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Endothelialization of embolized tumor cells during metastasis formation

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The reaction of the endothelial barrier to tumor cell extravasation has been studied using electron microscopy. The model system was pulmonary metastases produced by intravenous injection of B16-F10 melanoma cells. A striking difference was observed in the behavior of the endothelial lining of arterioles versus that of capillaries.

In capillaries, partial retraction of endothelial cells took place following the attachment of tumor cells. The tumor cells then immediately attached to the basement membrane and the basolateral surface of the retracted endothelial cells. The endothelial cells extended to cover the tumor cells prior to complete extravasation.

In the arterioles, on the other hand, endothelial retraction did not occur following tumor cell attachment. Instead the attached tumor cell emboli became encompassed by endothelial cells, outgrowing from the intact endothelial lining of the arteriole. Owing to the proliferation of the tumor cells, tumor colonies encompassed by endothelial cells expanded within the lumen. When these intravascular growths completely filled the lumen, the tumor cells extravasated from the vessel only after the original endothelial layer became mechanically disrupted and the tumor cells thereby came into contact with the basement membrane.

Introduction

One of the earliest investigations of hematogenous metastasis formation was reported by Iwasaki in 1915 who concluded that arterioles were the main site of the extravasation. He also recognized that in some cases intravascular proliferation preceded extravasation [8, 25]. Warren, however, in 1936 [28], argued that such a transvascular proliferation of tumor cells through the walls of arterioles was rather improbable and favored capillaries as the site of extravasation. The next systematic study was conducted by Baserga *et al.* in 1955 [1]. Based on light microscopic studies, they reported that extravasation of tumor cells took place at the level of both arterioles and capillaries, although the latter seemed to be more frequent (66 per cent).

In more recent (post-1960) studies of hematogenous metastasis formation, attention was focused on the fate of tumor cells arrested in the capillaries. According to these studies [2–6, 9–11, 14–16, 19–22, 24, 26, 27, 29], extravasation of tumor cells from the capillaries can take place by tumor cells moving between endothelial cells

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[14, 22, 29], by destruction of the endothelial and basement membrane barriers [2, 4, 9–11, 16, 19, 21, 26, 27], and rarely, by mechanical disruption of the capillary walls caused by the intravascular proliferation of tumor cells [15, 16].

These previous studies have not completely addressed the question of endothelial integrity during and following tumor cell extravasation. This information is relevant to the question of whether vessels remain patent and functional during and after extravasation. We therefore followed the fate of the endothelial barrier during extravasation in the lung, following intravenous (i.v.) administration of tumor cells. We also directly compared the participation of arteriole versus capillary endothelium during the extravasation process.

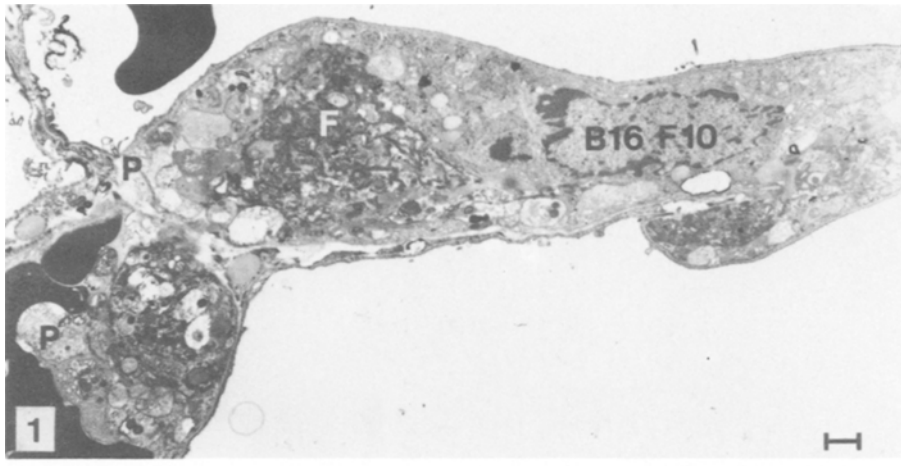
Materials and methods

B16-F10 mouse melanoma tumor cells were the kind gift of Dr I. J. Fidler. The tumor cells were cultured and harvested as described previously. A suspension of B16-F10 cells in culture Medium 199 at a concentration of 10^6 cells/ml in a volume of 0·2 ml (2×10^5 cells) was injected into a lateral tail vein of C57 B16 mice. Altogether, 40 mice received tumor cell suspension intravenously. Recipient mice were killed by nembutal anesthesia at various time points 1, 5, 10, 15 and 30 min, 1, 3, 6, 12, 14 and 48 h and 3, 4, 5, 6, 7, 10, 12, 14 and 21 days following tumor implantation successively. The lungs were fixed *in situ* injecting 2·5 per cent cold glutaraldehyde solutions into the trachea by a syringe until each lobe of the lung became evenly enlarged and pale. The lungs were then submerged in 2·5 per cent glutaraldehyde solution for 2 h. Following that, the upper lobes of the lungs were cut into 1/2–1 mm^3 pieces, which were postfixed for 2 h in 2 per cent OsO_4 prepared in buffer. Following dehydration, the tissue pieces were embedded in Poly-B Poly-science, cut by an LKB Ultratome and studied and photographed with a JEOL 100 B electron microscope. Two mice composed a representative group for each time point and six samples were studied from each mouse. Two semithin sections were cut routinely from each sample, stained with toluidine blue and studied by light microscopy for the presence of tumor cells in lung microvessels. Ultrathin sections, two to three from each lung, were prepared and photographed.

