Redirection of Tumor Metastasis by Expression of E-Selectin In Vivo

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Summary

The selectin class of adhesion molecules plays a critical role in facilitating leukocyte adhesion to and subsequent transmigration of endothelium. On this basis, selectins have been suggested to promote tumor cell attachment to endothelium, thereby facilitating metastasis of certain types of tumors, although direct evidence for such a role is lacking. To explore this hypothesis, two sets of transgenic mice were developed: TgnES, which constitutively expresses cell surface E-selectin in all tissues, under the control of the β-actin promoter; and TgnEsol, which expresses truncated, soluble E-selectin in the liver, under the control of the α1 antitrypsin promoter. B16F10 melanoma cells were stably transfected with α(1,3/1,4) fucosyltransferase-specific cDNA (B16F10ft), allowing them to express E-selectin ligands or with hygromycin resistance selection vector only (B16F10*hygro*). Normal mice injected with B16F10ft and B16F10*hygro* and transgenic mice injected with B16F10*hygro* developed lung tumors exclusively. In contrast, TgnES mice injected with B16F10ft cells developed massive infiltrating liver tumors. B16F10ft cells injected into TgnEsol mice also formed liver tumors, but these grew more slowly, with a well-delineated, noninfiltrating distinct histologic pattern. These observations provide direct evidence that expression of E-selectin can redirect metastasis of tumor cells expressing appropriate ligands in vivo.

Tumor metastasis is a multistep process requiring detachment of malignant cells from the primary tumor mass, penetration of blood and/or lymph vessels, evasion of immune surveillance, attachment to endothelium of distant organs, penetration of the secondary host tissue, and formation of new tumor colonies. The ability of tumor cells to selectively adhere to endothelium of certain organs may be responsible in part for preferential metastatic patterns associated with different tumor types. Interaction between tumor cells and host tissue endothelium is thought to be mediated by adhesion receptor–ligand pairs, some of which are involved in physiologic leukocyte–endothelial interactions (1). Tumor cells express various combinations of cell surface molecules that may serve as ligands for endothelial cell surface receptors (2–7), which are typically induced upon stimulation by mediators of inflammation (2–4). A local inflammatory response may therefore facilitate circulating tumor cell adhesion and arrest.

Two members of the selectin family of adhesion molecules, E- and P-selectin, are transiently expressed on endothelial cells after stimulation by IL-1 and TNF-α. Both receptors recognize sialylated fucosylated lactosaminoglycans on the surface of various leukocyte subsets and promote leukocyte rolling on the cytokine-activated endothelial surface, facilitating subsequent leukocyte arrest and extravasation to sites of injury (4). E-selectin has been proposed to facilitate attachment of tumor cells to endothelium in similar fashion (4). This suggestion is based on observations that several types of malignancy express elevated levels of a fucosyltransferase required for the synthesis of E-selectin–binding oligosaccharides and that adhesiveness of several colon carcinoma cell lines to E-selectin–expressing endothelial cells in vitro correlates with their metastatic proclivity in vivo (3, 7, 8). However, direct evidence of involvement of E-selectin in tumor metastasis in vivo is lacking.

To address the possible role of E-selectin expression in tumor metastasis in vivo, B16F10 melanoma cells, which typically form tumors primarily in the lung and lack cell surface expression of E-selectin ligands, were transfected with α(1,3/1,4) fucosyltransferase cDNA and tested for their pattern of hematogenous dissemination in transgenic mice constitutively expressing cell surface or soluble E-selectin. Our results indicate that expression of E-selectin can redirect metastasis of tumor cells expressing appropriate ligands in vivo.

L. Biancone and M. Araki contributed equally to this work.

Materials and Methods

Development of Transgene Constructs. To make the cell surface E-selectin transgene, a BamHI fragment of mouse E-selectin cDNA (nucleotides 83–2,108, EMBL accession number M80778) was inserted into the Klenow polymerase–filled EcoRI site of the pCAGGS expression vector (9). For microinjection, the SnaBI/BamHI fragment was isolated (see Fig. 1).

