Morphological Study of the Interaction of Intravascular Tumor Cells with Endothelial Cells and Subendothelial Matrix

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ABSTRACT

Multiple steps or events have been described as essential in the metastatic cascade. Tail vein injection of single cell suspensions was used to study the ultrastructural details of the events involved in the initial arrest and attachment of circulating tumor cells. Lewis Lung Carcinoma (3LL) and a mammary adenocarcinoma (16c) were compared to a previous ultrastructural study of B16 amelanotic melanoma (B16a) detailing morphological events in the initial arrest and attachment of tumor cells in lung. The three murine tumors followed similar steps and varied only slightly in the time sequence of the steps. We observed the following steps: (a) initial arrest of tumor cells was characterized by an intimate tumor endothelial cell contact; (b) platelet activation and aggregation was noted by two minutes. Platelet aggregation continued for 1–4 h until a thrombus formed; (c) after approximately 4 h endothelial cell separation with extension of the tumor cell to the subendothelial matrix was noted; (d) at approximately 24 h the tumor cell associated thrombus dissipated and the attached tumor cells were exposed to a reestablished circulation. (e) mitoses were observed after 24 h with cell division and the development of intravascular tumor nodules; (f) the final step in the extravasation sequence was dissolution of the basement membrane by the attached tumor cells.

INTRODUCTION

A complex sequence of interrelated events must occur in order to permit the successful establishment of lung colonies after i.v. injection of tumor cells. This experimental metastasis assay is recognized as artificial in the sense that local tumor invasion of normal tissue and intravasation are not required. However, the i.v. model is accepted for the study of the tumor cell arrest and extravasation portions of the metastatic cascade. The basic steps which are necessary for circulating tumor cells to extravasate include (a) initial arrest, (b) penetration through or between vascular endothelial cells, and (c) dissolution and penetration of the subendothelial matrix. The exact sequence in which these steps occur and the mechanism(s) involved in the successful arrest and extravasation of circulating tumor cells remain controversial.

Active migration of tumor cells from the vascular space to the perivascula" space was reported by Wood (1) using the rabbit V2 carcinoma. In rabbit ear chambers, circulating V2 carcinoma cells were observed to arrest and form platelet thrombi. Arrest was followed by an immediate migration of tumor cells out of the vascular space in a manner similar to that observed for leukocytes. The in vivo observations of Wood (1) did not agree with those previously reported in a light microscopic study by Baserga and Safi-Otti (2). The latter study reported intravascular arrest and proliferation of anaplastic carcinoma (T10S) cells prior to extravasation. However, both reported that the circulating tumor cells were arrested in small arterioles coincident with thrombus formation (1, 2). Intravascular proliferation of the arrested tumor cells was observed over several days in the report of Baserga and Safi-Otti (2). This tumor growth resulted in the destruction of the vessel and subsequent tumor cell extravasation. These latter observations have been confirmed in other ultrastructural studies of tumor cell arrest and extravasation (3–5).

The major controversies regarding characterization of the steps involved in extravasation of tumor cells include (a) the mechanism of initial arrest of the circulating tumor cells, (b) the role of platelet (and fibrin) thrombi, both in the initial stage of tumor cell arrest and as a protective “cocoon” during the early stages of the extravasation portion of the metastatic cascade, and (c) the time sequences and mechanism by which tumor cells cross endothelial and subendothelial barriers to reach the extravascular space (extravasation). We recently reported our ultrastructural observations of the events involved in arrest, extravasation, and lung colony formation from i.v. injected B16 amelanotic melanoma (B16a) cells (3). Our observations defined the following orderly and reproducible sequence for this tumor cell line: (a) initial tumor cell arrest with extensive contact between tumor cell and endothelial cell plasma membranes during the first 8 h; (b) immediate (<10 min) development of a platelet thrombus in association with arrested tumor cells; (c) penetration by the arrested tumor cells through vascular endothelial cells junctions and initiation of contact with subendothelial matrix commencing at 4 h; (d) dissolution of the tumor associated thrombus starting at 8 and completed by 24 h; (e) intravascular proliferation of tumor cells as reflected by the presence of mitotic figures. Proliferation was first noted 24 h after the initial arrest of the circulating tumor cell; (f) penetration through the subendothelial matrix by the expanding intravascular tumor nodule after 48–72 h.

