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Interactions of tumor cells with vascular endothelial cell monolayers: A model for metastatic invasion

(adhesion/extracellular matrix/malignancy/migration)

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ABSTRACT **The interactions of tumorigenic and nontumorigenic human and rodent cells with vascular endothelial cells and their underlying extracellular matrix were studied in culture. The abilities of various cells to attach to endothelial monolayers and cause morphologic changes, such as rupture of endothelial–endothelial cell interactions leading to retraction of endothelial cells and exposure of extracellular matrix, as well as their propensities to invade and underlap retracted endothelial monolayers and continue migration were assessed by time-lapse and phase-contrast microscopy as well as scanning and transmission electron microscopy. In general, highly malignant or highly invasive cells *in vivo* were capable of attachment, invasion, and migration under endothelial cells *in vitro*. This system may be useful for elucidating mechanisms of tumor cell arrest and extravasation.**

Malignant cells possess the abilities to invade into surrounding normal tissues and disseminate to form secondary tumors (metastases) at near and distant host sites (1–3). Two critical stages during blood-borne metastatic spread are circulating tumor cell (*a*) arrest or attachment to the vascular endothelium and (*b*) extravasation or invasion of the vascular endothelium and underlying basement membrane. Although little is known concerning the mechanism of blood-borne tumor cell arrest (2, 3), several theories have been proposed for extravasation: (*i*) endothelium penetration by breakage of endothelial intercellular junctions (4, 5), (*ii*) cytotoxic or enzymatic destruction of endothelial cells adjacent to tumor cells (6, 7), (*iii*) extension of tumor cell pseudopodia through endothelial cell cytoplasm (8), and (*iv*) endothelial vessel rupture due to proliferation of tumor cells at the site of arrest (4). Due to the difficulties in studying tumor cell extravasation *in vivo* we have developed an *in vitro* model to evaluate malignant tumor cell interactions with vascular endothelial cells. This model utilizes cultured vascular endothelial cells, which form a uniform cell monolayer, intercellular junctions, and a basal lateral extracellular matrix (9–12).

METHODS

Cells. Cloned calf bovine aortic endothelial cells (BAE) and human umbilical cord endothelial cells were obtained from D. Gospodarowicz (9–11). Endothelial cells were used at passage 4–8. BAE cells were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% calf bovine serum (Irvine Scientific), and human umbilical cord endothelial cells were cultured in medium 199 plus 20% fetal bovine serum. Fibroblast growth factor was purified as described (11) and added every other day at a concentration of 100–500 ng/ml. At confluency the serum concentration was reduced to 5% and fibroblast growth factor at 5 ng/ml was added every other day. Tumorigenic and nontumorigenic cell lines were obtained and grown as referenced in Table 1. Human foreskin and mouse embryo fibroblasts were obtained from D. Cunningham and cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

Assays and Electron Microscopy. The adhesion and invasion assays were performed as follows: Completely confluent monolayers of BAE or human umbilical cord endothelial cells were seeded with suspensions of tumorigenic or nontumorigenic cells at 37°C in Dulbecco's modified Eagle's medium plus 10% calf serum (or, in the case of human endothelial cells, in medium 199 plus 20% fetal calf serum) at 2–5 × 10^5 cells per 16-mm culture dish. Single cell suspensions of adherent cell lines were prepared after a 10- to 15-min incubation with 2 mM EDTA in Ca^{2+},Mg^{2+}-free Dulbecco's phosphate-buffered saline (28). After incubation for various times at 37°C, the culture dishes were examined by time-lapse or phase-contrast microscopy (see legend to Table 1). Some of the endothelial monolayers were carefully washed with phosphate buffered saline (28) at 37°C by aspiration and fixed in phosphate-buffered saline/1.5% glutaraldehyde for 10 min at 37°C and then for 1–3 hr at 22°C. The glutaraldehyde-fixed monolayers were prepared for scanning or transmission electron microscopy after postfixation in 1% osmium tetroxide/1 mM $CaCl_2$/0.1 M sodium phosphate, buffer, pH 7.2, for 0.5 hr at room temperature. For scanning electron microscopy, monolayer samples were dehydrated through a graded series of ethanol, transferred to Freon 113, and critical-point dried (29). After they were coated with 50–100 Å of gold/palladium (Hummer II, Technics), the samples were observed in a Hitachi model S500 scanning electron microscope. For transmission electron microscopy, osmium-fixed monolayer samples were rinsed in 0.1 M sodium phosphate buffer (pH 7.2), dehydrated in ethanol to 70%, and stained in block with 0.5% uranyl acetate/70% ethanol. Samples were further dehydrated in ethanol and embedded in 1 mm of Epon 812 (30). Epon-embedded cell monolayers were stripped from plastic tissue culture dishes and sectioned perpendicular to the monolayer plane. Thin sections (300–600 Å) were stained with uranyl acetate and lead citrate and observed in a Hitachi model HU-12 transmission electron microscope.

