TUMOR ANGIOGENESIS

Rapid Induction of Endothelial Mitoses

Demonstrated by Autoradiography

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ABSTRACT

Walker ascites tumor cells and an extract derived from such cells (tumor angiogenesis factor, TAF) were injected into the subcutaneous tissue of rats by using a dorsal air sac technique. At intervals thereafter, thymidine-^{3}H was injected into the air sac and the tissues were examined by autoradiography and electron microscopy. Autoradiographs of 1μ thick Epon sections showed thymidine-^{3}H labeling in endothelial cells of small vessels 1–3 mm from the site of implantation, as early as 6–8 hr after exposure to live tumor cells At this time interval endothelial cells appeared histologically normal. DNA synthesis by endothelium subsequently increased and within 48 hr new blood vessel formation was detected. The presence of thymidine-^{3}H-labeled endothelial nuclei, endothelial mitoses, and regenerating-type endothelium was confirmed by electron microscopy. TAF also induced neovascularization and endothelial cell DNA synthesis after 48 hr. A similar response was not evoked in saline controls. Formic acid, which elicited a more intense inflammatory response, was associated with less endothelial labeling and neovascularization at the times studied. Pericytes and other connective tissue cells were also stimulated by live tumor cells and TAF. The mechanism of new blood vessel formation induced by tumors is still unknown but our findings argue against cytoplasmic contact or nonspecific inflammation as prerequisites for tumor angiogenesis.

INTRODUCTION

A variety of human and experimental tumors have the capacity to induce new blood vessel formation (1–18), and it has been suggested that a property of tumor cells is their ability to stimulate the continuous growth of new capillaries (2). Recently, Folkman et al. (17) reported the isolation of an extract derived from tumor cells that is capable of inducing vascular proliferation in vivo. The factor has been designated tumor angiogenesis factor (TAF) and it has been postulated that TAF may be responsible for neovascularization and growth of tumors beyond the size that can be attained due to nutrition by diffusion alone (17). Since the initial study (17) suggested that TAF was a potent endothelial mitogen and induced only a mild inflammatory response, it seemed worthwhile to study in detail the time-sequence of endothelial cell proliferation and morphogenesis of new blood

vessel formation elicited by TAF. Although endothelial regeneration plays an important role in such processes as wound healing, closure of large vessel defects, and organization of thrombi, our understanding of the kinetics of endothelial turnover and the stimuli for endothelial regeneration is incomplete.

Mitoses are rarely encountered in normal adult vascular endothelium, and it has long been assumed that mature endothelial cells have extremely slow turnover rate and rarely divide (2, 19, 20). Autoradiographic studies with thymidine-^{3}H have in general confirmed these views (21–27), but have been hampered by difficulties in sampling sufficient numbers of endothelial cells, especially in tissue sections. Engerman et al. (27) emphasized the variability of endothelial labeling among different tissues. For example, the labeling index in retina was 0.01 % and in myocardium 0.13 %. More recent studies employing autoradiography of *en face* preparations of aortic endothelium (28–31) have indicated that a small proportion of endothelial cells synthesize DNA under normal conditions; the labeling appears to be greater in areas of bifurcations and branching of the aorta (31, 32), and in younger growing animals (30). Increased endothelial labeling, representing endothelial regeneration (33, 34), has been reported after physical (26, 32, 35), chemical (26), and cold (36) injury to the aorta, in aortic endothelial cells of swine fed cholesterol for 3 days (29); and in aorta of rabbits after a single injection of endotoxin (36). Sade et al (30) demonstrated DNA synthesis by endothelial cells in cultured rat aortic segments after in vitro crush injury.

Endothelial regeneration in *small blood vessels* has been studied principally in wound healing (37–40) where endothelial mitoses and ultrastructural evidence of regenerating endothelium appear 3 days after wounding. In the course of autoradiographic experiments on the origin of fibroblast proliferation in wound healing the presence of labeled endothelial cells was briefly mentioned and illustrated (41), but not dealt with in detail. Spector and Lykke (42) described thymidine-^{3}H-labeled vessel wall cells in foci of local inflammation induced by Freund's adjuvant, however, they emphasized the difficulty in differentiating endothelial from periendothelial cells or emigrating monocytes by light microscopy in paraffin sections.

In the present paper we report the results of experiments employing thymidine-^{3}H autoradi-

ography to determine the kinetics of endothelial proliferation after exposure to tumor cells and TAF. Autoradiographic studies were performed on 1 μ thick Epon sections in which labeled endothelial cells can be identified with relative ease It will be shown that endothelial cells can be stimulated to undergo mitosis within 6 hr after exposure to live tumor cells, and that TAF and tumor cells cause considerable endothelial mitotic activity and new blood vessel formation after 48 hr with a relatively mild inflammatory reaction.

MATERIALS AND METHODS

Materials

(a) Live washed Walker 256 ascites tumor cells were harvested from the peritoneal cavities of rats as described elsewhere (17). In some experiments cells were heated at 56°C for 1 hr.

(b) "Crude" TAF was obtained by disrupting tumor cells by nitrogen cavitation at 850 psi for 30 min as in step 3 of Table I in our previous report (17). The nuclei were separated by centrifugation at 900 g for 20 min and discarded. The supernate was centrifuged at 360,000 g for 1 hr. The pellet contained a TAF moiety termed crude because delipidation and further purification were not carried out These maneuvers increase the activity of TAF but diminish its stability in vivo. The lipid in the crude TAF pellet appears to act as a slow release vehicle, so that TAF activity is prolonged after a single injection.

