

Endothelial P-Selectin as a Target of Heparin Action in Experimental Melanoma Lung Metastasis

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ABSTRACT

Spontaneous and experimental metastasis can be effectively inhibited by the widely used anticoagulant heparin in different tumor models. At the cellular level, many of the antimetastatic effects of heparin *in vivo* are due to its action on P-selectin-mediated binding. Whereas previous attention has focused on P-selectin-dependent tumor-cell–platelet interactions in blood-borne metastasis, we sought to address the potential contribution of endothelial P-selectin expression to adhesive events between the microvasculature and melanoma cells *in vivo*. Transplantation of bone marrow from P-selectin-deficient into wild-type mice conveyed inhibition of experimental melanoma metastasis. However, the extent to which bone marrow-conferred lack of platelet P-selectin expression attenuated melanoma lung metastasis was significantly less than that seen in P-selectin-deficient mice, suggesting that endothelial P-selectin expression may additionally contribute to formation of hematogenous metastases. This assumption was supported by our intravital microscopy studies, in which a significant proportion of melanoma cells were capable of directly interacting with postcapillary venules of the murine ear in a P-selectin-dependent manner. Heparin not only inhibits P-selectin-mediated melanoma cell rolling but also attenuates melanoma metastasis formation *in vivo*, further supporting the concept that endothelial P-selectin expression may represent an additional target of heparin action in experimental melanoma lung metastasis.

INTRODUCTION

The formation of metastases depends on a series of events, including the arrest and extravasation of cancer cells in the capillary bed of secondary organs and the continued survival and initial proliferation after extravasation (1, 2). Most circulating cancer cells are thought to eventually arrest by mechanical factors, preferentially as a result of blood-flow pattern and size restriction.

Ample evidence supports the concept that blood-borne metastasis is significantly facilitated by interactions between disseminating tumor cells and blood platelets (3–5). Interference by antiplatelet agents or anticoagulants in tumor-cell–platelet association potentially inhibits both spontaneous and experimental metastasis. In addition, measures that lower circulating platelet counts have resulted in a decrease in distant metastases. At the cellular level, tumor-cell–platelet interactions are assumed to both stabilize tumor cell arrest and assist cancer cell survival in secondary organs. In addition, platelet–tumor cell aggregates can protect from immune surveillance (6) and may supply tumor cells with critical stimulatory growth factors and cytokines, which are released on platelet activation (7, 8).

Important molecular mechanisms of tumor-cell–platelet interac-

tions have recently been identified (7). P-selectin, a member of the selectin family of adhesion molecules, has been demonstrated to mediate crucial cancer cell interactions with platelets and endothelial cells. *In vitro*, P-selectin binds to numerous tumor cell lines via sialylated fucosylated glycans present on mucin and nonmucin structures (9, 10). P-selectin is constitutively present in the α -granules of platelets and in the Weibel–Palade bodies of endothelial cells and can be rapidly translocated to the cell surface on activation to support adhesive interactions (11). The proposed role of P-selectin in platelet–tumor complex formation and metastasis was established by the use of genetically engineered P-selectin mutant mice. Both efficient tumor-cell–platelet aggregation and the development of experimental lung metastasis after *i.v.* injection of colon carcinoma cells were significantly diminished in P-selectin-deficient mice (12, 13). These observations indicate that P-selectin is critical for lung colonization of hematogenously borne tumor cells, supporting the concept that P-selectin-mediated tumor-cell–platelet interactions assist the process of metastasis.

On the other hand, the extent to which endothelial P-selectin expression may additionally contribute to formation of metastases has yet to be elucidated. Detailed ultrastructural analyses revealed that involvement of platelets occurred only after tumor cells had arrested, suggesting that early tumor cell arrest in the capillary bed proceeds independently of platelets (14, 15). Although cancer cells in general seem not to follow the adhesion cascade with eventual arrest to vessel wall endothelium in a manner comparable to that seen with leukocytes in inflammation, interactions with endothelial P-selectin have previously been demonstrated to mediate adhesion of melanoma cells under flow *in vitro* (16, 17). Thus, P-selectin-mediated adhesive events between the microvasculature and tumor cells may provide critical activation signals that facilitate tumor cell survival and initial proliferation after extravasation (18), whereas definite arrest of cancer cells is accomplished by blood flow pattern and mechanical entrapment (1).

Several independent studies have indicated that tumor metastasis can be inhibited by the widely used anticoagulant heparin in different experimental models (19). In addition to its functions as an anticoagulant drug, many biological effects of heparin and its derivatives have been elucidated over the past years that potentially account for their therapeutic actions in spontaneous and experimental metastasis. In particular, the effects of heparin in cancer have been attributed to interference with immune surveillance (20), with the action of heparin- or heparan sulfate-bound angiogenic growth factors (21), to obstruction of the activity of the extracellular matrix-degrading heparanase (22), and to inhibition of cancer cell adhesion to platelets and vascular endothelium (19). At the molecular level, strong evidence suggests that many of the established heparin effects are mediated by blockade of P-selectin binding (23, 24). This assumption has recently been substantiated *in vivo* by use of inflammatory and metastatic disease models (10, 25).

Here we show that transplantation of bone marrow (BM) from P-selectin-deficient into wild-type (wt) mice conveys inhibition of

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experimental lung metastasis. However, the extent to which BM-conferred lack of platelet P-selectin expression attenuated melanoma lung metastasis was significantly less than that seen in P-selectin-deficient mice. Because we demonstrate that a significant fraction of melanoma cells directly interact with postcapillary venules in a P-selectin-dependent manner, endothelial P-selectin expression may additionally contribute to formation of hematogenous metastases. Heparin is shown to inhibit both P-selectin-mediated melanoma cell rolling and lung melanoma metastasis formation *in vivo*, providing further evidence that endothelial P-selectin expression may represent an extra target of heparin action in experimental melanoma lung metastasis.