Although these observations were reproducible and appeared to represent a well defined sequence of steps for B16a tumor cell extravasation, these observations may be unique to the B16a tumor line. Therefore, in the present study, we extend our observations to two additional murine tumor lines which are histologically distinct from the B16a, the Lewis lung carcinoma (3LL) and a highly metastatic mammary adenocarcinoma (16c).

MATERIALS AND METHODS

The Lewis lung carcinoma and B16 amelanotic melanoma cell lines were obtained initially from the tumor bank of the NIH. The tumor lines were obtained from frozen stock and propagated by s.c. injection of tumor cells into the axillary region of C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME). The 16c murine mammary tumors were obtained from Dr. Tom Corbett of the Division of Medical Oncology, Department of Medicine, Wayne State University, Detroit, MI and...
propagated by s.c. injection of tumor cells into the axillary region of C3H mice (The Jackson Laboratory). The tumor cells used for tail vein injection were disaggregated from 1- to 2-g s.c. tumors grown in appropriate host mice. The tumors were minced and dispersed at 37°C in minimum essential medium (Gibco, Grand Island, NY) containing 0.8% type III collagenase, 0.007% DNase II, 0.10% soybean trypsin inhibitor, and 1% fatty acid free bovine albumin (Sigma Chemical Co., St. Louis, MO). Disaggregated cells were elutriated using a Beckman JE-6 centrifuge with elutriator rotor at 1460 rpm as previously described (6). Tumor cell numbers were counted using a Coulter Model ZB1 particle counter. Tumor cell viability was determined by trypan blue dye exclusion. We have previously established that enzymatic disaggregation of solid tumors followed by elutriation results in a >98% pure tumor cell population with >90% viability. Tumor cells (1 x 10^6) viable cells/100 µl minimum essential medium) were injected into the lateral tail vein of unanesthetized mice. Three mice in each time group (2, 10, and 30 min; 1, 2, 4, 8, and 16 h; and 1, 2, 3, 5, and 8 days) were sacrificed by cervical dislocation after tumor cell injection. The lungs were removed immediately and placed in Trump's fixative (1% glutaraldehyde-4% formaldehyde in 0.1 M phosphate buffer, pH 7.2). The lower lobes were diced in 1-mm³ pieces. Ten pieces of lung/mouse were randomly chosen, routinely processed, and embedded in plastic resin (Embed 812, Electron Microscopy Sciences, Fort Washington, PA). Semithin (l.tun) sections were prepared from end pieces of lung stained with toluidine blue and evaluated by light microscopy. Thin sections were cut with a diamond knife, stained with uranyl acetate followed by lead citrate, and examined with a Zeiss EM 10 CA electron microscopy. The tumor cells were identified during the early time periods by their relatively large size, enlarged pleomorphic nuclei, and abundant cytoplasm. At later (2-5 days) time intervals pulmonary tumor colonies were readily identified. Toluidine blue stained the platelet and fibrin thrombi in an amorphous pattern.

Ultrastructural observations included the association of the arrested tumor cells with endothelial cells, platelets, erythrocytes, macrophages, leukocytes, and lymphocytes. The presence and distribution of platelet and/or fibrin thrombi and the displacement/retraction of the endothelial cells with adherence of the tumor cells to subendothelial matrix were also noted. In addition, evidence of tumor cell proliferation was judged by the number of cells present in each colony and the frequency of mitotic figures. Extravasation was defined as dissolution of the endothelial basement membrane with simultaneous contact of the tumor cells with collagen fibers of the lung interstitium.

**RESULTS**

The sequence of arrest and extravasation is similar for both the Lewis lung carcinoma (3LL) and mammary adenocarcinoma (16C) (Table 1). In addition, the chronological sequence of the steps involved in extravasation of these two lines is similar to our previous observations for B16a (data included in Table 1 for comparison) (3). A summation of the steps is as follows.