Results

Capillary extravasation

The earliest event in capillary extravasation was fibrin deposition on the free surface of tumor cells arrested on the luminal surface of the endothelium. Thrombocytes, most of which were degranulated, were attached to the fibrin-coated cell surface (figures 1 and 18 (a)). Such thrombi surrounding the tumor cells persisted from 5 to 30 min until 6–12 h. The aggregation of platelets then abated, followed by lysis of the deposited fibrin. In many cases,the tumor cells did not appear to attach tightly to the endothelial cells. A narrow slit was observed between the two cell types (figures 2 and 22 (a)). Mitotic tumor cells were not seen in the capillaries. Up to 6 h following the i.v. injection of the tumor cell suspension, the capillary endothelial lining appeared to remain intact. Although tumor cell processes protruding between the endothelial cells were never observed, after 6–12 h, tumor cells were seen in tight contact with the basement membrane, over a rather small area at first (figures 3 and 22 (b)), but the surface area of the contact increased with time (figures 4, 4 (a), 5, 5 (a) and 22 (c)). The tumor cells attached to the basement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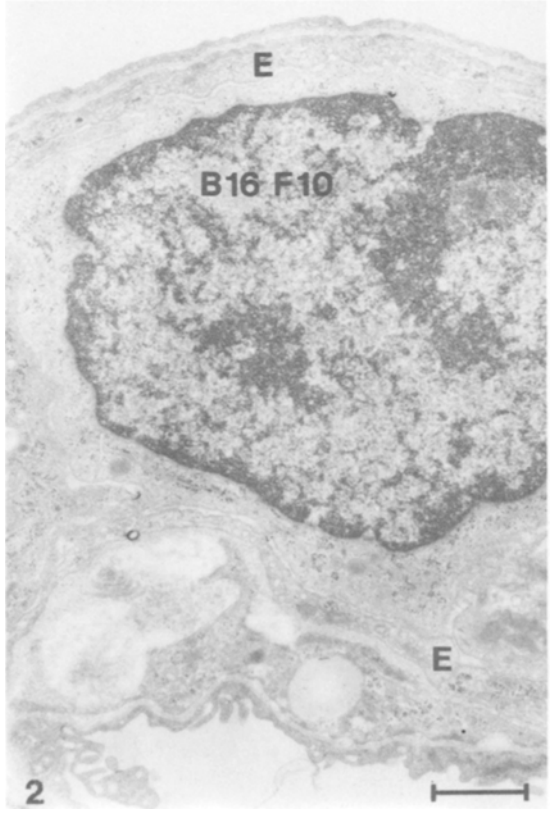
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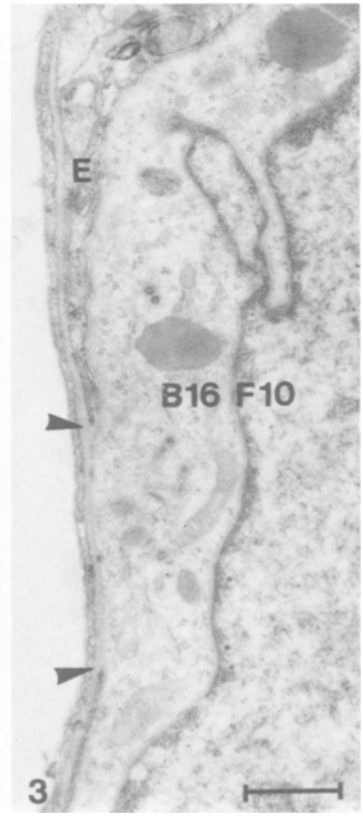
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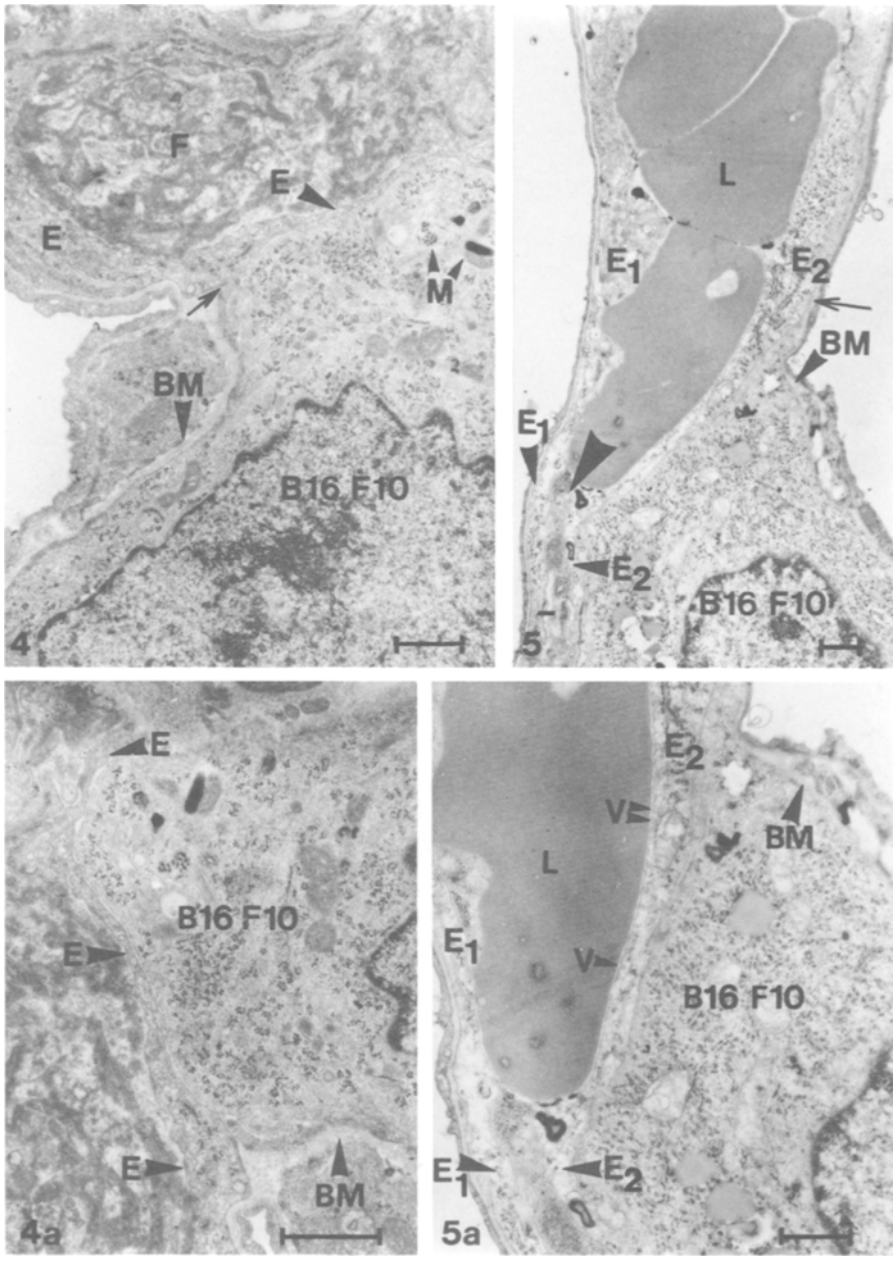
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Figure 1. A B16-F10 tumor cell in a lung capillary 3 h following i.v. inoculation. Non-polymerized fibrin (F) and platelets (P) are adherent to the tumor cell. Scale bar: 1 μm, 4250 ×.

Figure 2. At 6 h following i.v. inoculation the endothelium (E) adjacent to the attached tumor cell is still intact. A narrow slit can be observed between the tumor cell and the endothelium. Scale bar: 1 μm, 12 800 ×.