MATERIALS AND METHODS

Mice. P-Selectin-deficient (P-sel^{-/-}) mice on a C57BL/6J background (homozygous for the *Selp*^{tm1Bay} mutation) and control wt C57BL/6J mice were obtained from Charles River Deutschland GmbH (Sulzfeld, Germany) and were maintained on a 12-h light–dark cycle at the animal facility of the J. W. Goethe University. Mice were fed acidified drinking water and standard chow *ad libitum*. All protocols were approved by the governmental administration (Darmstadt, Hessen).

Cells and Culture Conditions. The human melanoma cell lines NW624 and NW1539 were generated and cultured as described previously (26). The murine melanoma cell line B16F10, originally derived from spontaneous melanoma of C57BL/6 origin, was from American Type Culture Collection (Rockville, MD). B16F10 cells were cultured at 37°C and 5% CO₂ in DMEM (Gibco Invitrogen Corp., Karlsruhe, Germany) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Gibco), 100 units/ml penicillin, 100 µg/ml streptomycin, and 250 µg/ml amphotericin B (all from Sigma Chemicals, Deisenhofen, Germany). For intravital microscopy, cells were fluorescently labeled with 10 µM carboxyfluorescein diacetate, succinimidyl ester [CFDA-SE; Molecular Probes, Eugene, OR] or with 2.5 µM Vybrant DiI cell-labeling solution (Molecular Probes) for dual-color intravital microscopy according to the manufacturer's instructions. For platelet isolation, blood was drawn from healthy volunteers through a 21-gauge needle. Blood was collected in 3-ml tubes containing sodium citrate (Sarstedt, Nümbrecht, Germany) and was centrifuged immediately at 110 × g for 15 min at 24°C to obtain platelet-rich plasma. Platelets were then separated from plasma proteins by gel filtration (27) through a Sepharose 2B column (Pharmacia, Uppsala, Sweden) and activated by the thrombin receptor-activating peptide TRAP-5 at 2.5 nM (Bachem, Heidelberg, Germany). Human peripheral blood mononuclear cells were prepared by Ficoll-Paque PLUS density gradient sedimentation (Amersham Biosciences, Freiburg, Germany), stained with 2.5 µM Vybrant DiI cell-labeling solution (Molecular Probes), and adjusted to 3 × 10⁷ cell/ml in AIM V cell culture medium (Gibco).

Experimental Lung Metastasis Assay. To prepare B16F10 cells for inoculation, cells were harvested by brief exposure to a 0.25% trypsin–0.02% EDTA solution (Sigma). Cell viability in single-cell suspensions was determined by trypan blue exclusion. A total of 3 × 10⁵ B16F10 cells (>90% viability) in 100 µl of PBS were injected into the lateral tail vein of 8–12-week-old male syngeneic C57BL/6J mice (Charles River). As indicated, selected mice received commercially available unfractionated sodium heparin [12.5 or 60 IU (100 µl) in 0.9% sodium chloride; Liquemin N; Hoffmann-La Roche AG, Grenzach-Wyhlen, Germany] 30 min before inoculation or every second day starting 24 h after tail vein injection. Animals were sacrificed 11 days postinjection, and lungs were excised and fixed in phosphate-buffered 10% formaldehyde. Metastatic foci at lung surfaces were counted by two observers in a blinded fashion.

Murine BM Transplantation Model. BM cells were obtained by flushing the femurs of donor P-selectin-deficient mice in C57BL/6J background (Charles River) as described previously (28). Recipient C57BL/6J mice received lethal irradiation doses at 4–6 weeks of age, and animals received *i.v.* injections containing 2–3 × 10⁶ BM cells. BM cells from normal C57BL/6J mice were taken as controls. At 6 weeks post-transplantation, by which time the BM of recipient mice was reconstituted, melanoma cell inoculation was performed.

Analysis of Platelet P-Selectin Expression. When mice were sacrificed at day 11 postinjection for analysis of lung metastases, blood was obtained by heart puncture and anticoagulated with sodium citrate (Sarstedt). Five µl of blood were immediately diluted in 50 µl of PBS containing calcium and magnesium. Platelets were activated with epinephrine (Aventis, Bad Soden, Germany) followed by staining with phycoerythrin-labeled anti-CD41 (clone MWReg30; BD Biosciences, Heidelberg, Germany) and FITC-labeled anti-CD62P-antibody (Ab; clone RB40.34; BD Biosciences) to identify platelets and P-selectin expression, respectively. Subsequently, cells were washed twice and analyzed by a BD FACScan Cytometer (Becton Dickinson, Mountain View, CA), equipped with CellQuest software for data acquisition and analysis (BD Biosciences). We collected 10,000 CD41⁺ cells for analysis of P-selectin expression.

Adherence of Platelets to Melanoma Cells. The adherence assay was performed according to the description by Kim *et al.* (29). Platelets were isolated as detailed above and were subsequently fluorescently labeled with 10 µM CFDA-SE. Before seeding, B16F10 cells were stained with 2.5 µM Vybrant DiI for dual-color fluorescence microscopy; B16F10 cells were grown to 50% confluency in 6-well plates overnight, washed with PBS, and incubated with TRAP-5-activated CFDA-SE-labeled platelets (3 × 10⁶ cells/well) in the presence of blocking P-selectin Ab (5 µg/ml; clone WAPS12.2; Pierce Biotechnology, Rockford, IL) or isotype-matched control Ab (5 µg/ml; rat IgG1, clone 43414; R&D Systems, Wiesbaden, Germany) or in the presence of unfractionated sodium heparin (5 IU/ml). After shaking for 15 min at room temperature, wells were washed twice with PBS. CFDA-SE-labeled platelets and DiI-stained B16F10 cells were visualized by fluorescence microscopy of corresponding representative fields.

