

ISOLATION OF A TUMOR FACTOR RESPONSIBLE FOR ANGIOGENESIS*

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Algire suggested that an attribute of tumor cells is their capacity to elicit continuously the growth of new capillary endothelium *in vivo* (1). Subsequently, Greene observed that tiny tumors implanted for more than a year in the anterior chamber of the guinea pig eye would not grow because they could not become vascularized (2). When these tumors were reimplanted in the muscle of a rabbit where they could become vascularized, they grew to a large size.

The growth of tumors which have been implanted in any one of several different organs and maintained by a long-term perfusion stops when the tumor reaches a diameter of 3–4 mm (3). Further growth of tumor tissue in *in vitro* organ cultures cannot be sustained without neovascularization of the tumor (4). Neovascularization does not require direct contact by tumor cells since vessels have been elicited from the hamster cheek pouch by tumors contained in a Millipore filter (5, 6). Similar outgrowth of new blood vessels was observed by us when Millipore chambers containing cells of B-16 melanoma or Walker carcinoma were implanted into the dorsal air sac of rats. In the present communication, the isolation of a soluble factor from human and animal neoplasms which is mitogenic for capillary endothelium is described. This factor induces growth of new capillaries, and it is proposed that it is responsible for tumor angiogenesis.

Materials and Methods

*Isolation of Tumor-Angiogenesis Factor (TAF).*¹—Walker 256 ascites tumor was harvested from 21-day old Sprague-Dawley rats which had been injected with 2×10^6 tumor cells 4–5 days previously. About 5 ml of bloody ascites were removed aseptically from the exposed

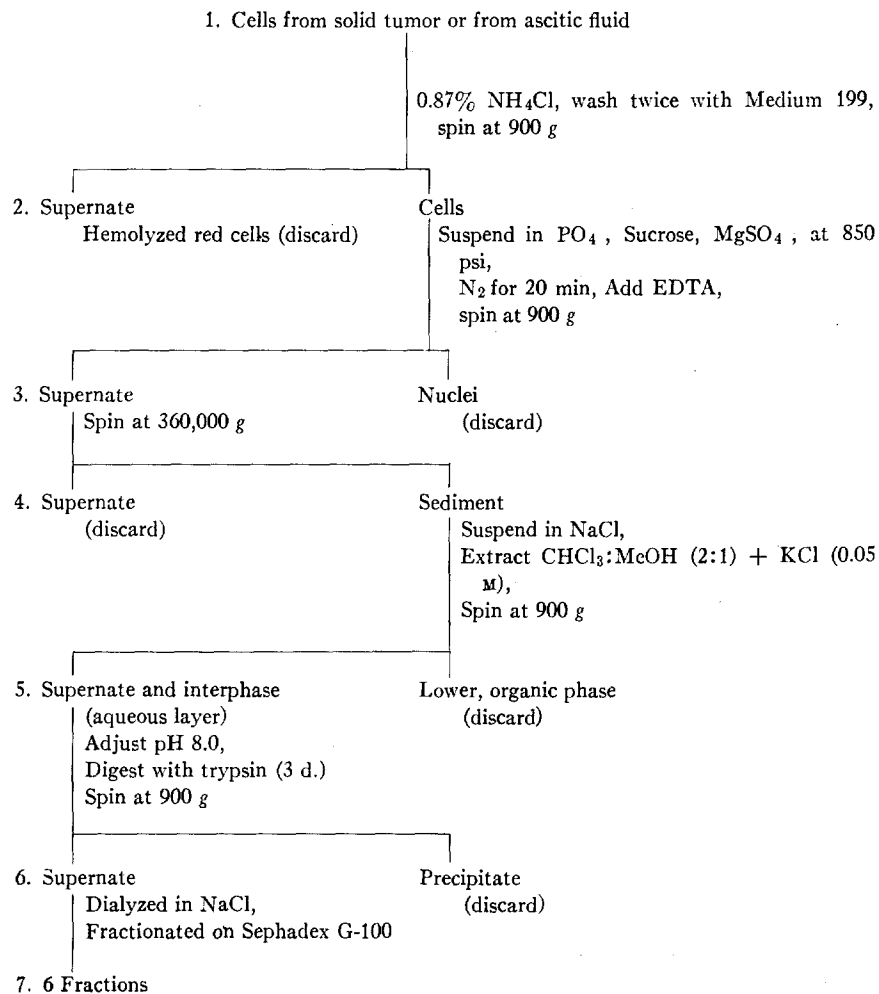
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¹ *Abbreviation used in this paper:* TAF, tumor-angiogenesis factor.

abdominal cavity of 10–15 rats. 1 mg heparin was added to each 10 ml of ascites (Table I, step 1). Solid Walker tumors were removed aseptically when they were about 1.5 cm in diameter. Necrotic portions were discarded. Tumor tissue was passed through a Snell cytosieve (Foster

TABLE I



Steps 1 through 6 are carried out under sterile conditions. Reagents are sterilized by millipore filtration. Glassware is sterilized by steam autoclave.

