L-Selectin Facilitation of Metastasis Involves Temporal Induction of *Fut7*-Dependent Ligands at Sites of Tumor Cell Arrest

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Abstract

Hematogenous carcinoma metastasis is supported by aggregated platelets and leukocytes, forming tumor cell emboli. Early tumor cell-platelet interactions can be mediated by P-selectin binding to tumor cell surface ligands and this process is blocked by heparin. We previously showed that L-selectin deficiency also attenuates experimental metastasis. However, the mechanisms and timing of L-selectin action remained unknown. Here, we study how L-selectin facilitates establishment of pulmonary metastatic foci in syngeneic mice by using experimental metastasis to time events following entry of tumor cells into the bloodstream. Although L-selectin deficiency did not affect platelet aggregation or initial tumor cell embolization, the association of leukocytes with tumor cells was reduced and tumor cell survival was diminished 24 hours later. Temporal inhibition of L-selectin by a function-blocking antibody reduced metastasis. Moreover, although selectin blockade by heparin 6 to 18 hours after tumor cell injection was synergistic with P-selectin deficiency in reducing metastasis, there was no further effect in L-selectin-deficient animals. Thus, heparin apparently works at these time points primarily by blocking L-selectin. Endogenous L-selectin ligands were concomitantly induced adjacent to established intravascular tumor cell emboli in a similar time window when leukocytes were also present. Metastasis was attenuated in mice missing these induced endogenous L-selectin ligands due to fucosyltransferase-7 deficiency. Thus, L-selectin facilitation of metastasis progression involves leukocyte-endothelial interactions at sites of intravascular arrest supported by local induction of L-selectin ligands via fucosyltransferase-7. These data provide the first explanation for how leukocyte L-selectin facilitates tumor metastasis. (Cancer Res 2006; 66(3): 1536-42)

Introduction

Substantial evidence indicates that carcinoma cells entering the blood circulation from solid tumors rapidly interact with platelets and leukocytes, forming tumor cell emboli that contribute to establishing metastatic foci (1, 2). Although the mechanism of platelet aggregation on tumor cells and its contribution to metastasis have been studied (3–6), the facilitating role of leukocytes remains poorly understood. Leukocyte responses to cancer are not unique and have many parallels with inflammation and wound healing, as leukocytes undergo regional and intratumoral activation (7–9). Leukocytes are now thought to not only have potential antitumor roles but also seem to be supporting tumor progression. The contribution of leukocytes to tumor progression seems to be dependent on temporal and spatial stimulation, which is defined by the tumor microenvironment (7, 10, 11). During early tumorigenesis, inflammatory leukocytes can be powerful tumor promoters, producing chemokines, growth factors, and cytokines required for tumor growth, angiogenesis, and migration (7). These causal relationships are now widely accepted but many cellular and molecular mechanisms remain unresolved.

The contribution of vascular cell adhesion molecules P-selectin and E-selectin to metastasis is known, involving selectin binding to tumor cell ligands (3, 5, 12, 13). However, the possibility of endogenous (nontumor) selectin ligand involvement in facilitation of metastasis was not studied. In peripheral blood, L-selectin is constitutively expressed on all neutrophils and monocytes and on subsets of T cells, B cells, and natural killer (NK) cells (14). L-selectin supports recruitment of leukocytes into sites of inflammation via endothelial ligands (14, 15). Leukocyte rolling along blood vessel walls is also mediated by L-selectin recognition of P-selectin glycoprotein ligand-1 (PSGL-1) on already adherent leukocytes (16).

Heparin, a commonly used anticoagulant in the clinic, is an excellent inhibitor of P-selectin and L-selectin (17). Furthermore, heparin treatment of cancer patients was shown to improve survival of cancer patients by mechanisms not explainable by anticoagulation (18, 19). In mouse models, heparin was shown to effectively inhibit P-selectin-mediated binding of platelets and endothelia to tumor cells, thereby attenuating metastasis (3, 5, 20).

Previously, we have observed attenuation of metastasis in L-selectin-deficient mice, thus implicating leukocytes in this process (20). The effect of L-selectin was noted even in T- and B-cell-deficient mice, indicating that granulocytes and/or monocytes are the likely effectors. The present study was undertaken to elucidate the role of L-selectin in facilitating metastasis, using selectin- and fucosyltransferase-deficient mice, as well as treatment with heparin and anti-L-selectin antibodies.

Materials and Methods

Syngeneic mouse/tumor model. P-selectin-deficient mice (Psel−/−) in a C57BL6 background <Selp tm1Bay> and control wild-type (wt) C57BL6 mice were from The Jackson Laboratory (Bar Harbor, ME). L-selectin-deficient mice (Lsel−/−) were backcrossed into the C57BL6 background as described previously (20). Fucosyltransferase 7-deficient mice (FucT7−/−) in a C57BL6 background were kindly provided by Dr. J.B. Lowe (University of Michigan, Ann Arbor, MI). For experimental metastasis studies, syngeneic MC-38 murine colon carcinoma cells, derived from C57BL6 mouse strain and isolated as a mixed population of cells stably expressing green fluorescent protein (GFP; MC-38GFP), were used (20).

