

# Cytoplasmic Dye Transfer Between Metastatic Tumor Cells and Vascular Endothelium

Marwan E. El-Sabban and Bendicht U. Pauli

Cancer Biology Laboratories, Department of Pathology, Cornell University, College of Veterinary Medicine, Ithaca, New York 14853

**Abstract.** Metastatic colonization of a secondary organ site is initiated by the attachment of blood-borne tumor cells to organ-specific adhesion molecules expressed on the surface of microvascular endothelial cells. Using digital video imaging microscopy and fluorescence activated cell sorting techniques, we show here that highly metastatic cells (B16-F10 murine melanoma and R3230AC-MET rat mammary adenocarcinoma cells) previously labeled with the fluorescent dye BCECF begin to transfer dye to endothelial cell monolayers shortly after adhesion is established. The extent of BCECF transfer to endothelial cell monolayers is dependent upon the number of BCECF-labeled tumor cells seeded onto the endothelial cell monolayer and the time of coculture of the two cell types, as visualized by an increase in the number of BCECF-positive cells

among cells stained with an endothelial cell-specific mAb. Dye transfer to BAEC monolayers proceeds with a progressive loss of fluorescence intensity in the BCECF-labeled tumor cell population with time of coculture. The transfer of dye is bidirectional and sensitive to inhibition by *l*-heptanol. In contrast, poorly metastatic B16-F0 melanoma cells and non-metastatic R3230AC-LR mammary adenocarcinoma cells do not efficiently couple with vascular endothelial cells. It is inferred from these experiments and from the amounts of connexin43 mRNA expressed by tumor cells that tumor cell/endothelial cell communication is mediated by gap junctional channels and that this interaction may play a critical role in tumor cell extravasation at secondary sites.

**M**ETASTASIS is the colonization of selective, secondary organ sites by dissemination of tumor cells from their primary location. This event is dependent upon distinct tumor and host cell characteristics and is achieved in multiple, highly coordinated steps (1, 32, 35, 43, 44). Early steps in the metastatic cascade include the shedding of cancer cells from the primary neoplasm, the invasion of enzymatically and compositionally altered host extracellular matrices, and the intravasation of tumor cells into lymph and/or blood vessels (18, 27, 29, 34). Upon circulation, many cancer cells are eradicated by physical forces exerted onto them during passage through the microvasculature of secondary organs (41, 42) and by host defence mechanisms. The few surviving tumor cells are thought to colonize selective organ sites by recognizing organ-specific adhesion molecules expressed on the surface of vascular endothelial cells (1, 2, 4, 32, 33, 35, 37). The events that follow tumor cell adhesion to the endothelium and lead to the establishment of secondary tumor colonies are poorly understood.

A widely supported hypothesis is that upon tumor cell adhesion to endothelium a retraction of the endothelial cell occurs which exposes its basement membrane to the arrested tumor cell (21–23, 40). Numerous studies have shown that tumor cells equipped with specific ligands recognize and bind to components in the vascular basement membrane (26), thereby initiating extravasation and the beginning of

new growth at secondary organ sites. In studying the organ-specific arrest of cancer cells at secondary organ sites, we have recently observed that highly metastatic tumor cells upon adhering to the vascular endothelium establish cytoplasmic conduits with endothelial cells. Using digital video imaging microscopy and timed fluorescence activated cell sorting (FACS, a registered trademark of Becton Dickinson Co., Mountain View, CA), we show here that highly metastatic tumor cells transfer cytoplasmic dye *via* gap junction-like channels to endothelial cells. Dye transfer is significantly diminished by an inhibitor of gap junction-mediated communication and in poorly metastatic tumor cells.

## Materials and Methods

### Materials

DME, Rosewell Park Memorial Institute-1640 medium (RPMI-1640),<sup>1</sup> and magnesium- and calcium-free HBSS were purchased from Gibco Laboratories, (Grand Island, NY); FBS from Hyclone Laboratories, Inc. (Logan, UT); 2',7'-bis(carboxylethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester (BCECF-AM) and the acid form (BCECF) from Molecular Probes (Eugene, OR). Biotinylated goat anti-mouse IgG F(ab')<sub>2</sub> and phyco-

1. *Abbreviations used in this paper:* BCECF-AM, 2',7'-bis(Carboxylethyl)-5-(and-6)carboxyfluorescein acetoxymethyl ester; PE, phycoerythrin; RPMI-1640, Rosewell Park Memorial Institute-1640.

erythrin-avidin conjugate were from Accurate Chemical and Scientific Corp. (Westbury, NY), respectively; and Falcon 35-mm tissue culture dishes from Becton-Dickinson (Oxnard, CA). All other reagents were of the highest grade available from Sigma Chemical Co. (St. Louis, MO).

### Cell Cultures

Bovine aortic endothelial cells (BAEC) were isolated from thoracic aortas of 18-mo-old steers, essentially as described by Booyse et al. (6). BAEC were grown and maintained in DME supplemented with 2-mM *L*-glutamine and 10% heat-inactivated FBS. Aliquots of BAEC were frozen at passage 2 and used between passages 4 and 6. BAEC were selected for this study over lung-derived capillary endothelial cells for the following reasons: (a) the ease of isolating and maintaining primary BAEC cultures (6); (b) the consistency of growth and relative phenotypic stability of cultured, primary BAEC (3,33); and (c) the strong adherence of metastatic tumor cells to BAEC monolayers (20,35). Since capillary endothelial cells, like BAEC, do not represent endothelia of the primary, postcapillary extravasation site of malignant tumor cells (45) and are often difficult to grow as phenotypically stable cells (33), they are a clearly less desirable cell system for the present studies.

The tumor cells selected for our studies were B16 murine melanoma cell variants (12) obtained from D. I. J. Fidler (University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, Texas) and R3230AC rat mammary carcinoma cell variants (31) obtained from Dr. J. A. Kellen (Sunnybrook Medical Center, University of Toronto, Toronto, Canada). Both B16-F10 and R3230AC-MET were selected for high lung colonization from their respective parental cell lines, while B16-F0 (parental cell line) were low lung-metastatic and R3230AC-LR (WGA- and Con A-resistant variant of R3230AC) were non-metastatic (12,31). Tumor cells were maintained in RPMI-1640 supplemented with 10% FBS. MDCK cells served as controls for BAEC, since they are deficient in intercellular communication under conditions of high cell density (36). MDCK cells were cultured and maintained according to the instructions of the distributor (American Type Culture Collection, Rockville, MD).