Induction of Angiogenesis

Tumor cells, TAF, and control solutions were assayed for their ability to induce vascular proliferation, using the rat dorsal air sac technique (17) in restrained animals Subcutaneous air sacs were produced in 35–54 day old ether-anesthetized rats by injecting, aseptically, 30 ml of air through a No. 25 gauge needle.

(a) When live or heated tumor cells were used, they were injected with a No. 25 needle in a volume of 0 03 ml containing 1 × 10⁶ cells. The injection was made after the air sac was opened with a transverse incision (see below) The needle was placed in a very superficial plane of fascia on the floor of the air sac and produced a bleb of 1–2 mm in diameter.

(b) TAF was delivered in the following manner A Millipore tube (Millitube) was prepared by sliding a 0.8 cm segment of Millipore tubing (Millipore Corp , Bedford, Mass., 0 4 μ pore size) over a 1.5 cm length of silicone tubing (0 065 × 0.030 inches) which had previously had one or two large "V"-shaped holes cut out of the wall at its center. The silicone tube acted as a mandrel for the filter, and the ends of the

filter were sealed to it with Silastic cement (Dow Corning Corp., Midland, Mich.). Each end of the silicone tube was also plugged with Silastic cement. Millitubes were heat sterilized at 80°C for 24 hr. 0.02 ml of TAF was then injected into the Millitube through the Silastic cement.

With the rat under ether anesthesia, the shaved skin over the dorsal air sac was cleaned with Betadine solution, and a transverse incision was made at the cephalad extent of the air sac as described in Fig. 1 of our previous report (17). Aseptic technique was used throughout. The Millitube was implanted in each rat toward the posterior portion of the air sac over a white triangle of fascia. A 5–0 silk suture was placed around each end of the silicone tube to hold the Millitube at a fixed site. The sutures were therefore very distant from the diffusion zone around the Millipore filter.

(c) Saline controls consisted of (i) air sac preparation with millitubes (as in b) containing saline, (ii) injections of 0.03 ml of saline in the fascia of the air sac, as for tumor cells.

(d) 0.2% formic acid was delivered as (i) a single injection of 0.03 ml in the fascia of the air sac, (ii) 0.03 ml contained in a Millitube, as for TAF.

In experiments with saline or formic acid, the site of delivery was identified by marking its periphery with small deposits of colloidal carbon (Pelikan-Werke, Hanover, Germany).

Experimental Groups

Over 200 animals were used for gross, histologic, autoradiographic, and ultrastructural studies.

GROUP I: Animals received washed Walker ascites tumor cells by subfascial injection.

GROUP II: Animals received tumor extract (crude TAF) from Walker ascites tumor cells through a Millitube.

GROUP III: Animals received saline either by injection of 0.03 ml into the air sac (controls for group I) or through a Millitube placed in the air sac (controls for group II).

GROUP IV: Animals received 0.03 ml of a solution of 0.2% formic acid either by injection (controls for group I) or through a Millitube as for TAF (controls for group II).

Other controls consisted of random samples of fascia from the contralateral side of the air sac, not traumatized either by Millipore filter or injection of test material, but exposed to thymidine-^{3}H.

Tritium-Labeled Thymidine Studies

To assess DNA synthesis in endothelial cells, a single pulse of 50 μCi of thymidine-^{3}H (New England Nuclear Corp., Boston, Mass., specific activity 6.7 μCi/mole) in 2 ml of saline was injected into the air

sac 6, 12, 24, and 48 hr after the test material. In all experiments, animals receiving the radioactive label were sacrificed 2 hr later.

Morphologic Studies

8, 14, 26, and 50 hr after testing (2 hr after thymidine-^{3}H), the animals were lightly anesthetized with ether, the skin was widely incised and reflected, and the floor of the air sac was observed under a stereomicroscope ($\times$20). The Millitube was removed, and tissues were excised under a dissecting microscope from the fascia surrounding the tumor injection site or Millitube as well as from the soft tissue underlying the tumor bed. For conventional histology, tissues were fixed in buffered formalin, embedded in paraffin, cut at 4 μ and stained with one of the following: hematoxylin and eosin or periodic acid–Schiff (PAS)-Luxol-fast-blue-hematoxylin (43) (E. I. du Pont de Nemours & Co , Wilmington, Del.). For thick Epon section light microscopy and electron microscopy, tissues were fixed in a solution containing 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.025% calcium chloride, pH 7.4 (44). The fixative was dripped onto the tissue surface after opening of the air sac. In some experiments the fixative was also injected underneath the subcutaneous fascia

SAMPLING: When vascular proliferation was clearly visible, areas were selected from such sites, when no newly formed vessels could be detected under the stereomicroscope, samples were removed in the following manner: Group I: A 1.0 cm square centered by a 2–3 mm nodule of tumor cells was cut out and initially fixed in a large drop of fixative. After 10 min, it was trimmed into ± 1 mm thick strips. Specimens were selected including the edge of the tumor and from distances of 1, 2, and 3 mm from the tumor edge. Group II: Upon removal of the Millitube, the site of delivery of TAF consisting of an approximately 1.5 $\times$ 0.2 cm surface was exposed. A 1.0 cm square was cut out and briefly fixed. Two 1 mm strips were obtained from the center and parallel strips were cut from distances which varied from 1, 2, and 3 mm from the delivery site; Group III From animals receiving saline by injection, tissues of the site of injection and in a radius of 1, 2, and 3 mm were obtained; when saline was delivered through a Millitube, specimens were sampled as for TAF, Group IV When formic acid was injected, specimens were obtained in a manner similar to that of animals injected with saline or tumor cells; from those receiving formic acid through a Millitube, sampling was as for TAF. In all experiments, the specimens were further trimmed into 1 mm cubes and transferred to fresh fixative.