Step 1. Initial arrest (2-min time interval) of the circulating tumor cells was characterized by endothelial cell-tumor cell membrane contact (Figs. 1 and 2). Cells from all three tumor lines demonstrated intimate contact with endothelial cells at the time of initial arrest (data not shown). A limited number of activated platelets were associated with the surface of arrested tumor cells as early as 2 min postinjection (30% of LLC cells, 55% of 16C cells). The plasma membranes of tumor and endothelial cells were smooth and in close approximation without any intervening platelets or other identifiable structures. The observation that platelets were associated with tumor cells at the earliest time period (2 min) might suggest that tumor cell platelet contact is initiated while the tumor cell is circulating. However, heterotypic aggregates of sizes appreciable enough to result in mechanical embolization in the capillary bed were not identified at the early time intervals. The initial arrest of the circulating tumor cells appeared to be partially mechanical because the tumor cells are larger than circulating erythrocytes. However, the majority of radioisotope tagged tumor cells lodge temporarily but eventually pass through the pulmonary capillary bed in the first 24 h (data not shown). Although homotypic aggregates of tumor cells were occasionally observed, especially with the 16C adenocarcinoma, the majority arrested as single cells.

Step 2. The next identifiable step in the sequence was, in part, a continuation of step 1. Platelet activation (7) and deposition around the arrested tumor cell continued to result in eventual occlusion of the vascular lumen (Fig. 3). In tumor cell associated thrombi, platelets which were in proximity to the tumor cell membrane were activated as evidenced by shape change and degranulation. As previously reported (8), the arrested tumor cells developed characteristic cell extensions or processes which were intimately associated with and projected into the platelet thrombi (Fig. 3). The full development of platelet tumor cell interaction occurred between 1 and 4 h postinjection (data not shown). Occasional foci of fibrin were also present at the periphery of the platelet thrombi and were readily identified by light microscopy. The extent of contact between tumor cell and endothelial cell appeared to increase with time but never reached 100%.

Step 3. This identifiable step was first observed 4 h following arrest. It involved retraction and/or displacement of the endothelium by the tumor cell with apparent attachment of the tumor cell to the subendothelial matrix. There was marked

<table>
<thead>
<tr>
<th>Time after tail vein injection</th>
<th>Light microscopy cells or aggregates of cells/mm²</th>
<th>Mitotic figs.</th>
<th>Electron microscopy relative contact with basement membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>17 ± 10.6</td>
<td>35 ± 24</td>
<td>-</td>
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<tr>
<td>10 min</td>
<td>10 ± 7.2</td>
<td>20 ± 20</td>
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<tr>
<td>30 min</td>
<td>10 ± 7.6</td>
<td>31 ± 25</td>
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<td>1 h</td>
<td>15 ± 4.1</td>
<td>54 ± 36</td>
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<td>2 h</td>
<td>13 ± 10.0</td>
<td>26 ± 14</td>
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<td>4 h</td>
<td>12 ± 9.4</td>
<td>33 ± 16</td>
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<td>16 h</td>
<td>ND²</td>
<td>74 ± 108</td>
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<td>24 h</td>
<td>10 ± 5.5</td>
<td>55 ± 22</td>
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<tr>
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<td>12 ± 3.7</td>
<td>112 ± 140</td>
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<tr>
<td>192 h</td>
<td>ND</td>
<td>222 ± 106</td>
<td>-</td>
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</tbody>
</table>

* Mean ± SD.

+ - no contact; ++, less than 25%; +++ less than 50%; ++++, less than 75%; ++++, 100% of cells in contact.

ND, not done.
thinning of the endothelial cell cytoplasm adjacent to the arrested tumor cell prior to its attachment to basement membrane (Fig. 4A). The tumor cells appeared to gain access to the basement membrane by separating/displacing endothelial cell-cell junctions (Fig. 4B). Penetration of endothelial cells by tumor cell cytoplasmic processes was not observed. The degree or extent of surface attachment by any individual tumor cell to the subendothelial extracellular matrix increased with time. At approximately 24 h, all of the arrested B16a, 3LL, and 16c tumor cells had displaced the lining endothelial cells and were in contact with the basement membrane. Degenerating endothelial cell and their remnants were occasionally identified adjacent to the attached tumor cells, but displacement, not degeneration, of the endothelial cells appeared to be the primary event in this portion of the extravasation sequence (Fig. 5).