### BCECF Labeling of Cells

Tumor cells were labeled with the membrane permeable, pH-sensitive dye BCECF-AM (668 kD). Labeling was achieved with 1.5  $\mu$ M BCECF-AM in RPMI-1640, pH 7.4, for 30 min at 37°C in 5% CO<sub>2</sub> air essentially as described by Guinan et al. (16). Upon entry of the dye into the cell, intracellular esterases rapidly cleaved the molecule to the fluorescent, membrane impermeable acid form (BCECF: 520 kD). Labeled cells were washed twice with serum-free medium, incubated in complete growth medium for 30 min at 37°C to allow any non-deesterified dye to leave the cells, washed again, and used immediately in dye transfer experiments. The same labeling procedures were employed for BAEC to study bidirectional transfer of BCECF and for MDCK substituting for BAEC in control experiments.

### Fluorescence and Digital Video Imaging Microscopy

Confluent endothelial cell monolayers were cocultured for various periods of time at 37°C with tumor cells that had been labeled with BCECF. Unbound tumor cells were removed by washing with serum-free medium. BAEC monolayers and adherent tumor cells were fixed in situ in 1% formaldehyde in PBS and observed under a phase contrast or fluorescence microscope. BCECF fluorescence was imaged with a Nikon Diaphot inverted microscope using a Nikon 40 $\times$  objective lens (Nikon Inc., Garden City, NJ). Illumination at 490 nm was accomplished with a 150 W Xenon source. Images were acquired with a microchannel plate image intensifier (model KS-1380; Video Scope Intl. OPELCO, Washington, DC), coupled to a silicon diode video camera (model 70; Dage-MTI Inc., Wabash, MI), digitized, and averaged in a Tracor-Northern TN-8500 image processor and transferred to a hard disk. Background image from a different area (nonfluorescent) was collected and subtracted from each image.

### FACS Analyses of BCECF Transfer

Confluent BAEC monolayers grown in 35-mm petri dishes were seeded with BCECF-labeled tumor cells and cocultured for various periods of time at 37°C. After having removed unbound tumor cells by washing with serum-free medium, endothelial cells, and adherent tumor cells were released from the growth surface with 0.05% trypsin and 0.02% EDTA in HBSS. The cell suspension was washed twice with DME and incubated for 20 min at 4°C with mAb directed against an endothelial cell-specific surface epitope (45).

The cells were washed four times with DME and incubated with the biotinylated F(ab')<sub>2</sub> portion of the secondary antibody (goat anti-mouse IgG). After an incubation period of 20 min at 4°C, cells were washed and phycoerythrin-avidin conjugate added for 10 min. The cells were thoroughly washed with PBS, fixed in 1% formaldehyde in PBS, and examined for both fluorescein (BCECF) and phycoerythrin (PE) fluorescence in a fluorescence activated cell sorter (Coulter Epics Profile; Coulter Electronics, Hialeah, FL). Results were plotted as log BCECF fluorescence vs log PE fluorescence. Inhibition of communication between tumor cells and endothelial cells was studied in the presence of 3.5 mM 1-heptanol. Bidirectional dye transfer was investigated using cocultures of BCECF labeled endothelial cell monolayers and unlabeled tumor cells. Control experiments were conducted with MDCK cells as a communication-deficient replacement for endothelial cells. Also, control experiments were performed to verify that the secondary antibody as well as the PE-avidin did not label cells in the absence of the primary antibody. Histogram printouts from the FACS analyses were graphically represented for enhanced appearance.

### Northern Blot Analyses

Total cellular RNA was isolated from B16 melanoma cells (F0 and F10) according to the method of Chomczynski and Sacchi (9). RNA samples were denatured with formaldehyde and subjected to 1% agarose/formaldehyde gel electrophoresis. Separated RNA was transferred onto Hybond membranes (Amersham Corp., Arlington Heights, IL) and cross-linked onto the membranes by exposure to a 300-nm UV source for 2 min. High stringency blots were prehybridized at 65°C for 4 h in 0.5 M phosphate buffer, pH 7.4, 1% BSA, and 7% SDS and then hybridized in the same buffer containing 10% formamide at 65°C for 24–48 h with connexin43 cDNA probe obtained from Dr. D. Shalloway, Cornell University, Ithaca, NY. This probe is the Pml1-Sall fragment excised from the dol43 plasmid, which was prepared from plasmid p43 (5). It was purified by gel electrophoresis and labeled with <sup>32</sup>P]dCTP by random primer extension using the Klenow fragment of DNA polymerase I and hexonucleotide primers (11). Unbound probe was removed by three 10-min washes with 1 $\times$ SSC/0.1% SDS at room temperature, followed by a 15–20-min wash with 0.1 $\times$ SSC/0.1% SDS at 65°C. Hybridized mRNA species were identified by autoradiography, and relative amounts of mRNA estimated by computer assisted video scanning (Bioimage Visage 110; Millipore Corp., Ann Arbor, MI) after verifying equivalence of loading (10  $\mu$ g per lane, estimated as the 280/260-nm absorbance ratio) by scanning of ethidium bromide stained rRNA. Northern blot analyses were repeated at least twice.