Fixation was carried out for 4 hr at room temperature. Tissues were washed overnight in 0.1 M cacodylate buffer, pH 7.4, postfixed with 2% osmium

tetroxide for 2 hr, dehydrated in graded ethanol, infiltrated with Epon 812-propylene oxide, and embedded with Epon 812. Thick Epon sections (1 μ) were cut in an LKB III ultrotome type 8801 A (Laboratorie och Kemikaliska Produkter, Stockholm) and stained with 1% toluidine blue. From selected areas, thin sections were obtained, double stained with uranyl acetate and lead citrate, and examined in a Philips EM 200 electron microscope.

Autoradiography

Autoradiographic studies were performed on both paraffin and thick Epon sections using standard techniques (45). Areas were selected including the site of injection of tumor cells and surrounding vessels. This was done with the purpose of assessing the relationship between the tumor and growing capillaries and to evaluate the contribution of the inflammatory component to vascular proliferation. 8–30 thick plastic sections and two paraffin sections were prepared by autoradiography from each animal. The slides were exposed in groups for 10, 14, and 21 days at 4°C, developed with D-19 developer, and fixed with Kodak fixer (Eastman Kodak Co., Rochester, N. Y.). Paraffin section autoradiographs were stained with hematoxylin and eosin, PAS-hematoxylin, or PAS-Luxol-fast-blue-hematoxylin (43). Thick Epon section autoradiographs were similarly prepared, exposed in groups for 7, 14, 18, and 21 days, developed, and stained with 1% toluidine blue. Negative and positive controls were run for all experiments. Thin sections from selected blocks exhibiting endothelial labeling by light microscopy were prepared essentially according to Baserga and Malamud (45), and examined with the electron microscope.

RESULTS

General

In the original study of TAF it was found that gross evidence of new blood vessel formation could be detected as early as 48 hr after implantation of Millitube containing TAF (17). The biological assay system for TAF activity thus involved assessing the extent of gross vascular proliferation at 48 hr on a scale of 0–5 +. With this technique a variety of control preparations, including heat-killed tumor cells, failed to induce angiogenesis.

For the present studies initial pilot experiments were conducted to determine the earliest period after exposure to live tumor cells when histologic or autoradiographic evidence of endothelial proliferation occurred. Since labeled endothelial cells were identified when the label was injected after 6 hr, detailed autoradiographic studies at

this time interval were conducted and compared with findings at 48 hr.

Since thymidine-^{3}H labeled endothelial cells could be confused with labeled pericytes, macrophages, or perivascular connective tissue cells in paraffin sections, all observations were confirmed in 1 μ thick Epon sections in which identification of individual endothelial cell nuclei could be made with relative ease (Fig 1). Although the problem of sampling enough tissue in thick Epon sections precluded adequate quantitation of the degree of endothelial labeling in different groups, an attempt at semiquantitative assessment was made by recording the number of labeled cells per 1 mm^2 block The findings, which should be taken to be descriptive rather than quantitative, are summarized in Table I.

Groups I and II

Except for slight variation in the extent of inflammation the results in groups I and II (live tumor cells and TAF) were similar and will be described together. No observations were made at 6–8 hr after TAF (Table I).

8 hr after injection of tumor cells, vessels in the fascia around and underneath the implantation were prominent due to engorgement with blood. There was slight edema but no gross evidence of new blood vessel formation, i.e., no new capillaries, sprouts, loops, or buds (46). Histologically, the vessels were slightly dilated and exhibited well-spaced endothelial cells with flattened nuclei. The interstitium showed focal, mild to moderate infiltration with leukocytes, but in many sections there was no evidence of inflammation. In autoradiograms, some labeled endothelial cells were found in almost all blocks examined from animals sacrificed 8 hr after tumor explant (thymidine-^{3}H having been injected locally at 6 hr). The labeled cells were well-spaced and, except for clear-cut evidence of thymidine-^{3}H labeling, could not be distinguished from the usual "resting" endothelium (Figs. 1–3). On the average, 3–4 definite endothelial cells were labeled in each block. In many blocks there was minimal or no evidence of inflammation in the presence of labeled endothelial nuclei.

