo*. *A*, visualization of the rolling of a CD24/FucTVII cell within 1 s. Pictures from video micrographs of the same vessels (from consecutive 0.1 frames) were pasted below each other to demonstrate the rolling behavior of the cell in fluorescence microscopy. The *bar* represents 50 μm. *B*, interaction of transfectants in TNF-α-treated wild-type mice with endothelium of cremaster muscle venules after blockade of E-selectin with mAb 9A9. The graph represents the rolling flux of transfectants as a percentage of passing transfectants through the vessel (SE). Data represent six measurements from two independent experiments. *C*, cumulative frequency of rolling velocities of rolling A125-CD24/FucTVII transfectants on microvascular endothelium of mouse cremaster muscle. Rolling velocity of 50 individual cells was measured for 0.5–1.5 s of rolling time.

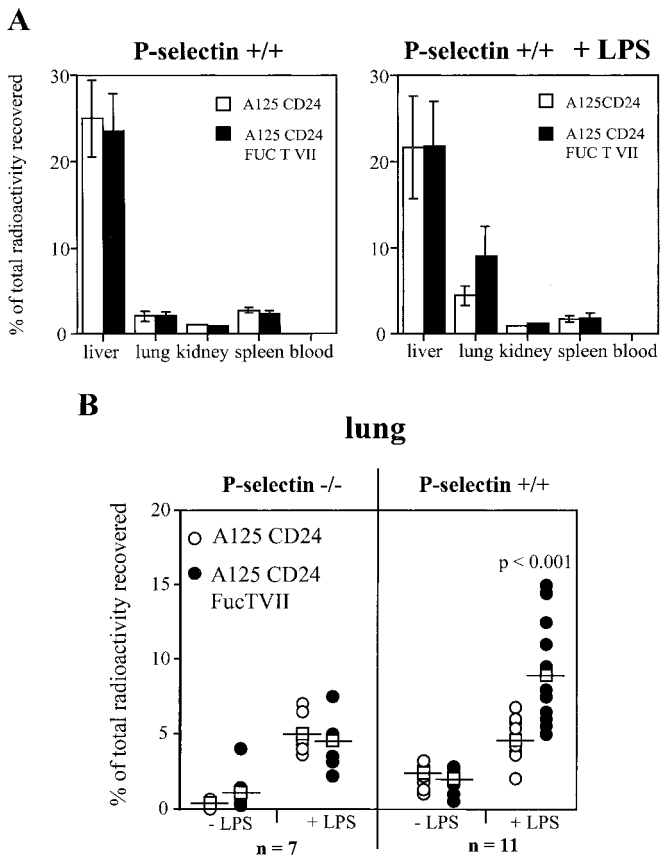

Fig. 5. Role of P-selectin in lung retention of tumor cells. *A*, A125-CD24 and A125-CD24/FucTVII double-transfected cells were labeled with ^{51}Cr, and 10^6 cells were injected into LPS-pretreated or nontreated P-selectin+/+ mice. Organs of tumor cell-bearing animals were removed and counted after 6 h. The distribution of radioactivity in individual organs is given as a percentage of total input. *B*, comparison of lung retention of A125-CD24 and A125-CD24/FucTVII double-transfected cells in P-selectin+/+ or P-selectin−/− mice. The experiments were performed as described, and data from *A* are included for statistical analysis.

lature. P-selectin was also detectable on platelets in the vascular lumen of the mice as well as on platelet aggregates detected in the lungs and other tissues.

