**Summary**

Tumor emboli were produced in lungs of Sprague-Dawley rats by i.v. injection of Walker 256 tumor cells into the tail vein. Tissues were examined by electron microscopy at periods from 30 sec to 72 hr after tumor injection. Two methods of conventional staining were used, in addition to immunoperoxidase techniques, with antifibrin antibodies produced in rabbits.

Tumor cells accompanied by a platelet mass were seen in pulmonary arterioles at the earliest time period (30 sec). By conventional staining, small amounts of fibrin were detected within the platelet clumps by 5 min after inoculation. Periodicity indicating stable fibrin was not seen by this technique until 15 to 45 min. When peroxidase-labeled antibody was applied to tissue, sections showed fibrin-positive material at 30 sec, and periodicity of fibrin was detected by 5 min. Fibrin reached a maximum by both techniques at about 1 hr and disappeared, along with the platelets, at about 9 hr. When fibrinolysin was injected prior to the tumor cell inoculation, platelets and fibrin were either absent or present only in traces, and no stable fibrin was detected. These observations show that fibrin occurs very early in small amounts in association with tumor cell emboli, and is removed while the cells are still intravascular.

**Introduction**

The role of fibrin in metastases continues to be a controversial subject in spite of the large numbers of studies that have been carried out with a variety of experimental models over many years. Some workers, including Warren and Gates (26), Baserga and Saffiotti (2), and Wood (27, 29), have described a fibrin thrombus in the early stages of tumor cell arrest, using light microscopic observations. Other investigators, including Ludatscher et al. (17), Coltmore and Carter (6), Locker et al. (16) and, most recently, Sindelar et al. (21), in recording the initial events in tumor cell embolization, have not reported any fibrin in association with the arrested cells. In an earlier paper (13) a report from our laboratory described Walker tumor cell emboli in rat lungs and noted large platelet aggregations accompanying the tumor cells for about 8 hr but no polymerized fibrin was seen by light or electron microscopy, although it was detected immunologically in the emboli with the use of fluorescein isothiocyanate-tagged antifibrin antibodies. It was suggested that the immunologically positive material represented fibrin monomers or some form of unpolymerized fibrin. Subsequently, in an abstract (4), we reported the detection of fibrin by electron microscopy with peroxidase-labeled antibodies, nonpolymerized fibrin being seen by 30 sec and stable fibrin within 5 min after i.v. injection of the tumor cells.

The continuing uncertainty in the literature concerning the presence or absence of fibrin with arrested tumor cell emboli makes it imperative to describe fully our technical methods and observations in these experiments. They establish clearly that, at least with the tumor system used, fibrin formation is an early and either common or constant event in tumor cell embolization, arrest, and adherence to pulmonary vessels, and that it is a temporary and sometimes inconspicuous feature of the event that can be easily missed.

**Materials and Methods**

**Tumor System**

All of the studies described here were done with the Walker 256 tumor and young adult Sprague-Dawley rats, 250 to 275 g, as hosts. This is an allogeneic system, but the short duration of the experiments makes it unlikely that an immune response would complicate the picture to any extent unless the animals were preimmunized. A total of 190 rats were used in the studies, and for each technique and time interval studied, 5 rats were most often used. Tumor cell suspensions were prepared from extramuscular grafts by mincing the tumor in Hanks’ solution. Cell concentrations were adjusted to 15 x 10⁶ cells/ml, and 3 x 10⁶ cells were given in 0.2 ml. Injections were made into the tail vein, and rats were killed at intervals from 30 sec to 72 hr in these studies. Lung tissue fragments were fixed in 2.5% glutaraldehyde for ordinary staining procedures and in special fixative for immunoperoxidase techniques, as described below.

**Immunological Methods**

**Source of Antigen.** Early trials were made with rat fibrin prepared in our own laboratory. In the studies reported here, rat fibrinogen, Fraction I, was purchased from Pentex, Inc., Kanhabee, III. No appreciable difference was found in the results between our fibrin and commercial fibrinogen.
Antiserum. The antiserum was prepared by immunization of male Norway albino rabbits, as previously described (13). Antiserum obtained was characterized by double-gel diffusion, and after absorption with normal rabbit serum, a single line was obtained against fibrin and fibrinogen. This serum was used in an indirect method with enzyme-labeled antibody.

Enzyme-labeled Antibody. This was prepared by the method of Avrameas (1). Five mg of goat anti-rabbit y-globulin (Pentex); 12 mg of horseradish peroxidase well dissolved in 1 ml of 0.1 M phosphate buffer, pH 6.8; and 0.05 ml of 1% aqueous glutaraldehyde were added with stirring. After standing at room temperature for 2 hr, the enzyme mixture was dialyzed against 2 changes of 5 liters of phosphate-buffered saline (NaCl, 8 g/liter; Na2HPO4 anhydrous, 1.07 g/liter; and NaH2PO4·2H2O, 0.39 g/liter), overnight at 4°. The precipitate was removed by centrifugation at 20,000 rpm and the supernatant conjugate was stored at —20°. This conjugate was also tested in double-gel diffusion against rat y-globulin and gave a single wide precipitin line against rabbit anti-rat fibrinogen antibody.

