An Electron Microscopic Study of Pulmonary Tumor Emboli from Transplantable Morris Hepatoma 5123

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SUMMARY

Lungs of 20 Buffalo rats with Morris hepatoma 5123 transplanted to the thigh were examined 5 to 12 weeks after transplantation. Metastases in the lungs as well as the thigh tumor were examined by light and electron microscopy. Pulmonary tumor emboli first were observed 6 weeks after transplantation. Metastases and tumor emboli were evident in lungs of all animals at 7 and 8 weeks. Naked tumor emboli without an endothelial covering were seen in intimate contact with the vascular lumen, and apparently sending out pseudopods in preparation for penetration between the endothelial vascular lining. Elsewhere they were seen immediately behind leukocytes that had already separated the vascular endothelial cells. It is postulated that embolic tumor cells penetrate vessels in the same manner as leukocytes. Some arrested endothelialized emboli were so altered as to appear nonviable, whereas others were well preserved. The cytologic details of the tumor, whether in emboli, metastases, or in the thigh transplant were reminiscent of normal liver.

INTRODUCTION

The slow-growing transplantable Morris 5123 hepatoma (6, 11) differs only minimally from normal liver either morphologically or enzymatically (19, 24) although it has a high incidence of pulmonary metastases (19, 22). Mechanisms involved in metastasis were studied extensively in 1903 by Schmidt (21), and later by Iwasaki (12), who traced the fate of circulating tumor cells in both human and experimental tumors. Iwasaki (12) described encapsulation of the sarcoma cells in a thrombotic mass in pulmonary vessels within ten minutes after injection of a suspension of Jensen’s rat sarcoma into the femoral veins of rats. He noted that endothelial cells from the blood vessel wall proliferated to cover the thrombi. Among others who have contributed to the understanding of circulating neoplastic cells and their resultant metastases were: Willis (26); Baserga et al. (2); Baserga et al. (3); Greene and Harvey (9); Zeidman and Buss (29); Wood (27); Wood et al. (28); Griffith and Salsbury (10); and Kinsey et al. (13). In spite of the importance of tumor emboli in the biology of tumor spread, little attention has been directed toward either their ultrastructure or their mode of implantation, however, the ultrastructure of some other hepatomas has been reported in detail (1, 7, 14, 28). The purpose of this investigation is to describe the ultrastructure of pulmonary hepatoma emboli.

MATERIALS AND METHODS

The Morris hepatoma 5123 originally was obtained from a hepatoma induced by feeding Buffalo rats a diet containing 0.042% N-(2-fluorenyl)phthalamic acid for a period of 10 months (20). Our line was obtained in 1961 from Dr. Harold Morris and has been maintained by subcutaneous transplantation into the thigh of adult Buffalo rats. The experimental animals, from different transplant generations, were killed by decapitation 5 to 3 months after transplantation. The trachea was immediately injected with Dalton’s chrome-osmium fixative. Under a dissecting microscope, small pieces then were removed from subpleural nodules in each lung and fixed for 1.5 hours. Small blocks of the transplanted tumor in the thigh also were fixed in chrome-osmium. After ethanol dehydration, blocks were embedded in Dow epoxy resin (15). Sections approximately 1 micron thick were cut on a Porter-Blum microtome for examination with the phase microscope. Serial phase sections 0.5 micron thick were used to follow emboli. Some sections were stained with methylene blue or with hematoxylin-phloxine. Thin sections were mounted on uncoated copper grids and stained with lead acetate or uranyl acetate prior to examination in an RCA EMU-2B or 3C electron microscope.