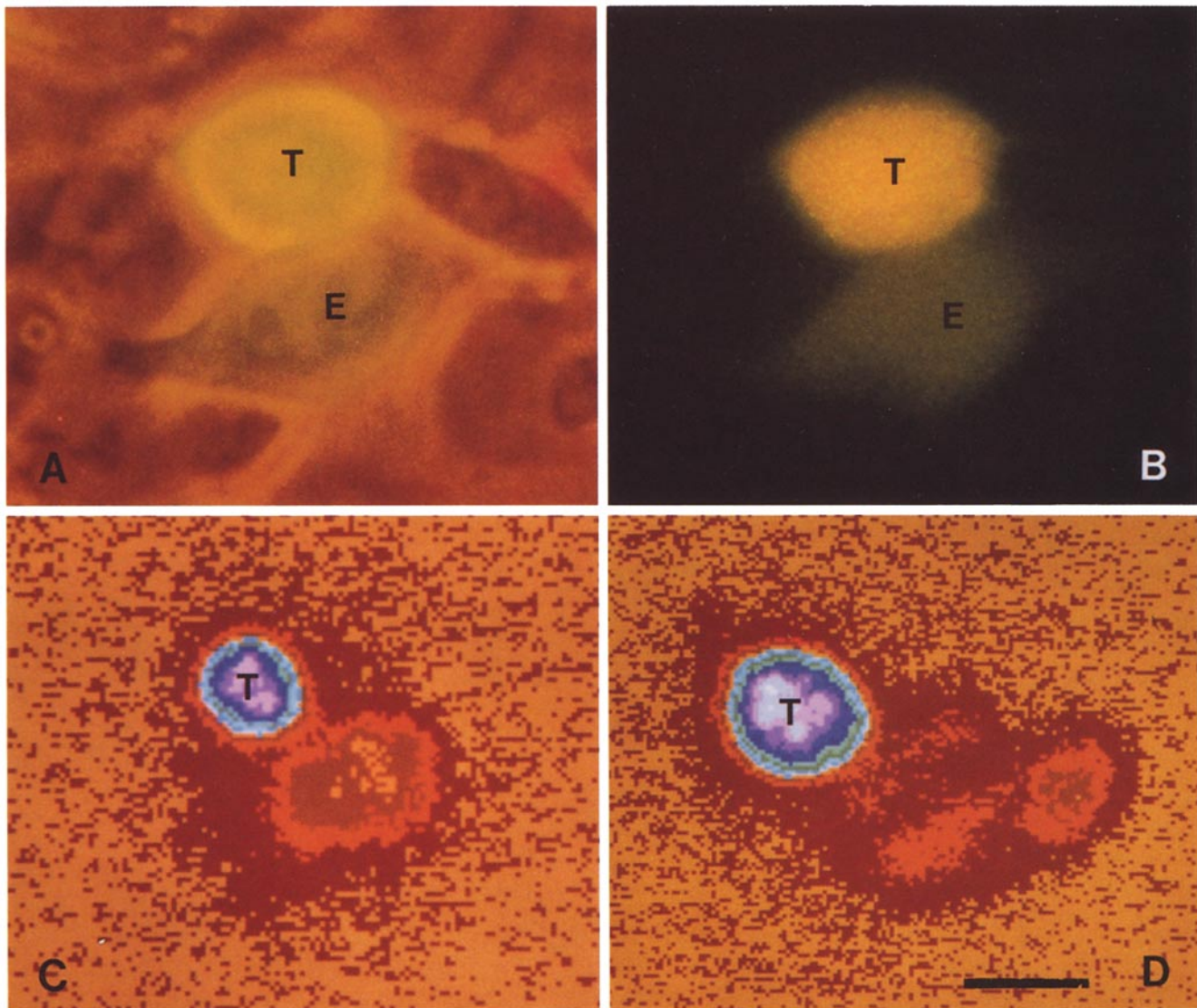
### Results

#### BCECF Uptake and Cell Morphology

Monolayers of BAEC, MDCK cells, B16 melanoma cells, and R3230AC carcinoma cells readily took up the membrane permeable dye BCECF-AM and processed it in their cytoplasm to the membrane permeable fluorescent acid form. BCECF had no adverse effect on cell morphology, growth, and survival. BCECF-labeled BAEC and MDCK monolayers exhibited a significantly lower fluorescence intensity than B16 melanoma cells and R3230AC mammary carcinoma cells. BAEC and MDCK cells usually displayed a pale greenish fluorescence, while tumor cells emitted an intense green/yellow fluorescence. This difference in the extent of BCECF labeling of various cell types can be attributed to many factors including differences in BCECF-AM permeability rates, amounts or activities of cytoplasmic esterases, cytoplasmic pH levels, as well as cell shape. During a 6-h culture period cells maintained their original fluorescence intensity and there was no fluorescent dye leakage into the extracellular compartment and no fluorescent vesicles were observed to be shed into the medium.

#### BCECF Transfer Visualized by Light Microscopy

BCECF transfer from unlabeled B16 melanoma cells to endothelial cells was visualized using epifluorescence micros-



**Figure 1.** BAEC monolayer seeded with BCECF-labeled B16-F10 melanoma cells and cocultured for 3 h: endothelial cells form a confluent monolayer to which brightly fluorescent tumor cells are attached. Dye transfer from a B16-F10 melanoma cell (*T*) to a BAEC is depicted (*E*). (*A*) Phase-contrast and fluorescence; (*B*) fluorescence only; (*C* and *D*) color-enhanced digital video images (pseudocolor map) of BCECF transfer from a labeled B16-F10 melanoma cell to a BAEC (*C*) and lateral homotypic dye spread to neighboring BAEC (*D*). Bar, 10  $\mu\text{m}$ .

copy combined with digital video imaging techniques. Transfer of BCECF fluorescent dye from metastatic B16-F10 melanoma cells to unlabeled BAEC monolayer was evident by the appearance of pale greenish fluorescent endothelial cells next to brightly fluorescent, adherent tumor cells (Fig. 1, *A* and *B*). In digitized, color-enhanced images, tumor cells were readily identified as brightly colored spheres sitting on top of the endothelial cell monolayer. Dye transfer was generally limited to endothelial cells that were in direct contact with the BCECF-labeled tumor cells. Endothelial cell dye uptake was often depicted as a fluorescent area adjacent to one side of the tumor cell, outlining the confines of the endothelial cell and confirming the previous observation that tumor cells predominantly adhered to the marginal zones of endothelial cells (Fig. 1 *C*). This area of fluorescence was significantly weaker and was in a clearly different focal plane than that of the tumor cell. Occasionally a secondary transfer of cytoplasmic dye to neighboring en-

dothelial cells that were not in immediate contact with the tumor cells was observed. Lateral dye transfer proceeded without leakage into the culture medium and was reflective in morphology of the typical cobble-stone appearance of the endothelial cell monolayer (Fig. 1 *D*). Such dye transfer to neighboring endothelial cells was limited to three or four cells.