Tumor cells were also fluorescence labeled before injection into LPS-treated mice and identified in organ sections using fluorescence microscopy. Fluorescent tumor cells were abundant in the lungs of wild-type animals 6 h after injection. Cells were localized mostly in capillaries of alveolar septa. In P-selectin-deficient mice, the frequency of fluorescent tumor cells was much smaller in the lungs, in agreement with the radioactivity data. We determined the number of tumor-platelet aggregates using a combination of fluorescence and histological staining with a mAb to CD41, a specific marker for mouse platelets. The results are summarized in Fig. 6*B*. Mixed aggregates consisting of platelets and fluorescent tumor cells were observed in lung sections of LPS-treated P-selectin-deficient or wild-type mice. However, the percentage of tumor cells surrounded by a few or more platelets was rather small (approximately 15%). Most of the tumor cells in the lungs were devoid of platelet association. There was only a small (if any) difference in the percentage of tumor-platelet aggregates observed in lungs of P-selectin−/− *versus* P-selectin+/+ animals. This observation suggested that under the experimental conditions, P-selectin was unnecessary to form tumor rosettes *in situ*. Similar observations were made in the liver and other organs of tumor-injected mice (data not shown).

PIPL-C Treatment of Carcinoma Cells Affects Lung Retention. CD24 is a GPI-anchored cell surface molecule that can be removed by PIPL-C treatment. We have shown previously that the treatment affects P-selectin binding and P-selectin-mediated rolling of tumor cells (40). We therefore tested whether the treatment would influence the lung arrest of A125-CD24/FucTVII double transfectants. FACS analysis of the cells before and after PIPL-C treatment for 1 h showed a reduction of CD24 cell surface expression (a decrease of mean fluorescence from 30 to 5; see Fig. 7*A*). The staining of the transmembranal MHC class I antigen was not affected by PIPL-C treatment (data not shown). As shown in Fig. 8, the PIPL-C treatment reduced the lung arrest of labeled tumor cells to the level seen with A125-CD24 cells. The reduction was specific for the lung because the arrest in the kidney (Fig. 8) or spleen and liver (data not shown) remained unaffected.

FucTVII Transfection Is Not Sufficient for Lung Retention. These results suggested that sialylLe^x carbohydrates presented on PIPL-C-sensitive ligands were involved in the lung arrest of A125 tumor cells. To further investigate a particular role of CD24 in this process, we established A125-FucTVII transfectants devoid of CD24. As shown in Fig. 7*B*, these cells did not react with a mAb to CD24 (ML-5) but did react with the sialylLe^x-specific mAb AM-3. When analyzed for lung arrest *in vivo*, the A125-FucTVII cells behaved in a manner similar to that of A125-CD24 or PIPL-C-treated double-transfected A125 cells (Fig. 8). These observations indicated that sialylLe^x *per se* was not sufficient to initiate lung arrest.

To confirm our results in another tumor cell system, we used the CD24- and sialylLe^x-positive breast carcinoma cell line KS. These cells were previously shown to promote CD24 and P-selectin-dependent rolling *in vivo* that was sensitive to PIPL-C treatment (40). We injected PIPL-C treated or nontreated cells into LPS-pretreated mice and analyzed the organ distribution of retarded cells. Enzyme treatment reduced CD24 expression (40) and, as shown in Fig. 9, also diminished the recruitment of cells in the lungs. Collectively, these data suggest an important role for sialylLe^x-modified CD24 and P-selectin in the accumulation of tumor cells in the lungs.

DISCUSSION

The present study was undertaken to gain insight into molecular interactions that are potentially important for the dissemination of tumor cells. We demonstrate that the ability of A125 tumor cells to interact with P-selectin can be altered by expression of CD24 and FucTVII cDNAs. Transfected cells expressed CD24 and/or sialylLe^x at the cell surface. We established by biochemical means that CD24 in double-transfected cells was modified with sialylLe^x. Thus, the CD24 mucin-type glycoprotein was readily accepted as a substrate for the addition of sialylLe^x glycans. Altered expression of P-selectin ligands was detected in static binding assays to immobilized P-selectin. Only double-transfected cells showed strong interaction. We then determined whether transfection had changed P-selectin-mediated rolling on vascular endothelium. Again, only double-transfected cells were able to show rolling interactions. These results are consistent with our previous observation using KS breast carcinoma cells, showing that in inflamed endothelium, tumor cell rolling was predominantly mediated by P-selectin (40). Transfected cells were also tested for their ability to colonize the organs of mice after i.v. injection. To induce selectin expression, the animals were pretreated with LPS. A glycosylation-dependent arrest of injected tumor cells was observed in the lungs of LPS-treated mice and was barely detectable in nontreated animals. Arrest in this organ appeared to be P-selectin dependent because it was only observed in P-selectin wild-type but not P-selectin-deficient mice. Importantly, CD24 had to be