Intravital Microscopy. Intravital microscopy was performed as described previously (30, 31). In brief, mice were anesthetized by *i.p.* injection of ketamine (Ketamin; Schwabe-Curamed, Karlsruhe, Germany) and xylazine (Rompun; Bayer AG, Leverkusen, Germany) and were placed on a homothermic blanket. The right common carotid artery was prepared microsurgically, and a catheter was inserted for retrograde injection of cells and heparin. The left ear of the mouse was gently placed between a microscope slide and a coverslip. Vascular architecture and fluorescently labeled cells were visualized during their passage through vessels under fluorescent epi-illumination by either a Zeiss-09 filter system (Zeiss, Oberkochen, Germany) for single-color intravital microscopy or a multiband filter system (XF 53; Omega Optical, Brattleboro, VT) for two-color intravital microscopy. Continuous video recordings of the microcirculation were obtained from a 1/3-inch DSP-3 charge-coupled device camera (DXC-390; Sony, Cologne, Germany) mounted on a modified (32) Zeiss microscope (Axioteck Vario 100 HD; Zeiss) equipped with a ×10 saltwater immersion objective (Nikon, Düsseldorf, Germany). Images were stored on tape by use of a Panasonic AG 7355 recorder. The velocities of cells in individual vessel segments were determined by off-line analysis of videotapes. Cells were considered noninteracting when they moved at the velocity of the mean blood flow (V_{free}), whereas cells moving at lower velocities were defined as interacting or rolling, respectively. To calculate V_{free} , we determined the mean velocity of at least 20 noninteracting cells for each vessel.

RESULTS

Transplantation of BM from P-Selectin-Deficient Mice Attenuates Lung B16F10 Melanoma Metastasis. Previously, the development of experimental lung metastasis after *i.v.* injection of colon carcinoma cells has been demonstrated to be significantly diminished in P-selectin-deficient mice (12, 13). To determine whether P-selectin deficiency also affected syngeneic B16F10 melanoma lung metastasis, we analyzed lung colony formation in P-selectin-deficient mice compared with wt controls (Fig. 1A). We found that the number of metastatic foci at lung surfaces was significantly reduced in P-selectin-deficient mice (median, 48 *versus* 246 in wt controls), supporting the assumption that a critical role of P-selectin for lung colonization is not restricted to particular subsets of tumor cells.

At present, primarily P-selectin expression by platelets has been implicated in assisting the process of metastasis via tumor-cell–