Figure 3. A tumor cell in a capillary 12 h after i.v. inoculation. Over a small surface area, the tumor cell is tightly adherent to the basement membrane (arrow heads). E: endothelium. Scale bar: 1 μm, 12 800 ×.

Figure 4. Tumor cell in a lung capillary 12 h after inoculation. The tumor cell is adherent to the basement membrane (BM), over a large surface area. An endothelial cell (E) has detached from the basement membrane (arrow) and moved over the tumor cell, separating it from the fibrin (F) deposit. M: melanosomes. Scale bar: 1 μm, 9600 ×.

Figure 4 (*a*). Part of figure 4 at a higher magnification. The tumor cell is partly covered by endothelial cell (E). Note the basement membrane (BM) contact. Scale bar: 1 μm, 13 500 ×.

Figure 5. B16-F10 tumor cell located between the endothelium (E) and the basement membrane (BM), 12 h following i.v. inoculation. The detached endothelium (arrow) (E_2) overlying the tumor cell comes into close contact with the endothelial lining (E_1) of the opposite side of the capillary (large arrow head). Scale bar: 1 μm, 5280 ×.

Figure 5 (*a*). Part of figure 5 at a higher magnification. The tumor cell is in a subendothelial (E_2) position in connection with the basement membrane (BM). L, capillary lumen; V, pinocytotic vesicles. Scale bar: 1 μm, 9600 ×.

Figure 6. Tumor cells (*) 24 h after i.v. inoculation between the endothelium (E) and basement membrane (in an 'extraluminal' position). One of them (double arrow head) is necrotic. Scale bar: 1 μm, 3200 ×.

Figure 7. Situation is the same as in figure 6. The tumor cell is covered by endothelial cell (E_1), which is in close contact with the endothelial cell of the vessel wall (E_2). L, vessel lumen. Scale bar: 1 μm, 15 000 ×.

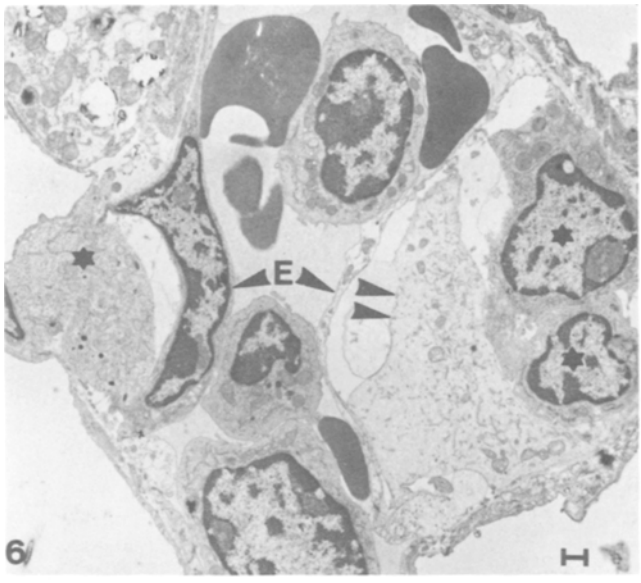
Figure 8. The same cell as in figure 7 in another sectioning plane. The tumor cell lying underneath the endothelium (E) is penetrating into the perivascular connective tissue via dehiscencies (arrow heads) in the basement membrane (BM). Scale bar: 1 μm, 8000 ×.

Figure 9. The tumor cell in figure 6 can be seen in another cutting plane. A cell process (Pr) of the tumor cell is piercing the basement membrane (BM) (arrow head). M, melanosome. Scale bar: 1 μm, 12 800 ×.

Figure 10. Tumor cells located in extravascular spaces 5 days following i.v. inoculation. The tumor cells are in direct contact with alveolar epithelial cells (Ep) and compress a type II pneumocyte (Pn). C: capillary. Scale bar: 10 μm, 2660 ×.

Figure 11. Light micrograph of semithin section prepared from lung 4 days after i.v. inoculation of B16-F10 cells. Note in the arteriole the tumor cell containing thrombus (TR) attached to the vessel wall. Scale bar: 100 μm.