50 hr after tumor or TAF, the vessels were still grossly engorged but in addition there were small, fine, tortuous capillary sprouts beneath the site of injection or Millitube implant. There were focal areas of hemorrhage amidst these new vessels In
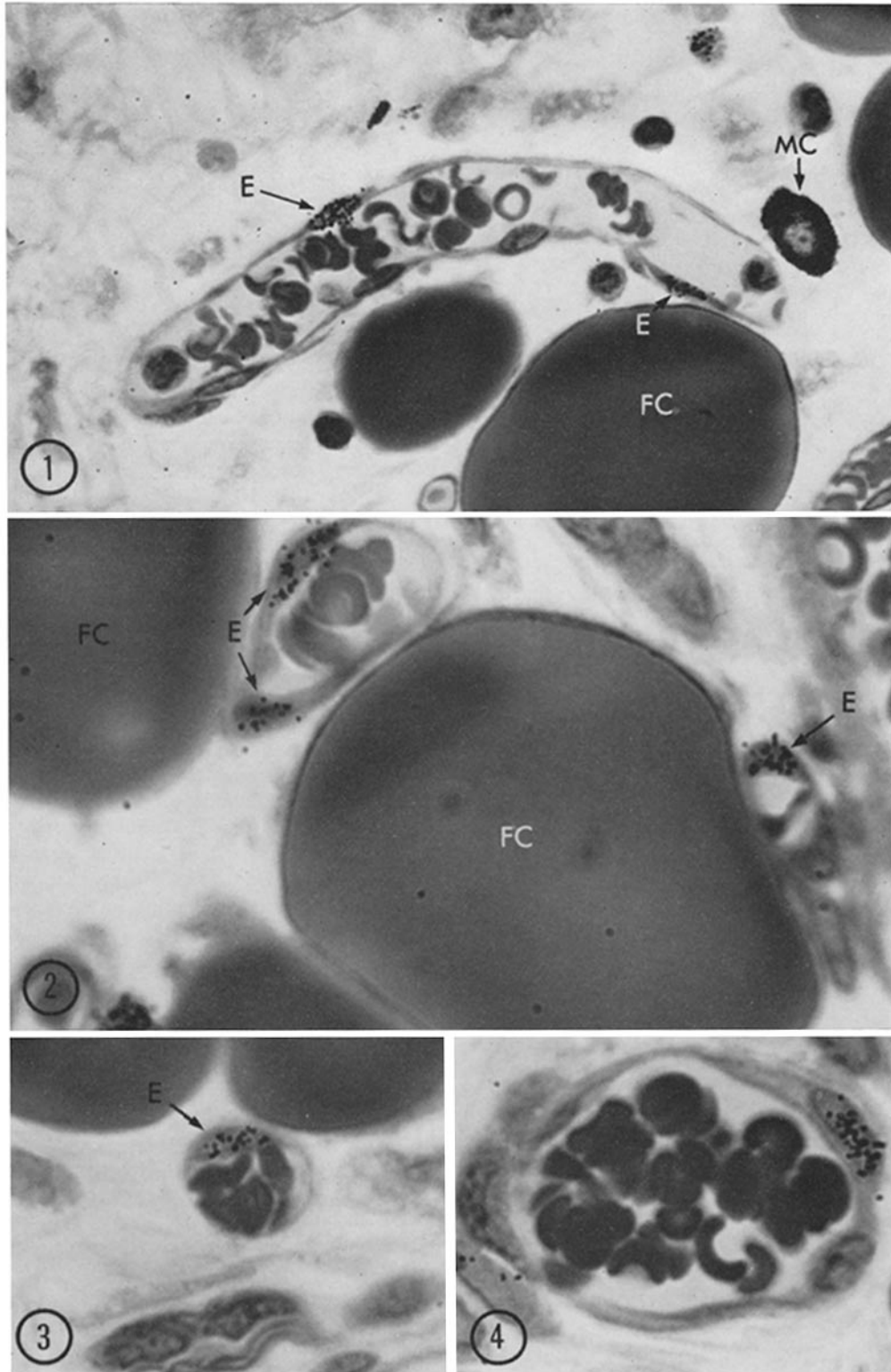
Figures 1–3   Endothelial cells of small blood vessels exhibiting DNA synthesis. Walker tumor cells (1.0×10^6) were injected 8 hr earlier into the adjacent fascial areolar tissue. A single pulse of thymidine-^{3}H was given 2 hr before sacrificing. Several endothelial cells (E) are labeled. FC, fat cell; MC, mast cell. Fig. 1, $\times$ 950; Figs. 2 and 3, $\times$ 1500.

Figure 4   Small blood vessel depicting DNA synthesis in a vascular wall cell, possibly a pericyte. From the same experiment as indicated in Figs. 1–3. $\times$ 1500.

Group	Number of animals*	Hr after initial stimulus	Endothelial cell labeling‡
I	6	8	4 2
(Walker tumor cells)	5	50	11 2
II (TAF)	7	50	12 2
III (Saline)	5	8	0
	3	50	0
IV (0.2% formic acid)	3	8	0
	5	50	2 4

* Refers exclusively to the number of animals in which thick plastic section autoradiographs were scored A larger number of animals were used for other studies (see Methods).

‡ Number of labeled cells per number of 1 mm² blocks examined.

general, there was more edema and focal hemorrhage with live tumor cells than with TAF Histologically, newly formed vessels were irregular, exhibiting areas of extreme thinness of the vascular wall particularly close to the growing tips. Often the endothelial cells in such vessels were crowded and presented irregularities of their contours, causing the lumen to be narrowed or virtually absent Several endothelial cells in mitosis were identified in thick Epon sections and by electron microscopy (Fig 5); these were present in apparently newly formed vessels, but also in larger "parent" venules. Many endothelial cells had prominent nucleoli Collections of macrophages occasionally occupied a perivascular distribution, but neutrophils were rare at this stage. Foci of hemorrhage were present. Mitosis in perivascular and interstitial cells were also seen. By autoradiography there was extensive labeling of endothelial cells. As a rule, vessels exhibited one or several labeled endothelial cells (Figs 6 and 7) Both large engorged vessels and small capillaries exhibited labeling. Some vessels with labeled endothelial cells were sometimes surrounded by foci of hemorrhage and inflammatory cells, but many

were from areas with minimal or no inflammation (Fig. 7). On the average, there were 11–12 endothelial cells labeled in each 1 mm² block. Electron microscopic autoradiography on selected blocks confirmed labeling in endothelial cell nuclei (Fig 8).