Electron Microscope Methods

Three techniques were used: (a) Phosphotungstic acid staining of the dehydrated blocks, followed by staining of thin sections on the grid with 6% uranyl acetate and Reynolds' lead citrate; (b) simultaneous double staining of the uncut blocks by uranium nitrate and lead citrate, followed by cutting of thin sections and further staining of these with uranyl acetate and lead citrate; (c) immunoenzyme staining using the peroxidase-labeled antibody. For this purpose, a method modified from Pierce et al. (20) was used. Small pieces of rat lung (5 × 3 × 2 mm) were fixed in 1% formalin (freshly prepared from paraformaldehyde in 0.25 M sucrose and 0.2 M cacodylate buffer, pH 7.2 to 7.4) for 1 hr (15). The blocks were then washed in several changes of the sucrose-cacodylate buffer and were quick frozen in acetone-dry ice and sectioned in a cryostat at 20 to 30 μm. These thick cryostat sections were exposed to 1 ml of absorbed rabbit anti-rat fibrinogen antiserum, undiluted, overnight at 4°. After being washed for 8 hr in cacodylate buffer, they were then exposed to the peroxidase-labeled goat anti-rabbit y-globulin in 1/6 dilution with phosphate-buffered saline, total volume 1 ml, for 8 hr. After washing the sections with several changes of the cacodylate buffer, they were fixed in 2.5% glutaraldehyde in cacodylate buffer at pH 7.3 for 2 hr, and again washed them with several changes of the cacodylate buffer. Sections were then incubated in complete Karnovsky's solution (10), slightly modified, as described by Nakane and Pierce (18). Two control tissue groups were used; one group reacted with peroxidase-labeled antibody but not with Karnovsky's solution; the other reacted with unlabeled goat anti-rabbit y-globulin and with Karnovsky's solution.

After the immunoperoxidase treatment, the thick sections were used as blocks for thin section cutting. Thin sections were stained on the grid with uranyl acetate and Reynolds' solution.

Fibrinolysin Studies

Animals in 1 group were subjected to the same studies as outlined above except that, 30 min prior to tumor inoculation, 6000 units of fibrinolysin per kg (Thrombolysin, streptokinase activated; Merck, Sharp & Dohme, Dorval 700, Quebec, Canada) were given by tail vein.

RESULTS

Light Microscopic Observations. These observations were essentially the same as those previously described (13). Tumor cells accompanied by a platelet mass were detected in pulmonary arterioles at the earliest time periods (30 sec), and the general pattern over the various time periods did not differ from those in our earlier reports.

Conventional Electron Microscopy. These observations were also in keeping with our earlier descriptions, with the important exception that when simultaneous double staining in the block was used, small amounts of fibrin were detected within the platelet clumps as early as 5 min after tumor cell arrest. The deposits were scanty and often present at one level of a block and absent at another. Periodicity was very rarely detected in the fibrin at this stage, indicating that stable fibrin polymer was not yet formed. By 15 min, fibrin was more abundant, but periodicity was still difficult to detect (Figs. 1 to 3). By 45 min after injection of cells, fibrin strands were plentiful and periodicity was distinct, indicating polymerized fibrin. From 1 to 6 hr, fibrin became smaller in amount and was absent from sections taken at 9 to 72 hr. During the same period, platelets associated with the tumor cell emboli also steadily diminished and disappeared by 9 hr (Figs. 4 to 6).

Observations with Peroxidase-labeled Antibody. The general quality of cytology in the preparation was inferior to that obtained with ordinary electron microscopic fixatives and stains; but it was quite adequate for identification of endothelium, basement membrane, tumor cells, and platelets. Peroxidase-positive material was detected among the platelet masses at the earliest time period (30 sec). This material was amorphous, small in amount, and variable. There was no periodicity. The material was never seen in the control sections. By 2 min, this material was quite plentiful; but no periodicity was seen (Figs. 7 and 8). Periodicity was seen for the 1st time at 5 min and first appeared as a faint but definite lining up of granules in the positive material. The periodicity was measured at 195 Å (Figs. 9 to 11). The amount of peroxidase-positive material increased to a maximum at 1 hr and decreased rapidly after 3 hr.

Fibrinolysin-treated Animals. These animals developed tumor cell emboli similar to the other animals; however the tumor cells were markedly reduced, and very few platelets and no stable fibrin were seen by electron microscopy with any technique. Small amounts of peroxidase-positive material were seen at 3 and 6 hr, but no periodicity was detected.