Figure 12. Lung arteriole 2 weeks after i.v. inoculation of tumor cells. The lumen is completely filled by tumor tissue (T) with central necrosis. There is pronounced tumorous infiltration (TI) in the oedematous periarteriolar connective tissue. Scale bar: 100 μm.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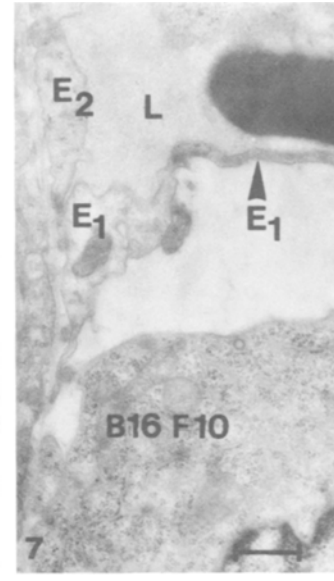
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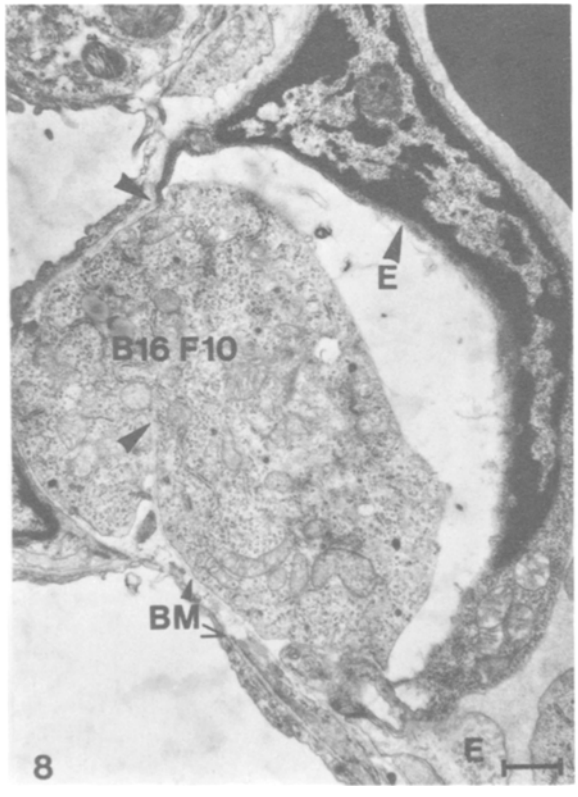
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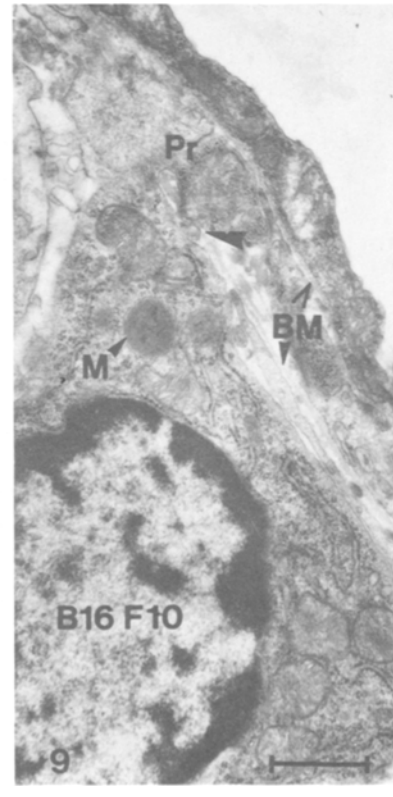
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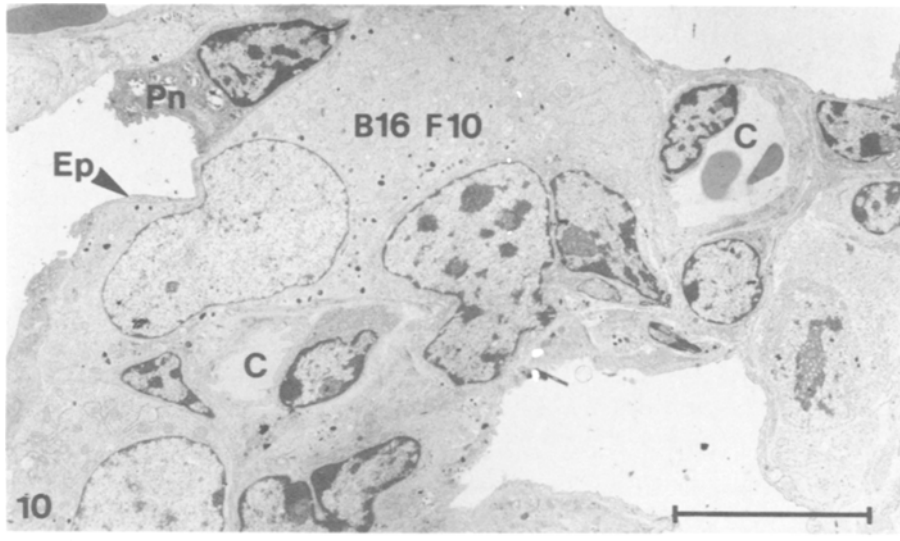
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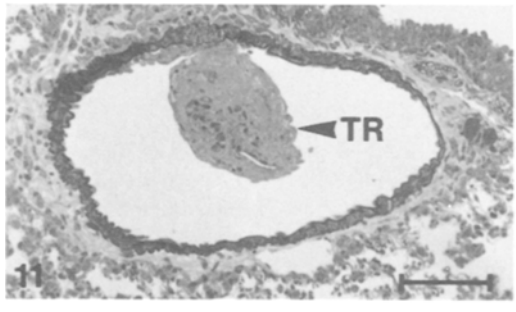
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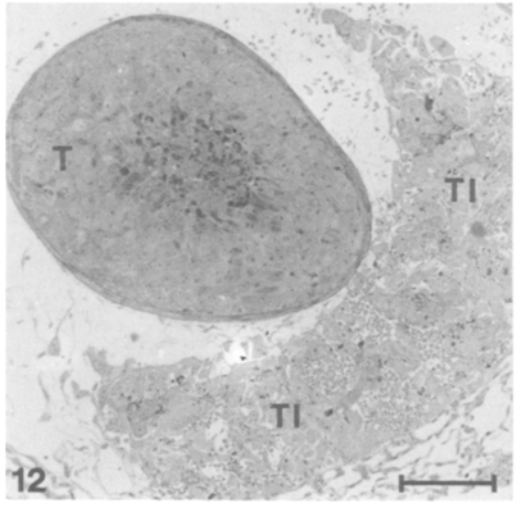
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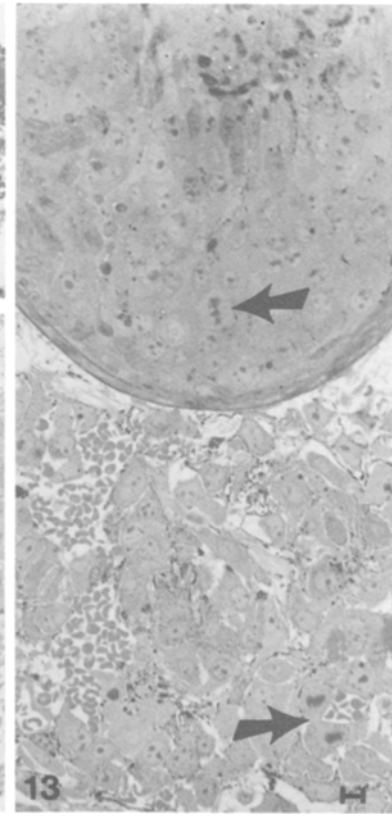
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Figure 13. Part of figure 12 at a higher magnification. Note that mitotic tumor cells in the vessel lumen and in the perivascular connective tissue (arrows). Scale bar: 10 μm.

Figure 14. A tumor cell containing thrombus completely endothelialized in a small venule 24 h following i.v. inoculation of tumor cells. E, endothelium; M, melanosome. Scale bar: 1 μm, 4250 ×.

Figure 14 (*a*). Enlarged part of figure 14. Intercellular junctions (arrow heads) can be seen between the endothelial cells covering the thrombus. Scale bar: 1 μm, 15 000 ×.

Figure 15. An endothelialized thrombus attaching to the vessel wall (arrow heads), 24 h after inoculation of tumor cells. Part of a tumor cell (*) and fibrin (F) can be seen in the thrombus. E, endothelium. Scale bar: 1 μm, 2100 ×.

Figure 15 (*a*). Part of figure 15 at a higher magnification. Endothelial cell covering the thrombus in the vessel lumen. Note the presence of pinocytotic vesicles in the cytoplasm (arrow heads). L, vessel lumen. Scale bar: 0·1 μm, 22 500 ×.

Figure 15 (*b*). A portion of figure 15 at a higher magnification. The endothelium (E) covering the thrombus extends out from the endothelial lining of the vessel wall (arrow heads). The apical surface of the endothelium is facing (in direct contact) the thrombus. Scale bar: 1 μm, 6400 ×.