D. Snell, Inc., New York) into cold saline containing heparin (1 mg/10 ml). B 16 melanoma from C57 BL/6 mice, human hepatoblastoma, neuroblastoma, and Wilms tumors were handled in the same way, as were tissues used in control experiments. To lyse red cells, ammonium

chloride (0.87%) was added in equal volume to a suspension of cells containing 10^8 cells/ml (step 2). The cell suspension was centrifuged at 200 g for 10 min at 4°C. The supernatant fluid was discarded and the ammonium chloride wash repeated. The cells were washed twice with 40 ml of Medium 199 at 4°C, and resuspended in 10 volumes of phosphate buffer ($\mu = 0.15$, pH = 7.2), 0.25 M sucrose, and 10^{-2} M $MgSO_4$. They were exposed to nitrogen gas and maintained at a pressure of 850 psi for 20 min at 4°C while they were stirred (7). The nitrogen was suddenly released. 2 drops of decyl alcohol and 0.25 ml of 0.02 M disodium ethylenediaminetetraacetic acid (EDTA) were added for each 10 ml of suspension which was placed in a Vortex shaker

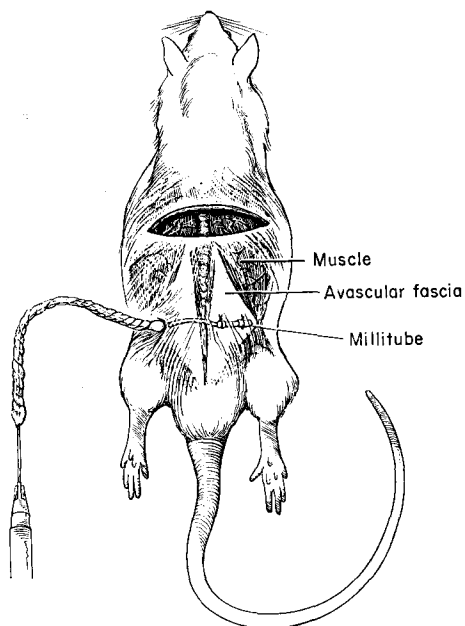


FIG. 1. Schematic representation of rat dorsal air sac assay.

(Scientific Industries, Inc., Springfield, Mass.) to disperse the EDTA among the subcellular components.

Nuclei were removed by centrifugation at 900 g for 20 min (step 3). The supernatant fluid was centrifuged at 360,000 g for 1 hr. The pellet was then resuspended in 5–10 ml NaCl (step 4).

Lipids were removed from step 4 by the addition of a 2:1 mixture of $CHCl_3/MeOH$ (20 volumes), followed by 0.2 volume KCl (0.5 M). The mixture was centrifuged at 900 g for 5 min. The upper aqueous layer and the solid interphase were aspirated and concentrated by evaporation at 37°C for 30 min (step 5). The concentrate was resuspended in dilute NH_4HCO_3 (pH 8.0). Trypsin was added² (1:100 w/w) and the solution was overlaid with toluene. The mixture was allowed to stand at room temperature for 3 days. Bacteriological cultures taken at this time were sterile. After centrifugation at 900 g for 10 min at 4°C, the supernatant fluid was dialyzed against NaCl and tested for its biological activity (step 6).

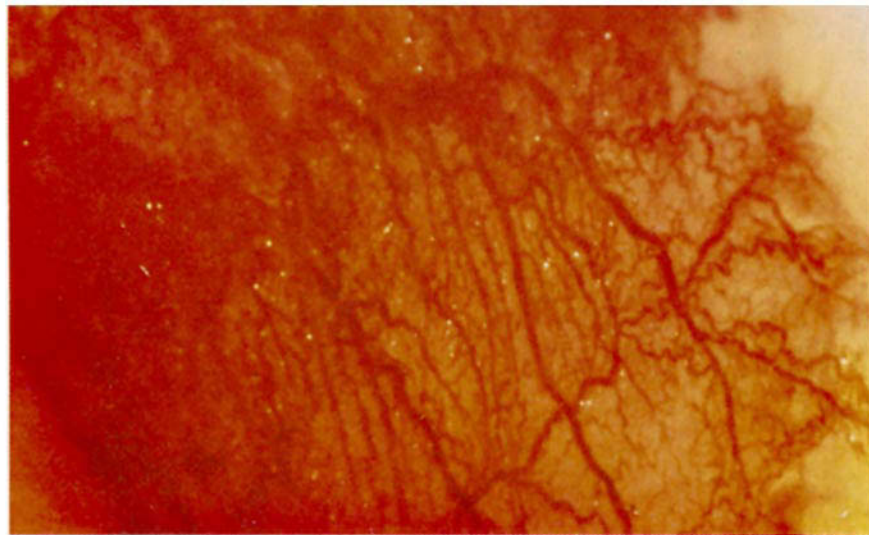
² Bovine pancreatic trypsin, 2 × recrystallized (Worthington Biochemical Corp., Freehold, N. J.)