#### **FACS Analyses of BCECF Transfer**

BCECF-labeled B16 melanoma cells began to transfer fluorescent dye to the cytoplasm of confluent, nonlabeled BAEC monolayers shortly after adhesion was established. Within the first 15 min of coculture BCECF-labeled B16-F10 melanoma cells seeded at a density of  $4.5 \times 10^6$  cells onto confluent BAEC monolayers in 35-mm dishes transferred dye to 40% of the endothelial cells, compared to 50% at 60 min of coculture (Table I). Highly metastatic B16-F10 melanoma

**Table I. BCECF Transfer from B16-F10 Melanoma Cells to BAEC Monolayers as a Function of Time**

Time (min)	Percent BAEC-labeled BAEC	Tumor cells fluorescence*
0	—	141.4 ± 1.9
15	39.4	122.5 ± 2.0
30	42.4	114.6 ± 2.1
60	50.3	107.4 ± 2.3

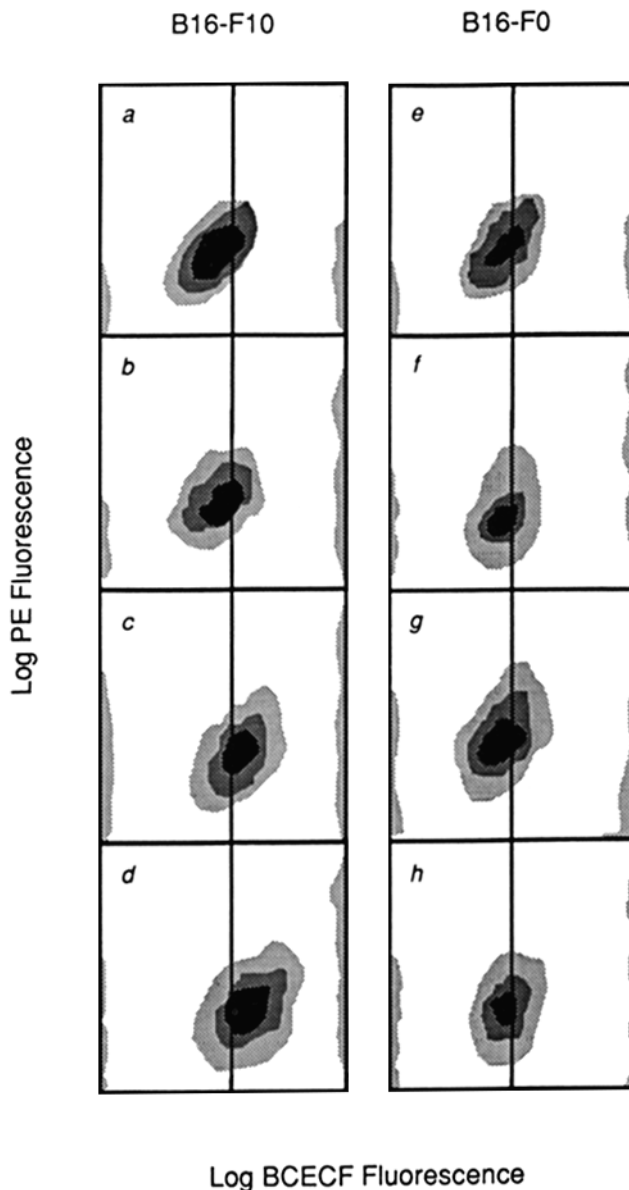
BAEC monolayers grown to confluence in 35-mm petri dishes ( $4.5 \times 10^5$  cells) were seeded with  $5.0 \times 10^5$  BCECF-labeled B16-F10 melanoma cells and cocultured for the indicated times. Cells were released from the growth surface and analyzed by flow cytometry.

\* Mean ± SD

cells transferred BCECF to three to five times as many BAEC than an equal number of low metastatic, BCECF-labeled B16-F0 cells cocultured for the same length of time with unlabeled BAEC (Fig. 2; and Table II). Similar observations were made with the R3230AC mammary carcinoma cell variants. BCECF-labeled, high lung metastatic R3230AC-MET seeded in increasing numbers onto confluent BAEC monolayers transferred dye to increasing numbers of endothelial cells. In contrast, the non-metastatic R3230AC-LR tumor cells were unable to transfer dye to BAEC at any of the seeding concentrations tested (Fig. 3). These data correlated with the abilities of these tumor cells to adhere to endothelial cells and with their lung colony formation potential when injected into syngeneic hosts.

Direct transfer of dye from the cytoplasm of tumor cells to the cytoplasm of BAEC was verified by the following experiments: (a) BAEC were unable to take up the acid form of BCECF, even when added to BAEC at double the labeling concentration ( $3 \mu\text{M}$ ), thus, making it impossible that BCECF was taken up by endothelial cells from the media into which dead, labeled tumor cells had released their cytoplasmic dye; (b) BCECF fluorescence was detected in cells stained with an endothelial cell-specific mAb after coculture of BCECF-labeled B16 melanoma cells (or R3230AC-MET carcinoma cells) with unlabeled BAEC monolayers (Figs. 2 and 3); (c) the average fluorescence intensity of the BCECF-labeled B16 melanoma cells decreased with time of coculture with BAEC monolayers, while the percent of BAEC to which BCECF was transferred increased (Table I); (d) The addition of increasing numbers of BCECF-labeled, metastatic tumor cells to BAEC monolayers resulted in increased numbers of labeled BAEC (Table II; and Figs. 2 and 3); and (e) BCECF-labeled tumor cells were unable to transfer their cytoplasmic dye to communication-deficient MDCK monolayers and, thus, did not lose any fluorescence over a 3-h co-culture period with these control cells.

Cytoplasmic dye transfer between B16-F10 melanoma cells (or R3230AC-MET) and BAEC monolayers was inhibited by the addition of 3.5 mM of 1-heptanol into the culture medium (Fig. 4). The classic inhibitor of gap junctional communication impeded the transfer of BCECF from metastatic tumor cells to endothelial cells by 70–80%. This finding strongly suggests that dye transfer between tumor cells and endothelial cells was achieved by gap junctional channels and was thus limited to molecules of less than 1,000 daltons in molecular weight (13).



**Figure 2.** BCECF transfer from labeled B16-F10 (b–d) or B16-F0 (f–h) to unlabeled BAEC monolayers during a coculture period of 1 h: endothelial cell monolayers ( $4.5 \times 10^5$  cells) are seeded with (a) no tumor cells, (b)  $6.25 \times 10^4$ , (c)  $1.25 \times 10^5$ , (d)  $2.5 \times 10^5$  BCECF-labeled B16-F10 melanoma cells; (e) no tumor cells, (f)  $6.25 \times 10^4$ , (g)  $1.25 \times 10^5$ , and (h)  $2.5 \times 10^5$  BCECF-labeled B16-F0 melanoma cells. The number of BAEC to which dye is transferred increases parallel with the number of BCECF-labeled B16-F10 melanoma cells seeded onto the endothelial cell monolayers. In contrast, increasing numbers of BCECF-labeled B16-F0 cells seeded onto unlabeled BAEC monolayer yielded no significant increase in the numbers of labeled BAEC.