Figure 16. A group of tumor cells completely surrounded by an endothelial covering (E) in the lumen of an arteriole, 2 weeks after inoculation of tumor cells. Scale bar: 10 μm, 1060 ×.

Figure 17. A group of tumor cells within an arteriole 2 weeks after i.v. inoculation of the tumor cell. The tumor cells are nearly completely covered by endothelial cells (E). The endothelial lining of the arteriole being in contact with the tumor colony base is well preserved (arrow). SM: smooth muscle cell, EI: elastica interna, EE: elastica externa. Scale bar: 10 μm, 1600 ×.

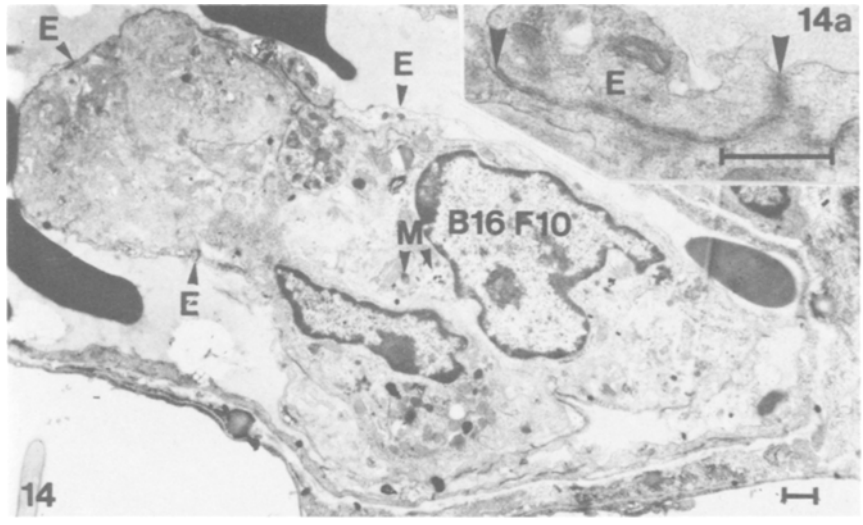
Figure 17 (*a*). Enlarged part of figure 17. The cell covering the surface of the intravascular tumorous growths (T) contains pinocytotic vesicles (arrow heads). L, vessel lumen. Scale bar: 0·1 μm, 22 500 ×.

Figure 18. A portion of a tumor colony in an arteriole 2 weeks after i.v. inoculation. One of the tumor cells is penetrating into the vessel wall via the natural slit (arrow heads) of the elastica interna (EI). M, melanosomes. Scale bar: 1 μm, 9600 ×.

Figure 19. Cross section of tumor colony in an arteriole 2 weeks after i.v. inoculation of tumor cells. On the luminal side of the arteriole (upper part of the micrograph) densely packed tumor cells can be seen. Tumor cells (*) are present also in the arteriole wall between the lamina elastica interna (EI) and externa (EE). Scale bar: 1 μm, 1500 ×.

Figure 20. Part of a tumor cell between the lamina elastica interna (EI) and externa (EE). M: melanosomes. Scale bar: 1 μm, 6000 ×.