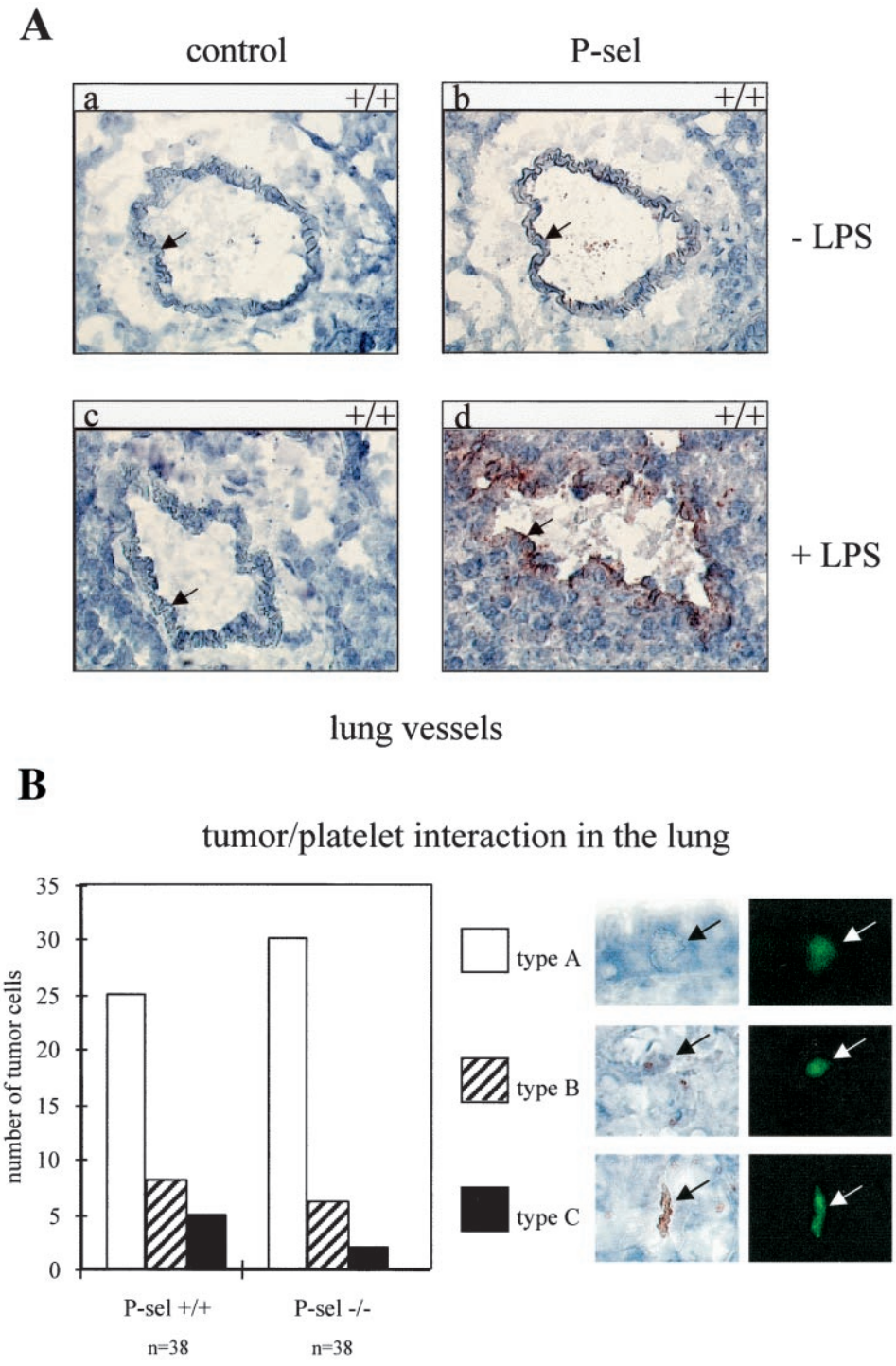

Fig. 6. Histological examination of injected tumor cells in lung tissue. *A*, histological examination of mouse lung vessels for P-selectin expression after injection with LPS. Two h after injection, lungs were removed, and frozen sections were stained with an antibody to P-selectin followed by a secondary antibody (*b* and *d*). Control staining was done by using the secondary antibody alone (*a* and *c*). Note the strong induction of P-selectin staining by LPS on the lung endothelium indicated by *arrowheads*. *B*, quantification of mixed tumor-platelet aggregates in lung sections. Fluorescence-labeled A125-CD24/FucTVII double transfectants were injected into LPS-pretreated P-selectin−/− or +/+ mice. Tumor cells were detected by fluorescence microscopy, and platelets were detected by CD41 staining. Tumor cells (identified by fluorescence) were examined for the number of associated platelets and classified in three groups: type A, no detectable platelets; type B, <10 platelets associated; and type C, large tumor-platelet aggregates. *n* refers to the number of tumor cells examined in the lung sections of P-selectin−/− or +/+ mice. Note that for P-selectin−/− animals, many more lung sections had to be examined to score the same number of tumor cells.

present at the cell surface because the sole expression of sialylLe^x was not sufficient for accumulation of cells in the lungs. Additional experiments using PIPL-C treatment to remove CD24 from the cell surface confirmed the requirement for this molecule in the process. We concluded that a P-selectin-CD24 interaction was responsible for initiating the arrest of tumor cells in the lungs and that CD24 had to be modified by sialylLe^x to exert the effect.