RESULTS

Interactions of tumorigenic and nontumorigenic cells with vascular endothelial cells were monitored by time-lapse, phase-contrast microscopy and scanning and transmission electron microscopy. We measured the abilities of added cells to (*a*) adhere or attach to endothelial monolayers, (*b*) cause morphological changes in endothelial cells (retraction) leading to rupture of endothelial cell–endothelial cell interactions (intercellular junctions), and (*c*) invade the endothelial cell mo-

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Abbreviation: BAE, cloned calf bovine aortic endothelial cells.

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Table 1. Interactions of tumorigenic and nontumorigenic cells with BAE *in vitro*

Cell type/origin* (ref.)	Observation		
	Adhesion†	Retraction‡	Invasion–migration§
Tumorigenic			
B16-F1 melanoma/M (13)	+	+	+
B16-F10 melanoma/M (13)	+	+	+
B16-B10n melanoma/M (14)	+	+	+
S91 melanoma, clone 2/M (15)	+	+	+
Hs939 melanoma/H (16)	+	+	+
HT 1080 fibrosarcoma/H (17)	+	+	+
MSV-3T3 sarcoma/M (18)	+	+	+
HeLa S3 adenocarcinoma/H (19)	+	+	±
13762 adenocarcinoma/R (20)	+	+	±
SK-Br-3 carcinoma/H (16)	+	+	±
EL4 lymphoma/M (21)	±	±	±
RAW117-P lymphosarcoma/M (22)	±	−	−
RAW 117-H10 lymphosarcoma/M (22)	±	−	−
P815 mastocytoma/M (23)	±	±	±
Nontumorigenic			
Foreskin fibroblasts/H	+	+	−
Embryonic fibroblasts/M	+	+	−
Lung fibroblasts/M	+	+	−
Peritoneal macrophages/M (24)	+	+	−
Splenic lymphocytes/M (25)	±	−	−
PMN leukocytes/H (26)	+	+	+
Monocytes/H (27)	+	+	+
Erythrocytes/H	−	−	−
Platelets/H	−	−	−

MSV, murine sarcoma virus; PMN, polymorphonuclear.

* Species origin: M, mouse; H, human; R, rat.

† Adhesion was measured under nonshear conditions (31) after a 3-hr incubation at 37°C. +, >70% adhesion to endothelial cell monolayer; ±, <70% adhesion; −, <1% adhesion.

‡ Retraction of endothelial cells from, and exposure of, underlying extracellular matrix due to interactions with cells adherent to the endothelial monolayer after a 5-hr incubation at 37°C. +, A majority of the adherent cells produced endothelial cell reaction; ±, some evidence of retraction; −, no evidence of retraction.