2



3



4

The remainder of the fluid was chromatographed on a column of G-100 Sephadex resin (2.5×100 cm) in NaCl. Six fractions were obtained (step 7). The column effluents were monitored at 280 nm. Each fraction was dialyzed against distilled water, concentrated by lyophilization, and reconstituted to 5 ml in NaCl.

Biological Assay of TAF.—A dorsal air sac was produced in 42-day old rats by injecting, aseptically, 30 ml of air through a 25 gauge needle. The air was evenly distributed by manual compression of the skin. The air dissected into a plane of areolar tissue and separated skin from fascia without trauma. In early experience, Millipore filter chambers (0.45μ pore size) containing live tumor cells were placed within these air sacs. This procedure was altered in the following way for the testing of tumor cell fractions.

Silicone rubber tubing ($8 \times 0.065 \times 0.030$ inches) was occluded at one end with Silastic cement.³ Several holes were cut about 1 cm from the occluded end. Millipore tubing⁴ (Millitube), 0.8 cm in length, was threaded over the tubing and cemented in place to cover the holes. The Millitube was not used as a filter but as a diffusing wick because it was found to be the least reactive of all materials tested and by itself caused no vasoproliferation. Pin holes were made in it; a 21 gauge hypodermic needle was then cemented into the long end of the silicone tube. Silastic cement was applied thickly over the remaining silicone tubing and allowed to cure. This prevented kinking and the formation of air bubbles in the tubing caused by gas diffusion through the silicone (Fig. 1).

Rats were restrained on boards by holding their feet with rubber bands. Each tube was implanted into the air sac of one rat, using sterile conditions and light ether anesthesia, after shaving the back and cleaning the skin with Betadine. A transverse skin incision was made in the most cephalad portion of the air sac. The Millitube was inserted into the sac through a small hole made 2 cm caudad to the incision. The Millitube was fixed to the white triangle of avascular fascia (always present in the floor of the air sac and extending in the caudad direction) with two fine sutures. The skin was closed with clips. The skin incision was never placed near the Millitube. A 2 ml plastic syringe was filled with a solution of fraction to be tested which had previously been filtered through a bacteriological filter (0.45μ pore size). Approximately 0.1 ml of solution was injected every 4 hr except at night when only one injection was made. Food and water were supplied ad lib.

Reading the Air Sac Assay.—At 48 hr, the animals were removed from their restraints and anesthetized lightly with ether. The skin was incised widely over the Millitube. This area was observed under a stereoscope ($20 \times$), after which the tube was removed and histological sections were taken. The extent of vascular proliferation was graded on a scale of 0–5+ (Figs. 2–4).

³ Medical adhesive, Dow Corning Corp., Midland, Mich.

⁴ Millipore Corporation, Bedford, Mass. (# CH 00 200 40).

FIG. 2. Negative response in rat dorsal air sac following 48 hr exposure to millitube without T.A.F. The size of the grid is 2.5 mm. $\times 18$.

FIG. 3. 3+ response in the rat dorsal air sac following 48 hr exposure to millitube with T.A.F. Neovascularization is seen in an area of 0.5 cm^2 from which the millitube has been removed. The size of the grid is 2.5 mm. $\times 18$.

FIG. 4. 5+ response in the rat dorsal air sac following 48 hr exposure to millitube with T.A.F. The millitube was removed from the left margin of this field. The vasoproliferative response is more dense in proximity to the millitube. There are dilated serpentine veins and dense, papular capillary beds. Neovascularization extends 1.5 cm^2 . $\times 18$.

RESULTS

Tumor Tissue Fractions

Walker 256 Carcinoma.—Following nitrogen cavitation of Walker 256 carcinoma cells, which had been grown either in the ascitic or solid forms, the cellular lysate was centrifuged at 900 g. The nuclear pellet was positive (3+), but since it was difficult to delipidate, filter, and purify, it was not used as a source of TAF.

The remaining supernatant fluid after removal of the nuclei also produced

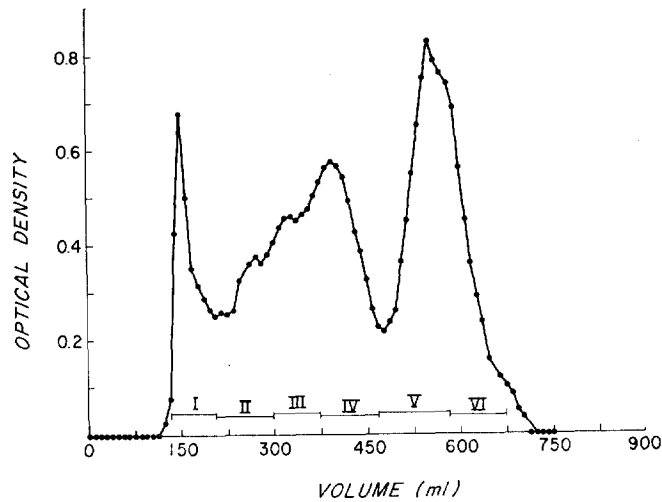


FIG. 5. Separation on a G-100 Sephadex column of the 360,000 g pellet following delipidation and trypsin digestion. Elution with NaCl, 0.15 M. OD at 280 nm vs. eluting volume.

neovascularization (3+) (Fig. 3). In order to ascertain the optimal conditions for separation of TAF, the supernatant fluid was subjected to differential centrifugation.