RESULTS

Light and Phase Microscopy

The occurrence of pulmonary tumor emboli (Figs. 1, 2) and metastases of Morris hepatoma 5123 is shown in Table 1. Hepatoma first was evident in the lung as embolic nodules within small blood vessels 6 weeks after transplantation. At this time emboli were sparse and small, disappearing after only a few 1-micron sections. By 7 to 8 weeks, intravascular tumor was more common and small metastatic nodules were sometimes present around blood vessels. By 10 to 12 weeks, pulmonary metastases were large compact nodules but vascular emboli no longer could be identified as such. Tumor emboli frequently occurred in arteries near a bronchus, as well as in the smaller blood vessels. Morphologically, the embolic cells were indistinguishable from those of the main tumor. A thin layer of flattened cells often enveloped the embolus.

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These cells had abundant ergastoplasm in comparison with regions, tumor was in intimate contact with endothelium. Much as they do in the normal space of Disse. In other spaces between the tumor cells and their endothelial coat (Fig. 1). Here there is continuity between the intima and the covering of the embolus with extension of an intimal endothelial cell along the embolus. This cell interdigitated with adjoining ones to form a coat around the tumor embolus. Contiguity between the vessel lining and cells enclosing embolus, however, was common (Figs. 14, 15), the plasma membranes of adjacent cells either touching each other or being separated by only a narrow space (Fig. 14). Occasionally, tumor cytoplasm even indented that of the endothelial cell (Fig. 15).

Electron Microscopy

Three distinct types of emboli were present: (a) an embolus without an endothelial covering; (b) an endothelial covered embolus usually without apparent attachment to the intima; and (c) an arrested inactive embolus covered by many layers of proliferated endothelial cells and with obvious intimal attachment.

**Emboli without an Endothelial Covering.** These rare emboli were composed of active neoplastic cells, some even in mitosis (Fig. 3), and usually filled the lumen. Where the neoplastic cells were separated from the vascular wall, their protoplasmic membranes were intact with short microvilli (Fig. 4). Serial sections, however, would be necessary for complete clarification of topographic relations between such emboli and the vessel. Tumor appears able to penetrate between endothelial cells (Fig. 5) as well as escape through the openings between endothelial cells made by leukocytes (Fig. 6), suggesting that these emboli are the site of formation of the metastatic nodules as well as sometimes undergoing dissolution or becoming enclosed by endothelium.

**Relation of Endothelial Covering to Emboli.** Emboli may be enclosed by a single layer (Figs. 7, 8, 14) or a multilayered covering of endothelial cells (Figs. 9, 10). The external layer of this capsule was composed of typical endothelial cells with elongated nuclei, prominent ergastoplasm, numerous pinocytotic vesicles, and overlapping cell junctions (Fig. 9). In contrast the internal layers (Fig. 10) may be extremely flattened. Basement membrane surrounds the inner endothelial cells about such emboli (Fig. 9) filling the spaces between layers. Often short microvilli of the tumor cytoplasm projected into the space between the tumor cells and their endothelial coat (Fig. 8), much as they do in the normal space of Disse. In other regions, tumor was in intimate contact with endothelium.

Some cells in arrested endothelialized emboli had long processes that extended toward the center of the nodule (Fig. 14). These cells had abundant ergastoplasm in comparison with either endothelial or tumor cells. Their exact identity is in question, but their basement membrane covering and the desmosomes at their cell junctions suggested altered endothelial cells rather than fibroblasts. Serial sections showed that emboli often were split into two or more pieces by proliferating endothelium (Figs. 7, 12) to form new vascular channels within the embolus. Such channels may be equated with the newly formed capillaries seen by light microscopy during recanalization of tumor thrombi.

**Relation of Emboli to Vascular Wall and to Blood Stream.** Emboli were frequent in vessels with a lumen of 50 to 250 microns and either touched the vessel wall or were separated by a channel of variable width. Contrary to expectations based upon light microscopy, thrombi per se seldom were encountered although this probably was only a reflection of the evanescent nature of such thrombi (27). Rarely clusters of degenerating platelets were interposed between endothelialized emboli and the vascular wall (Fig. 13).