§ Invasion and migration under endothelial cell monolayer monitored by phase-contrast microscopy (scoring of nuclear overlap) and transmission electron microscopy after a 5-hr incubation at 37°C. +, A majority of the adherent cells have undergone invasion and migration; ±, some evidence of invasion and migration; −, no evidence of invasion and migration.

nolayer and actively migrate *under* the monolayer. Differences in cell adherence, retraction, invasion, or migration under human or calf endothelial cells were not found in the present assays.

Adhesion. Attachment or adhesion of cells to endothelial cell monolayers was assessed during a 3-hr incubation at 37°C. Quantitative measurements of rates or strengths of initial adherence under shear forces (31, 32) were not performed in the present experiments. In general, epithelial and connective tissue cells adhered to the endothelial cell monolayers under the assay conditions, whereas fewer lymphoid cells attached, and these adhered only very weakly (Table 1). Also, under these conditions different rates of adhesion of high- or low-metastatic tumor cells to endothelial cell monolayers were less than previous reports (32), although in this assay these differences were not recorded. For example, B16-F1 melanoma cells attached rapidly to endothelial monolayers at 37°C (Figs. 1*A* and 2*A*)

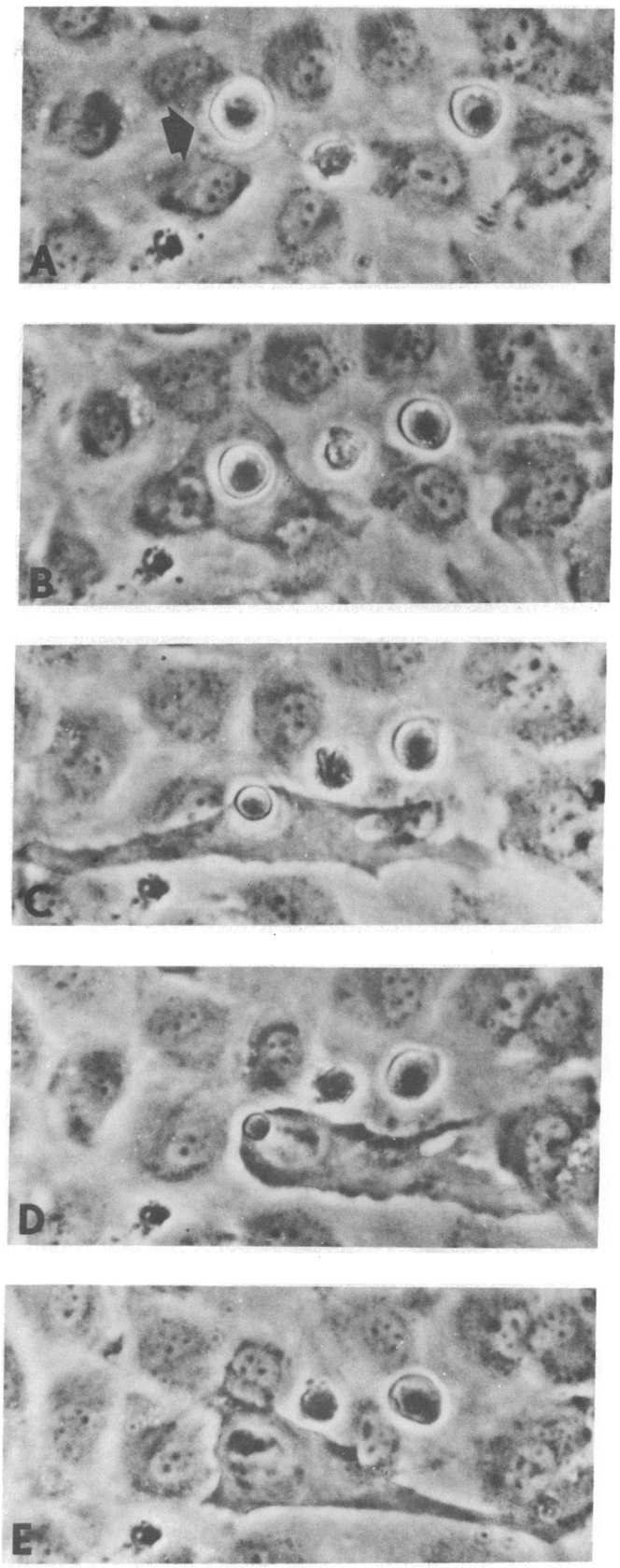

FIG. 1. Time-lapse, phase-contrast microscopy of attachment, invasion, and migration of B16 melanoma cells under vascular endothelial cell monolayer. Melanoma cells (arrow) were seeded on confluent BAE cell monolayers and examined at the following time intervals: *A*, 30 min; *B*, 60 min; *C*, 120 min; *D*, 180 min; *E*, 240 min. (×525.)

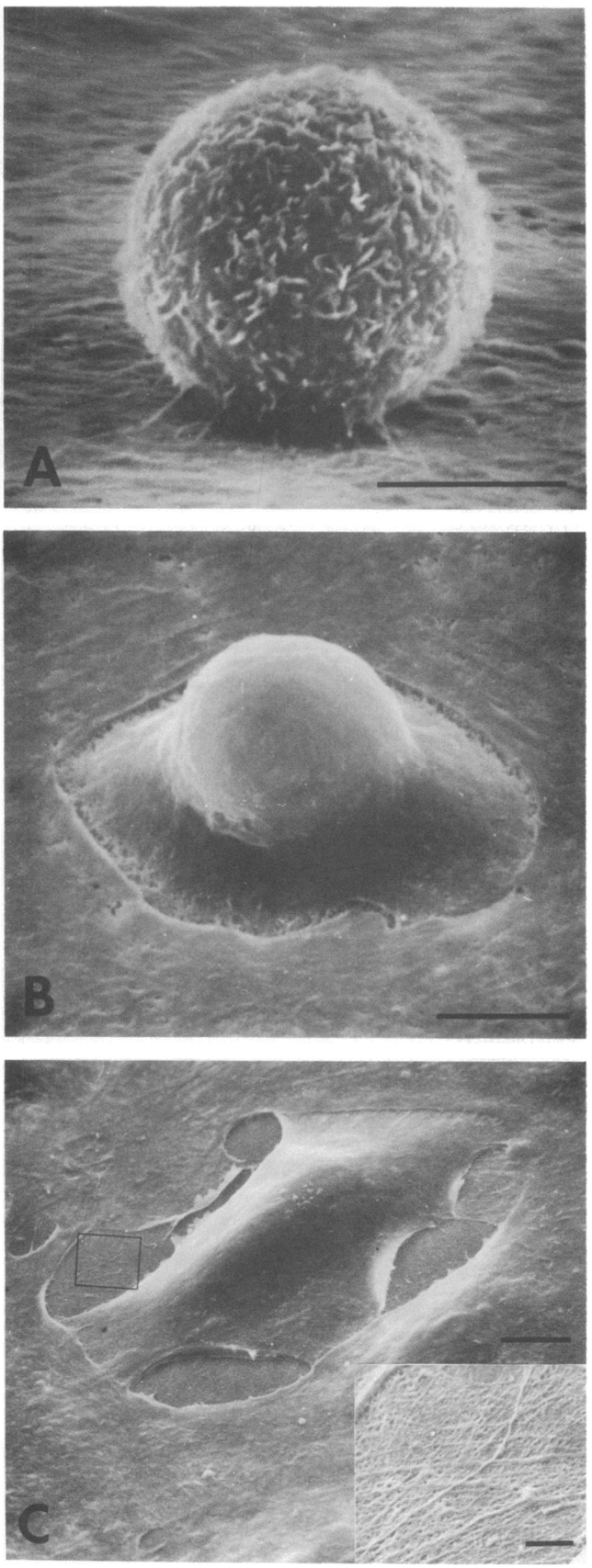

FIG. 2. Scanning electron microscopy of attachment, invasion, and migration of B16 melanoma cells under vascular endothelial cell monolayer. Melanoma cells were seeded on a confluent BAE cell monolayer and examined at the following time intervals: *A*, 30 min; *B*, 1.5 hr; *C*, 3 hr. (Bars equal 5 μm; Fig. 2*C*, *Inset*, 1 μm.)