Centrifugation at 80,000 g for 1 hr removed small amounts of TAF, but did not result in any appreciable loss of activity from the supernatant fluid. A white precipitate gradually developed if the supernatant fluid was left in the cold (4°C). Delipidation and trypsinization of this precipitate yielded active TAF. When EDTA was added to the remaining clear fluid after spontaneous precipitation, further precipitation appeared. This precipitate contained no TAF.

Centrifugation at 165,000 g for 1 hr inconsistently yielded a pellet containing TAF activity; however, centrifugation at 360,000 g for 1 hr reliably caused sedimentation of TAF in a pellet. This pellet was delipidated, digested with

trypsin, and chromatographed on G-100 Sephadex. Fraction II (Fig. 5) of the eluate produced the most positive results in the neovascularization assay (4+ to 5+) (Fig. 4). Fractions I and III were weaker in their activity, and fractions IV-VII were usually negative in the bioassay (Fig. 2).

Mouse B-16 Melanoma.—Neovascularization was produced by fractions I and II obtained from the G-100 Sephadex column. Activity varied from 1+ to 3+. The type of vascular response was similar to that of fractions from the Walker tumor.

Human Neuroblastoma.—Neovascularization was also produced by fractions I and II from the Sephadex column (3+ to 4+).

Wilms Tumor.—This tumor also demonstrated angiogenesis activity. However, activity was found in specimens removed from the periphery and not from tissues taken from the central necrotic part of the tumor.

Hepatoblastoma.—Neovascularizing activity was positive in fraction I (4+ to 5+) and slightly less in fraction II (2+).

Mitogenic Effect upon Endothelial Cells.—Histologic sections of subcutaneous tissue exposed to TAF revealed the presence of dilated, serpentine veins and venules (Figs. 3 and 4). No hemorrhages were noted and the areas of venular and capillary engorgement blanched upon inhalation of ether. As the reactions become progressively more intense, dense capillary beds, resembling hemangiomas, obliterate the fascia (Fig. 4). New capillaries are crowded together with plump, dark-staining nuclei (Fig. 6), compared with sections of capillaries from fascia not exposed to TAF (Fig. 7). The histologic characteristics of the new capillary walls resemble regenerating endothelium. Mitotic figures are encountered frequently in endothelial cells and pericytes (Fig. 8), but not in the surrounding connective tissue.

In several experiments, during the last 12 hr of the 48 hr assay, 50 μ Ci of ^3H -thymidine were injected into the air sac along with the TAF. Control rats received the same injection of ^3H -thymidine into the air sac without TAF. In areas of TAF-induced neovascularization, endothelial cells and pericytes incorporated ^3H -thymidine (Fig. 9). No incorporation of tritium was observed in the control microsections. No cells other than endothelium in either set of experiments incorporated the ^3H -thymidine, except for occasional fibroblasts. In these experiments, microsections showed no accumulations of polymorphonuclear leukocytes or round cells within vessels or in the interstitial tissue. Exudate cells and fibrin deposits were sometimes noted when the source of TAF had not been dilapidated and chromatographed. In all cases, mast cells were observed in their normal positions and retained their normal granulation.

Control Tissue.—Nonmalignant tissues did not produce neovascularization except for rat and calf thymus, which gave mild neovascularization reactions (1+ to 2+). Human placenta from fetuses aborted at 18 wk produced moderately strong neovascularization (3+). Rat kidney, liver, and regenerating