Figure 21. Portion of an arteriole completely filled with tumor tissue 2 weeks after i.v. inoculation of tumor cells. The expansive growth of the tumor cells have caused a rupture of the elastica externa (EE) and the tumor cells proliferate into the perivascular connective tissue. Scale bar: 10 μm, 2100 ×.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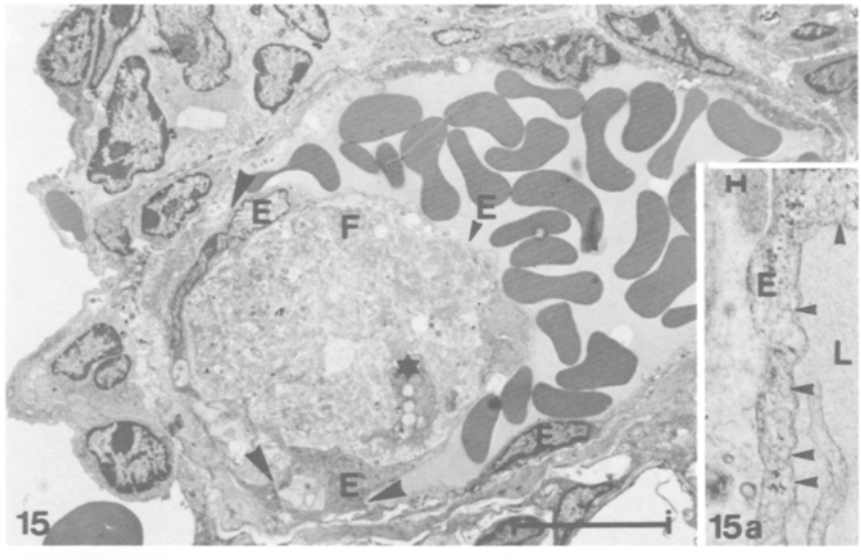
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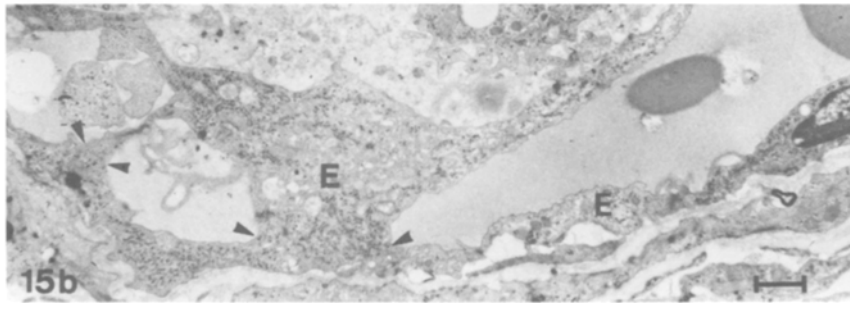
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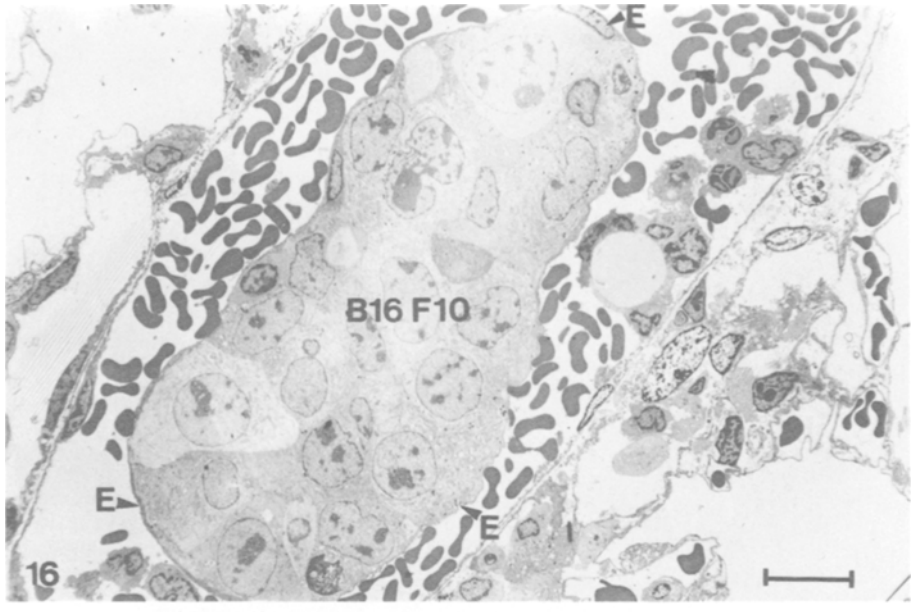
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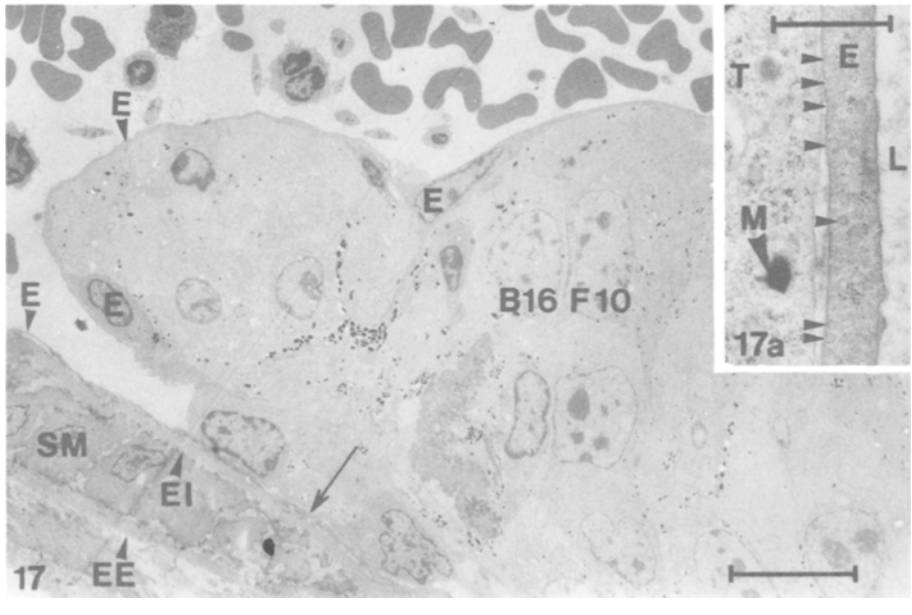
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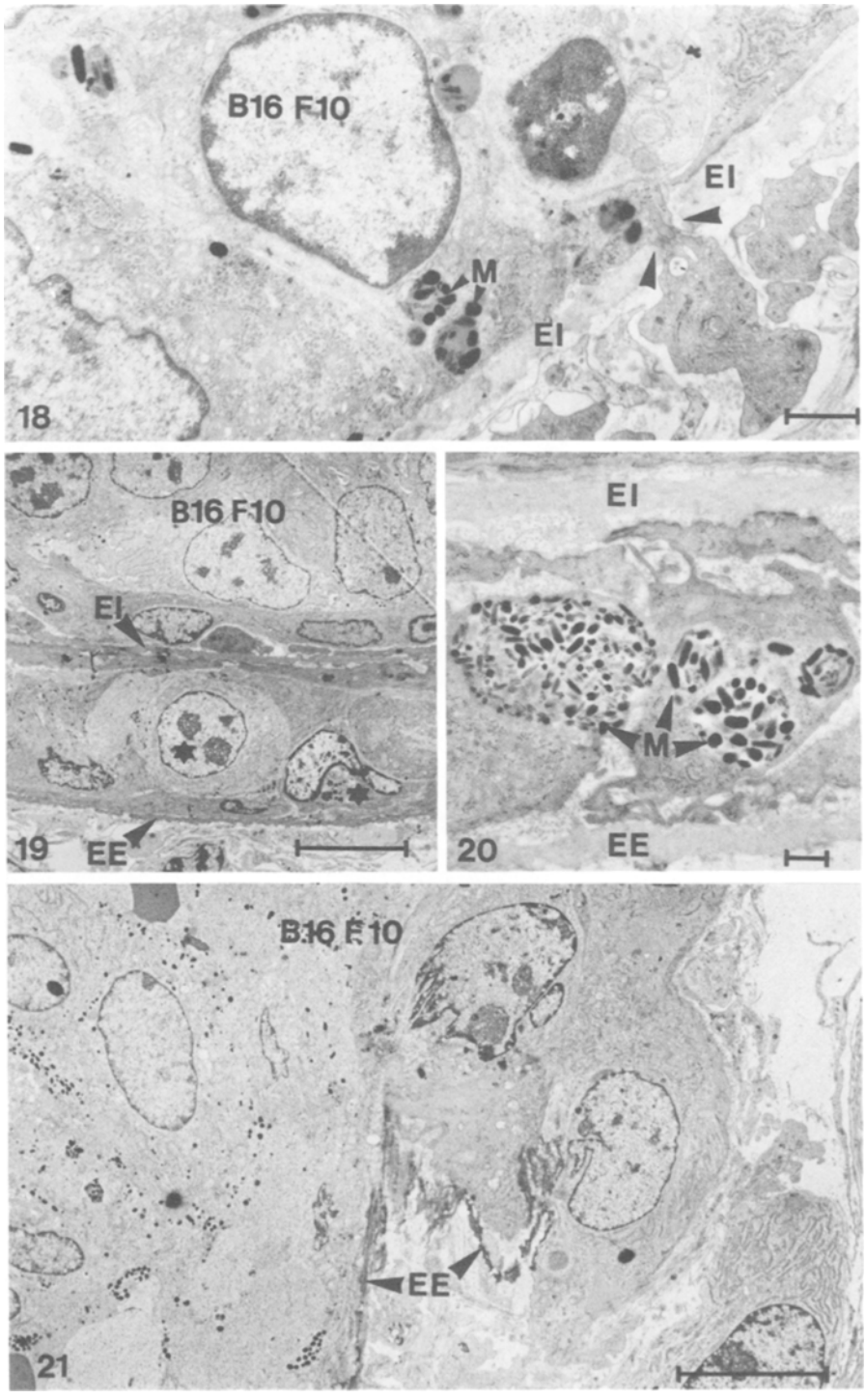
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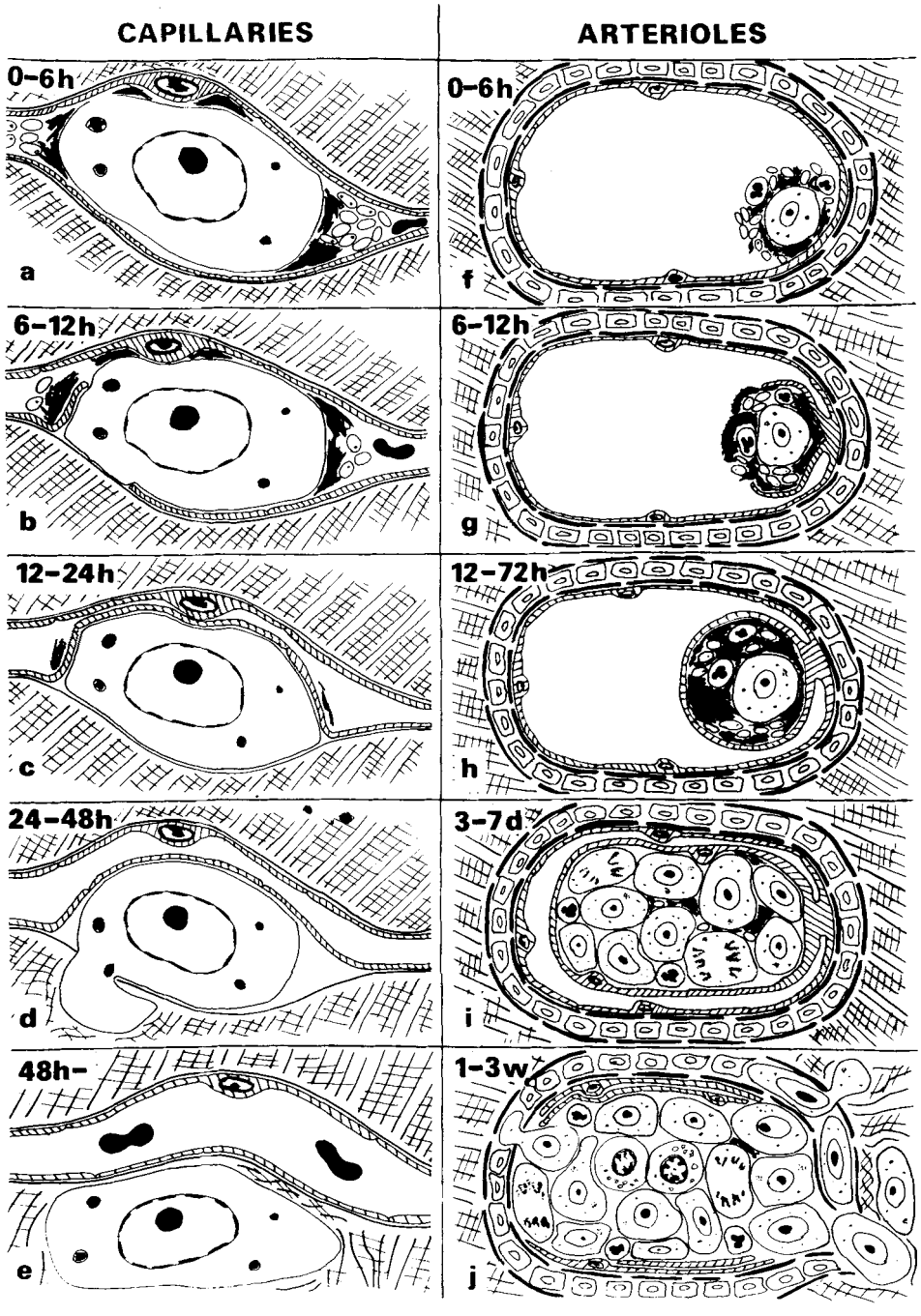
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Figure 22.