(>70% adherent by 3 hr); these same cells failed to attach at 4°C (<5% adherent). In general, a majority of the seeded cells adhered to the endothelial monolayers at or near junctional regions between adjacent endothelial cells.

Cells attached to endothelial monolayers remained spherical and were capable of motile migration on the dorsal surfaces of endothelial cells (Fig. 1 *A* and *B*). At this stage endothelial monolayers were completely confluent, and only rarely were gaps found between adjacent endothelial cells. Initial adhesions of B16 melanoma cells to endothelial cells appeared to involve microvilli that attached to the endothelial cell surface as well as more intimate plasma membrane contacts between adherent and endothelial cells (Figs. 2*A* and 3*A*).

Retraction. Cells that adhered strongly to endothelial cell monolayers were capable of causing intercellular junction disruption and endothelial cell retraction. After strong attachment, adherent cells were observed to emit microvilli and small lamellopodia, which often penetrated beneath neighbor endothelial cells (Figs. 1*B*, 2 *B* and *C*, and 3*B*). Endothelial cells frequently underwent extensive cell retraction at the site of adherent cell contacts, exposing regions of underlying extracellular matrix (Fig. 2*C*).

Invasion–Migration. After endothelial cell retraction most adherent cells penetrated to and spread on the underlying extracellular matrix and invaded under neighboring endothelial cells. The penetration and invasion of vascular endothelial monolayers was seen with almost all of the adherent cell lines, but only those cells that had adhered near endothelial junctions appeared to be capable of subsequent invasion. Nontumorigenic cell lines such as mouse embryonic fibroblasts were unable to penetrate beneath and migrate under endothelial cell monolayers (Table 1). However, tumor cell lines such as B16 melanoma which possess high malignant potential *in vivo* (33) and high invasive potential in cell culture (34) rapidly penetrated the endothelial cell monolayers (Figs. 2 *B* and *C*) and extended cellular processes under adjacent endothelial cells between the extracellular matrix and the dorsal endothelial cell surface (Fig. 3*B*). In time many of the invading tumor cells penetrated completely under the endothelial monolayers, spread, and began actively migrating (Fig. 1*D*). Examination of the endothelial and tumor cells by transmission electron microscopy indicated that the endothelial cell monolayers reformed and eventually reestablished extensive intercellular junctions, resulting in a walling-off of the migrating malignant cells (Fig. 3*C*). The entire sequence of events from initial adhesion to walling off of invading cells was variable but often occurred in less than 3 hr.

DISCUSSION

Tumor cell attachment to and invasion of endothelial monolayers in tissue culture appeared to be similar to blood-borne tumor arrest and extravasation *in vivo*. Various studies (4, 5, 35, 36) on tumor cell extravasation have suggested that neoplastic cells invade blood vessels at sites near or at endothelial intercellular junctions that are broken and subsequently reseal after tumor cell penetration, in a manner analogous to leukocyte emigration (37). However, in contrast to some reports (38–40), tumor cells in our studies were never observed to penetrate directly through endothelial cytoplasms. The time required for tumor cell adhesion, invasion, and complete migration under endothelial cells *in vitro* was quite variable and occurred in most experiments in less than 3 hr depending on the tumor cell–endothelial cell combination. Various times (2–48 hr) have been recorded *in vivo* for tumor cell extravasation (36, 41, 42). Once tumor cells have invaded and migrated under endothelial cells, the latter reform previously broken intercellular junctions,