Note: H. Läubli and J.L. Stevenson contributed equally to this work.

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***In vivo* analysis of tumor cell thrombi formation and tumor cell survival.** Mice were i.v. injected with 4×10^5 MC-38GFP cells and lungs were prepared at various time points as described previously (3). Frozen lung sections were stained with a polyclonal antifibrin antibody (Nordic Immunological Lab, Tilburg, the Netherlands) or with a monoclonal anti-CD41 antibody (Becton Dickinson, San Jose, CA) followed by donkey–anti-goat–Alexa568 or goat–anti-rat–Alexa660 antibodies, respectively (Molecular Probes, Eugene, OR). Sections were analyzed on a Leica SP1 confocal laser scanning microscope. Serial images in the *z* axis through the tissue section were taken, and the resulting stack of images was analyzed using Imaris software (Bitplane AG, Zurich, Switzerland). At least eight images for every lung/time point were analyzed. The number of surviving cells was determined on 240 view fields at ×400 magnification from at least two mice at every time point for each genotype (Lsel+/+ or Lsel−/−). Statistical significance was determined by the Student's *t* test.

***In vivo* detection of leukocytes associated with tumor cells.** Lungs were prepared from mice injected with MC-38GFP cells as described above. Frozen lung sections were stained with a monoclonal anti-CD11b (Mac-1) antibody (Becton Dickinson), followed by goat–anti-rat–Alexa568 antibody (Molecular Probes). Serial section image stacks, representing 4.5 μm thickness of a tissue section acquired by Leica SP1 confocal laser scanning microscope were analyzed by Imaris software (Bitplane). At least 30 independent images for every lung/time point were analyzed. Statistical significance was determined by the Student's *t* test.

Experimental metastasis assays. Mice were i.v. injected with 3×10^5 to 5×10^5 MC-38GFP cells. Some received 100 IU of i.v. heparin (Liquemin 5,000 IU/mL, Roche Pharma, Reinach, Switzerland) 6 and 12 hours after tumor cell injection (hereafter called "late" heparin injection). In some experiments, L-selectin function was blocked *in vivo* by i.v. injection of 160 μg of MEL-14-F(ab′)$_2$ at given time points. The L-selectin function blocking monoclonal antibody (rat IgG2a) MEL-14 was purified from hybridoma supernatant (American Type Culture Collection, Manassas, VA) and processed to F(ab′)$_2$ fragments [Pierce Immunoglobulin F(ab′)$_2$ Preparation kit; Pierce Chemical Co., Rockford, IL]. Metastases were evaluated after varying amounts of time by counting lung foci and/or detecting GFP fluorescence in lung homogenate as described previously (20).

Detection of L-selectin ligands in the lung tissue. Lungs were prepared for analysis at various time points after i.v. injection of tumor cells essentially as described previously (3) but were first fixed for 15 minutes by inflation with 2% paraformaldehyde in HBSS (Sigma, Buchs, Switzerland) through the trachea. To detect selectin ligands, sections were treated for 30 minutes at 4°C in the dark with 2 mmol/L sodium periodate, which selectively oxidizes sialic acid side chains, thereby enhancing L-selectin binding to its ligands (21), followed by incubation with mouse L-selectin–Ig-Fc chimera at 30 μg/mL (Sigma) for 1 hour at room temperature. Control slides were incubated with L-selectin chimera in the presence of 10 mmol/L EDTA. Chimera binding was detected by goat–anti-human–Alexa568 antibody (Molecular Probes). Lungs were prepared from two independent experiments and at least five random images for each time point (11 images for 24-hour time points) were analyzed (see above).

Results

L-selectin supports the extended survival of hematogenously embolized tumor cells. All studies were done in a syngeneic mouse model by using experimental metastasis to precisely monitor the timing of events following entry of tumor cells into the bloodstream. To determine the time window during which L-selectin facilitates metastasis, we analyzed the survival of the i.v. injected tumor cells in lungs, the organ primarily colonized by such injected cells. Wt (Lsel+/+) and L-selectin-deficient (Lsel−/−) mice were injected with MC-38GFP syngeneic murine colon carcinoma cells and euthanized at different time points. Surviving GFP-labeled tumor cells were visualized in tissue sections in the lung microvasculature. Initial arrest of tumor cells in the pulmonary vasculature was similar in both groups (Fig. 1*A*). This contrasts with P-selectin deficiency, which causes a decrease in initial arrest (22). There was no difference in tumor cell survival during the first 12 hours, indicating that initial survival was also L-selectin independent (Fig. 1*A*). However, significantly fewer tumor cells were observed in the lungs of Lsel−/− mice at 24 hours postinjection and beyond (Fig. 1*A*). Thus, the absence of L-selectin reduced the long-term survival of tumor cells. These data implicate L-selectin (presumably expressed on leukocytes associated with the established tumor emboli) in supporting the eventual establishment of metastatic foci.