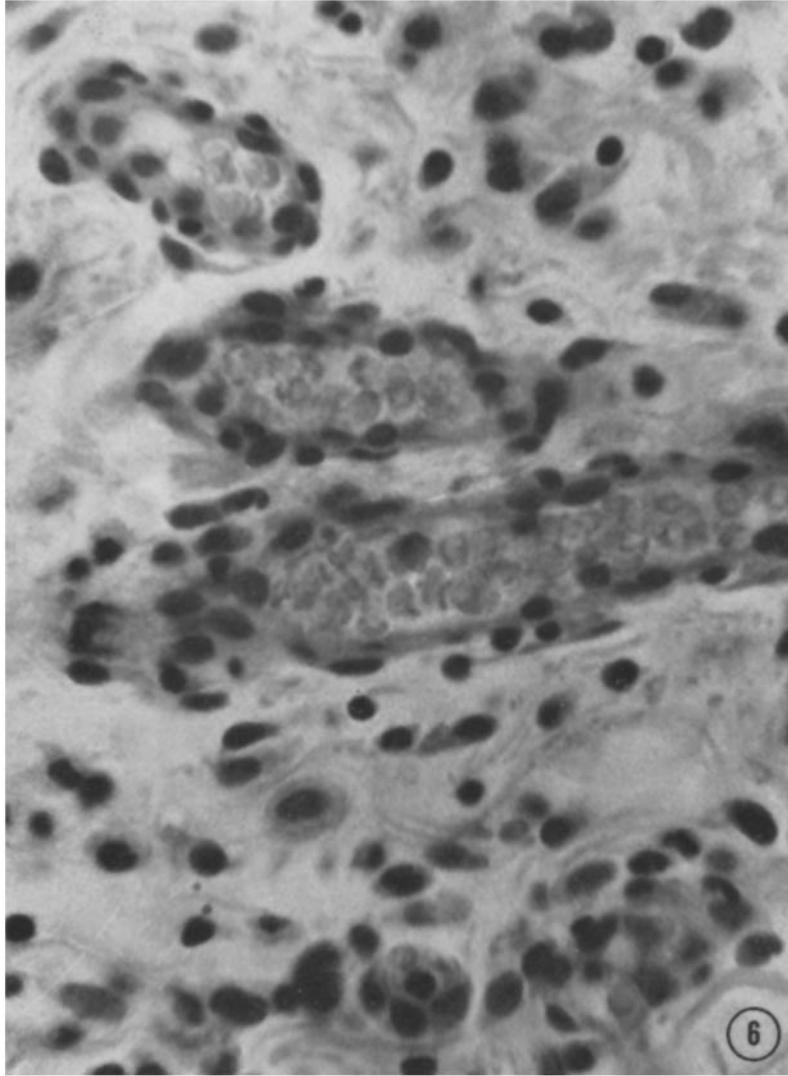


FIG. 6. Histologic section of subcutaneous fascia underlying millitube with T.A.F. after 48 hr exposure. Endothelial cells and pericytes are closely spaced. Nuclei are dark staining, plump, and contain large nucleoli. The appearance is that of regenerating endothelium. H & E stain. $\times 600$.

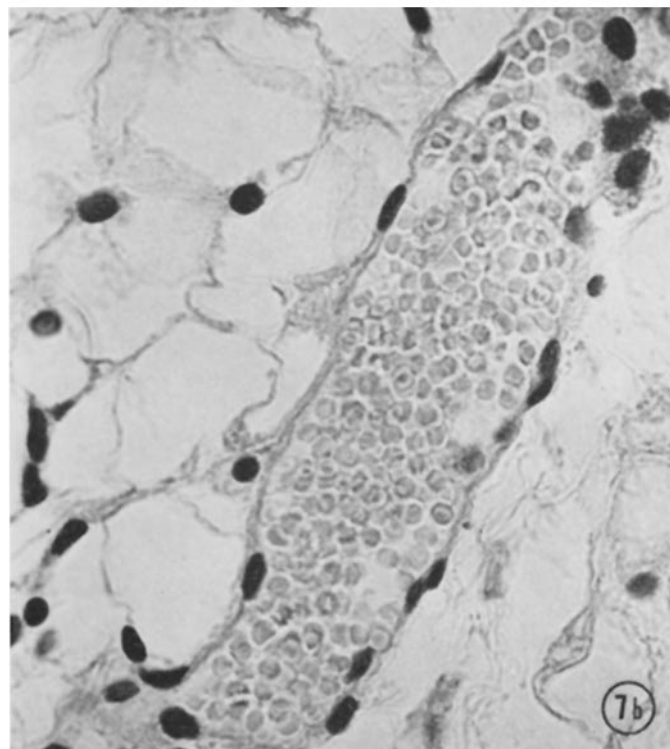
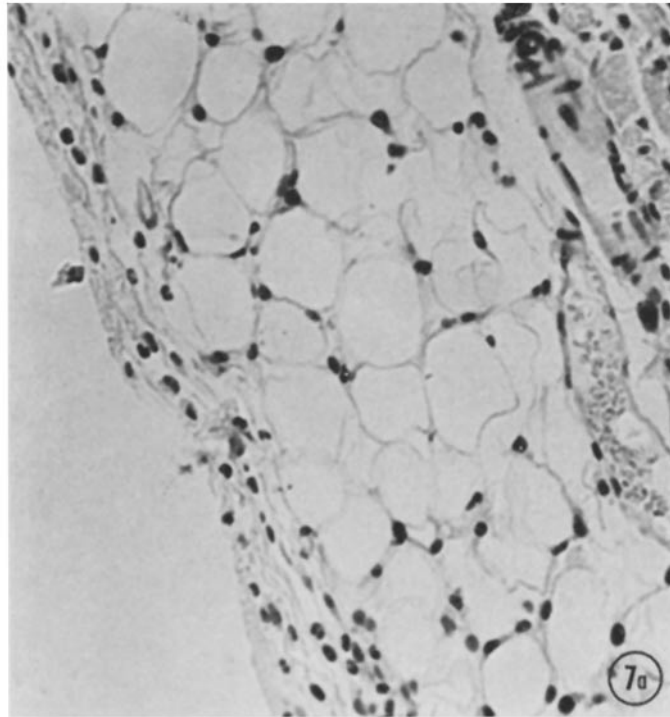