P-selectin can be expressed by activated platelets as well as inflamed endothelium, raising the question of which cellular interaction was responsible for the observed effect *in vivo*. To clarify this point, we carried out histological examinations showing that after LPS injection, the lung endothelium of LPS-treated mice strongly expressed P-selectin. As expected, this was not observed in the lungs of P-selectin-deficient mice, raising the possibility that endothelial P-selectin was a decisive factor by allowing tumor cell rolling in the lung vasculature. However, a contribution by platelet P-selectin to the observed lung arrest had to be considered. Indeed, platelets have been hypothesized to contribute to tumor dissemination (3, 49, 50). Many human or animal tumors possess procoagulant activity that can be due to the production of tissue factor, production of factor X activators, or the ability of the tumor cell to mediate the assembly of the prothrombinase complex, leading to the generation of active thrombin from

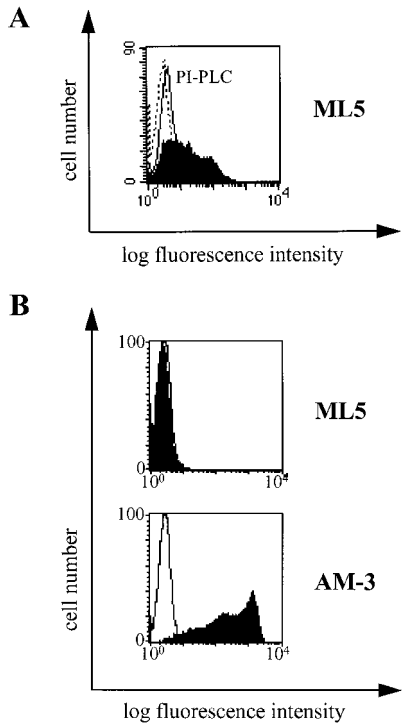

Fig. 7. Characterization of PIPL-C-treated A125-CD24/FucTVII and A125-FucTVII cells. *A*, Effect of PIPL-C treatment on the expression of CD24. A124-CD24/FucTVII transfectants were treated for 1 h with 500 milliunits of PIPL-C and analyzed for CD24 expression using the mAb SWA11. The *dashed line* represents staining with the secondary antibody only. *B*, A125-FucTVII cells were stained for the expression of CD24 or sialylLe^x using the respective mAbs and analyzed by FACS analysis.