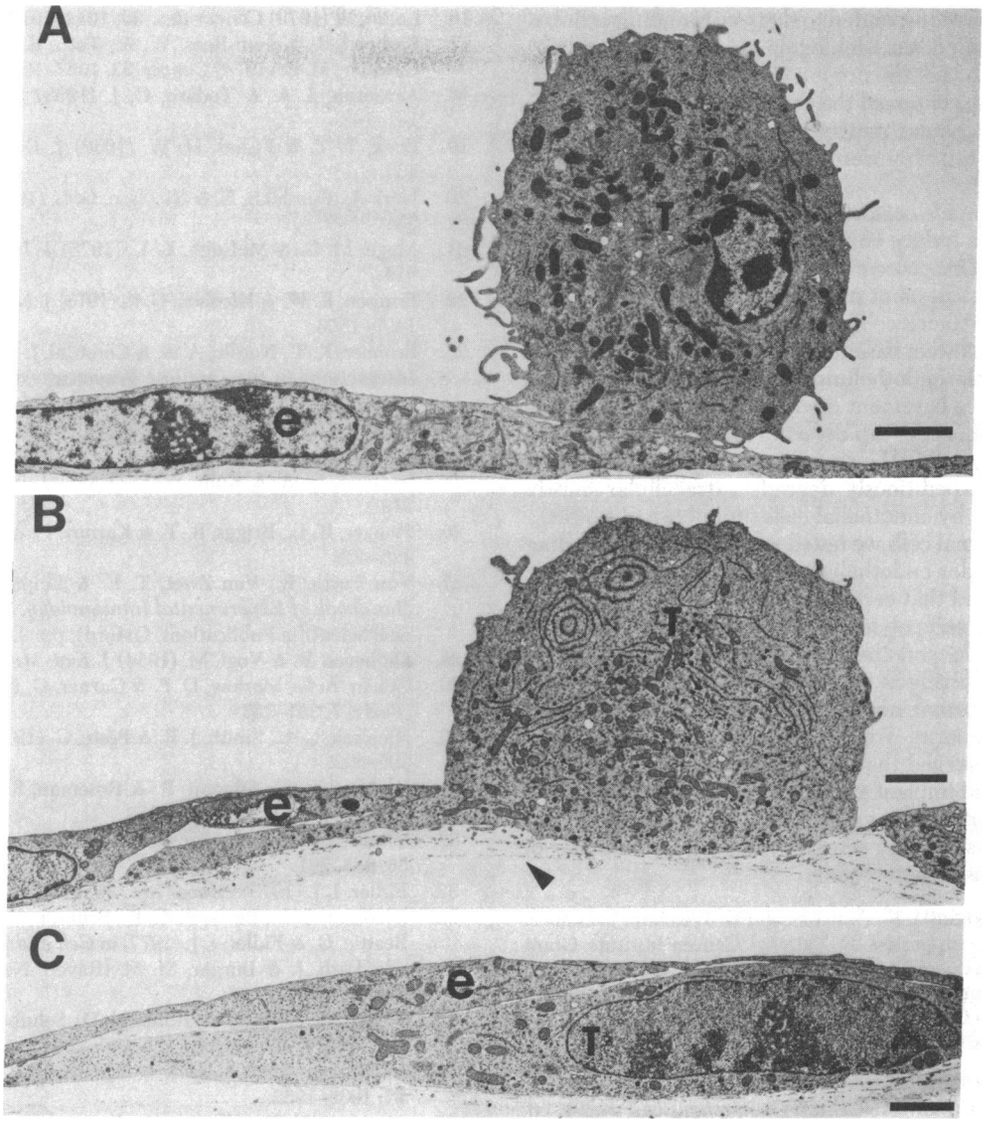

FIG. 3. Transmission electron microscopy of attachment, invasion, and migration of B16 melanoma cells under vascular endothelial cell monolayer. Melanoma cells were seeded on confluent BAE cell monolayers and examined at the following time intervals: *A*, 30 min; *B*, 1 hr; *C*, 3 hr. Arrow indicates BAE extracellular matrix. (Bars equal 2 μm.)

thereby walling off tumor cells from the extracellular environment. Similar observations have been made *in vivo* by Ludatscher *et al.* (36), who found tumor cells enveloped between resealed endothelial cells and basement membrane.