FIG. 7. Histologic section of subcutaneous fascia underlying millitube without T.A.F. There are no new capillaries. Endothelial cells are flat and widely spaced. H & E stain. (a) $\times 250$; (b) $\times 600$.

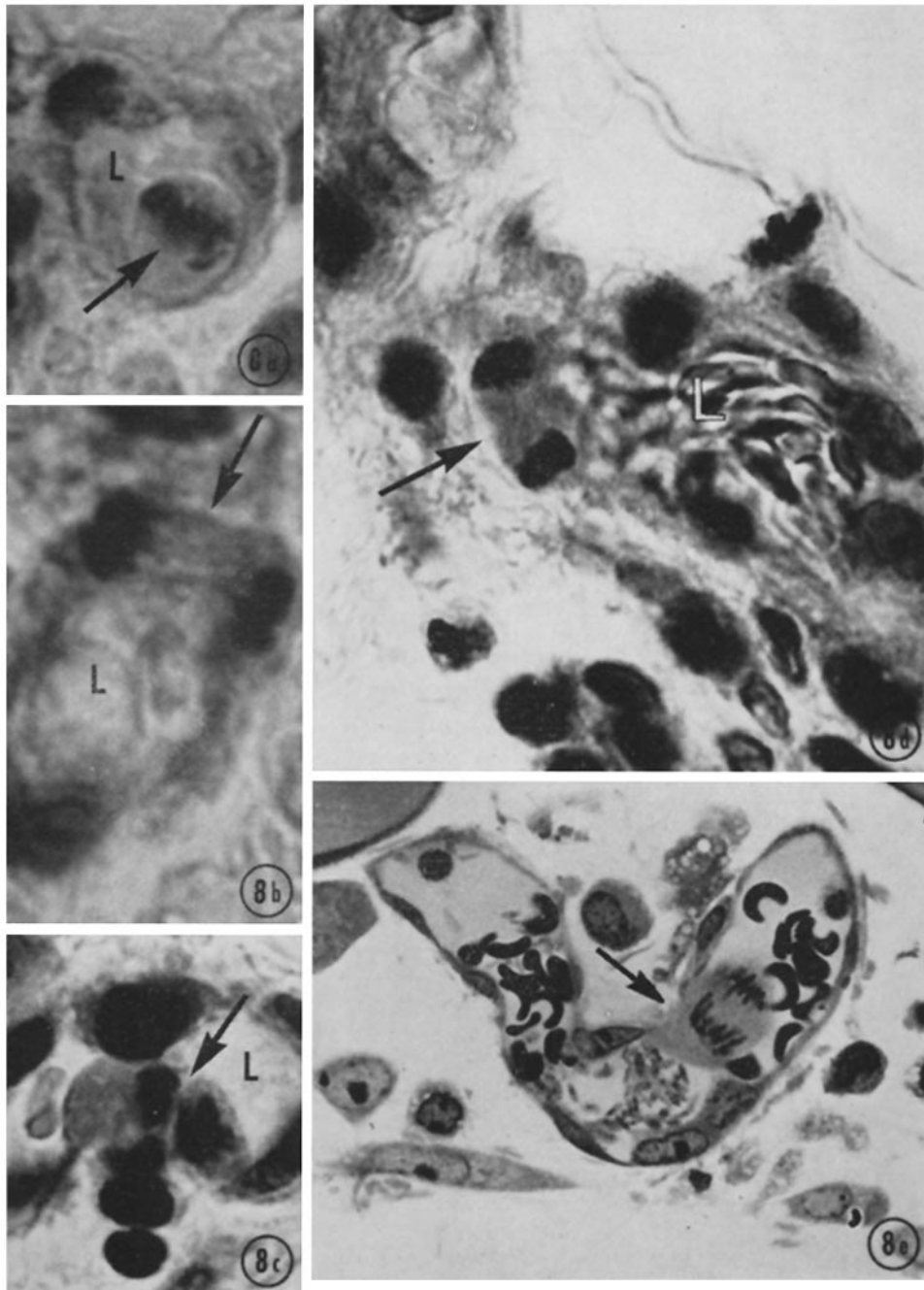


FIG. 8. Mitoses (arrows) in blood vessels of subcutaneous fascia underlying millitube with T.A.F. *L* indicates lumina of blood vessels. (*a*, *b*, and *c*) endothelial cell, H & E stain. $\times 1200$; (*d*) pericyte, H & E stain. $\times 1200$; (*e*) endothelial cell, 1μ thick Epon section, fixed with gluteraldehyde and stained with Toluene blue. $\times 800$.