DISCUSSION

We believe that the foregoing results establish clearly that fibrin is present in association with experimentally induced...
tumor emboli, that precursors of stable fibrin can be detected very early in the process by immunoperoxidase and later also by the double staining techniques, and that stable fibrin can be detected as early as 5 min after tumor inoculation. All of these points may apply only to the tumor model used. It is emphasized that both the unpolymerized and the stable fibrin may be present in very small amounts and is easily missed; it is quite possibly absent in some tumor platelet complexes, even with the model used. Moreover, as is also true with platelets, the presence of the fibrin is a transient event that may end in 6 to 9 hr. It is therefore not surprising that other workers have reported that no fibrin can be detected in early human and experimental metastases. We have, however, found similar fibrin deposits in experimental metastases of CSHB A tumor in C3H mice in liver and brain, as well as in Walker 256 tumor metastases in these sites (3, 4, 23).

Whether the fibrin formation has any effect on the process of embolization and subsequent growth is not clear to us. It is possible that fibrin, either polymerized or in monomer form, may form an amalgam with the platelet masses and may aid in tumor cell arrest and adherence to endothelium. Metastases occurred in fibrinolysin-treated animals but in smaller numbers; this has been the general experience with fibrinolysin and metastases (5, 7, 11, 28). Possibly, some tumors have an absolute requirement for a fibrin-platelet meshwork to arrest and multiply, while others such as the Walker 256 tumor may be able to metastasize in spite of its absence or reduction. It is also likely that, as long as the experimental methods used produce no endothelial damage at the point of cell arrest, the chances of large amounts of stable fibrin being formed are reduced. Tissue damage has been reported to increase local experimental metastases (8, 9) and might be expected to result in endothelial damage as part of the picture while, under normal circumstances, fibrinolysis from endothelium could cause dissolution of the thrombus. This important variable in the metastatic process has been studied and discussed by Warren (24, 25). Another variable is the presence of thromboplastin substances on the tumor cell. This has been discussed at length by various writers (12, 14, 19, 22). It is possible that some tumors may promote much greater fibrin formation while embolizing and impacting and, in such cases, the process we have discussed might be intensified to a marked degree. It may be necessary, however, to use sequential ultrastructural studies on a variety of tumors if a proper perspective of the problem is to be established.

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REFERENCES

Fig. 1. Walker 256 tumor cell (T) in a pulmonary arteriole 2 min after i.v. injection of a cell suspension. A large platelet mass (P) is adherent, and a neutrophil (N) and 2 lymphocytes (L) are also incorporated into the mass. No fibrin is seen by this technique at this early time period. Double-staining technique (see text). × 4,500.

Fig. 2. Area adjacent to an embolized Walker 256 cell in pulmonary arteriole 15 min after injection. Most of the field is occupied by platelets (P) that lie on endothelium (E). Two neutrophils are present (N). Small amounts of fibrin are present (arrows). Double-staining technique. × 11,000.

Fig. 3. Deeper section from the same block as Fig. 2, same area. No fibrin is seen at this level. Double-staining technique, × 11,000.

Fig. 4. Large mass of platelets (P) adherent to a tumor cell (not seen in figure) in a pulmonary arteriole 3 hr after i.v. injection. Fibrin (F) is abundant and showed periodicity. Most of the platelets are degranulated. Double-staining technique, × 8,500.
Fig. 5. Two Walker tumor cells (T) in pulmonary arteriole 6 hr after i.v. injection. No platelets are seen. A small rounded clump of residual fibrin (F) is present, and there may be phagocytosis of the material by endothelium (E). Double-staining technique, × 18,100.

Fig. 6. Walker tumor cell in pulmonary vessel 24 hr after injection. The cell is viable and in mitosis, but no fibrin or platelets are seen. Endothelium is obliterated at the bottom of the vessel. Double-staining technique, × 8,500.

Fig. 7. Two Walker tumor cells (T) in pulmonary vessel 2 min after inoculation using peroxidase-labeled antifibrin antibody technique, followed by staining of grids with uranyl acetate and lead citrate. Quality of material is poor compared to conventional processing, but platelet mass (arrows) can be seen and small clumps of positive staining material (F), × 15,000.

Fig. 8. Higher power view of positive material in Fig. 7 (arrows). No periodicity is seen, coarse granularity. Immunoperoxidase technique, × 77,000.
Fig. 9. High-power view of peroxidase-positive material adjacent to a tumor cell 5 min after injection. There is faint but definite lining up of the granules to a periodicity of approximately 195 Å. x 145,000.

Fig. 10. Platelet fibrin mass adjacent to a tumor cell (T) 15 min after i.v. injection. The fibrin (F) stains strongly positive and is quite abundant. Immunoperoxidase technique, x 10,500.

Fig. 11. Fibrin mass at 15 min, showing distinct periodicity. Immunoperoxidase technique, x 83,000.
Demonstration of Fibrin in Early Stages of Experimental Metastases

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