Besides incorporation of thymidine-³H by endothelial cells, there was labeling of variable numbers of perivascular cells in close apposition to the vessel wall. Some of these appeared to represent pericytes (Fig. 4), and ultrastructural studies showed mitoses in cells covered with a basement membrane (Fig. 9) In addition, there was labeling of interstitial cells (Fig. 6), which appeared to be both connective tissue cells and monocytes. About 80% of the tumor cell population showed the intensity of the label increasing centrifugally in the tumor deposits. Central portions of the tumor did not take up the label

Group III (Saline Controls)

The site of injection was marked by a pinpoint hemorrhage which was visible at 8 hr and barely so at 50. There was no gross edema, hemorrhage, or evidence of new blood vessel formation histologically in animals receiving saline by injection or through a Millipore tube By autoradiography, there was no labeling whatsoever of endothelial cells 8 and 50 hr after saline injection

Group IV (Formic Acid)

At 8 hr, and more prominently at 50 hr, the Millitube implant site exhibited fibrinous exudate, hemorrhage, dilated engorged vessels, and severe edema Histologically, at 8 hr there was considerably more inflammation than in groups I and II. The infiltrate was mostly composed of neutrophils, and there was exudation of fibrin, vascular congestion, and interstitial hemorrhages At 50 hr the infiltrate was severe and predominantly monocytic. Evidence of new blood vessel formation, as described for tissue of groups I and II, was equivocal, some vessels did exhibit endothelial cells with prominent nucleoli, and there were occasional suggestions of endothelial buds, but in general, these were slight By autoradiography there was no endothelial cell labeling 8 hr after injection of irritant. However, sections examined after 50 hr did show labeled endothelial cell nuclei and newly formed blood vessels, but they were less numerous than those of groups I and II (Table I)
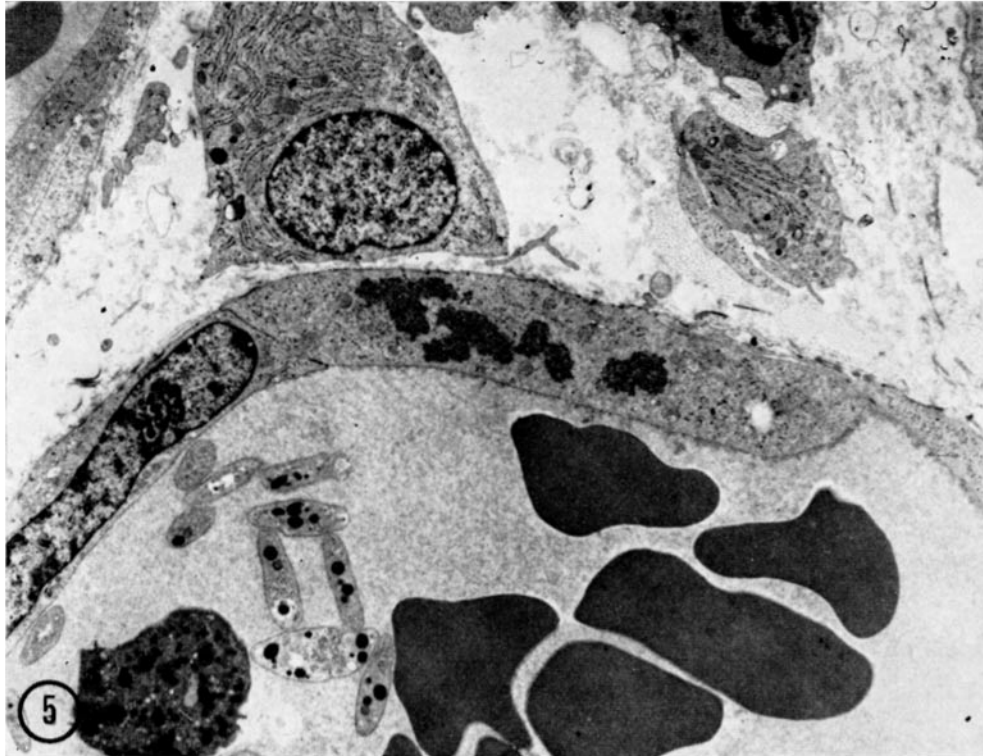
FIGURE 5 Electron micrograph of portion of small blood vessel 50 hr after injection of Walker tumor cells (1.0×10^6) into the areolar tissue of the dorsal air sac. This capillary was sampled from tissue surrounding the site of tumor implantation. One endothelial cell is in mitosis. A portion of a second cell shows prominent chromatin in nucleus and increase in cytoplasmic ribosomes. $\times$ 5000.

DISCUSSION

Our studies show that within 6–8 hr after implantation of live tumor cells, the endothelium of small blood vessels 1–3 mm from the implantation site is stimulated to undergo mitosis. Mitotic activity subsequently increases and, within 48 hr, evidence of new blood vessel formation is seen both grossly and microscopically. The use of thick Epon sections facilitated the identification of labeled endothelial cells, and the finding of endothelial mitoses (Fig. 5), regenerating-type endothelium (Fig. 5), and labeled nuclei by electron microscopy confirmed the light microscopic data. DNA synthesis in endothelial cells was associated with only mild inflammatory cell infiltrate, and especially at 6 hr (Figs. 1–3) many vessels exhibiting labeled endothelium appeared otherwise normal. A similar response was not evoked in saline controls. TAF induced a degree of neovascularization and endothelial labeling

that was comparable to that produced by tumor cells at 48 hr.