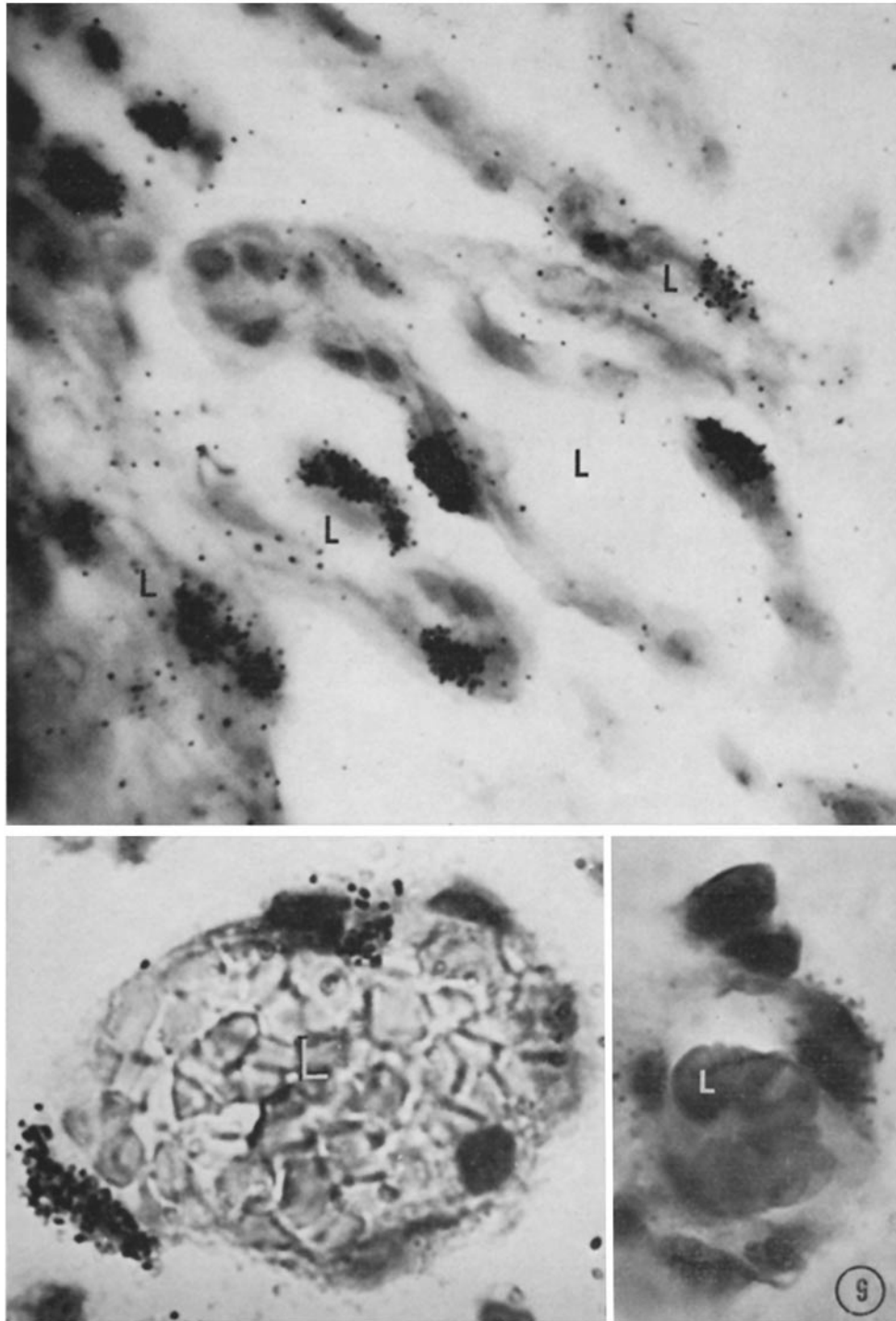


FIG. 9. Radioautograph of histologic section of subcutaneous fascia underlying millitube with T.A.F. *L* indicates lumina of blood vessels. Grains represent ^3H -thymidine in nuclei of endothelial cells and pericytes. Weigert stain. $\times 800$.

liver (1, 2, 3, and 4 days after hepatectomy) gave negative results. Neovascularization was not produced by fractions from tissue-cultured HeLa cells, hamster embryo cells, or hamster embryo cells transformed by SV 40 virus.⁵

Infected granulation tissue, intact *Escherichia coli* (*E. coli*), endotoxin,⁶ agar, sucrose, concentrated trypsin solutions, ribonuclease, rat plasma, hexachlorophene, heparin, gelfoam, silicone adhesive, and tissue culture media obtained from HeLa and hamster cell cultures all gave negative results.