Step 4. This step overlapped step 3. By 24 h, the tumor cell-associated thrombus had dissolved or was in the process of dissolving (data not shown) and the tumor cells were attached to the subendothelial matrix (Table 1; Fig. 3). In many instances, especially in larger blood vessels, the vascular lumen was reestablished as evidenced by a lumen containing erythrocytes, inactivated platelets, etc. (Fig. 6).

Careful analysis for lymphocytes, monocytes, and PMN cells was also performed. There was a slight increase in the number of lymphocytes associated with the arrested tumor cells during the first 24 h, but no direct interactions between lymphocytes and tumor cells were observed. The association of PMNs with arrested tumor cells was similar to that of mononuclear cells in the 16c (and B16a) tumor lines. However, an increased number of PMNs were found in association with 3LL cells at all time periods, being most prominent during the first 24 h (data not shown). Occasional degenerative changes were noted in arrested 3LL cells (Fig. 7). The PMNs might have induced these degenerative changes or, conversely, they might have been drawn to a thrombus in a chemotactic reaction to cell necrosis. We were not able to distinguish between these possibilities.

Step 5. The number of B16a tumor cells per mm² of lung was greatest at the earliest time interval (10 min), then declined to a relatively stable level thereafter (Table 1). In 3LL, the numbers fluctuated over time without statistical significance. In 16c, the number of tumor cells per mm² declined from an initial elevated level, then increased over time in the form of proliferating nodules. This increase correlated with the increase in the number of mitotic figures observed after 4 h. Once attachment to subendothelial matrix was established, the tumor cells appeared to be secure and after 4 h mitotic figures were also noted in the

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Fig. 1. A group of 16c tumor cells (¢) occludes the lumen of a small blood vessel at 10 min postinjection. The cells appear to be in contact with the endothelium, because no interposed blood cells are visible. Bar, 1 μm.

Fig. 2. A 3LL cell at 2 min postinjection lies in direct contact with the endothelium. The characteristic micropinocytotic vesicles (solid arrowheads) and tight junctions (¢) of the endothelial cell layer are evident. Bar, 1 μm.
3LL (and B16a) tumor cell lines (Table 1). The 16c adenocarcinoma is a rapidly proliferating tumor and occasional mitotic futures were found at the earliest time intervals with a substantial increase noted after 4 h. Intravascular tumor cell nodules were observed concomitantly with increased numbers of mitotic figures supporting the conclusion that the increased number of cells found in intravascular nodules were the result of cell proliferation. The proliferating cells maintained their attachment to the subendothelial basement membrane matrix and caused further displacement of the endothelial cells as they formed expanding intravascular tumor nodules. Penetration or breakdown of the endothelial basement membrane could not be identified during the initial phase of intravascular tumor nodule development.

The number of groups or nodules of tumor cells observed by light microscopic evaluations of semithin sections was similar at all time periods after 24 h. The number of colonies for 3LL were 0.52, 0.58, 0.68, and 0.32/mm² lung area and those observed for 16c were 4.8, 2.8, 4.4, and 4.4/mm² lung area at 1, 3, 5, and 8 days, respectively. This observation implies that a tumor cell which is arrested and has developed contact with the subendothelial matrix at 24 h is likely to remain in place, proliferate, and in our model develop into a successful tumor colony. Tumor cells mechanically trapped in the small capillaries which do not illicit endothelial interaction resulting in adherence to extracellular matrix are no longer found after 24 h.