To make the soluble E-selectin transgene, the α1 antitrypsin promoter, isolated from human genomic DNA (EMBL accession number D38257) by PCR using synthetic oligonucleotide primers, was ligated to rabbit β-globin splice (nucleotides 902–1,542) and polyA signal (nucleotides 1543–2180) sequences, derived from the pKCR vector (10), and the ligation product was inserted into a BssHII/XhoI–cut Bluescript II plasmid. Mouse E-selectin cDNA was subjected to EcoRI digestion, and the EcoRI-cut fragment, containing sequences encoding a portion of the extracellular domain (nucleotides 83–1506) were inserted into the EcoRI site between the β-globin intron and polyA sequences in the pBluescriptII vector containing the AAT promoter and β-globin intron/polyA segments (the 5′ EcoRI site was destroyed by blunting). To generate a soluble form of E-selectin composed of the entire extracellular domain, a stop codon was inserted by PCR into the E-selectin cDNA immediately upstream of sequences encoding the transmembrane domain, using a reverse primer designed to contain an EcoRI site at nucleotide position 1822 as follows: 5′ TTT GAA TTC AGG CCT GGC TGA CTG GGG CTT CAC A 3′. This and a forward primer spanning the EcoRI site at nucelotide 1,506 were used to PCR amplify sequences encoding the membrane-proximal portion of the extracellular domain. The amplified product was subjected to EcoRI digestion and ligated into the EcoRI-cut AAT/E-selectin vector (see Fig. 1 *B*). For microinjection, the BssHII/XhoI fragment was isolated (see Fig. 1). Detection of each transgene was achieved by PCR, using synthetic oligonucleotide primers corresponding to appropriate segments of promoter and E-selectin coding sequences, and by Southern blot using full-length mouse E-selectin cDNA as a probe.

Mice. C57Bl/6xDBA2 F1 and C57Bl/6 mice of either sex were purchased from IFFA Credo (L'Abresles, France). Transgenic founder mice were then backcrossed with C57/BL/6 mice in the animal facilities of the Centre Medical Universitaire, University of Geneva. To generate transgenic mice, the DNA fragments shown in Fig. 1 were excised with the restriction endonucleases indicated above and purified by agarose gel electrophoresis and passage through NACS PREPAC (GIBCO BRL, Gaithersburg, MD). The recovered DNA was resuspended in 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, at 5 ng/μl and used for microinjection. Founder mice were explored for the presence and number of copies of the transgene by Southern blot analysis, and their transgenic progeny were detected by PCR analysis.

RNA Blot Analysis. Total RNA from murine tissues obtained at autopsy was prepared using the guanidinium thiocyanate/cesium chloride method. 20 μg of each total RNA sample were loaded onto gels, subjected to electrophoresis, transfered to nylon filters, and hybridized to a full-length mouse E-selectin cDNA probe (11).

Detection of Soluble E-Selectin in Sera. Detection of soluble E-selectin in sera was performed using an immunodot blot system. 100 μl of PBS-diluted normal or transgenic mouse serum was blotted onto a nitrocellulose membrane using a 96-well filtration manifold (Hybri-dot; GIBCO BRL). The membrane was washed with PBS and TBS-T (0.1% Tween-20 in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl) and incubated with 1% blocking reagent (Boehringer Mannheim Corp., Indianapolis, IN) in TBS-T for 1 h at room temperature to block nonspecific antibody binding. A rat anti–mouse E-selectin mAb 21KC10 (12) followed by an AP-conjugated mouse anti–rat Ig κ chain mAb were used to detect soluble E-selectin. Serum concentrations were determined after the establishment of a standard curve by adding known amounts of E-selectin–Ig-Fc fusion protein (12) to normal mouse serum.