An important step in the process of hematogenous metastasis is the formation of tumor cell emboli (1). We previously showed that initial platelet-tumor cell interactions are mediated mainly by P-selectin (3). To address the possible role of leukocyte L-selectin at this step, we analyzed tumor cell emboli for the presence of platelets and fibrin by immunofluorescence microscopy, followed by three-dimensional image reconstitution. There was no significant difference in the tumor thromboembolus composition at any given time

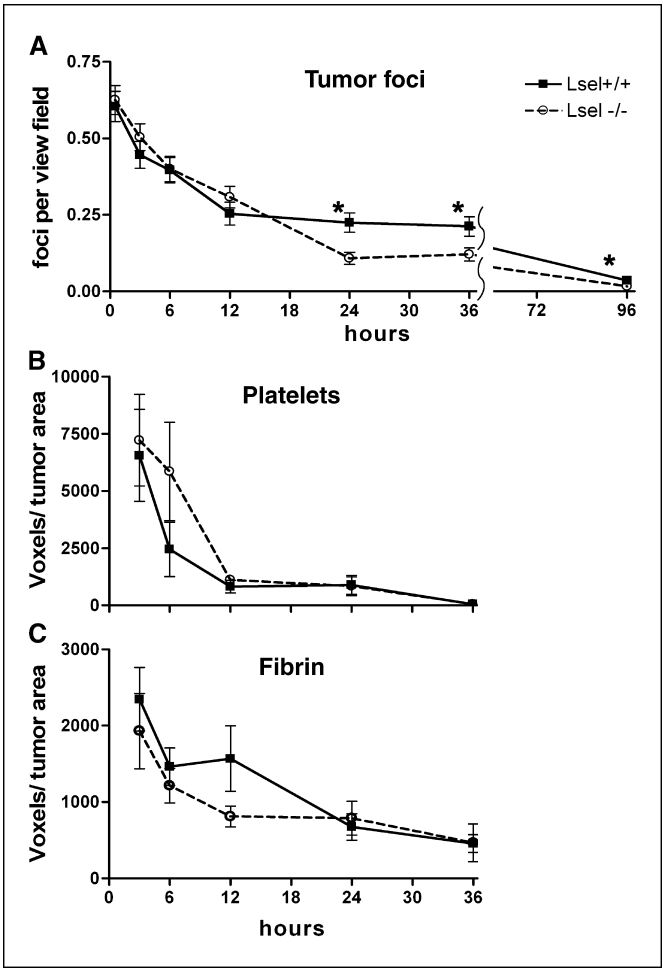
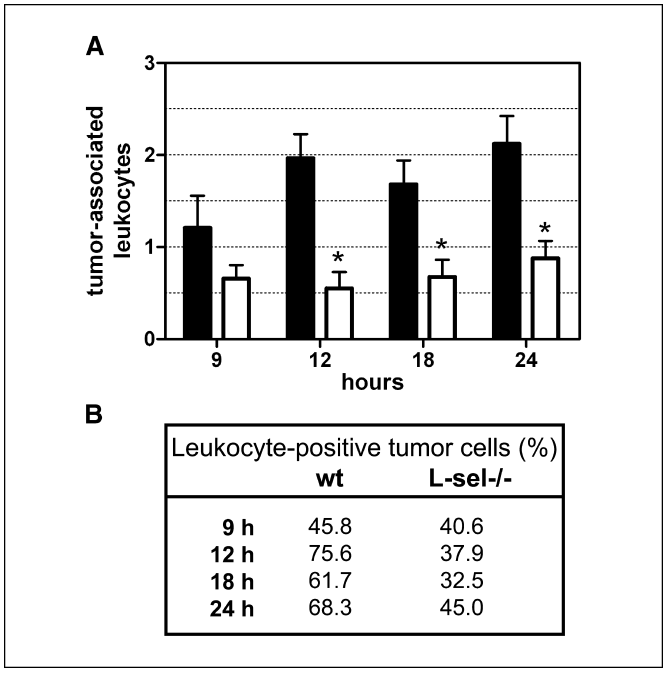


Figure 1. L-selectin deficiency reduces the number of surviving tumor cells in the lung vasculature but does not affect the formation of tumor cell thrombi. *A,* mice were i.v. injected with 4×10^5 MC-38GFP cells and euthanized at different time points between 6 and 96 hours. The number of viable, GFP-positive tumor cells (microfoci) was determined. The reduction of viable tumor cells in L-selectin-deficient mice was statistically significant at time points 24 hours and later (*, $P < 0.05$). *B* and *C,* tumor cell emboli were analyzed by staining with CD41 antibody detecting platelets and with antifibrinogen antibody. Analysis of tumor emboli was done by three-dimensional reconstruction using Imaris software. The signal in voxels is presented for the volume of 1,000 μm^3 surrounding each central tumor cell. None of the time points showed statistically significant differences. *Points,* mean; *bars,* SE.



B

Leukocyte-positive tumor cells (%)	wt	L-sel-/-
9 h	45.8	40.6
12 h	75.6	37.9
18 h	61.7	32.5
24 h	68.3	45.0

Figure 2. Association of leukocytes with tumor cell emboli is L-selectin dependent. Mice i.v. injected with MC-38GFP cells were euthanized at different time points, and lung frozen sections were stained with anti-CD11b antibody to detect monocytes and granulocytes. *A,* the number of leukocytes found near tumor cells in wt mice (*solid columns*) and in L-sel−/− mice (*open columns*) was found to be significantly different from 12 hours onward. *Columns,* mean; *bars,* SE; *, $P < 0.005$. *B,* the percentage of tumor cells associated with leukocytes at given time points was found to be significantly different from 12 hours onward ($P < 0.05$) between wt and L-sel−/− mice.

point between Lsel+/+ and Lsel−/− mice (Fig. 1*B* and *C*). Interestingly, platelets and fibrin were rapidly eliminated from surviving tumor cells within the first 12 hours, in agreement with previously observed rapid platelet elimination (6). Thus, the reduction of metastasis observed in Lsel−/− mice is mediated neither by effects on platelet aggregation nor on fibrin deposition.