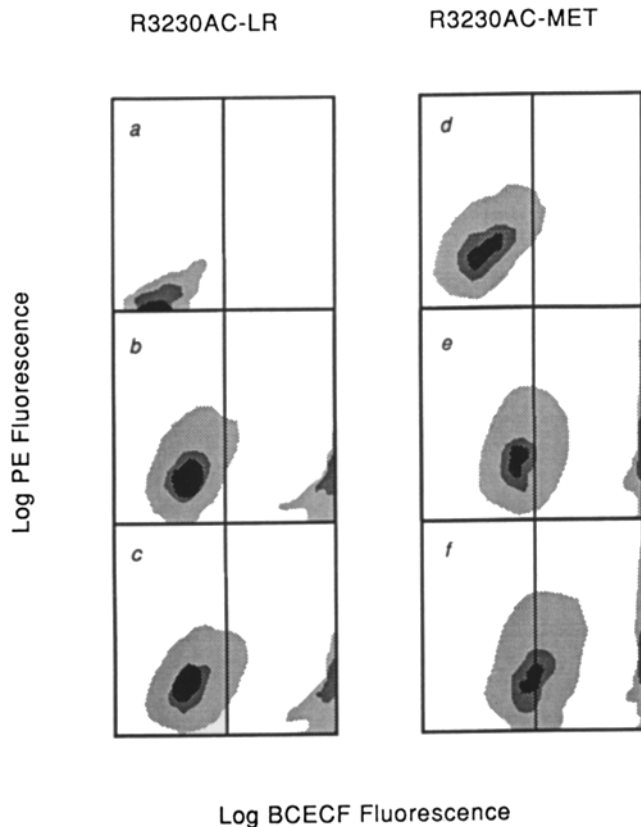
Cytoplasmic dye transfer between metastatic tumor cells and BAEC monolayers was bidirectional. When unlabeled metastatic B16 melanoma cells were added to BCECF labeled BAEC monolayers a generalized low level increase in fluorescence was observed in all adherent tumor cells (Fig. 5). No dye transfer was observed between control BCECF-labeled MDCK monolayers and tumor cells during a coculture period of 3 h.

**Table II. BCECF Transfer from B16 Melanoma Cells to BAEC Monolayers as a Function of the Number of Labeled Tumor Cells Added**

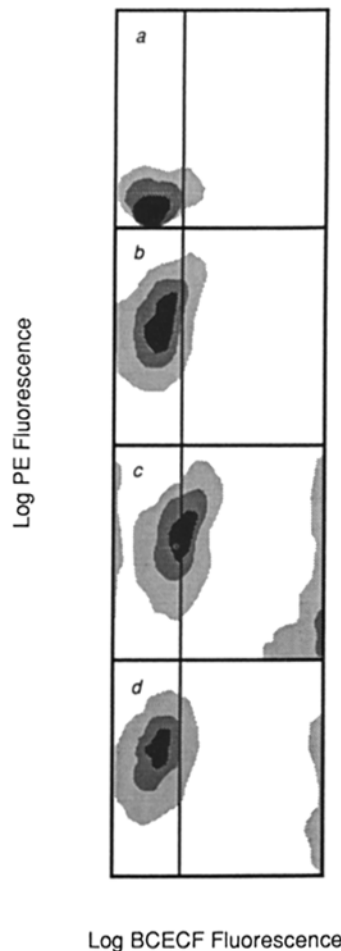
Number of tumor cells added	Percent BAEC positive for BCECF and PE Fluor.	BCECF* fluorescence	PE* fluorescence
<b>A B16-F10</b>			
—	2.2	20.0 ± 1.5	8.6 ± 1.6
6.25 × 10 <sup>4</sup>	2.4	22.0 ± 1.5	9.0 ± 1.6
1.25 × 10 <sup>5</sup>	32.3	40.2 ± 1.6	7.8 ± 1.7
1.875 × 10 <sup>5</sup>	40.7	51.7 ± 1.6	6.6 ± 1.8
<b>B B16-F0</b>			
—	2.3	20.0 ± 1.5	8.6 ± 1.6
6.25 × 10 <sup>4</sup>	2.5	18.9 ± 1.5	10.9 ± 2.0
1.25 × 10 <sup>5</sup>	10.4	27.8 ± 1.5	7.8 ± 1.7
1.875 × 10 <sup>5</sup>	8.9	33.6 ± 1.6	7.0 ± 1.8

Indicated numbers of BCECF-labeled B16-F10 or B16-F0 melanoma cells were added to BAEC monolayers as described earlier. After 60 min of coculture, cells were released from the growth surface, and incubated with mAb directed against a BAEC-specific surface epitope for 20 min. The cells are then washed and a second antibody (Goat anti-mouse IgG) conjugated to biotin is added and incubated for 20 min. The cell suspension is then incubated with avidin-Phycoerythrin conjugate and analyzed by FACS for both fluorescent dyes.

\* Mean ± SD



**Figure 3.** BCECF transfer from labeled high lung metastatic R3230AC-MET or non-metastatic R3230AC-LR carcinoma cells to BAEC monolayers during a coculture period of 1 h: (a) unstained BAEC, no tumor cells; (b and c) Transfer of BCECF from 3.2 × 10<sup>5</sup> (b) or 6.4 × 10<sup>5</sup> (c) labeled R3230AC-LR to unlabeled BAEC; (d) BAEC stained with monospecific antibody (PE positive); (e and f) transfer of BCECF from labeled 2.1 × 10<sup>5</sup> (e) or 4.2 × 10<sup>5</sup> (f) R3230AC-MET to unlabeled BAEC. The percentage of BCECF-labeled BAEC and mean BCECF fluorescence intensity was: (a) no BCECF fluorescence; (b) 3.0%, 12.6 ± 1.8; (c) 6.1%, 15.5 ± 1.8; (d) 3.2%, 10.0 ± 1.5; (e) 32%, 28.3 ± 1.9; and (f) 47%, 44.4 ± 1.9.