Cell Lines, Transfectants, and Injection of Cells into Mice. The murine B16F10 melanoma and the human myeloid leukemia HL60 were obtained from the American Type Culture Collection (Rockville, MD). Cells were transfected with πH3M vector containing the hygromycin resistance gene only or with πH3M vector containing α(1,3/1,4)fucosyltransferase cDNA (13). Transfectants were generated by electroporation (Gene Pulser; Bio-Rad Laboratories, Richmond, CA) at 250 V and 960 μF in 4-mm electroporation cuvettes. Clones were selected for hygromycin resistance in DMEM, 10% FCS, and 500 μg/ml hygromycin B (Boehringer Mannheim Corp.) and tested for sialyl Lewis^x (SLe^x) and sialyl Lewis^a (SLe^a) expression by flow cytometry.

For in vivo experiments, cells were detached from plates with EDTA, washed with PBS, and resuspended in saline. 0.2×10^6 cells in a total vol of 250 μl were injected through the tail vein. All animals were killed on day 22 after injection and subjected to autopsy. All organs were examined macroscopically for evidence of tumor growth. Portions of liver, lung, and brain tissue from each animal as well as any tissue containing visible tumor growth were fixed in formaldehyde or frozen in liquid nitrogen for light microscopy and immunofluorescence studies, respectively.

Antibodies and Immunofluorescence. Cells were detached from plates with EDTA, washed, resuspended in PBS, and incubated with mAbs for 45 min at 4°C followed by fluorescein-labeled goat anti–mouse affinity-purified antibody (Cappel Laboratories, Malvern, PA) for 30 min at 4°C. Binding of mAb to cells was analyzed by flow cytometry. Anti-SLe^a mAb CSLEA-1 and anti-SLe^x mAb CSLEX (14) were obtained from the Fifth Human Leukocyte Typing Workshop. Control isotype–matched antibodies were from PharMingen (San Diego, CA). For tissue staining, 5-μm frozen tissue sections were mounted onto slides, fixed in cold acetone for 5 min, air dried, and incubated with 10 μg/ml of 10E9.6 rat anti-murine E-selectin mAb (12) or control isotype–matched antibody (PharMingen) for 45 min at room temperature. The slides were washed in PBS, incubated with fluorescein-labeled goat anti–rat IgG affinity-purified antibody (Cappel Laboratories) for 30 min at room temperature, washed, and examined under an epifluorescence microscope (Nikon Inc., Melville, NY).

Results and Discussion

Development of E-Selectin–Transgenic (TgnES) Mice. TgnES mice were transgenic lines in which the transgene consisted of full-length murine E-selectin cDNA placed under the control of the chicken β-actin promoter (Fig. 1). Northern blot analysis of RNA from various organs of F1 mice showed expression of the expected 2.6-kb transcript in all tissues tested, including liver, kidney, brain, heart, skeletal muscle, spleen and intestine (Fig. 1). Interestingly, the liver displayed the weakest E-selectin expression among the tis-