L-selectin facilitates establishment of metastatic foci. The presence of L-selectin in wt mice led to better survival of tumor cells (Fig. 1*A*), suggesting that L-selectin can positively affect early steps in metastasis. This led us to hypothesize that L-selectin contributed to the recruitment of leukocytes to tumor cell emboli. Thus, we analyzed lung sections for the presence of leukocytes (monocytes and granulocytes) by staining with anti-CD11b (Mac-1) antibody (Fig. 2). A statistically significant difference in the number of leukocytes associated with tumor cells between wt and L-sel−/− mice was detected at 12 hours and beyond (Fig. 2*A*). Although a time-dependent increase in association of CD11b-positive leukocytes with tumor cells was observed in wt mice, no difference was detected in L-sel−/− mice (Fig. 2*B*). This finding suggests that L-selectin-mediated recruitment of leukocytes to tumor cell emboli might affect the initiation of tumor cell extravasation. To test this, we injected mice with L-selectin function blocking $F(ab')_2$ MEL-14 Ab at 6 and 12 hours after tumor cell injection. The selected dose of $F(ab')_2$ MEL-14 antibody lasted in circulation for up to 6 hours (data not shown). This temporal inhibition of L-selectin function for ~12 hours led to a significant attenuation of metastasis as evaluated by the number of metastatic foci and the extent of metastases (Fig. 3*A* and *B*). The time when the inhibition of L-selectin function affected metastasis (+6 hours up to +18 hours after tumor cell injection) corresponded to the time period when a difference in tumor cell survival was observed (Fig. 1*A*) and when L-selectin-dependent association of leukocytes with tumor cell was detected (Fig. 2). Inhibition of L-selectin function by the $F(ab')_2$ MEL-14 antibody given beyond 12 hours after tumor cell injection did not effect metastasis (data not shown), indicating the time restriction of L-selectin action in this process. Taken together, these data suggest that L-selectin-mediated interactions of leukocytes at the sites of embolization support the initiation of tumor cell extravasation. Thus, inhibition or absence of L-selectin is reflected in the reduced number of eventually established metastatic foci.

Heparin also attenuates metastasis at time points beyond the early inhibition of P-selectin. We previously showed that a single injection of unfractionated heparin, which remained in the circulation for <6 to 8 hours, significantly reduced metastasis both in syngeneic and xenogeneic mouse models (3, 20). This "early" heparin injection (given 30 minutes before tumor cell injection) inhibited P-selectin-mediated platelet-tumor cell interactions. Because heparin can inhibit not only P-selectin but also L-selectin (17), we asked whether heparin administration several hours after tumor cell injection would affect metastasis (23). Based on the results with temporal L-selectin function blocking by antibody administration (Fig. 3), we tested the effect of two heparin injections given at 6 and 12 hours after tumor cell challenge

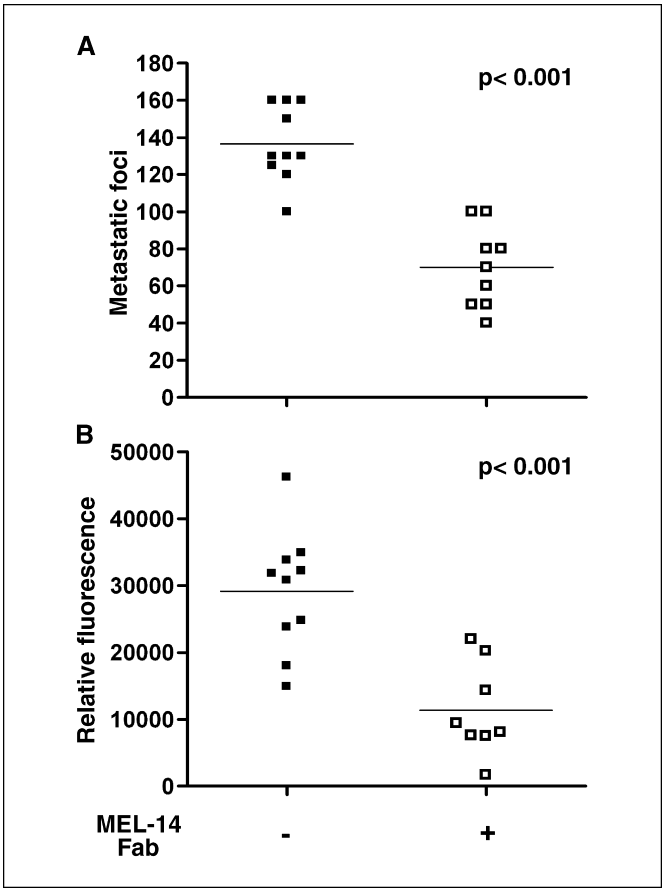


Figure 3. Temporal inhibition of L-selectin function *in vivo* attenuates metastasis. *A,* wt mice were injected with MC-38GFP cells, followed by injections of 160 μg of L-selectin function blocking antibody MEL-14 $F(ab')_2$ at +6 and +12 hours. Mice were euthanized after 28 days and dissected lungs were evaluated for metastatic foci. *B,* the extent of metastasis was analyzed by GFP fluorescence read out in lung homogenates.