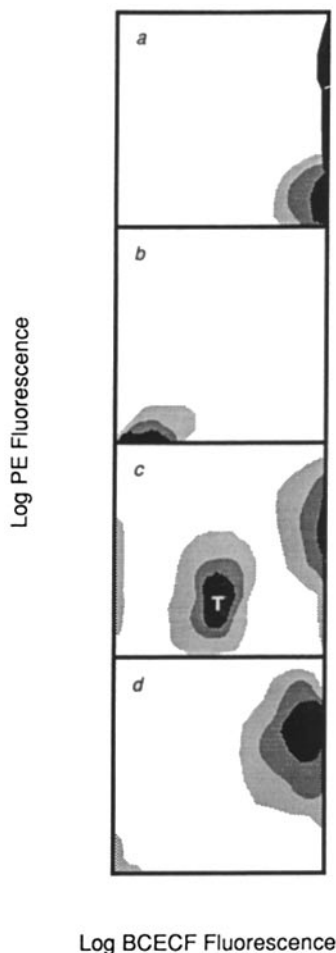
**Figure 4.** BCECF transfer from labeled B16-F10 melanoma cells to BAEC monolayers during a coculture period of 1 h: (a) BAEC, labeled without endothelial cell-specific mAb conjugated with phycoerythrin; (b) BAEC, labeled with the endothelial cell-specific mAb conjugated with phycoerythrin; (c) Transfer of BCECF from labeled B16-F10 cells (2.0 × 10<sup>5</sup>) to unlabeled BAEC; (d) transfer of BCECF from labeled B16-F10 cells (2.0 × 10<sup>5</sup>) to BAEC in the presence of 3.5 mM 1-heptanol. The percentage of BAEC to which BCECF was transferred was 27.6% (c) and 6.0% (d), indicating that 1-heptanol inhibits BCECF transfer by ~80%.

### Expression of Connexin43 mRNA

Both low-metastatic B16-F0 and high-metastatic B16-F10 melanoma cells contained message for the gap junctional protein connexin43, as determined by Northern blot analysis with a cDNA probe that contained ~65% of the carboxy-terminal coding region of rat heart connexin43 (Fig. 6). This message is expressed in two mRNA species, the conventional 3-kb band and an as yet unidentified ~9-kb band. Densitometric comparison of the 2 mRNA species between B16-F0 and B16-F10 yielded a three to four fold higher expression of the 3-kb RNA and a two- to threefold higher expression of the 9-kb mRNA in B16-F10 melanoma cells. This difference in connexin43 message between the low- and high-metastatic B16 variants is consistent with their ability to transfer cytoplasmic dye to BAEC monolayers.

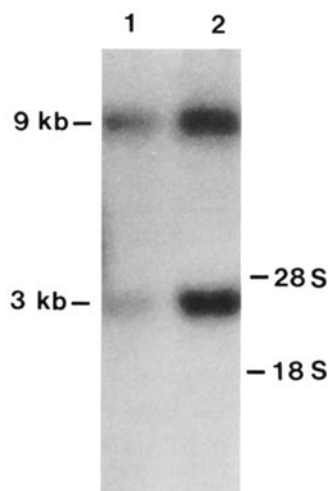
### Discussion

Adhesion of blood-borne tumor cells to vascular endothelial cells is a prerequisite for tumor cell extravasation and the formation of metastatic tumor colonies. Although the mechanisms by which tumor cells penetrate the endothelial cell lining have been associated with exposure of subendothelial matrix (10, 21, 22, 40), tumor cell adhesion, and destruction of basement membrane (26, 27, 29), and tumor cell migration (46), the initial steps of extravasation that follow tumor cell adhesion to the endothelium lining remain largely obscure. Morphological studies on tumor cell/endothelial cell



**Figure 5.** BCECF transfer from labeled BAEC to B16-F10 cells during a coculture period of 1 h: (a) BCECF-labeled BAEC; (b) unlabeled B16-F10 melanoma cells; (c) transfer from BCECF-labeled BAEC to unlabeled B16-F10 melanoma cells (T); (d) same as c in the presence of 3.5 mM 1-heptanol (see text for description). The ratio of BAEC to B16-F10 melanoma cells was 1:1 ( $5.0 \times 10^5$  cells). The percent B16-F10 labeled by the transfer of BCECF from labeled BAEC was  $\sim 40\%$  and was inhibited by 1-heptanol.

adhesion indicate that adhesion preferentially occurs at the apposition zone between neighboring endothelial cells (10, 35), and is followed by the retraction of endothelial cells and the recanalization of the blood vessel around the arrested tumor cell (21, 23). In this report, we show that an important event follows adhesion and precedes endothelial retraction that has not been recognized before. Using fluorescence imaging and flow cytometry techniques, we show here that highly metastatic tumor cells immediately upon adhesion to



**Figure 6.** Northern blot analysis of B16 total RNA with a connexin43 cDNA probe (see text): both B16-F0 (lane 1) and B16-F10 (lane 2) express two connexin43 mRNA species, a 3- and a 9-kb band. The 3- and 9-kb bands are significantly stronger in B16-F10. The positions of the 18S and 28S ribosomal RNA subunits are identified on the right-hand side of the blot. Each lane contains 10  $\mu$ g of total RNA.

the endothelium establish gap junctional channels between the cytoplasm of tumor cells and endothelial cells. This is verified by the transfer of a membrane impermeable cytoplasmic dye BCECF from tumor cells to endothelial cells. This dye transfer can be mediated by any of three mechanisms: (a) the formation of membrane channels between endothelial cells and adherent tumor cells (gap junctionlike channels) (13); (b) heterotypic cell fusion between tumor cell and endothelial cell; and (c) the transfer of tumor cell membrane vesicles to endothelial cells. The inhibition of dye transfer by alkanols (e.g., 1-heptanol), the failure of MDCK cells to acquire dye from B16 melanoma cells or R3230AC-MET carcinoma cells under high density culture conditions (36), as well as the absence of fluorescein tumor cell vesicles in the culture media and of endothelial cell-tumor cell fusions, strongly argue for the hypothesis that gap junctional channels are involved in the transfer of dye from the highly metastatic tumor cells to the endothelial cells. This conclusion is further supported by the ability of BAEC to synthesize the gap junctional protein connexin43 (24) and our finding that the communication-efficient cell types used in this study (e.g., BAEC [25], B16-F10, B16-F0, and R3230AC-MET) express mRNA for connexin43. Interestingly, the connexin43 message is present in higher amounts in highly metastatic tumor cells than in low metastatic cells.