there is accumulating evidence that the C-type lectins and the sialyl-Le^x oligosaccharide binding pathway may have versatile functions for cell-cell interaction (56). Ohayama *et al.* (57) have recently reported that sialylLe^x carbohydrates exposed at high density on short *N*-glycans of B16 melanoma cells can be targeted by natural killer cells *in vivo*, most likely through a receptor similar to C-type lectins. However, when expressed on poly-*N*-acetyllactosamines typical for O-linked glycans, these cells were highly metastatic, probably through an interaction with a C-type lectin on lung endothelial cells (57). Although our results are in agreement with the latter findings, we cannot at present rule out the possibility that although retained in the lungs, the accumulated tumor cells may have been the target of natural killer-mediated lysis at a later stage.

CD24 consists of a small protein core with many potential sites for O-linked glycosylation and can therefore be considered a mucin. CD24 is expressed in many human carcinomas including breast carcinoma, small cell lung carcinoma, neuroblastoma, rhabdomyosarcoma, and renal cell carcinoma (see Ref. 35). Mucin-type glycoproteins have been implicated to serve as ligands for selectins, and it was shown recently that all P-, E-, and L-selectins can bind to colon carcinoma cell lines and fresh tissue sections in a calcium-dependent fashion and in an *O*-sialoglycoprotease-sensitive fashion (39). However, individual selectin ligand molecules on tumor cells have not been identified. The results presented in this report indicate that CD24 modified by sialylLe^x can act as a P-selectin ligand and exert functions similar to those of PSGL-1 on leukocytes. It is evident from our study that CD24 modified by sialylLe^x could be an essential factor for the formation of tumor metastases.

plasma prothrombin (51, 52). Generation of thrombin or other tumor mechanisms activate platelets, leading to direct aggregation or secretion of ADP, serotonin, and/or intermediates of the arachidonate metabolism. LPS is the most potent stimulus for monocyte procoagulant activity, which can, in turn, activate the clotting protease cascade and stimulate platelet aggregation (53). Indeed, in our experiments, platelet aggregates were abundant in the organs of LPS-treated and tumor-injected animals. It was therefore quite surprising that the double-transfected A125 cells *in situ* were largely devoid of platelets. Most importantly, there was no significant difference in the number of tumor-platelet aggregates in P-selectin$-/-$ *versus* wild-type control mice. These observations suggested that under our experimental conditions, the ability of platelets to bind to tumor cells was not dependent on P-selectin. Nevertheless, the lung recruitment of tumor cells still showed a dependency on P-selectin. Although at present we cannot exclude additional effects of activated platelets beyond tumor cell binding, it is more likely that P-selectin on endothelial cells was the most important parameter in our experiments. It is interesting to note that this effect was seen most clearly in the lungs, but not in the liver and other organs. This could indicate that lung endothelium expressed higher amounts of P-selectin or was more rapidly up-regulated. Interestingly, activation of P-selectin on microvascular endothelium also plays a major role for the initial up-regulation of the inflammatory response occurring in hemorrhagic shock that is accompanied by injury to the liver and lungs (54, 55).