The endothelial origin of cells enclosing emboli could on occasion be demonstrated by serial sections. Fig. 11 shows such an embolic nodule within the lumen of the 30-micron intrapulmonary arteriole shown in Fig. 1. Here there is continuity between the intima and the covering of the embolus with extension of an intimal endothelial cell along the embolus. This cell interdigitated with adjoining ones to form a coat around the tumor embolus. Contiguity between the vessel lining and cells enclosing emboli, however, was common (Figs. 14, 15), the plasma membranes of adjacent cells either touching each other or being separated by only a narrow space (Fig. 14). Occasionally, tumor cytoplasm even indented that of the endothelial cell (Fig. 15).

### Ultrastructural Aspects of Hepatoma Emboli

Intravascular tumor closely resembled transplanted tumor in the thigh as well as that of already formed pulmonary metastases. Hepatoma cells were of two types, pale and dark (Fig. 12). Their nuclei usually were ovoid but some were deeply infolded and occasionally even contained intranuclear inclusions of lipid or invaginations of cytoplasm (Fig. 14). Pale hepatoma cells had abundant smooth endoplasmic reticulum with scant ribosomes whereas rough cisternae, free ribosomes, and even polyribosomes forming rosettes or spirals were conspicuous in dark cells. Mitochondria of intravascular hepatoma cells were similar in shape and internal structure to those in the pulmonary metastases and in the thigh transplant. Although mitochondrial peculiarities occurred in hepatoma cells, it should be stressed that for the most part the mitochondria and their relation to rough-surfaced endoplasmic reticulum were remarkably similar to those of normal liver. Degenerative changes, and especially myelin figures, were common in hepatoma cells. Often the myelin figures enclosed mitochondria, lipid droplets, or other cytoplasmic material. Glycogen also was present. There was no difference in distribution or morphology of bile canaliculi in emboli as compared to metastases or thigh tumor. Desmosomes occurred at bile canaliculi, between neighboring cells, and even between a pulmonary alveolar cell and a hepatoma cell.

Degeneration of cytoplasmic organelles was prominent in endothelialized emboli. Dilation of ergastoplasmic saes in marginal tumor cells, swollen mitochondria with short retracted cristae, or even total degradation of centrally located cells occurred. These changes were in large emboli, whereas in small vessels degeneration of emboli was rare except in uncovered extensions from a large embolus into the lumen of ramifying small vessels (Fig. 16). In some arrested endothelialized emboli, regressive changes were complete (Fig. 11). The center of such

<table>
<thead>
<tr>
<th>No. of animals</th>
<th>Weeks after transplantation</th>
<th>Results</th>
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<tr>
<td>3</td>
<td>5</td>
<td>No evidence of hepatoma in lungs</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>Pulmonary hepatoma emboli in 3 of 5 animals</td>
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<tr>
<td>3</td>
<td>7</td>
<td>Pulmonary metastases and emboli evident in all animals</td>
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<td>4</td>
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<td>Pulmonary metastases and emboli evident in all animals</td>
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<td>2</td>
<td>10</td>
<td>All have pulmonary metastases</td>
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<tr>
<td>3</td>
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<td>All have pulmonary metastases</td>
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**TABLE 1**

Occurrence of Pulmonary Tumor Emboli and Metastases of Morris Hepatoma 5123
DISCUSSION