Step 6. The final step in the extravasation of all three tumor cell lines studied was the focal dissolution of the subendothelial matrix and contact of the tumor colony with extravascular structures. This process began as early as 3 days and continued through the 8 days of observation. Dissolution or breakdown of subendothelial matrix occurred after the initiation of intravascular tumor cell proliferation and formation of tumor nodules. At no time did we observe the penetration of the subendothelial matrix by single migrating cells. Instead, the subendothelial matrix which was in direct contact with the tumor cell membrane of the intravascular nodule became irregular, indistinct, and eventually disappeared. This sequence resulted in tumor cell contact with the extravascular collagen fibers of the lung interstitium.

We have previously reported that arrested B16a cells do not exhibit changes in the size, number, or cytoplasmic distribution of their organelles (3). Likewise, these were no differences observed in these organelle parameters for arrested 3LL or 16c tumor cells at all time intervals (data not shown).

DISCUSSION

The steps identified in the arrest and extravasation of 3LL and 16c tumor cells were in the same sequence and with a similar chronology to those identified earlier in the B16a tumor line (Fig. 8) (3). The events (initial adherence to endothelium,
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Fig. 4. 3LL tumor cell at 8 h postinjection showing a small area of contact with the endothelial basement membrane (arrows). The nearby endothelium is markedly thinned. B, a process of a 3LL tumor cell at 8 h postinjection penetrates the junction (open arrow) between adjacent endothelial cells (solid arrow). Bars, 1 μm.

The initial arrest of circulating tumor cells appears to involve tumor cell-membrane endothelial cell membrane interaction(s) as evidenced by their close apposition. In the present study specific membrane structures (i.e., pseudopodia) or specific points of attachment could not be identified and initial tumor cell arrest appeared to be secondary to endothelial and tumor cell surface interactions. Pseudopodia or specific sites of attachment between tumor cells and endothelium were described in the ultrastructural studies of Sindelar et al. (9) but the published electron micrographs display only undulation of the tumor cell surfaces and thus are not convincing. Close approximation of the tumor cell-endothelial cell membranes during the initial phase of arrest was also reported by Chew et al. (5). The mechanism of initial arrest for circulating tumor cells is not clear but may be mechanical with trapping of the large tumor cells in the smaller capillary vessels. This initial arrest allows adequate opportunity for the interaction of specific membrane receptors on the surface of the endothelial cell and/or tumor cell. Nicolson et al. (10) have presented experimental evidence for tumor cell surface components which are implicated not only in the determination of malignant behavior but also in the specific organ site of tumor cell arrest. There is a growing body of experimental data suggesting that there are specific cell surface mediated interactions between circulating tumor cells and the microvascular endothelium of particular host tissues (11, 12). Our data support the role of tumor cell-endothelial cell membrane surface interactions as a potentially important mechanism in maintaining the initial arrest of circulating single tumor cells.

The development of platelet thrombi in the initial phases of tumor cell arrest has been known for many years (13). However, the specific role of tumor cell-associated thrombosis in the arrest and extravasation steps of tumor metastasis remains unsettled. The most conclusive evidence to date for platelet involvement in metastasis are experiments in which induced thrombocytopenia results in decreased numbers of lung colonies after tail vein injection of tumor cells (14) and fewer spontaneous metastases from s.c. tumors (15). The majority of ultrastructural studies of tumor cell arrest and extravasation document the coexistence of platelet thrombi during the arrest phase of the metastatic cascade. The duration of platelet association with arrested tumor cells is variable but generally is limited to 8–24 h post-i.v. injection of the tumor cells (4, 5, 16). The presence of fibrin in the thrombus is also reported to be variable; however, in studies using immunostaining, fibrin is usually present (4). This apparent contradiction may be explained by the fact that fibrin cannot be reliably identified by transmission electron microscopy and therefore was not identified in the tumor-associated thrombi observed in many of the ultrastructural studies of tumor cell arrest and extravasation.

The third step in the extravasation process involves displacement of adjacent endothelial cells followed by tumor cell attachment to the subendothelial matrix. Penetration or fragmentation of endothelium and tumor cell attachment to the base-
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Fig. 6. Reestablishment of lumen 16c cell attached to subendothelial matrix after dissi-
pation of associated thrombus. Several circu-
lating platelets and RBC are evident. One
platelet touches the tumor cell but retains its
granules (nonactivated).