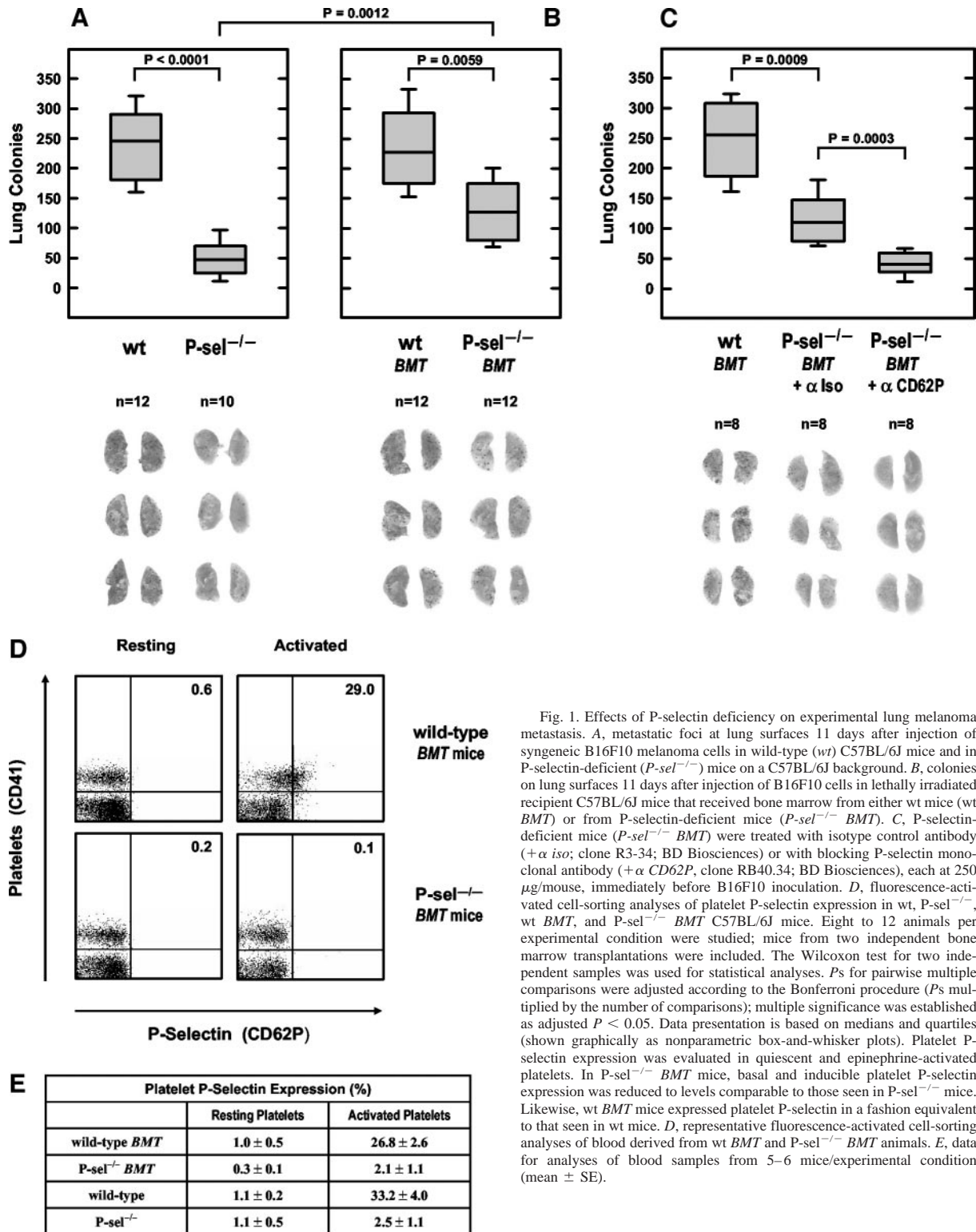


Fig. 1. Effects of P-selectin deficiency on experimental lung melanoma metastasis. *A*, metastatic foci at lung surfaces 11 days after injection of syngeneic B16F10 melanoma cells in wild-type (*wt*) C57BL/6J mice and in P-selectin-deficient (*P-sel*^{-/-}) mice on a C57BL/6J background. *B*, colonies on lung surfaces 11 days after injection of B16F10 cells in lethally irradiated recipient C57BL/6J mice that received bone marrow from either *wt* mice (*wt BMT*) or from P-selectin-deficient mice (*P-sel*^{-/-} *BMT*). *C*, P-selectin-deficient mice (*P-sel*^{-/-} *BMT*) were treated with isotype control antibody (+ α *iso*; clone R3-34; BD Biosciences) or with blocking P-selectin monoclonal antibody (+ α *CD62P*, clone RB40.34; BD Biosciences), each at 250 μ g/mouse, immediately before B16F10 inoculation. *D*, fluorescence-activated cell-sorting analyses of platelet P-selectin expression in *wt*, *P-sel*^{-/-}, *wt BMT*, and *P-sel*^{-/-} *BMT* C57BL/6J mice. Eight to 12 animals per experimental condition were studied; mice from two independent bone marrow transplantations were included. The Wilcoxon test for two independent samples was used for statistical analyses. *Ps* for pairwise multiple comparisons were adjusted according to the Bonferroni procedure (*Ps* multiplied by the number of comparisons); multiple significance was established as adjusted $P < 0.05$. Data presentation is based on medians and quartiles (shown graphically as nonparametric box-and-whisker plots). Platelet P-selectin expression was evaluated in quiescent and epinephrine-activated platelets. In *P-sel*^{-/-} *BMT* mice, basal and inducible platelet P-selectin expression was reduced to levels comparable to those seen in *P-sel*^{-/-} mice. Likewise, *wt BMT* mice expressed platelet P-selectin in a fashion equivalent to that seen in *wt* mice. *D*, representative fluorescence-activated cell-sorting analyses of blood derived from *wt BMT* and *P-sel*^{-/-} *BMT* animals. *E*, data for analyses of blood samples from 5–6 mice/experimental condition (mean \pm SE).

platelet interactions (7). Comparable to previous findings on LS-180 colon cancer cells (29), activated platelets adhered to murine B16F10 melanoma cells in a P-selectin-dependent fashion (Fig. 2). In a manner similar to the effect seen with blocking P-selection Ab (Fig. 2B), the presence of heparin almost entirely suppressed adherence of platelets to B16F10 cells (Fig. 2C). To provide further evidence for the significance of P-selectin expression by platelets, we transplanted BM from P-selectin-deficient into *wt* mice to explore whether the lack

of platelet P-selectin expression would convey inhibition of experimental lung metastasis. BM from either *wt* mice [*wt BMT* transplantation (*BMT*)] or from P-selectin-deficient mice (*P-sel*^{-/-} *BMT*) was transplanted in lethally irradiated *wt* recipient C57BL/6J animals (Fig. 1B). To confirm BM reconstitution, peripheral blood from each animal was analyzed for P-selectin expression on platelet activation (Fig. 1, *D* and *E*). Analyses of colonies at lung surfaces 11 days after injection of B16F10 cells revealed significant attenuation of meta-

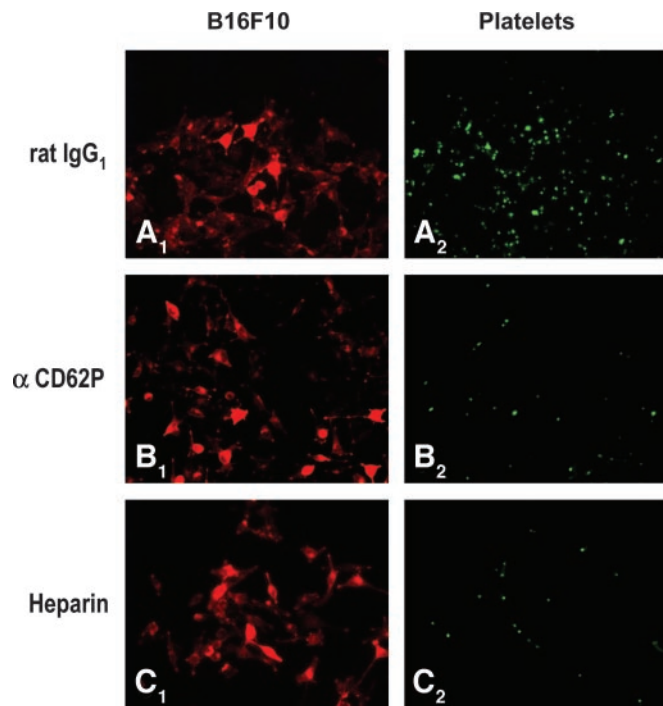


Fig. 2. P-selectin-dependent adherence of activated platelets on B16F10 melanoma cells. Adherence of freshly isolated platelets to cultured B16F10 melanoma cells in the presence of isotype control antibody (*rat IgG₁*; 5 μ g/ml; *A*), in the presence of blocking P-selectin antibody (*α CD62P*; 5 μ g/ml; *B*), or in the presence of unfractionated sodium heparin (*Heparin*; 5 IU/ml; *C*). DiI-stained B16F10 cells (*left panels*) and CFDA-labeled platelets (*right panels*) were visualized by fluorescence microscopy of identical fields. Data displayed are representative of three experiments that were performed, revealing comparable results.

static foci in mice that received BM from P-selectin-deficient mice (median, 138 *versus* 228 in wt *BMT* controls). These data substantiate the current view that P-selectin expression by platelets represents an important factor of blood-borne metastasis. However, the extent to which the BM-conferred lack of platelet P-selectin expression attenuated melanoma lung metastasis was significantly lower than that seen in P-selectin-deficient mice (Fig. 1, *A* and *B*). These data suggest that P-selectin expression by cells other than platelets may contribute to metastasis of experimental melanoma. This interpretation is also supported by our experiments in which the effects of an anti-P-selectin Ab on lung metastasis formation were tested in mice that received P-selectin-deficient BM (Fig. 1*C*). These analyses revealed that the extent to which the BM-conferred lack of platelet P-selectin expression attenuated melanoma lung metastasis was further decreased by anti-P-selectin Ab. We thus hypothesized that endothelial P-selectin expression may additionally assist formation of hematogenous metastases.