The fact that dye transfer precedes extravasation and is far more pronounced in highly metastatic B16-F10 and R3230AC-MET than in low metastatic B16-F0 and is absent in non-metastatic R3230AC-LR implies that metabolic coupling may play an important role in tumor cell extravasation. For example, low-molecular weight components synthesized by tumor cells could be transferred to endothelium without risking the dilution to inactive concentrations in the blood to initiate locally confined retraction of endothelial cells from their basement membrane, thereby allowing tumor cells to adhere to subendothelial matrix and to extravasate. A candidate molecule for such action is the arachidonic acid metabolite 12(S)-HETE, which has been shown not only to induce endothelial cell retraction in vitro, but also to be synthesized in increased amounts by highly metastatic tumor cells (15, 17). Beyond mediating retraction, the establishment of gap junctionlike channels between tumor cells and endothelium may induce, by as yet unknown signaling events, other endothelial cell responses known to be associated with extravasation. One such effect may be the direct stimulation of endothelial cells by the adherent tumor cell to induce lysis of its basement membrane. Although degradation of basement membrane has been associated unilaterally with a tumor cell function (27, 29, 30), it is conceivable that endothelial cells which have been shown to synthesize basement membrane-degrading proteolytic enzymes (14, 19), may have a more active role in basement membrane degradation than is generally ascribed to them. By signal transfer from tumor cells, endothelial cells may be stimulated to synthesize and/or secrete increased amounts of basement membrane-degrading enzymes and, thus, actively participate in tumor cell extravasation. Although this hypothesis is speculative and is currently under investigation in our laboratory, it is attractive as it once more represents a locally confined effect that is restricted to the endothelial cell to which the tumor cell adheres and communicates with.

The importance of cell-cell communication in tumor cell

extravasation is further substantiated by three observations. Firstly, Braunder and Hulser (7), using an *in vitro* invasion assay developed by Mareel et al. (28) have shown that only tumor cells which are able to communicate with cells of chick embryo heart fragments are able to invade this tissue, while tumor cells which are unable to do so form a cellular capsule around the heart fragments. Secondly, low metastatic tumor cells transfer cytoplasmic dye to endothelium to a significantly lesser extent than their highly metastatic counterparts. Thirdly, heterotypic coupling is also observed between lymphocytes and endothelial cells (16). During their homing to selective lymphoid tissues, lymphocytes use many of the same steps as blood-borne cancer cells do during the colonization of secondary organs. Arrest of lymphocytes in lymphoid tissues is initiated by the recognition of specific adhesion molecules expressed on the surface of high endothelial venules and is followed by the extravasation of the lymphocyte from the venular lumen into the lymphoid tissue (8, 38, 39). Since extravasation of lymphocytes is similar in nature to that of tumor cells, it is only logical that they may be governed by similar mechanisms. Gap junction formation may therefore be a key event in the initiation of extravasation, and the ability to communicate with the endothelium may represent a critical characteristic of successfully metastatic tumor cells.

The authors wish to thank Dr. Andrew Yen for his advice and helpful discussions, Dr. Duzhang Zhu, Ursula Nana, James Slatery, and Joseph Platko for their expert technical assistance.

This work was supported by a Public Health Service grant CA47668 from the National Cancer Institute.

Received for publication 12 February 1991 and in revised form 2 August 1991.