Fig. 7. 3LL tumor cells which have under-
gone considerable degeneration (cytoplasmic
vacuoles and myelin bodies denoted by arrows)
ilie adjacent to several PMNs. Bar, 1 μm.

membrane has been reported previously (4, 5) and has
been thought to represent the first major step in the extravas-
sation sequence (5). In vitro studies have demonstrated that the
addition of tumor cells to endothelial monolayers results in
endothelial cell retraction and adherence of the tumor cells to
the underlying subendothelial matrix (17). Our study and those
of others (5, 17) observed that the tumor cells penetrate or
displace endothelium at cellular junctions, suggesting that the
endothelium plays an interactive role in this step. The attach-
ment of the tumor cell to the subendothelial matrix appears to
represent an important and apparently stable adhesion step.
There is evidence that laminin receptors on the tumor cell
membrane are important in the establishment of successful lung
colony formation or metastases and may be most important at
this step in the extravasation sequence (18–20).

It has been suggested that tumor cell adhesion to the endo-
thelial cell is a weak association (21, 22) and the development
of a platelet thrombus may be protective against mechanical
forces which would tend to dislodge the tenuously attached
tumor cell (23). Stabilization of tumor cell-endothelium contact
after initial arrest may be an important step in the successful
development of extravasation since reentry of the tumor cell
into the circulation usually results in cell death. This hypothesis
is supported by the observation that the platelet thrombus
remains until the tumor cell achieves contact with the suben-
dotheelial matrix, presumably a more secure attachment (24).
Another possible role for the platelet in tumor cell arrest is

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presumably the facilitation of endothelial cell retraction and exposure of the subendothelial matrix. While this is an attractive hypothesis, in vitro studies of tumor cell-endothelial cell interactions in the absence of platelets (or plasma) suggest that endothelial displacement or retraction can occur in the absence of a platelet thrombus (17).

Most experimental studies of tumor cell arrest and extravasation have observed the presence of platelets, and to a lesser extent, fibrin in association with the arrested tumor cell. In this study, platelet thrombi are present for limited periods (24 h), similar to those reported in other tumor systems (4, 5, 9). However, it appears that tumor-associated platelets are not required for successful arrest and extravasation. Although the majority of experimental models of extravasation report either tumor cell associated thrombosis or a correlation between metastatic potential and ability to aggregate platelets (25) exceptions have been noted (26). For example, Kinjo (16) compared a thrombogenic (AH130) and nonthrombogenic AH130F (N) hepatoma cell line and found that both developed lung colonies after i.v. injection of the tumor cells.

Contact with the endothelial matrix appears to establish a secure tumor cell attachment which increases in area as the intravascular thrombosis dissipates. At this point, blood flow is often reestablished and the tumor cells attached to vascular basement membrane begin to proliferate. All three murine tumor lines proliferated within the vascular space along the vascular basement membrane forming intravascular nodules. Mitotic figures were common after 4 h in the 16c mammary adenocarcinoma and after 24 h in the 3LL and B16a tumor lines. This intravascular proliferation is consistent with the observations of Baserga and Sali-Otti (2) at the light microscopic level and similar to those of others at the ultrastructural level (4, 5, 9, 16). The intravascular proliferation of tumor cells eventually results in the development of multiple interruptions or defects in the vascular basement membrane. The destruction of the last vestiges of the vascular structures is the final step in the extravasation cascade. The time interval after tumor cell injection and the breakdown of vascular basement membrane is quite variable in the tumor cell lines previously reported and includes Walker 256, 9 h (5), fibrosarcoma, 24 h (9), and AH13 hepatoma, 12 h (16).

The steps involved in the arrest and extravasation of i.v. injected tumor cells were similar for the three tumors examined. The role of thrombus formation appeared important but still incompletely defined. Endothelial retraction and adhesion to vascular basement membrane was an important and probably critical step, but distinct from the initial arrest of circulating tumor cells. Intravascular tumor cell proliferation was an unexpected but unequivocal observation in all three tumor lines. Digestion of the basement membrane occurred after 24 h and only after formation of intravascular tumor nodules.

REFERENCES

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