membrane were soon covered by a continuous endothelial layer, lacking a basement membrane (figures 4, 4(a), 5, 5(a), 6, 7, 8 and 22(c)). The endothelial cells were identified based on their ultrastructural features. These features were the following: absence of melanosomes or premelanosomes, presence of intercellular junctions, presence of coated pits and vesicles and presence in serial sections of Weibel–Palade bodies. Within the next 24 h, the tumor cells (lying between the endothelial lining and the basement membrane of the capillaries) induced focal basement membrane lysis by or around their protruding cell processes and thereby moved to a complete extravascular position in the connective tissue matrix surrounding the involved capillaries (figures 6, 8, 9 and 22(d)). In this process, amoeboid locomotion of tumor cells may have a role (figure 8). At 48 h following the i.v. injection, no tumor cells were seen in the capillaries. All of them were in an extravascular position. Within the next 2–3 days, with the appearance of mitotic figures, the proliferation of these extravascular tumor cells began and within 4–5 days after extravasation micrometastases consisting of 2–8 cell colonies could be seen in the pericapillary connective tissue (figures 10 and 22(e)).

Arteriole extravasation

In the case of the tumor thrombi forming in the arterioles and larger venules with a diameter of from 50 to 300 μm, a completely different series of events took place. The thrombi consisted of tumor cells, fibrin and platelets. Within 6–12 h following the i.v. injection of the tumor cells, the amount of the aggregated platelets in the thrombi decreased and some of the thrombi were dissolved. At the same time, the endothelial cells in contact with the persisting thrombi quickly covered the surface of these thrombi, first by extending cell processes and later by proliferation resulting in the formation of a continuous endothelial covering lacking a basement membrane (figures 14, 15, 15(b), 22(g) and 22(h)). The newly formed endothelial covering remained as a monolayer and in continuous connection with the endothelial lining of the involved vessel at one or more points (figures 15(b) and 22(h)). The endothelial nature of the covering cells was proven by definitive ultrastructural features mentioned above (figures 14(a), 15(a) and 17(a)).

Beneath the thrombi and in the local neighbourhood, the endothelial lining of the involved vessel proved to be completely intact. In the majority of such thrombi

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Figure 22. Schematic representation of the endothelialization and extravasation of B16-F10 cells inthe lung capillaries versus arterioles. Capillaries: (a) A tumor cell arrested within the capillary lumen is attached to the endothelial surface. The surface of the tumor cell facing the lumen is coated with fibrin and platelets. (b) The tumor cells has stimulated a retraction of the endothelium and has attached to the exposed basement membrane. (c) The endothelial layer has extended to cover the tumor cell which now occupies a position between the endothelium and the basement membrane. (d) Local dissolution of the basement membrane occurs followed by the protrusion of a tumor cell pseudopodia. (e) The tumor cell has completed the extravasation to occupy an interstitial location.