In the present report, we have not addressed tumor growth parameters because we wished to focus on molecular interactions that are standing at the beginning of organ colonization. Our finding that tumor cells endowed with sialylLe^x-CD24 are particularly well suited to interact with platelets, to roll on endothelial cells, and have an advantage in colonizing the lungs is of great importance. However,

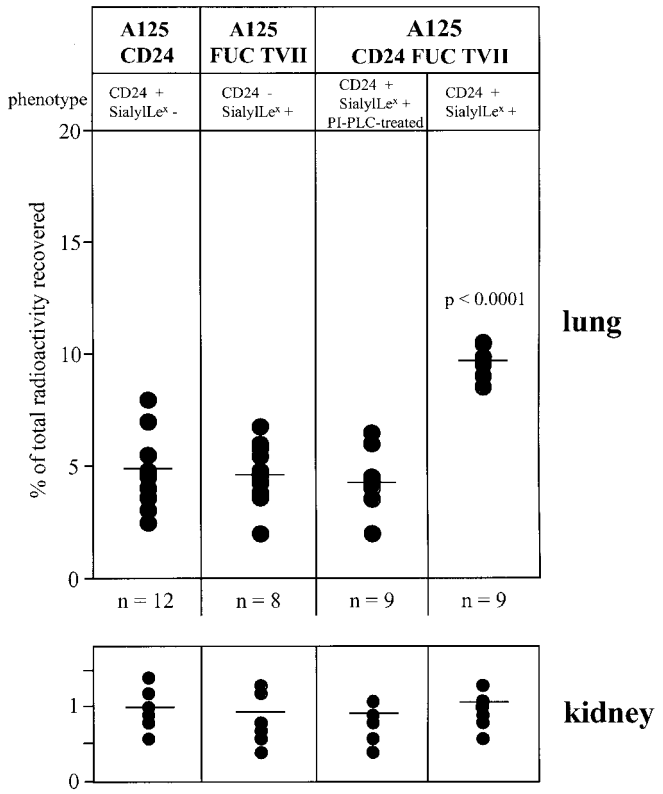

Fig. 8. In A125 cells, sialylLe^x-modified CD24 is required for lung arrest. Analysis of tumor cell arrest in the lungs. A125-CD24/FucTVII double-transfected cells untreated or treated with PIPL-C and A125-CD24 or A125-FucTVII single transfectants were labeled with ^{51}Cr, and 10^6 cells were injected into LPS-pretreated P-selectin$+/+$ mice. Organs of tumor cell-bearing animals were removed after 6 h, and the radioactivity content was determined by gamma counting.

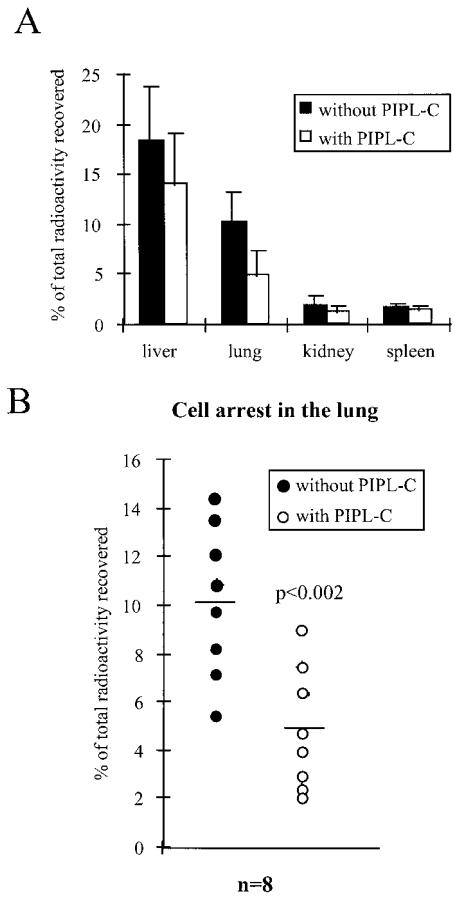

Fig. 9. Effect of PIPL-C treatment on lung arrest of KS breast carcinoma cells. *A*, KS breast carcinoma cells were not treated or treated with PIPL-C and labeled with ^{51}Cr, and 10^6 cells were injected into LPS-pretreated P-selectin+/+ mice. Organs of tumor cell-bearing animals were removed and counted after 6 h. The distribution of radioactivity in individual organs is given as a percentage of total input. *B*, the radioactivity content of the lungs is shown for each individual mouse.

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