Whereas most experiments have dealt with artificially produced emboli and metastases (2, 3, 13, 25, 27), this study is concerned with spontaneous hemogenous tumor emboli. In the experiments of Wood (27) and of Baserga et al. (3), the fate of injected tumor emboli was followed closely. Baserga et al. found that only 1 to 8 per thousand injected tumor cells survived and proliferated at the site of arrest. Wood, who followed the fate of arterially injected stained tumor cells by serial cinephotomicrography in rabbit ear chambers, showed the sequential interaction of cancer cells and capillary wall in the living animal. He noted cancer cells to adhere to the capillary endothelium within minutes, and soon thereafter thrombi formed about them only to break up and reform later. Within several hours or even sooner, leukocytes accumulated, penetrated the endothelium, and tumor cells were seen to migrate through the same endothelial defects. This is most interesting in view of recent experimental work on the mode of migration of leukocytes in which Marchesi and Florey (17, 18) have shown that they crawl between endothelial cells. In Fig. 6, a leukocyte immediately in front of a tumor cell has caused separation of the endothelial cells of the capillary. Wood (27) believed emigration of tumor cells was permitted by vascular damage from a histamine-like substance released by breakdown of tumor cell protein, a theory in keeping with the recent work of Majno and Palade (16), who have shown that histamine, as well as serotonin, increases permeability in venules by separation of endothelial cells at their sites of junction, exactly the site of penetration by emigrating leukocytes. Baserga et al. (3) reviewed the work of Flaks and Grynkraut (8) and of Cirio and Balestra (5), who reported an increased incidence of metastases after whole body irradiation. Baserga attributed this to inhibition of the reticuloendothelial system. Alternatively, we would suggest that irradiation caused an increased leukocytic emigration, as was observed by Luse (manuscript in preparation) in the nervous system after whole-body irradiation with high-energy protons. Baserga et al. (3) also considered exhaustion of the reticuloendothelial system to play a role in the increase in metastases after injection of Thorotrast. This too could be explained on the basis of increased capillary permeability at the site of attachment of endothelial cell to endothelial cell. Casley-Smith (4) has shown Thorotrast, ferritin, India ink, and chylomicrons to cross lymphatic endothelium between cells in a manner analogous to that of the leukocyte, as well as by pinocytosis, or through fenestra.

In our electron micrographs, no tumor cells were midway in the actual process of escape through the vessel wall, although early stages were observed (Figs. 5, 6). In all endothelialized emboli, tumor cells were completely covered, which suggests that invasion occurs only from a naked portion of an embolus. However, additional ultrastructural studies of uncovered emboli will be required before this point is established. Numerous microvilli usually occurred on the surfaces of tumor cells whether they faced their endothelial cover or the vascular lumen. Surface plasma membranes of hepatoma cells of some emboli were smooth, the microvilli being absent where tumor cells contacted the vascular intima. Most emboli observed in this investigation were endothelialized by either a single or layered covering. Many were close to the vessel wall and by phase microscopy appeared attached to the wall. Electron micrographs of the emboli revealed either a narrow rim of empty lumen or only close contiguity between the endothelial cover of the embolus and intima (Fig. 14). In our material, arrested emboli with obvious attachment to the intima were rare. This fact may be explained in part by inherent technical difficulties in obtaining the point where endothelialization of an embolus begins. Certainly such a point exists somewhere on the embolus, as demonstrated in the one shown in Fig. 11.

Within 24 hours after injection, Wood (27) observed that tumor emboli had become sheathed by endothelial cells thus preventing, at least temporarily, invasion. This ensheathment perhaps also protects embolic tumor cells from the disintegrative effects of blood plasma. As Warren and Gates (25) have pointed out, tumor emboli may become organized either to disappear or remain latent only to produce metastases at a later time. Emboli that persist are covered by a cellular coating. Iwasaki (12), Baserga et al. (3), Willis (26), and Wood (27) have all agreed that most arrested tumor cells fail to develop into metastases. The relative importance of such a cover in inhibition of growth in comparison to the direct lytic effect of blood on naked tumor cells is unknown. It is highly likely that the largest number of cells are destroyed prior to this stage and that endothelialization acts as a protective mechanism to the tumor cell, not to the organism. Cells so covered may remain viable and under proper stimulus may escape from their cocoon of endothelium. Endothelialized emboli are composed of intact tumor cells interspersed among degenerating cells. In no instance could we see in such emboli extension or penetration of neoplastic cells toward the vascular wall through its well-established endothelial capsule, although after 7 or 8 weeks from transplantation, some pulmonary emboli were associated with perivascular metastases.