(late heparin injection), presumably blocking P-selectin- and L-selectin-mediated interactions for ~16 hours, starting from 6 hours until ~20 hours after tumor cell injection.

To discount any effect of late heparin administration on P-selectin-mediated interactions, we first analyzed the effect of such treatment in P-selectin-deficient mice (Psel−/−). Because the level of observed metastases in Psel−/− mice is considerably lower than in wt mice (20), we euthanized them at a later time point than usual (after 44 days) and counted the number of metastatic foci (Fig. 4*A*). Mice receiving late heparin injections showed significantly fewer metastatic foci compared with those receiving PBS injections. The attenuation was confirmed by measuring GFP fluorescence in the whole lung homogenate (Fig. 4*B*). In fact, late heparin injection in Psel−/− mice further attenuated metastasis to a level comparable

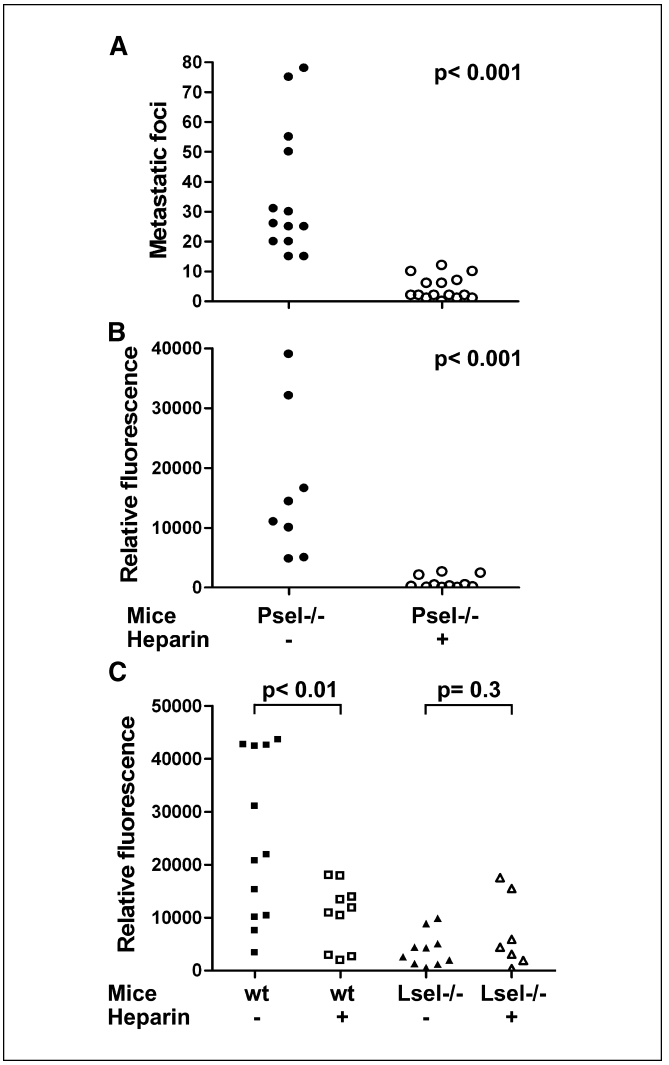


Figure 4. Late heparin injection attenuates metastasis in a P-selectin independent manner, but has no further effect in the absence of L-selectin. *A,* Psel−/− mice were injected with MC-38GFP cells, followed by injections of 100 IU of heparin at +6 and +12 hours. Mice were euthanized after 44 days and dissected lungs were evaluated for metastatic foci. *B,* the extent of metastasis was analyzed by GFP fluorescence read out in lung homogenates. *C,* wt and Lsel−/− mice were injected with MC-38GFP cells, followed by an i.v. injection of 100 IU unfractionated heparin at +6 and +12 hours. Heparin blocked selectin-dependent interactions for a period between +6 and +18 hours after tumor cell injection. Mice were euthanized after 28 days, and lungs were dissected and homogenized for quantitation of metastasis by GFP fluorescence detection.