The ability of tumors to induce neovascularization has long been known (1–18), and there have been numerous descriptions of the development and morphologic features of tumor vessels. In most studies new vessels become manifest 4–5 days after implantation of tumor (10, 11), and in some reports as early as 48 hr (13). In a series of studies, Warren (5, 7, 9, 11, 13) described in detail the ultrastructural morphology of new vessels induced by transplantable tumors in the hamster cheek pouch.

In contrast to the large number of descriptive studies on tumor angiogenesis, there have been few reports on the possible mechanisms involved. In 1968 Greenblatt and Shubik (10) reported neovascularization in hamster cheek pouch stroma adjacent to a melanoma tumor transplant, despite separation of the two tissues by Millipore
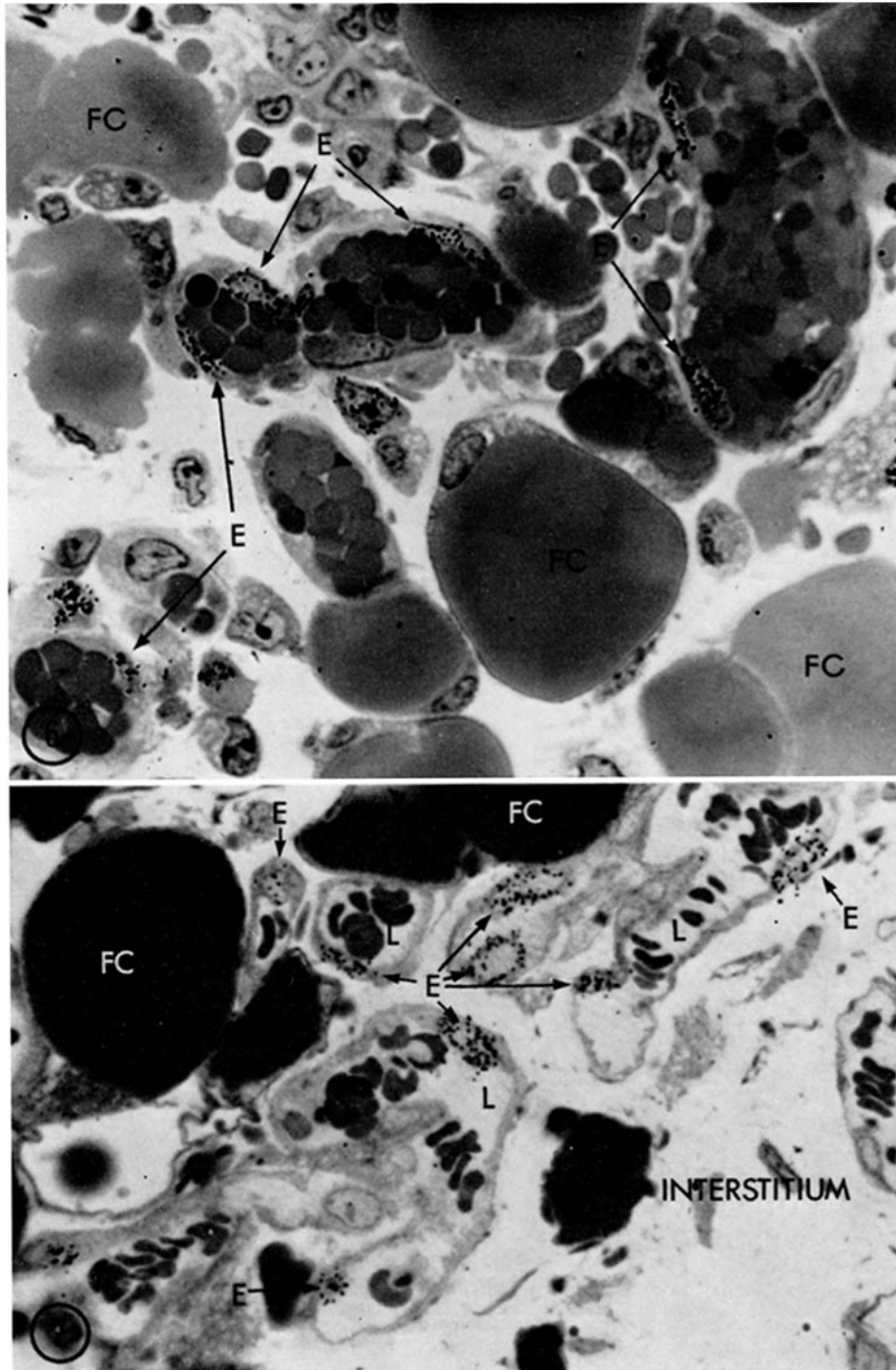
FIGURE 6   Small blood vessels 50 hr after TAF. This area of neovascularization underneath the site of TAF shows intense labeling of endothelial cells (*E*). Some vessels are irregular and endothelial cells are numerous and more prominent than at 8 hr. A few interstitial cells are also labeled. Inflammatory infiltrate is mild and predominantly composed of histiocytes. Red blood cells from fragile regenerating capillaries have spilled into the interstitium. *FC*, fat cells. × 700.