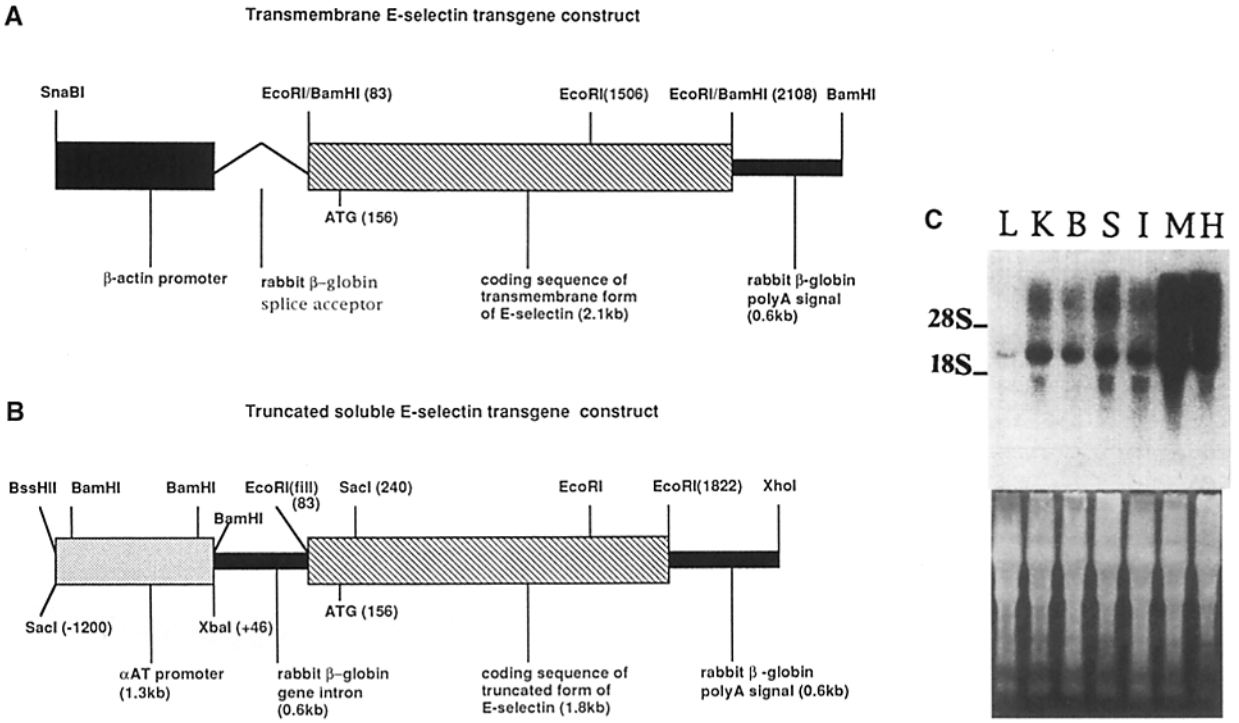

Figure 1. Development of E-selectin transgenes and expression of E-selectin in transgenic mice. (*A*) Schematic representation of the cell surface E-selectin transgene contruct. (*B*) Schematic representation of the soluble E-selectin construct. (*C*) RNA blot analysis of cell surface E-selectin transgene expression. 20 μg of total RNA was loaded per lane, subjected to electrophoresis, transferred to nylon filters, and hybridized to a ^{32}P-radiolabeled E-selectin cDNA probe (*top*). Comparative loading of RNA samples onto the gel is shown in the lower panel. The origin of RNA samples is denoted by letters: *L*, liver; *K*, kidney; *B*, brain; *S*, spleen; *I*, intestine; *M*, muscle; *H*, heart.

sues examined. No E-selectin expression was detected in tissues from normal mice (data not shown).

TgnEsol mice were transgenic lines in which the transgene consisted of a cDNA sequence encoding only the extracellular domain of E-selectin, placed under the control of the α1 antitrypsin promoter, which is hepatic tissue specific (Fig. 1). Analysis of F1 mice using a dot blot assay revealed expression of soluble E-selectin in the serum in the range of 5–50 μg/ml. All mice used in this work belonged to a single transgenic line of, respectively, TgnES and TgnEsol transgenic mice raised on a C57Bl/6 background.