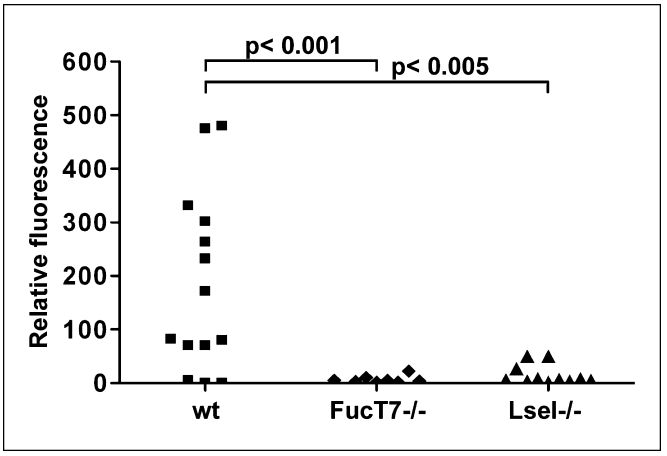


Figure 5. Absence of endogenous selectin ligands leads to attenuation of metastasis. FucT7−/− mice were i.v. injected with 5×10^5 MC-38GFP cells. Parallel injections of MC-38GFP cells were also done in wt and Lsel−/− mice. Mice were euthanized after 34 to 39 days, and lungs were dissected and homogenized for the detection of metastasis by GFP fluorescence.

with the extreme reduction of metastasis previously noted in P-selectin and L-selectin double-deficient mice (20). Thus, the late heparin treatment (6 and 12 hours after tumor cell injection) is not working by inhibiting P-selectin, but is synergistic with it. Rather, it is likely to be working by blocking L-selectin.

To exclude the possibility that late heparin injection exerts other major biological activities leading to reduction of metastasis, we also tested this regimen in Lsel−/− mice. However, we observed no further alteration of metastasis (Fig. 4*C*). Indeed, the late heparin injection in wt mice resulted in attenuation of metastasis to a similar extent as observed in L-sel−/− deficient mice (Fig. 4*C*). The extent of metastases in Lsel−/− mice was low but clearly distinguishable as evaluated by metastatic foci (data not shown). Taken together, these results indicate that late heparin injection primarily affects L-selectin-mediated interactions, which are necessary for successful establishment of metastatic foci.

L-selectin binding to endogenous selectin ligands facilitates metastasis. Although L-selectin plays a role in hematogenous metastasis (current data; ref. 20) it is unknown whether this is mediated via L-selectin interactions with tumor cell ligands or with endogenous ligands. To investigate whether endogenous selectin ligands play a role, FucT7−/− and wt mice were injected with tumor cells. FucT7 is the primary enzyme that generates endogenous selectin ligands in mice (24, 25). A marked reduction in formation of metastatic foci, comparable with that observed with L-selectin deficiency, was observed in FucT7−/− mice (Fig. 5). Attempts were made to distinguish between hematopoetic and endothelial selectin ligands by creating chimeras using wt and FucT7−/− mice but results were not interpretable due to unexpectedly extensive fibrosis of the lungs following lethal irradiation in this system (data not shown). Regardless, the attenuation of metastasis in FucT7 deficiency indicates the importance of selectin interactions with endogenous ligands. Meanwhile, preliminary studies indicate that mice lacking the major leukocyte P-selectin and L-selectin ligand PSGL-1 (26) do not show attenuation of experimental metastasis with the MC-38GFP colon carcinoma cells.[4] Thus, the relevant endogenous selectin ligands

[4] Unpublished observations, in collaboration with Dr. R.P. McEver.

missing in FucT7−/− mice are likely to be on endothelial cells. Taken together, the data indicate that the contribution of selectin binding to mediation of metastasis is dependent not only on recognition of tumor cell ligands but also some endogenous ligands.

FucT7-dependent ligands are up-regulated adjacent to tumor emboli 12 to 24 hours after injection. To identify the timing and possible expression sites of endogenous selectin ligands relevant for metastasis, we stained lung sections with a recombinant soluble mouse L-selectin probe at 6, 12, 24, and 48 hours after tumor cell injection. Control lungs from mice with saline injection showed no staining (data not shown). L-selectin ligands were first detected 12 hours after tumor cell injection and only in close proximity to tumor cells (Fig. 6*A*). Although there was virtually no staining at 6 hours, maximum binding of L-selectin was detected 24 hours after injection of tumor cells (Fig. 6*A*). This L-selectin ligand expression around the tumor cell emboli was transient and significantly reduced by 48 hours (Fig. 6*B*). Nevertheless, the presence of L-selectin ligands at 48 hours was elevated when compared with the 6-hour time point and was found statistically relevant ($P < 0.01$). The fact that specific L-selectin staining was detected exclusively in the vicinity of tumor cell emboli suggests an endothelial origin of selectin ligand expression. Interference contrast images were merged with immunofluorescence images, confirming that L-selectin ligands were not on the tumor cells themselves (Fig. 6*C*). In keeping with this, we have confirmed prior studies indicating that tumor cells indeed remain intravascular at this time point (27).[5] These findings show temporally induced expression of endogenous L-selectin ligands, which is likely caused by activation of local endothelium, due to the presence of tumor and/or inflammatory cells (28). This induced L-selectin ligand staining around tumor cells was absent in FucT7−/− mice at 24 hours, indicating a requirement for α1-3–linked fucose in sialyl Lewis X–like structures in induction of endogenous ligands (Fig. 6*C*). The punctuate pattern of L-selectin ligands is similar to the staining of activated endothelial cells *in vitro* (29).