P-Selectin-Dependent Melanoma Cell–Endothelial Cell Interactions *in Vivo* Are Blocked by Heparin Treatment. To further assess the potential role of endothelial P-selectin in melanoma metastases, we performed intravital microscopy experiments, which permitted direct analyses of interactions between injected tumor cells and murine endothelium of postcapillary venules *in vivo* (Fig. 3). These experiments revealed that a highly reproducible proportion of different melanoma cell lines interacted with skin microvessels (Fig. 3, *A*, *E*, and *F*; Table 1). The proportion of interacting tumor cells seemed not to be affected by P-selectin expression by circulating platelets; proportions of rolling melanoma cells in *P-sel^{-/-}BMT* chimeric mice were equivalent to those observed in both wt *BMT* and in wt mice (Fig. 3, *C* and *D*). Melanoma cell rolling was diminished by blockade of mouse

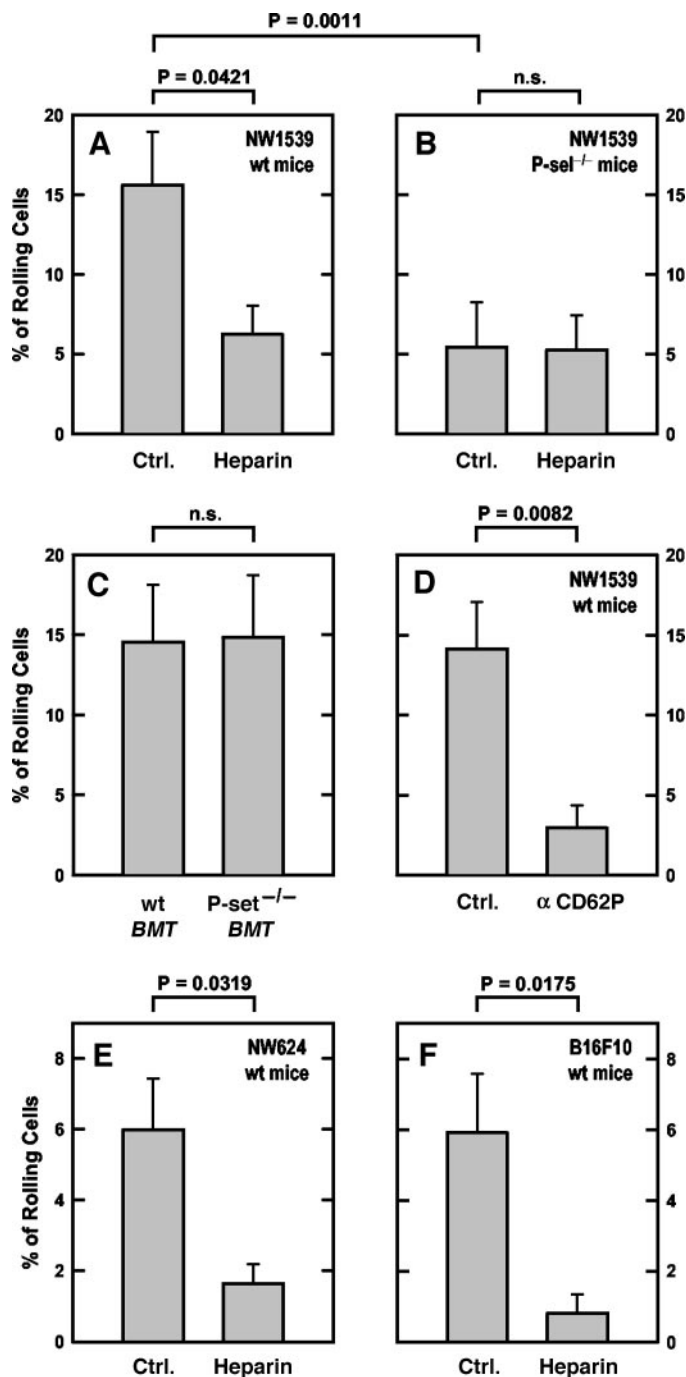


Fig. 3. Interactions of melanoma cells with postcapillary venules are P-selectin-dependent. Fluorescently labeled melanoma cells were injected into wild-type (*wt*) and P-selectin-deficient (*P-sel^{-/-}*) mice and were sequentially analyzed in the absence (*Ctrl*) and presence of heparin (*Heparin*; 100 IU). Interactions of tumor cells with postcapillary venules of the murine ear were assessed by intravital microscopy. Rolling interactions of the human melanoma line NW1539 in wt mice (*A* and *D*), in *P-sel^{-/-}* mice (*B*), and in mice that received transplanted bone marrow from either wt mice (*wt BMT*; *C*) or from P-selectin-deficient mice (*P-sel^{-/-} BMT*) are shown in graphic format at the top. The fraction of rolling cells was significantly reduced in wt mice in the presence of heparin (*A*) or blocking P-selectin monoclonal antibody [*D*; *α CD62P* (clone RB40.34; BD Biosciences), compared with isotype control antibody (clone R3-34, BD Biosciences); each at 150 μ g/mouse]. NW1539 melanoma cell-endothelial interactions were diminished in *P-sel^{-/-}* mice (*B*) compared with wt controls (*A*). Reduction of rolling in the presence of heparin was also demonstrable for the human NW624 line (*E*) and the murine B16F10 line (*F*). Data include results from 14 vessels/5 mice in *A*, 11 vessels/3 mice in *B*, 9 vessels/2 mice in *C*, 10 vessels/3 mice in *D*, 12 vessels/3 mice in *E*, and 7 vessels/2 mice in *F* (mean \pm SE; bars). The Wilcoxon test for two independent samples was used for statistical analyses; significance was established as $P < 0.05$. *n.s.*, not significant.