Arterioles: (f) A tumor cell coated with fibrin and platelets attaches to the intact endothelial surface. (g) No endothilial retraction occurs. An intact endothelial layer devoid of a basement membrane begins to extend over the tumor cell. (h) The endothelium has completely encompassed the tumor cell. (i) Tumor cell proliferation expands the colony, but the endothelial surface layer remains intact. (j) Once the tumor colony fills the lumen, the previously intact endothelium is broken exposing the basement membrane. Extravasation then occurs as shown in (c)–(e) above.

covered by an apparently continuous endothelial lining, tumor cells could be demonstrated. However, in some thrombi, the presence of viable tumor cells could not be identified even by studying serial sections. The thrombi without viable tumor cells contained cell debris and leukocytes in addition to fibrin and platelets. From the third day on, the proliferation of the tumor cells was noted (by the appearance of mitotic figures) and the size of the thrombi started to increase (figures 11, 12, 13 and 22 (i)). In parallel, the endothelial cells covering the surface of the thrombi also started to proliferate, resulting in maintenance of the continuous endothelial covering of the enlarging thrombi (figures 16, 17 and 22 (i)).

Owing to the proliferation of the tumor cells in the thrombi (figures 11, 12, 13 and 22 (i)), tumor colonies of different size bulged into the lumen of the involved vessels, until they filled the lumen completely (figures 12, 13 and 22 (j)). This process could last up to two weeks in the case of the larger arterioles or small arteries. In the meantime, in the center of these bulding tumor cell growths, necrosis took place (figures 12 and 13). When these proliferating tumor colonies filled the vessel completely, the continuity of both the endothelial lining of the vessel and that of endothelial covering of the tumor thrombi was noted to break at several points. The tumor cells then came into contact with the denuded basement membrane and even with the elastic interna of the involved vessel (figures 18 and 19). Some of the tumor cells penetrated quasi per diapedesim via the natural slits of the elastica interna (figures 18 and 22 (j)). They penetrated among the smooth muscle cells located between the elastica interna and externa (figures 19, 20 and 22 (j)), then penetrated further on invading the perivascular tissue. Here, further proliferation indicated by mitotic figures, lead to metastatis formation. In some cases, the tumor cells proliferating within the lumen disrupted the weakened walls of the vessels (figures 21 and 22 (j)).

Discussion

We have studied the process of extravasation of tumor cells from the capillaries and arterioles in the lung. A striking difference in pattern for arterioles versus capillaries was observed. In the capillaries, a partial retraction of the endothelial cells took place, following the attachment of tumor cells. In this way, the tumor cells came into contact both with the basement membrane of the involved capillaries and with the basolateral surface of the retracted endothelial cells. The phenomenon of the partial retraction of the endothelial cells has been observed in an *in vitro* system using the same B16 melanoma cell line [12, 17, 18]. In the arterioles, on the other hand, we observed no retraction of endothelial cells at the attachment sites of the tumor cells. The tumor cells come into contact only with the luminal surface of the endothelial cells and the tumor cell emboli became quickly covered by endothelial cells originating from the endothelial lining of the arteriole.

No mitotic tumor cells were seen in the capillaries, while in the arterioles the arrested tumor cells proliferated and formed colonies bulging into the lumen of the involved arterioles, encompassed by endothelial cells.

The extravasation through the capillary walls took place within 48 hours, while the process of the estravasation via the walls of arterioles lasted at least two weeks. However, in both sites of extravasation, the arrested tumor cells became covered by an endothelial layer prior to invasion of the basement membrane.

Groups of arrested tumor cells bulging into the vessel lumen were observed by Iwasaki [8] in human material as early as 1915. According to Iwasaki, the endothelial

covering provided a kind of protection for the encompassed tumor cells compared to those which happened not to be encapsulated by endothelium. According to Wood's [29] observation, the endothelial covering of the arrested tumor cells occurred only if the extravasation of tumor cells did not take place within the first 24 hours. Ludatscher [14], studying the spontaneous metastasis formation of experimentally induced hepatomas, also observed the endothelialization of tumor cell thrombi. Among the layers of the multilayered endothelial covering of the emboli or thrombi, even the presence of basement membrane was observed [14] at some places, indicating that the basolateral surface of the covering endothelial cells is facing the thrombi. However, extravasation of the tumor cells from these laminated thrombi or emboli were never seen. Ludatscher [14] concluded that extravasation took place only from so-called nude tumor emboli or thrombi which happened not to be surrounded by endothelial cells. In contrast to Ludatscher's observations, in our studies using the B16-F1O melanoma cells, the surface of the tumor cell thrombi or emboli was covered always only by one layer of endothelial cells, and no sign of basement membrane formation was seen, but extravasation from such encompassed thrombi did occur regularly.

Earlier, it was reported that in connection with the metastasis formation, phagocytosis of the fibrin surrounding the tumor cells took place [3, 19). In our case, the proliferation of endothelial cells over the tumor cell emboli is a prevailing process, in which the fibrin coating of the tumor cell emboli might have provided guidance to the endothelium. On the other hand, tumor cells can secrete factors which have a direct effect on endothelial cells (7). One observation which supports this suggestion is the occurrence of fibrin thrombi containing no viable tumor cells but covered by endothelial cells. The tumor cells might have been lysed in these thrombi.

Between 12 and 72 h following the arrest of tumor cells in arterioles, no proliferation was observed. During this period, the tumor cells were protected by the endothelial covering against the mechanical injuries and cellular host defenses [20]. In spite of this protective covering, many such thrombi were seen on the first day following the i.v. injection of tumor cells, compared to the number of intravascularly growing tumor cell colonies seen in the later phases. The proliferating tumor cells in the thrombi covered by endothelial cells could be stimulating, by mechanical or biochemical means, the proliferation of the covering endothelial cells. With the increasing pressure caused by the enlarging intravascular tumor cell colonies, the endothelial lining of the involved vessel suffer injury, possibly a kind of compression atrophy. Retraction of the endothelial cells took place at several points and the tumor cells obtain free acess to the denuded basement membrane. Following the effect of this close contact with the basement membrane, the process of extravasation commences. We can conclude this because, while the proliferating tumor cells were covered by endothelial cells, no signs of invasion of the vascular wall were observed.

Following the lysis or destruction of the basement membrane, the tumor cells leave the arteriole vessels via diapedes through the natural slits in the lamina elastica interna. The later ensuing disruption of the elastica interna and externa appears mostly mechanical in nature, although participation of a proteolytic process cannot be excluded [13, 18, 23].

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