Development of B16F10 Melanoma α(1,3/1,4) Fucosyltransferase Transfectants. B16F10 melanoma cells have been extensively studied in metastasis assays and have been observed to form predominantly lung tumors after intravenous injection through the tail vein (15, 16). In preliminary experiments, injection of B16F10 cells into normal, TgnES, or TgnEsol mice resulted in a comparable pattern of exclusively lung metastasis (data not shown). Because B16F10 cells do not express E-selectin ligands (data not shown), B16F10ft variant cells were developed that stably express a cDNA encoding an α1-3/4 fucosyltransferase that catalyzes transglycosylation reactions, which yield both Fucα(1,3)- and Fucα(1,4)-glycosidic bonds (13) and direct expression of E-selectin ligands, including the tetrasaccharides SLe^a and SLe^x (17). B16F10ft cells were found to react with CSLEA-1 mAb, specific for SLe^a, but not with CSLEX-1 mAb, specific for SLe^x (Fig. 2). Both antibodies stained HL60 cells, which express both tetrasaccharides (data not shown). Recent studies have shown that some melanoma cell lines that express SLe^a but not SLe^x, bind E- but not P-selectin (18). Expression of SLe^a but not SLe^x has been suggested to correlate with melanoma metastatic proclivity (19).

TgnES and TgnEsol Mice, in Contrast to Normal Mice, Develop Liver Metastasis, but of a Different Macroscopic and Histologic Pattern. Wild-type TgnES and TgnEsol mice (Table 1) were injected with 2 × 10^5 B16F10ft or B16F10*hygro* cells (transfected with the hygromycin resistance selection vector only) through the tail vein and killed 22 days later. Wild-type C57Bl/6 mice injected with B16F10ft as well as B16F10*hygro* displayed multiple tumor nodules in the lungs but no detectable tumor growth in the liver or in other organs (Table 1 and Fig. 3). Similarly, lungs were the only site of tumor growth in TgnES and TgnEsol mice injected with B16F10*hygro* cells. In contrast, all of the TgnES mice injected with B16F10ft cells developed large hepatic tumor masses with diffuse infiltration of melanoma cells in the liver parenchyma (Fig. 3). Small tumor nodules were also present in the lung, and in one case tumor infiltration of mesenteric lymph nodes and the pancreas was observed (Table 1).

TgnEsol mice injected with B16F10ft cells also displayed hepatic tumors. However, contrary to the tumors observed in TgnES animals, hepatic tumors in TgnEsol mice pre-

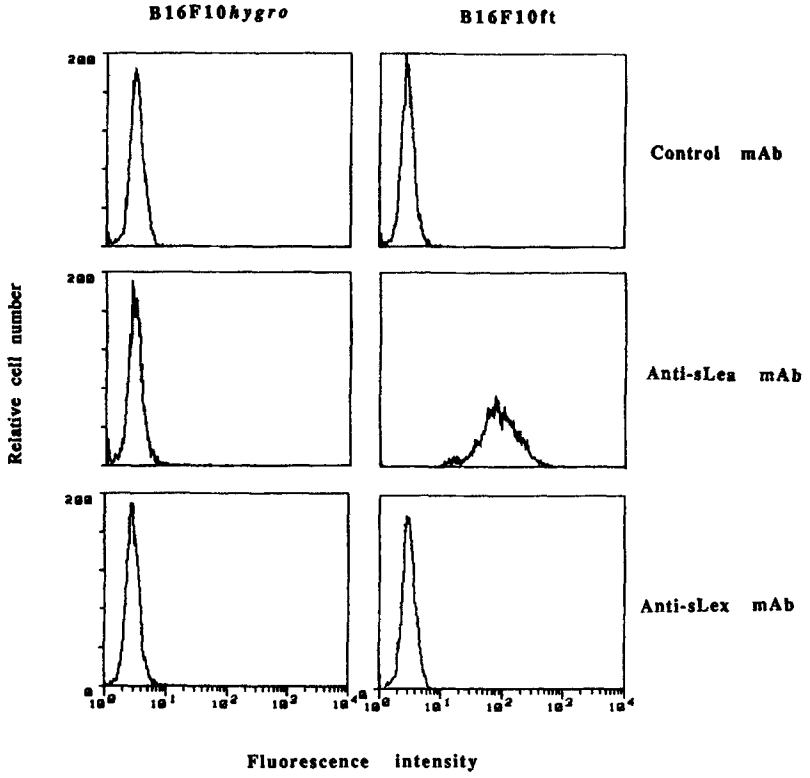

Figure 2. Expression of SLe^a and SLe^x in B16F10*hygro* and B16F10ft cells. The cells and antibodies used are indicated. An unrelated, isotype-matched antibody was used as a control. Staining was performed with CSLEA-1 and CSLEX mAbs.