Table 1 Blood flow during intravital microscopy experiments under different experimental conditions

Mouse	Cell line	Treatment	V_{free} ($\mu\text{m/s}$)	<i>P</i>
Wild-type	B16F10	None	959 \pm 204	NS
Wild-type	B16F10	Heparin	808 \pm 135	
Wild-type	NW624	None	544 \pm 263	NS
Wild-type	NW624	Heparin	545 \pm 270	
Wild-type	NW1539	None	547 \pm 150	NS
Wild-type	NW1539	Heparin	586 \pm 244	
P-sel ^{-/-}	NW1539	None	821 \pm 302	NS
P-sel ^{-/-}	NW1539	Heparin	656 \pm 216	

^a NS, not significant; P-sel^{-/-}, P-selectin-deficient.

P-selectin binding (monoclonal Ab RB40.34; BD Biosciences; Fig. 3D). Adhesive interactions were also sensitive to heparin, which is known to act as a potent P-selectin inhibitor (23, 24). On the other hand, diminished rolling in P-selectin-deficient mice was not further reduced by heparin treatment (Fig. 3, A and B), further supporting the interpretation that tumor cells contacted murine ear postcapillary venules primarily in a P-selectin-dependent fashion. Observed differences of cell velocities were not due to changes in blood flow (Table 1).

Because we recently observed that activated platelets can form aggregates with leukocytes *in vivo*, resulting in increased rolling of leukocytes along vascular endothelium of the murine ear in a P-selectin-dependent fashion (33), we aimed to determine whether melanoma-endothelial cell interactions would be enhanced in the presence of activated platelets in a comparable manner. To discriminate between melanoma cells and platelets *in vivo*, we used two-color intravital microscopy. Activated platelets were stained with the green fluorescent dye CFDA-SE, and NW1539 cells were fluorescently labeled with the red dye DiI. Cells were incubated for 5 min before injection to allow aggregate formation *in vitro*, which was confirmed by fluorescence-activated cell-sorting analyses (data not shown). These experiments revealed neither considerable platelet-tumor cell interactions *in vivo* nor increased melanoma cell rolling in postcapillary venules of the murine ear (Fig. 4). Therefore, in contrast to platelet-mediated leukocyte delivery to skin endothelium, NW1539 melanoma cell adhesive interactions with endothelium may not be assisted by activated platelets.

Heparin Inhibits Experimental Melanoma Metastasis only if Administered before B16 Melanoma Cell Inoculation. Various independent studies have revealed that blood-borne metastasis can be effectively attenuated by the anticoagulant heparin in different tumor models (19). Our results confirmed these previous findings (13, 34): a single dose of unfractionated sodium heparin before tumor challenge resulted in a dose-dependent reduction of experimental B16F10 melanoma metastasis (Fig. 5). Significant inhibition by heparin was already observed at a dose of 12.5 IU, with further reduction seen at 60 IU. To address whether heparin may additionally affect formation and growth of lung colonies after melanoma challenge, metastatic foci at lung surfaces were additionally assessed in mice that had been treated only after melanoma inoculation or in which heparin was administered both before and after tumor challenge. Initiation of heparin treatment 24 h after melanoma challenge had no effect on blood-borne metastasis (Fig. 4). Correspondingly, intermittent injection of heparin every second day after tumor cell inoculation failed to induce additional or synergistic antimetastatic effects compared with mice treated before tumor challenge only. Heparin thus appears to mainly interfere with the establishment of metastasis rather than the initiation or maintenance of metastatic tumor growth.

DISCUSSION

Accumulating evidence indicates that many of the *in vivo* anti-metastatic effects of heparin reflect its action on P-selectin-mediated binding (10, 29). Whereas previous attention has focused mainly on P-selectin-dependent cell-platelet interactions as important determinants of blood-borne metastasis, the potential contribution of endothelial P-selectin expression to adhesive events between the microvasculature and tumor cells has yet to be elucidated. Several cell surface glycoconjugates bearing selectin-binding sites have previously been detected in melanoma cells. Lewis antigens (*e.g.*, sialyl Lewis X and sialyl Lewis a; Refs. 35, 36) and heparin sulfate-like proteoglycans have been identified as potential P-selectin ligand determinants (16). The interaction of tumor cell glycoconjugates with P-selectin expressed by platelets and endothelial cells may therefore facilitate extravasation and survival of circulating melanoma cells (12). Significantly, in the present study we showed that transplantation of BM from P-selectin-deficient mice conveys inhibition of experimental lung metastasis, further establishing the dependence of metastasis formation on P-selectin expression. At the same time, these experiments revealed that P-selectin-dependent attenuation of lung metastasis in deficient mice can be explained only in part by the lack of platelet P-selectin expression (Fig. 1). Therefore, these results suggested to us that P-selectin expression by endothelial cells may also be involved in enhancing the metastatic efficiency of melanoma cells.