## References

- Alby, L., and R. Auerbach. 1984. Differential adhesion of tumor cells to capillary endothelial cells *in vitro*. *Proc. Natl. Acad. Sci. USA* 81:5739-5743.
- Auerbach, R., W. C. Lu, E. Pardon, F. Gumkowski, G. Kaminska, and M. Kaminski. 1987. Specificity of adhesion between murine tumor cells and capillary endothelium: an *in vitro* correlate of preferential metastasis *in vivo*. *Cancer Res.* 47:1492-1496.
- Augustin-Voss, H. G., R. C. Johnson, and B. U. Pauli. 1991. Modulation of endothelial cell surface glycoconjugate expression by organ-derived biomatrices. *Exp. Cell Res.* 192:346-351.
- Belloni, P. N., and R. J. Tressler. 1990. Microvascular endothelial cell heterogeneity: interactions with leukocytes and tumor cells. *Cancer Metastasis Rev.* 8:353-389.
- Beyer, E. C., D. L. Paul, and D. A. Goodenough. 1987. Connexin 43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 105:2621-2629.
- Booyse, F. M., B. J. Sedlak, and M. E. Rafelson, Jr. 1975. Culture of arterial endothelial cells: characterization and growth of bovine aortic cells. *Thromb. Diath. Haemorrh.* 34:825-839.
- Brauner, T., and D. Hulser. 1990. Tumor cell invasion and gap junctional communication. II. Normal and malignant cells confronted in multicell spheroids. *Invasion & Metastasis.* 10:31-48.
- Butcher, E. C. 1990. Cellular and molecular mechanisms that direct leukocyte traffic. *Am. J. Pathol.* 136:3-11.
- Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium-thiocyanate-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Crissman, J. D., J. S. Hatfield, D. G. Menter, B. Sloane, and K. V. Honn. 1988. Morphological study of the interaction of intravascular tumor cells with endothelial cells and subendothelial matrix. *Cancer Res.* 48:4065-4072.
- Feinberg, A. P., and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132:6-13.
- Fidler, I. J. 1973. Selection of successive tumor lines for metastasis. *Nature New Biol.* 242:148-149.
- Flagg-Newton, J., I. Simpson, and W. R. Loewenstein. 1979. Permeability of the cell-to-cell membrane channels in mammalian cell junction. *Science (Wash. DC).* 205:404-407.
- Gross, J. L., D. Moscatelli, E. A. Jaffe, and D. B. Rifkin. 1982. Plasminogen activator and collagenase production by cultured capillary endothelial cells. *J. Cell Biol.* 95:974-981.
- Grossi, I. M., L. A. Fitzgerald, L. A. Umbarger, K. K. Nelson, C. A. Diglio, J. D. Taylor, and K. V. Honn. 1989. Bidirectional control of membrane expression and/or activation of the tumor cell IRGpIIb/IIIa receptor and tumor cell adhesion by lipoxygenase products of arachidonic acid and linoleic acid. *Cancer Res.* 49:1029-1037.
- Guinan, E. C., B. R. Smith, P. F. Davies, and J. S. Pober. 1988. Cytoplasmic transfer between endothelium and lymphocytes: quantitation by flow cytometry. *Am. J. Pathol.* 132:406-409.
- Honn, K. V., I. M. Grossi, C. A. Diglio, M. Wojukiewicz, and J. D. Taylor. 1989. Enhanced tumor cell adhesion to the subendothelial matrix resulting from 12(S)-HETE-induced endothelial cell retraction. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 3:2285-2293.
- Iozzo, R. V. 1985. Neoplastic modulation of extracellular matrix: colon carcinoma cells release polypeptides that alter proteoglycan metabolism in colon fibroblasts. *J. Biol. Chem.* 260:7464-7473.
- Kalebic, T., S. Garbisa, B. Glaser, and L. A. Liotta. 1983. Basement membrane collagen: degradation by migrating endothelial cells. *Science (Wash. DC).* 221:281-283.
- Korach, S., M. F. Poupon, J. A. Du Villard, and M. Becker. 1986. Differential adhesiveness of rhabdomyosarcoma-derived cloned metastatic cell lines to vascular endothelial monolayers. *Cancer Res.* 46:3624-3629.
- Kramer, R. H., R. Gonzalez, and G. L. Nicolson. 1980. Metastatic tumor cells adhere preferentially to the extracellular matrix underlying vascular endothelial cells. *Int. J. Cancer.* 26:639-645.
- Kramer, R. H., and K. G. Vogel. 1984. Selective degradation of basement membrane macromolecules by metastatic melanoma cells. *J. Natl. Cancer Inst.* 72:889-899.
- Lapis, K., S. Paku, and L. A. Liotta. 1988. Endothelialization of embolized tumor cells during metastasis formation. *Clin. Exp. Metastasis* 6:73-89.
- Larson, D. M., E. C. Beyer, and C. C. Haudenschild. 1991. Biosynthesis of the gap junction protein connexin-43 by bovine aortic endothelial cells. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 5:A528.
- Larson, D. M., C. C. Haudenschild, and E. C. Beyer. 1990. Gap junction messenger RNA expression by vascular wall cells. *Circulation Res.* 66:1074-1080.
- Liotta, L. A., C. N. Rao, and U. M. Wewer. 1986. Biochemical interactions of tumor cells with the basement membrane. *Annu. Rev. Biochem.* 55:1037-1057.
- Liotta, L. A., U. P. Thorgeirsson, and S. Garbisa. 1982. Role of collagenases in tumor cell invasion. *Cancer Metastasis Rev.* 1:277-288.
- Mareel, M., J. Kint, and C. Meyvisch. 1979. Methods of study of the invasion of malignant C3H-mouse fibroblasts into embryonic chick heart *in vitro*. *Virchows Arch. Abt. B. Zellpath.* 30:95-111.
- Nakajima, M., T. Irimura, D. Di Ferrante, N. Di Ferrante, and G. L. Nicolson. 1983. Heparan sulfate degradation: relation to tumor invasive and metastatic properties of mouse B16 melanoma sublines. *Science (Wash. DC)* 220:611-613.
- Nakajima, M., D. R. Welch, P. N. Belloni, and G. L. Nicolson. 1987. Degradation of basement membrane type IV collagen and lung subendothelial matrix by rat mammary adenocarcinoma cell clones of differing metastatic potentials. *Cancer Res.* 47:4869-4876.
- Ng, R., J. A. Kellen, and A. C. Wong. 1983. Plasminogen activators as markers of tumor colonization potential. *Invasion & Metastasis.* 3:243-248.
- Nicolson, G. L. 1988. Cancer metastasis: tumor cell and host organ properties important in metastasis to specific secondary sites. *Biochim. Biophys. Acta.* 948:175-224.
- Pauli, B. U., H. G. Augustin-Voss, M. E. El-Sabban, R. C. Johnson, and D. A. Hammer. 1990. Organ preference of metastasis: role of endothelial cell adhesion molecules. *Cancer Metastasis Rev.* 9:175-189.
- Pauli, B. U., and W. Knudson. 1988. Tumor invasion: a consequence of destructive and compositional matrix alterations. *Human Pathol.* 19:628-639.
- Pauli, B. U., and C. L. Lee. 1988. Organ preference of metastasis: the role of organ-specifically modulated endothelial cells. *Lab. Invest.* 58:379-387.
- Sepulveda, F. V., and J. D. Pearson. 1984. Deficiency in intercellular communication in two established renal epithelial cell lines (LLC-PK1 and MDCK). *J. Cell Sci.* 66:81-93.
- Sher, B. T., R. Bargatze, B. Holzmann, W. M. Gallatin, D. Mathews, N. Wu, L. Picker, E. C. Butcher, and I. L. Weissman. 1988. Homing receptors and metastasis. *Adv. Cancer Res.* 51:361-390.
- Springer, T. A. 1990. Adhesion receptors of the immune system. *Nature (Lond.)* 346:425-434.
- Stoolman, L. M. 1989. Adhesion molecules controlling lymphocyte migration. *Cell.* 56:907-910.
- Vlodavsky, I., Z. Fuks, M. Bar-Ner, Y. Ariav, and V. Schirrmacher. 1983. Lymphoma cell-mediated degradation of sulfated proteoglycans in

the subendothelial extracellular matrix: relationship to tumor cell metastasis. *Cancer Res.* 43:2704-2711.

41. Weiss, L. 1987. The hemodynamic destruction of circulating cancer cells. *Biorheology.* 24:105-115.
42. Weiss, L., and D. S. Dimitrov. 1986. Mechanical aspects of the lungs as cancer cell-killing organs during hematogenous metastasis. *J. Theor. Biol.* 121:307-321.
43. Weiss, L., F. W. Orr, and K. V. Honn. 1989. Interactions between cancer cells and the microvasculature: a rate regulator for metastasis. *Clin. Exp. Metastasis* 7:127-167.
44. Zetter, B. R. 1990. The cellular basis of site-specific tumor metastasis. *New Engl. J. Med.* 322:605-612.
45. Zhu, D., and B. U. Pauli. 1991. Generation of monoclonal antibodies against organ-specific endothelial cell surface determinants. *J. Histochem. Cytochem.* 39:1137-1142.
46. Zimmerman, A., and H. U. Keller. 1987. Locomotion of tumor cells as an element of invasion and metastasis. *Biomed. Pharmacother.* 41:337-344.