FIGURE 7   Small blood vessels 50 hr after injection of Walker tumor cells into the air sac floor. Area selected 3 mm away from the tumor deposit. Several labeled endothelial cells (*E*) are seen. Thin-walled vessels have irregular contours and prominent endothelial cells. Note absence of inflammatory reaction. *FC*, fat cells; *L*, lumen. × 750.
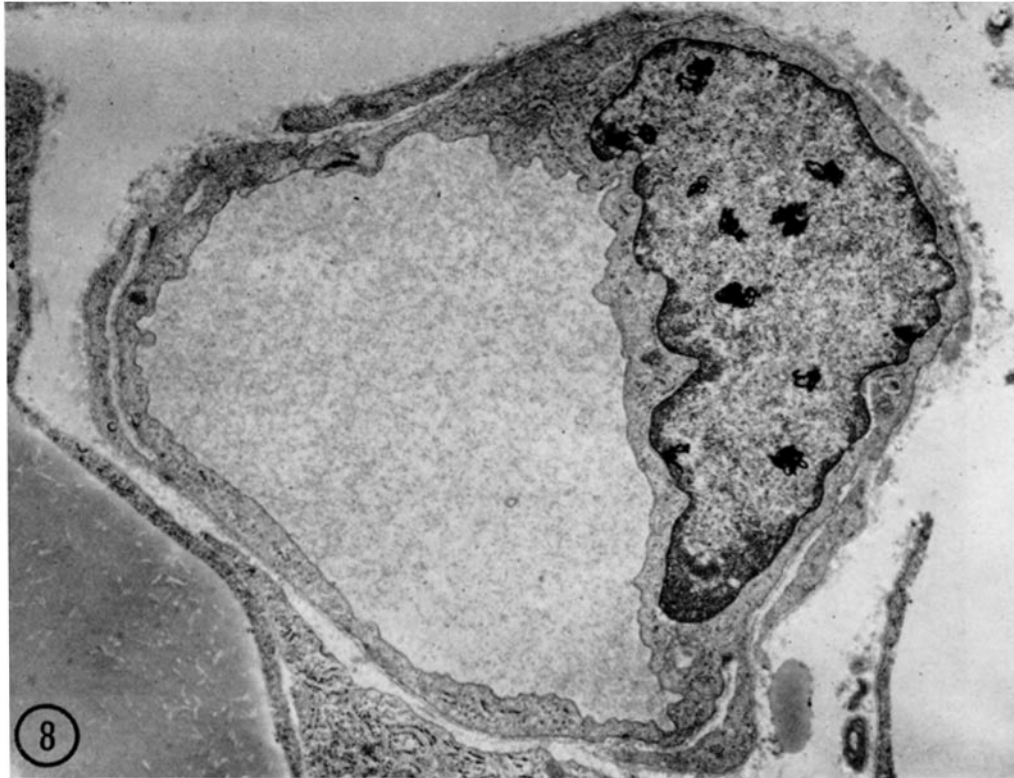
FIGURE 8   Electron microscopic autoradiograph of a capillary 50 hr after injection of 1.0×10^6 Walker tumor cells and 2 hr after injection of thymidine-^{3}H into the air sac. The endothelial cell nucleus is labeled. $\times$ 8700.

filter that blocked the passage of whole cells. Ehrmann and Knoth (12) confirmed these findings, using a transplantable choriocarcinoma. Both groups suggested that the tumor released a humoral factor responsible for angiogenesis. More recently, however, Greenblatt (47) re-examined the Millipore filter experiments and found that the ability of tumor to induce angiogenesis was related to the porosity of the filter; he further demonstrated cytoplasmic processes of tumor cells within the filter pores, and concluded that cytoplasmic contact is an essential feature in the mechanism of angiogenesis. The experiments with TAF (17) and the demonstration in the present study that live tumor cells induce DNA synthesis in endothelial cells within 6 hr at relatively great distance from the implantation site, however, argue against cytoplasmic contact as a necessary prerequisite for tumor angiogenesis.

Vascular proliferation is a major component of granulation tissue which forms as a result of the

local inflammatory response to necrotizing injury, and the question arises whether TAF stimulates endothelial mitosis by eliciting a nonspecific inflammatory response. Inflammation was not a prominent feature of the TAF response. To our knowledge, however, there are no comparable autoradiographic studies on the early proliferative endothelial response in acute inflammation. Our limited data with formic acid, which evokes a marked inflammatory response, show that this irritant elicits considerably less endothelial labeling and neovascularization at the time intervals studied. Previous control experiments with a variety of nonmalignant tissues and inflammatory agents failed to induce significant neovascularization at 48 hr (17) These findings indicate that live tumor cells and TAF possess vasoproliferative properties beyond eliciting a nonspecific inflammatory reaction.