Discussion

Hematogenous metastasis is facilitated by tumor cell-platelet-leukocyte emboli formation. The observed association of enhanced selectin ligand expression on tumor cells with cancer progression suggested involvement of vascular selectins in tumor progression (12, 30, 31). Endothelial E-selectin and P-selectin were found to contribute to metastasis in several mouse models (12, 13, 20) and our previous data particularly implicated platelet P-selectin during the early steps of metastasis (3, 20). In addition, the observation that L-selectin deficiency attenuated metastasis even in T- and B-cell-deficient mice indicated that granulocytes and/or monocyte L-selectin facilitates leukocyte participation in this process (20). Although the presence of leukocytes in primary tumors is well documented and their role is being elucidated, the mechanism of leukocyte contribution to metastasis remained poorly understood (7). Here, we provide the first evidence for the mechanism of leukocyte L-selectin action during metastasis. The finding that L-selectin promotes tumor cell survival and that this facilitation occurs 12 to 24 hours following tumor cell entry into the vasculature (Fig. 1*A*) indicates local involvement of leukocytes in establishment of metastatic foci. Indeed, an enhanced presence of CD11b-positive leukocytes associated with tumor cells was concomitantly detected, suggesting their involvement in this process (Fig. 2). One possible explanation for the observed

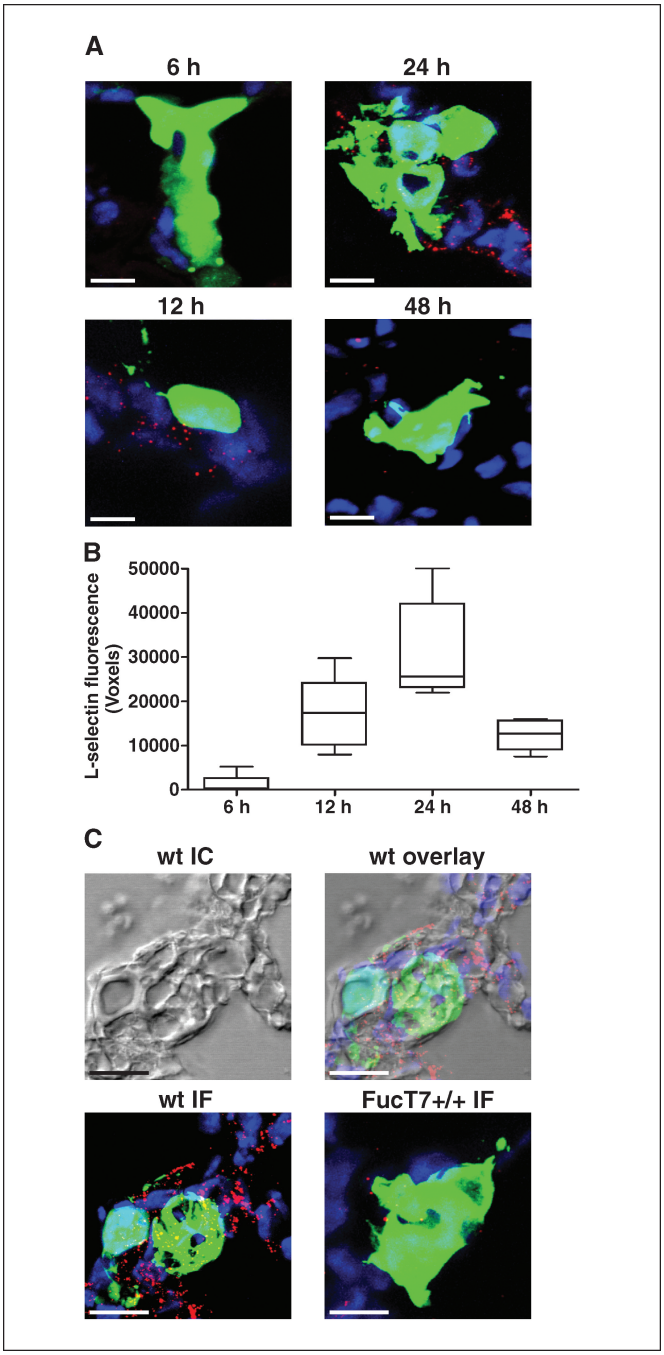


Figure 6. FucT7-dependent L-selectin ligands are induced locally at sites of tumor cell arrest. *A,* mice injected with MC-38GFP cells were terminated 6, 12, 24, and 48 hours after tumor cell injection, respectively. Representative images of lungs sections stained with mouse L-selectin chimera (*red*) and GFP tumor cells (*green*) and nuclei stained with 4′,6-diamidino-2-phenylindole (*blue*) are presented. Bar, 10 μm. *B,* quantification of mouse L-selectin ligand staining detected in area of 3,025 μm^2 in a stack of 22 sections (4 μm) in depth using Imaris software. The increase in L-selectin binding was statistically significant as determined by two-way ANOVA multiple compare test ($P < 0.01$). *C,* interference contrast (*IC*) and immunofluorescence image (*IF*) of lung section of wt mice in overlay show distinct L-selectin staining (*red*) apart from tumor cells in wt mice. No visible L-selectin ligand staining (*red*) was detected in FucT7−/− mice. Bar, 8 μm.