sented as several distinct, round 1–2-mm nodules (Fig. 4 *C*). These animals also displayed tumor nodules in the lung and, in one case, in the mesenteric lymph nodes (Table 1). At the completion of the study, only 33% of the TgnES animals injected with B16F10ft cells were alive compared with 100% of both TgnEsol and wild-type mice, whether they were injected with B16F10ft or B16F10*hygro* cells. The difference in survival probably reflects the more rapid growth of liver metastases in TgnES mice.

Histologic examination showed a striking morphologic difference between B16F10ft liver tumors in the two transgenic lines. In TgnES mice, tumor growth appeared to proceed by infiltration of liver sinusoids (Fig. 3). In contrast, in TgnEsol animals, tumors displayed spherical, well-circumscribed growth without infiltrating but rather compressing the liver parenchyma (Fig. 3). These distinct patterns of growth corresponded to the different distribution of E-selectin expression in the liver between TgnES and TgnEsol mice (Fig. 4). Immunohistochemical analysis revealed that E-selectin was predominantly localized on the endothelium of liver sinusoids and venules in TgnES animals (Fig. 4); expression on the hepatocyte membrane was more difficult to detect. In TgnEsol mice, E-selectin was detected within hepatocytes, as expected from the presence of the α1 antitrypsin promoter in the transgene (Fig. 3).

Table 1. *Experimental Groups and Tumor Distribution*

Mice	Injected transfectant	Metastasis	
		Localization	Incidence
Wild-type C57B1/6	B16F10*hygro*	Lung	8/8
Wild-type C57B1/6	B16F10ft	Lung	6/6
TgnES	B16F10*hygro*	Lung	3/3
TgnES	B16F10ft	Lung	6/6
		Liver	6/6
		Mesenteric LN	1/6
TgnEsol	B16F10*hygro*	Lung	4/4
TgnEsol	B16F10ft	Lung	6/6
		Liver	5/6
		Mesenteric LN	1/6

Taken together, our observations demonstrate the potential importance of carbohydrate ligand–E-selectin interaction in tumor metastasis in vivo. Although it is obvious that interactions between locally expressed E-selectin and melanoma cells bearing E-selectin ligands are directing the observed tumor growth pattern, differences in the gross and histologic appearance of liver metastases, which depend upon the site of E-selectin expression, indicate different mechanisms of interaction between tumor cells and host tissue in the two transgenic lines.

It may be asked why the liver appears to be the principal site of tumor colonization in TgnES mice; these mice express the transgene in most organs, with an especially high level of expression in the heart and skeletal muscle (which is likely to be related to the use of the actin promoter) and