REFERENCES

lial cells. The elastic lamella of the vessel is evident at EL. X 15,000.

mitosis. X 12,000.
The internal elastic membrane is well preserved. X 2,000.

space (arrows). The embolus has been split by proliferating endothelium to form a new vascular channel (L) within the tumor. The tumor cell at the top portion of the figure has microvilli on its luminal surface. X 20,000.

An overlapping cell junction is indicated at the arrow. X 15,000.

cell facing the endothelium (arrow) is identical in appearance with that of a normal liver cell at the space of Disse. X 12,000.

cells. The tumor cell at the bottom of the figure is covered by endothelium (TE) separated from the cell by a small subendothelial space (arrows). The characteristic microvilli on the surface of the hepatoma cell is for the most part smooth. The endothelial lining (VE) of the blood vessel contains numerous vesicles. An internal elastic membrane separates the endothelial from the smooth muscle of the vessel wall. X 16,000.

Fig. 5. Electron micrograph of another active tumor embolus that appears to be sending a pseudopod (PS) between vascular endothelium and tumor that appears to be adherent to the vascular endothelium. Seven weeks post-transplantation. X 600.

Fig. 6. Two pulmonary alveoli (AL) are evident. A small blood vessel is completely occluded by a tumor embolus. A leukocyte (LC), between tumor and vessel wall, has resulted in separation of the capillary endothelium (arrows) just prior to exiting from the vessel. X 7,500.

Fig. 7. Endothelialized embolus. Tumor cells are readily identified by their mitochondria, which are characteristic of normal liver cells. The tumor cell at the bottom of the figure is covered by endothelium (TE) separated from the cell by a small subendothelial space (arrows). The embolus has been split by proliferating endothelium to form a new vascular channel (L) within the tumor. The tumor cells at the top portion of the figure have microvilli on their luminal surface. X 20,000.

Fig. 8. Tumor embolus covered by a single layer of endothelium (TE). The characteristic microvilli on the surface of the hepatoma cell facing the endothelium (arrow) is identical in appearance with that of a normal liver cell at the space of Disse. X 12,000.

Fig. 9. Tumor embolus covered by 2 layers of endothelium, the inner layer labeled (IL), separated by basement membrane (BM). An overlapping cell junction is indicated at the arrow. X 15,000.

Fig. 10. Electron micrograph with multiple, closely packed, flattened layers of endothelium (TE) separated from the vascular endothelium (VE) by a narrow space (L). The elastic layer of the vessel is labeled EL. X 12,000.

Fig. 11. Arrested endothelialized tumor embolus (AE) with attachment of tumor endothelium to vascular endothelium at arrow. The vessel lumen is evident at L. This is the same arteriole as shown in Fig. 1, and here at the level of electron microscopy appears not to be viable. ENDO COV, endothelial covering X 8,000.

Fig. 12. Low magnification to demonstrate splitting of tumor emboli by their endothelial covering in such a way as to form new extensions of the vascular lumen (L). Dark and light tumor cells are present. X 4,500.
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Fig. 13. Tumor embolus with multiple layers of endothelium covering it (ENDO COV), separated from the vascular endothelium (VASC ENDO) only by platelets (arrows). Elastic lamella of the vessel is seen at EL. × 10,000.
FIG. 14. Endothelialized tumor embolus. Only a tiny space exists between tumor and vascular endothelium. Vacuoles due to invaginations of cytoplasm are evident in tumor nucleus (arrows). × 8,500.

FIG. 15. Electron micrograph of another tumor embolus where the neoplastic cell indents the endothelium (arrows). × 16,000.
FIG. 16. Low magnification of pulmonary vessel occluded by tumor embolus that extends out into capillaries. The embolus in the capillaries (arrows) is not covered by endothelium and is undergoing lysis. × 3,000.
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