[5] L. Borsig, unpublished observations.

leukocyte contribution to metastasis comes from the capacity of L-selectin-positive leukocytes to transmigrate through L-selectin ligand-positive endothelium (15, 32, 33). Thus, leukocytes may assist tumor cells to breach the endothelial barrier at sites of intravascular embolization, thereby facilitating metastasis (34–36). The L-selectin-positive leukocytes that promote metastasis are likely to be monocytes and/or granulocytes, which were found to be associated with tumor cells during metastasis initiation (Fig. 2). Although we cannot completely exclude a role for NK cells, there was no difference in the presence of NK cells in the lung sections of Lsel−/− and wt mice as detected by NK-1.1 antibody (data not shown). Also, no major alterations of NK cell counts or function have been reported in the setting of L-selectin deficiency (37, 38). It remains to be evaluated whether local L-selectin-mediated interactions of leukocytes with metastatic foci also contribute to subsequent tumor growth.

Inflammatory leukocytes can facilitate malignant outgrowth and potentiate metastasis (7, 35, 39, 40). The analysis of leukocyte populations associated with tumor cell emboli formation indicated a contribution of granulocytes and monocytes to this process, which correlates with the observed effect of L-selectin even in immunodeficient mice (Fig. 2; ref. 20). Tumor cell extravasation has a key role in metastasis and leukocytes could promote this process by mediating interactions with endothelium and/or chemokine production (35, 39, 40). The unexpected time restriction for L-selectin contribution to metastasis suggests involvement of leukocytes only after the early embolization event (Figs. 1 and 2). We found that metastasis was attenuated by a temporal inhibition of L-selectin function either by $F(ab')_2$ MEL-14 antibody or heparin injection during the time when tumor cells were still in the vasculature (Figs. 3 and 4). The observed facilitation of metastasis by L-selectin correlated with the time when the tumor cells were still intravascular, thus suggesting that L-selectin-positive leukocytes participate by establishment of metastatic foci (7).

Heparin is widely used as an anticoagulant treatment of venous thromboembolism in cancer patients (19). Improved cancer mortality after treatment with heparin, when compared with other anticoagulants (vitamin K antagonists), was observed in retrospective analysis of several clinical studies (18, 19, 41, 42). Although blood coagulation is thought to support metastatic progression, heparin affects several other biological activities (18, 22). In a recent study, selective inhibition of coagulation (hirudin) reduced colonization of mouse lungs by melanomas; however, heparin was not evaluated (6). Here, we show that heparin treatment within the first 6 to 22 hours after tumor cell challenge led to attenuation of metastasis primarily by inhibition of L-selectin-mediated interactions (Fig. 4) even in the absence of alterations in fibrin deposition or platelet-tumor cell aggregations (Fig. 1*B* and *C*). These results have clinical relevance because the tumor cells were already in circulation at the time of heparin treatment, similar to the situation in advanced-stage cancer patients.

The association of enhanced selectin ligand expression by tumor cells with poor prognosis originally led to a prediction of direct interaction among vascular selectins and tumor cells (12, 31). Here, we show for the first time the temporal appearance of endogenous L-selectin ligand expression in the vicinity of hematogenously borne tumor cells arrested in the vasculature (Fig. 6). The transient expression of endogenous selectin ligands around tumor emboli implies that L-selectin does not need direct interactions with tumor cells to promote metastasis. Furthermore, the absence of the putative endothelial ligands (Fig. 6*C*) and the reduction of metastases in FucT7-deficient mice (Fig. 5) support the involvement of endothelial L-selectin ligands in this process. Although FucT7−/− as well as PSGL-1−/− mice have moderately elevated total leukocyte counts (24, 26), the attenuation of metastasis was seen only in FucT7−/− mice, indicating that this effect cannot be explained by leukocytosis. The correlation of transient L-selectin ligand expression with the time when temporal inhibition of L-selectin resulted in attenuation of metastases strongly supports the interdependence of leukocyte recruitment with local selectin ligand expression. The precise nature of the L-selectin ligands remains unknown. Many studies on lymphocyte homing to lymphoid organs identified several mucosal "addressins" as ligands for L-selectin (for review, see ref. 43). In addition, L-selectin-mediated recruitment of leukocytes to sites of inflammation has been shown (32, 44–47). However, the nature of endothelial ligands induced during inflammation has been only partially characterized (44, 48, 49). The investigations of cultured endothelial cells led to prediction of candidate molecules that require appropriately sialylated, fucosylated, and sulfated forms of ligands carried on yet unidentified carrier molecules (43, 44). Furthermore, the induction of L-selectin ligands on endothelium is dependent on the type of inflammation (50). L-selectin ligands around tumor cell emboli were absent in FucT7-deficient mice and the binding of L-selectin was enhanced by mild periodate treatment of the tissues (Fig. 6). This is in agreement with the previously observed lack of L-selectin-dependent leukocyte recruitment to sites of inflammation in FucT7-deficient mice (25).

In conclusion, our study proposes L-selectin as one molecular link between recruitment of inflammatory leukocytes to the sites of tumor cell emboli in microvasculature and their potential to facilitate metastasis. The virtual lack of metastases in the absence of endogenous selectin ligands, due to FucT7 deficiency, provides strong evidence for the critical role of a tumor cell microenvironment during establishment of metastases.

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