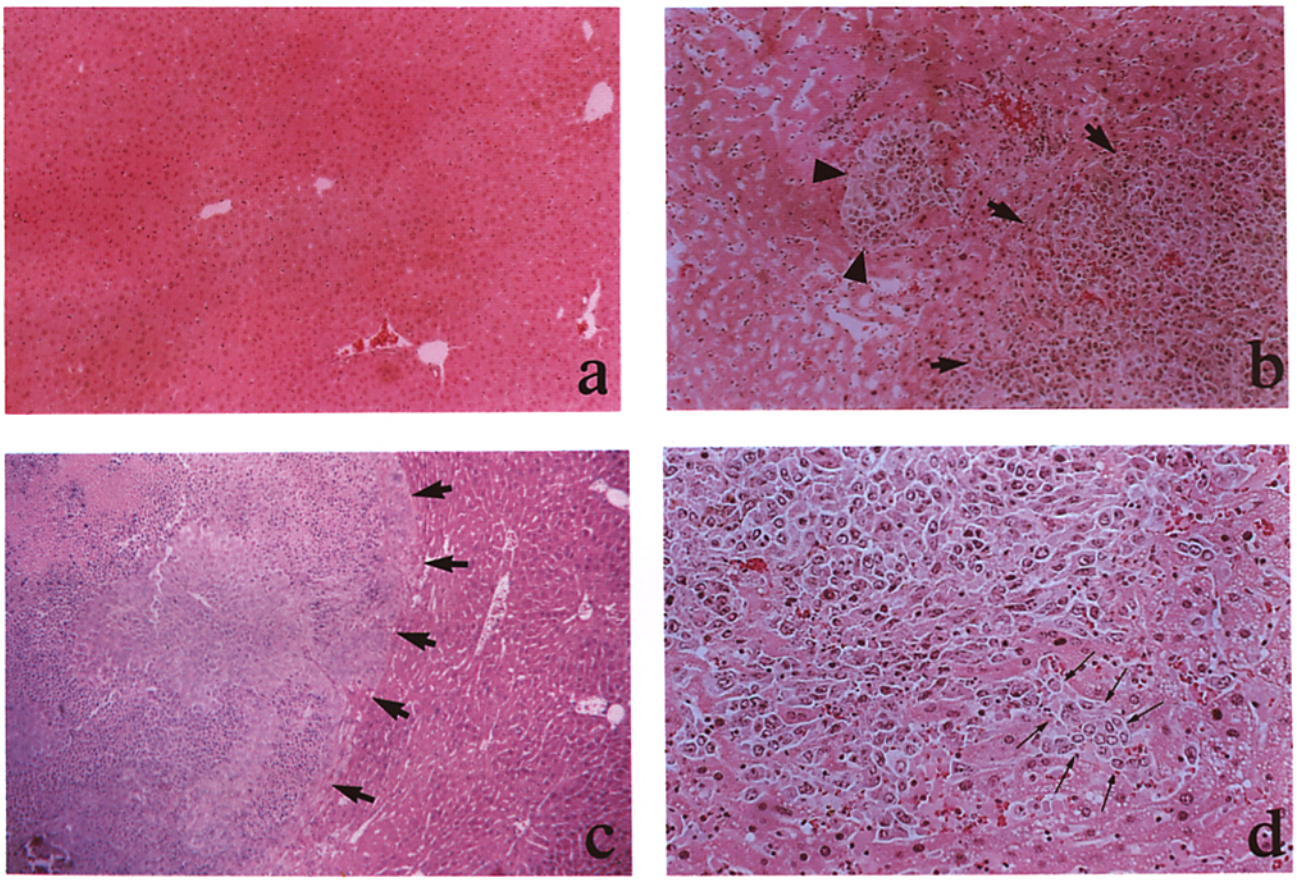

Figure 3. Histology (hematoxylin and eosin) of liver metastases from transgenic mice injected with B16F10ft. (*a*) Normal liver histology from a control C57B/6 mouse. ×4 (*b*) Liver metastasis in a TgnES mouse. Arrows indicate tumor margins; arrowheads denote a satellite tumor infiltrating liver parenchyma. ×4. (*c*) Liver metastasis in a TgnEsol mouse. Arrows indicate a well-defined tumor margin showing absence of sinusoid infiltration. ×4 (*d*) Higher magnification of tumor metastasis in TgnES mouse reveals diffuse tumor infiltration of the liver sinusoids (*arrows*). ×20.