Earlier studies on hematogenous metastasis have indicated that circulating tumor cells generally arrest in the microcirculation of secondary organs and may extravasate with high efficiency (37), regardless of the tumor type or the metastatic ability. Experimental *in vivo* observations and histological analyses revealed that the majority of cancer cells usually arrest in the first capillary bed because of size restrictions (38, 39). Despite efficient arrest and initial extravasation, however, significant differences are present relating to the subsequent propensity of cancer cells to survive extravasation and to proliferate to form metastatic colonies (1). We

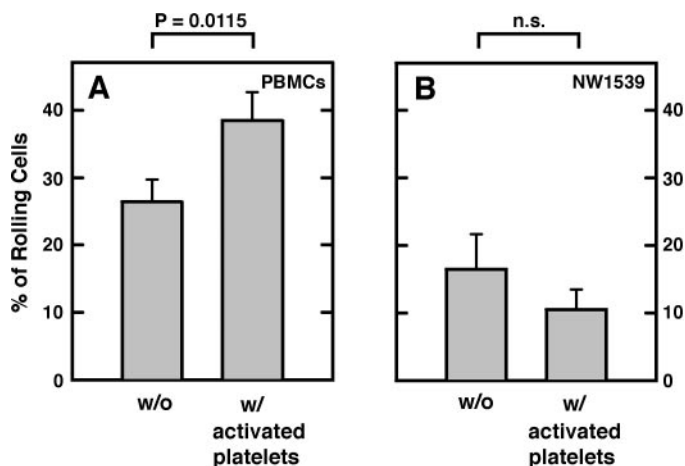


Fig. 4. Melanoma-endothelial cell interactions are not affected by the presence of activated platelets. Interactions of peripheral blood mononuclear cells (PBMCs) and NW1539 melanoma cells with murine endothelium of postcapillary venules in the absence (*w/o*) or presence (*w/*) of activated human platelets were analyzed by two-color intravital microscopy. PBMCs or NW1539 melanoma cells were simultaneously injected with activated platelets as indicated. In contrast to PBMCs (aggregate formation with platelets, 18.5 \pm 3.4%), NW1539 melanoma cells showed neither platelet-tumor cell interactions (aggregate formation with platelets, 0.0 \pm 0.0%) nor increased melanoma cell rolling *in vivo*. Data are from analyses of 10 vessels/4 mice in A and 6 vessels/3 mice in B (mean \pm SE; bars). The Wilcoxon test for two independent samples was used for statistical analyses; significance was established as $P < 0.05$. *n.s.*, not significant.

therefore hypothesized that endothelial P-selectin-mediated contacts to cancer cells may provide critical activation signals that potentially facilitate tumor cell survival and initial proliferation after extravasation. This assumption is supported by recent experimental data showing P-selectin-dependent adhesion of malignant melanoma cells *in vitro* (16, 17). Although postnatal vasculogenesis is less likely to be involved in the initial stages of metastatic growth, integration of P-selectin-deficient endothelial progenitors may have additionally contributed to P-selectin-dependent attenuation of lung metastasis in our BM transplantation studies.

We assessed probable adhesion pathways under shear flow conditions *in vivo* by use of intravital microscopy and demonstrated direct interactions between melanoma cells and murine ear postcapillary venules (Fig. 3). A highly reproducible proportion of different melanoma cell lines showed spontaneous P-selectin-dependent rolling in skin microvessels *in vivo*. As anticipated, the adhesive interactions were sensitive to heparin, which is known to act as a potent P-selectin inhibitor (23, 24). On the other hand, diminished rolling in P-selectin-deficient mice was not further reduced by heparin treatment (Fig. 3B), supporting the assumption that tumor cells make contact with murine ear postcapillary venules primarily in a P-selectin-dependent fashion. These observations are in line with results from our experimental metastasis assays, in which administration of heparin attenuated melanoma lung metastasis to levels comparable to those seen in P-selectin-deficient mice (Figs. 1 and 5). Similar findings were recently reported in an experimental lung metastasis model involving colon adenocarcinoma cell lines (10, 13), suggesting a more general significance of P-selectin in hematogenous metastasis. Because eventual arrest of tumor cells in secondary organs seems to be accomplished primarily by mechanical factors (1), P-selectin-dependent contacts with target cells before entrapment may mediate intercellular signaling events. In addition to its anchoring functions, P-selectin and its ligands have been shown to act as important signal transduction receptors (18). In the context of leukocyte extravasation, P-selectin-dependent binding of monocytes or neutrophils was demonstrated to trigger signaling that resulted in changes in cytokine expression or the integrin activation state (40, 41). Pertinent to these findings, adhesive interactions of melanoma cells with endothelial P-selectin could likely initiate signal transduction pathways that convey changes in gene expression and cytoskeletal arrangement, thereby affecting cellular survival and proliferation of single tumor cells during extravasation (37).

Whereas the inhibitory effects of heparin on experimentally induced metastasis have been repeatedly reported in distinct models over past years, the cellular and molecular mechanisms by which heparin mediates its actions have only recently been elucidated in greater detail (7, 19). Different heparin preparations were shown to act as ligands for P-selectin, thereby blocking binding of P-selectin to its natural and tumor mucin ligands (23, 24). Importantly, both tumor-cell-platelet aggregation and experimental metastasis of human carcinoma cells were seen to be significantly inhibited in P-selectin-deficient mice (29). In an equivalent fashion, we found that lung colonization of syngeneic B16 melanoma cells was markedly attenuated in P-selectin-deficient mice compared with wt controls (Fig. 1). Because L-selectin expression by leukocytes may also facilitate metastatic spread *in vivo* (10), the inhibitory effects of heparin on L-selectin binding may additionally convey the antimetastatic effects observed in our studies. However, both experimental lung metastasis formation (Fig. 5) and melanoma cell rolling (Fig. 3) are attenuated by heparin treatment to an extent comparable to that seen in P-selectin-deficient mice (Fig. 1), suggesting that the heparin effects detected in our experimental models are mediated primarily by inhibition of P-selectin-