Stable attachment of tumor cells to the vascular endothelium is the initial step in malignant cell arrest and extravasation. Malignant and nonmalignant cells of varied origin were tested for their abilities to adhere to vascular endothelial cells in culture. Tumorigenic and nontumorigenic cells of epithelial or connective tissue origin rapidly adhered to vascular endothelial cell monolayers, whereas some lymphoid cells (lymphosarcoma, lymphoma, and splenic lymphocytes) attached only very weakly to the endothelial cells and failed to elicit extensive endothelial cell retraction. In general, cells that attached to endothelial monolayers away from junctional regions remained rounded in shape unless they migrated to junctional regions, whereupon they evoked endothelial cell retraction from the underlying extracellular matrix and began spreading. Spreading of cells adherent to retracted or partially retracted endothelium appeared to be dependent on and occurred concomitantly with endothelial cell retraction and exposure of the endothelial extracellular matrix. The extracellular matrix of BAE and other endothelial cells is fibrous in nature (Fig. 2*B*, *inset*) and contains predominantly the glycoprotein fibronectin (12) and various glycosaminoglycans (unpublished results). Jones (43) has found basolateral extracellular matrix containing fibrillar collagen and elastin associated with endothelial cells grown on multilayers of smooth muscle cells in culture.

Cells that attached to BAE cell monolayers adhered more strongly to BAE extracellular matrix (unpublished results). This may explain why normal fibroblasts attached to BAE cell monolayers, stimulated BAE retraction, and migrated to the extracellular matrix. Differences between normal fibroblast adhesion to endothelial cells and to their extracellular matrix may have resulted in a net fibroblast cell movement to the extracellular matrix. Carter (44) has found that cells respond to adhesive gradients, and they direct their net movements toward substrates of high adhesive potential. Although normal fibroblasts inserted themselves into the spaces left by retracted endothelial cells to reform a confluent, quiescent cell monolayer, they failed to infiltrate and migrate under adjacent endothelial cells. Fibroblasts that have inserted themselves into endothelial

monolayers may have responded to their surrounding cellular environment through contact-inhibition of cell movement (45). Tumor cells, which lack the property of contact-inhibition of cell movement (46), continued their underlapping and invasion between the extracellular matrix and basolateral surfaces of endothelial cells until they were completely under the endothelial monolayer.

Once they have made contact with an endothelial monolayer, malignant cells can induce enzymatic destruction of extracellular matrix (47). This process may be analogous to the *in vivo* destruction of the basement membrane by metastatic tumor cells. That certain tumor cells *in vivo* remain between endothelial cells and an intact basement membrane for up to 24 hr after resealing of the endothelium may be a reflection of their inabilities to induce basement membrane destruction. Metastatic B16 melanoma cells rapidly extravasate the microcirculation *in vivo* (48), and in the presence of endothelial cells B16 melanoma cells expeditiously degrade extracellular matrix components made by endothelial cells (unpublished results).

Some of the normal cells we tested were capable of invading and migrating under endothelial monolayers in culture. We expected and found that certain highly invasive normal cells such as monocytes and polymorphonuclear leukocytes adhered to and invaded under endothelial monolayers in culture, similar to phenomena occurring *in vivo* (37). In fact, there are several instances where normal, nonhemopoetic cells colonize distant tissues via blood-borne arrest and invasion. For example, Traptiklis (49) observed that normal thyroid cells inoculated intravenously could implant and survive in extravascular spaces in the lungs and other organs, and it is well known that endometrial cells can spread to ectopic sites via local vascular or lymphatic systems (50).

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