Characterization.—The activity of TAF was destroyed by digestion with ribonuclease⁷ (45 min, 37°C, pH 6.5), or by heating at 56°C for 1 hr, or by incubation with subtilisin⁸ (1 hr, 37°C, pH 8.0).

TAF activity was not diminished by exposure to trypsin (for as long as 3 days), heating to 37°C, room temperature for at least 72 hr, or 4°C for up to 3 wk. Before delipidation, TAF could be stored for 3 months at 4°C.

TAF was found to contain approximately 25% RNA by direct analysis, 10% protein, 50% carbohydrate, the remainder being presumably lipoidal material. RNA extracted from Walker tumor cells with phenol (8) did not produce neovascularization. This implied that the protein moiety associated with RNA in TAF was essential to elicit the mitogenic effect of TAF upon capillary endothelium.

DISCUSSION

These experiments show that a soluble factor separable from human and animal tumors is mitogenic for endothelial cells and is responsible for formation of new capillaries. It is unlikely that TAF extracted from solid tumors originates from stroma or inflammatory cells rather than from tumor cells, since the amount of neovascularization from preparations of pure ascitic tumor cells produced an equivalent degree of neovascularization.

The rat dorsal air sac technique to measure neovascularization is cumbersome but very reliable. In over 900 rat assays during the past 2 yrs it has rarely given a false positive. Other methods were tried as substitutes but were found less reliable than the air sac assay. For example, the chicken egg chorioallantoic membrane (9) was too sensitive; a vasoproliferate response was observed even with minor trauma. Injections into the anterior chamber of the rabbit eye were difficult to interpret. Results comparable to those obtained with the rat dorsal air sac assay were obtained utilizing the rabbit ear chamber (10). This method was unsuitable for screening fractions because of the lengthy period of time required before the ear chamber was adequately vascularized for use in

⁵ We are grateful to Dr. Michael Oxman for these cultures.

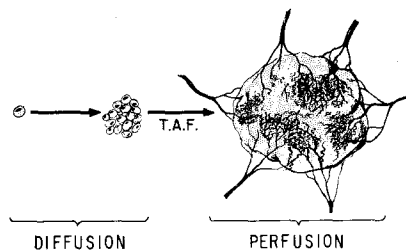
⁶ Endotoxin, *Salmonella typhi*, O 901 (phenol extracted).

⁷ Bovine pancreatic ribonuclease, 5 × recrystallized (Worthington Biochemical Corp., Freehold, N. J.).

⁸ Subtilisin; Nagarse strain of *Bacillus subtilis*.

an assay. Successful results with the air sac assay require that the transverse incision (Fig. 1) be far forward of the Millitube implant so that healing will not influence vascularity of the fascial floor. The Millitube must be implanted aseptically. Infection at this point obscures the neovascularizing reaction with a thick purulent exudate, or it may prevent neovascularization entirely. Occasionally, a thin areolar membrane or a delicate layer of fibrinous material overlies the new vascular reaction. This material is easily removed with fine scissors and forceps to disclose the new vessels beneath.

The presence of a tumor-angiogenesis factor suggests a transfer of information from tumor cells to capillary endothelial cells. The relationship between tumor cells and endothelial cells may be interdependent. Tannock has shown that the nutritional environment of tumor cells becomes poorer as the spacing between blood vessels increases, and this leads to a decreased rate of proliferation and cell death (11). His work implies that the rate of proliferation of endothelial cells may limit indirectly the rate of tumor growth.



It appears that most solid tumors, whether they originate from a cell transformed by virus or carcinogen, or whether they begin as a metastatic implant, must exist early as a small population of cells dependent upon nutrients which diffuse from the extravascular space. Such a pinpoint colony eventually expands to a size where simple diffusion of nutrients (and wastes) is insufficient (Fig. 10). New capillaries are elicited and the tumor then enters a phase in which nutrients arrive by perfusion. It is possible that TAF is responsible for this final stage. It is tempting to suggest that tumor growth might be arrested at a very small size if the angiogenesis activity of this factor could be blocked. This would be analogous to the cessation of growth of bacterial colonies when their size exceeds the diffusion of nutrients. The indirect evidence from isolated perfused organs and from the work of Greene suggests that interruption of angiogenesis results in cessation of tumor growth at an early stage. Thus, the understanding of the mechanism of tumor angiogenesis has potential therapeutic importance.

SUMMARY

Human and animal solid tumors elaborate a factor which is mitogenic to capillary endothelial cells. This factor has been called tumor-angiogenesis

factor. The important components of TAF are RNA and protein. It is suggested that blockade of this factor (inhibition of angiogenesis) might arrest solid tumors at a tiny diameter of a few millimeters.

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