There have been numerous theories to explain the stimulus for growth of new vessels, especially in
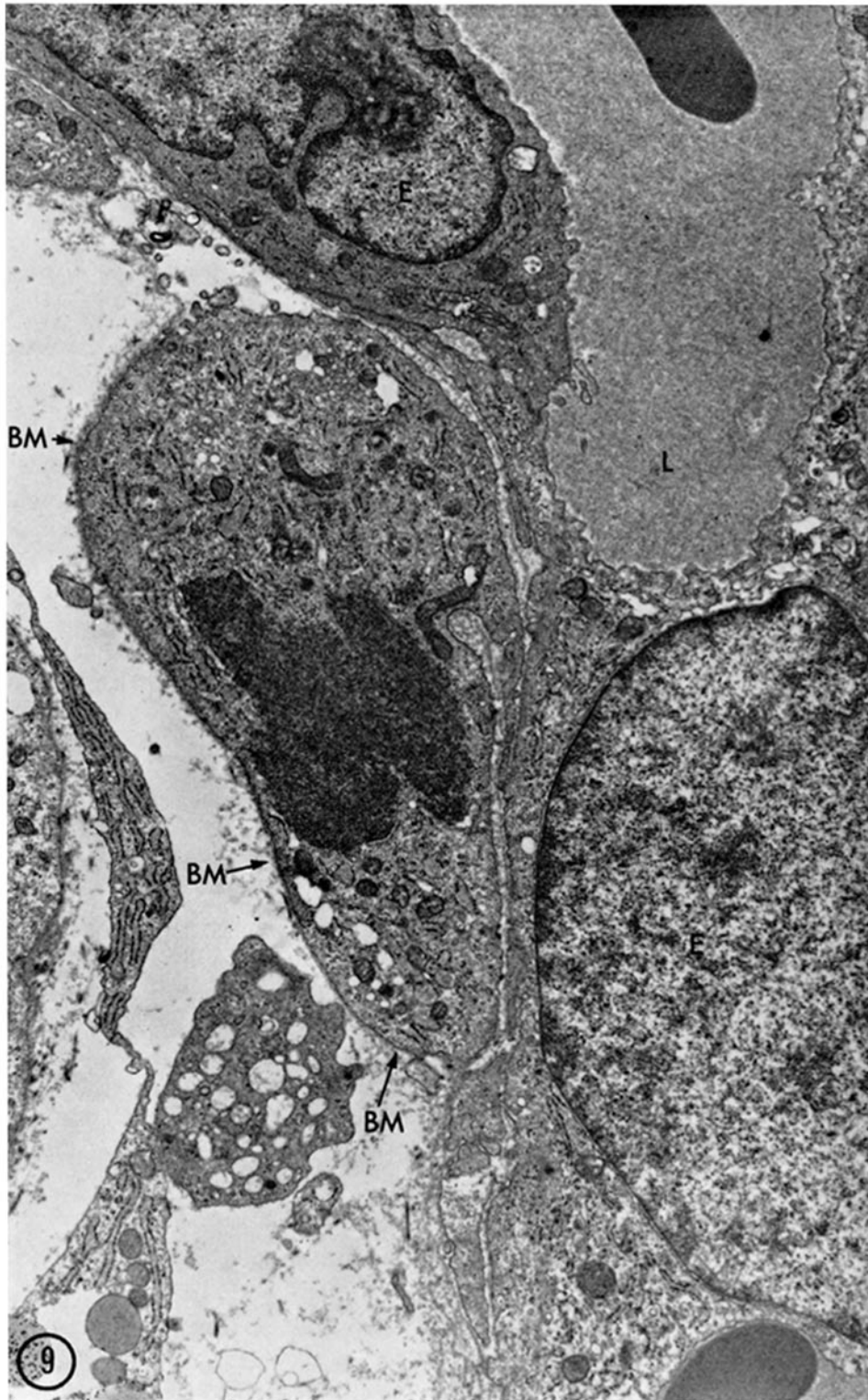
FIGURE 9   Electron micrograph of small blood vessel 50 hr after TAF. A mitosing adventitial cell is enveloped by basement membrane (*BM*) and is therefore considered to be a pericyte Note clumps of chromatin and lack of nuclear membrane. Regenerating endothelial cells (*E*) show large numbers of free ribosomes. *L*, lumen. × 9000.

wound healing (47, 48). These include release of materials from mast cells, vasoactive substances (49), local hypoxia or change in oxygen gradients (50), accumulation of cell metabolites, tissue extracts, and others (51). Although none of these theories has been proven, it could be that TAF acts indirectly on endothelial cells by activating some mechanism common with wound healing or inflammation. Indeed, it would be difficult to rule out an indirect effect in in vivo experiments on the microcirculation; thus, studies with an in vitro endothelial culture system have been initiated (30).

The present study examined the earliest vasoproliferative response to tumors and has not attempted to deal with the kinetics of endothelial turnover in already growing neoplastic tissue In a recent paper, Tannock (16) reported on the population kinetics of endothelial cells within a transplanted mouse mammary tumor at the height of tumor growth. Using paraffin sections, he found that 11.4% of capillary endothelial cells within the tumor were labeled 1 hr after a single pulse of thymidine-^{3}H. From repeated labeling experiments, he estimated the endothelial turnover time to be between 50–60 hr, and his results suggested that endothelial cells were not derived from a faster proliferating precursor population.

Our autoradiographic data as well as ultrastructural studies to be detailed separately indicated that not only endothelial cells but pericytes and connective tissue interstitial cells are also stimulated to undergo mitosis after implantation of tumor or crude TAF. The ability of pericytes to undergo mitosis has been questioned (27, 52, 53) In a recent study of wound healing, Crocker et al. (53) observed mitoses in perivascular mesenchymal cells which were distinguishable from pericytes by their lack of surrounding basement membrane, they suggested that such mesenchymal cells are then incorporated within a basement membrane to become pericytes. While such a phenomenon may occur, our studies with TAF show mitosis in cells that are enveloped by basement membrane as early as 48 hr after TAF. By autoradiography labeled cells in close apposition to the endothelium (e.g., Fig. 4) present in 6–8 hr lesions were also considered to be pericytes. That fibroblasts also undergo active proliferation within tumors has been long recognized. Tannock (16) studied fibroblast turnover within transplanted mouse mammary tumor by thymidine-^{3}H

autoradiography and found a 9.1% labeling index and a turnover time of 70–80 hr. In our studies, both tumors and crude TAF induced proliferation of fibroblasts. Whether stimulation of endothelial and connective tissue elements is caused by the same or different mediators is now conjectural and must await further purification of the active principle in tumor angiogenesis.

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