a lower level of expression in the liver. Interaction between endothelial cells bearing E-selectin and circulating tumor cells is most likely a determining factor in the localization of early metastases. Immunohistochemistry showed that E-selectin expression was high on endothelial cells of the liver, but it was also elevated on endothelial cells of a fraction of vessels in cardiac and skeletal muscle, lung, and kidney (data not shown). Because the liver has a larger vascular network than muscle and most other organs, it provides tumor cells with more potential attachment sites and the possibility to establish new colonies. In addition, the relatively low shear forces in the sinusoids and venules of the liver may provide a contributing factor, since expression of the E-selectin transgene was prevalent in medium and large vessels of other organs. Thus, the rapid appearance of liver metastases in TgnES mice may be explained in part by or-

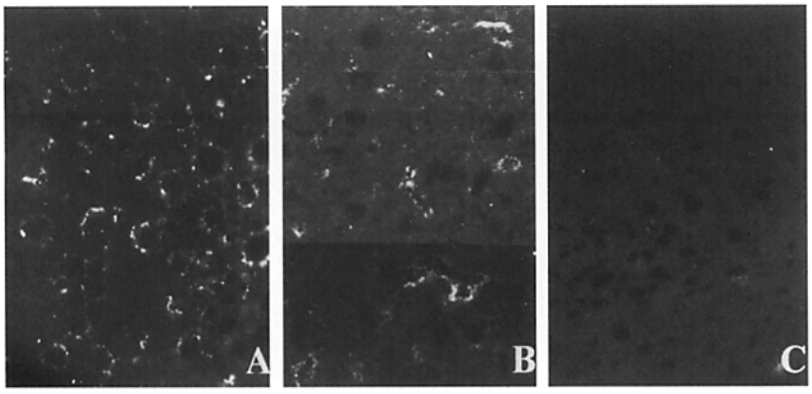

Figure 4. Expression of E-selectin in transgenic mice. Immunofluorescence staining for E-selectin in (*A*) TgnEsol mouse liver; (*B*) TgnES mouse liver; (*C*) normal C57Bl/6 mouse liver. E-selectin expression is predominantly localized along the sinusoids and veins in *B* and within the cytoplasm of the hepatocytes in *A*. Indirect immunofluorescence was performed by using rat anti–mouse E-selectin mAb clone 10E 9.6 (10 μg/ml) and a secondary fluorescein-conjugated affinity-purified goat anti–rat antibody.

gan-specific vascular anatomy and hemodynamics. However, differences in adhesiveness of tumor cells to organ-specific microvasculature may also play a role. It is possible that, similar to leukocytes, tumor cell arrest and endothelial transmigration require several sequential interactions between tumor-associated receptors and endothelial cell ligands. E-selectin may thus provide an essential accessory adhesion component that may facilitate interactions between other receptor–ligand pairs. Once the tumor cells are arrested in a given tissue, the pattern of metastatic growth is likely to become more dependent on interaction between the tumor and host tissue cells. Hepatocytes probably bore weak amounts of E-selectin in their membranes, which, together with preferential tumor cell adhesion to sinusoidal endothelium, may explain the infiltrating aspect of the liver tumor masses in TgnES mice, as well as their rapid growth, leading to early death in comparison with TgnEsol and nontransgenic mice.

It was expected that the circulating soluble E-selectin detected in TgnEsol mice would lead to a pattern of metastasis in which the E-selectin ligands borne by B16F10ft cells would play no role, i.e., comparable to that of B16F-10*hygro* cells. The well-delimited, noninfiltrating tumor masses found in the liver may have resulted from the fact that, despite the presence of circulating soluble E-selectin, a gradient of the transgene product released by the hepatocytes was likely to occur, with the highest existing concentration at the level of the hepatic sinusoids. It has been suggested that soluble E-selectin may act as a neutrophil chemoattractant (20), and the same possibility may apply to the situation of E-selectin ligand-bearing cells in the presence of any gradient of soluble E-selectin concentration. The lack of direct interaction between tumor cells and hepatocytes, as well as the lack of membrane-bound cell surface E-selectin on sinusoid endothelium, would be consistent with the noninfiltrating nature of the liver metastasis.

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