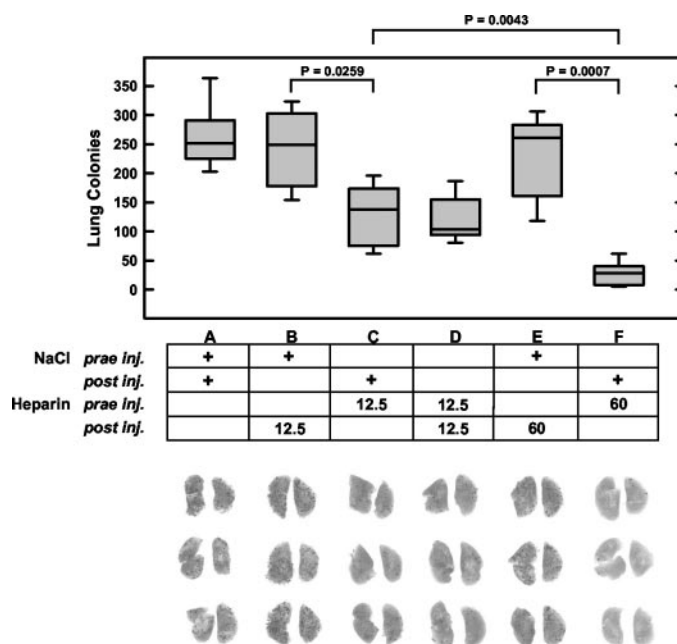


Fig. 5. Effects of different heparin treatment schedules on experimental lung melanoma metastasis. Metastatic foci at lung surfaces 11 days after injection of B16F10 melanoma cells in wild-type C57BL/6J mice. As detailed, mice received sodium heparin (12.5 or 60 IU in 100 μ l) 30 min before tumor challenge and every second day starting 24 h after tail vein injection (days 1, 3, 5, 7, and 9) or vehicle only (0.9% NaCl). Six to nine animals were studied per experimental group. The Wilcoxon test for two independent samples was used for statistical analyses; significance was established as $P < 0.05$. Data presentation is based on medians and quartiles (graphically as nonparametric box-and-whisker plots). *prae inj.*, before injection; *post inj.*, after injection.

dependent binding. This assumption is also supported by recent studies on colon carcinoma cell metastasis that indicated that the effect of a single heparin dose is accounted for by blockade of P-selectin function only (10). Heparin not only impaired P-selectin-dependent platelet-tumor cell interactions but also suppressed blood-borne metastasis in secondary organs, whereas in P-selectin-deficient mice no further inhibition by heparin treatment was seen (10, 13). Therefore, substantial evidence supports the concept that heparin mediates its antimetastatic effects to a great extent via inhibition of P-selectin-dependent interactions.

Activated platelets have been shown previously to facilitate leukocyte-endothelial cell interactions in a P-selectin-dependent fashion, providing an additional mechanism for leukocyte delivery to the microvasculature (42, 43). Evidence has been provided that leukocyte-platelet interactions may augment both direct adhesion of blood mononuclear cells to the endothelium (44) and indirect leukocyte adhesion via bridging platelets (42). Pertinent to these findings, we have recently observed that activated platelets can form aggregates with leukocytes *in vivo*, resulting in increased rolling of leukocytes along vascular endothelium of the murine ear in a P-selectin-dependent fashion (33). We hypothesized that melanoma-endothelial cell interactions would be enhanced in the presence of activated platelets in a comparable manner. Use of a two-color intravital microscopy that allows for discrimination between melanoma cells and platelets *in vivo* revealed neither considerable platelet-tumor cell interactions nor increased melanoma cell rolling in postcapillary venules of the murine ear (Fig. 4). Therefore, in contrast to platelet-mediated leukocyte delivery to skin endothelium, melanoma cell adhesive interactions with endothelium may not to be assisted by activated platelets.

Strikingly, a single dose of unfractionated heparin resulted in significant reduction of experimental blood-borne metastasis (13,

34). Previous studies have also indicated that both unfractionated and different fractionated heparin preparations may exert antimetastatic effects in a dose-dependent fashion (34). However, the extent to which heparin may additionally affect formation and growth of lung colonies after tumor challenge has yet to be resolved. Sciumbata *et al.* (34) reported a slight but insignificant prolongation of survival when heparin treatment was maintained on a twice/week cycle for 3 weeks after i.v. injection of B16 melanoma cells. The results from our heparin treatment studies clearly suggest that initiation of heparin treatment 24 h after tumor challenge has no effect on blood-borne metastasis (Fig. 4). Similarly, continued injection of heparin every second day after melanoma cell inoculation failed to produce additional or synergistic antimetastatic effects compared with mice treated before tumor challenge only. Thus, heparin seems to primarily interfere with the establishment of metastases rather than the initiation or maintenance of metastatic tumor growth.

Heparin comprises a complex set of glycosaminoglycan molecules that, in addition to the established anticoagulant effects, have been shown to modulate numerous cellular functions (19). Because inhibition of P-selectin represents a key mechanism by which heparin mediates its antimetastatic as well as its anti-inflammatory properties, specific oligosaccharide structures in heparin have been identified recently that may specifically interact with P-selectin (25). These studies indicated that inhibitory properties depend on 6-*O*-sulfated glucosamine residues, which are distinct from the binding site for antithrombin that confers the potent anticoagulant activity of heparin (45). Hence, chemically modified heparins are conceivable that exert diminished anticoagulant activity but retain antimetastatic properties. Alternatively, noncarbohydrate imidazole-based selectin inhibitors, which have been identified by use of a P-selectin ELISA-based assay system, may characterize an attractive class of compounds with potential therapeutic effects on metastasis formation (46). In addition, we have recently shown that efomycines, comprising a novel family of small molecules with inhibitory effects on selectin-dependent cell binding, exhibit considerable efficacy in different disease models of cutaneous inflammation (30). Hence, it will be of utmost interest to investigate whether these compounds will actually interfere with P-selectin-dependent tumor cell rolling and metastasis formation *in vivo*.

We here provide evidence that endothelial P-selectin expression may contribute to the formation of hematogenous metastases. This assumption is supported by transplantation studies with BM from P-selectin-deficient mice, revealing that P-selectin-dependent attenuation of lung metastasis in deficient mice can be explained only in part by the lack of platelet P-selectin expression. In addition, we demonstrated that melanoma cells directly interact with postcapillary venules in a P-selectin-dependent manner, an interaction that can be fully abrogated by heparin treatment. These results thus indicate that endothelial P-selectin expression may represent an additional target of heparin action in experimental melanoma lung metastasis.

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Endothelial P-Selectin as a Target of Heparin Action in Experimental Melanoma Lung Metastasis

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