TUMOR DORMANCY IN VIVO BY PREVENTION OF NEOVASCULARIZATION*

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The continued growth of solid neoplasms is always associated with neovascularization. A possible mediator is tumorangiogenesis factor (TAF), an endothelial cell mitogen which has been extracted from a variety of solid malignant tumors, in this laboratory (1). The specific blockade of this factor, "anti-angiogenesis," has been proposed as a new approach to the control of malignant growth (2, 3).

The consequences of failure of neovascularization for tumor growth have been fortuitously observed in two experimental situations. In isolated perfused organs, in which endothelial cell degeneration has occurred (4) and new vessel outgrowth is not possible, tumor implants grow to a small size (2–3 mm diameter) but no further (5). Similarly, during experiments with heterologous tumor transplantation in guinea pig eyes (6), Green observed that a few implants, which apparently did not vascularize, failed to grow for almost 2 yr. However, when these same tumors were reimplanted into their original host, they vascularized and grew progressively.

These observations suggested that a fundamental relationship might exist between angiogenesis and the ability of a tumor to grow malignantly. The following experiments now provide in vivo evidence that the malignant growth of a homologous solid tumor can be deliberately arrested at a small size by preventing neovascularization.

Materials and Methods

Animals.—Male New Zealand white rabbits, initially 6 wk old and weighing 2–3 kg, were used for propagation of tumor stock and all experiments.

Tumor.—A stock of Brown-Pearce rabbit epithelioma[1] was maintained by serial anterior chamber transfers. All stock and experimental iris implants yielded rapidly growing, richly

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[1] We are grateful to Mr. S. M. Poiley, National Cancer Institute, for this material.

vascular tumors which demonstrated a characteristic pseudoacinar histology (7). No instances of spontaneous regression were observed in serial passages through more than 12 generations (36 animals) during a 4 month period of observation (October through January).

Implantation Technique.—Intravenous pentobarbital[2] anesthesia (25 mg/kg) was supplemented by retrobulbar infiltration with 0.50 cc 2% Xylocaine.[3] The eye was proptosed and secured in position by clamping a fold in the lower lid. Stock tumor was harvested from mature (10–14-day old) vascular iris implants and finely diced with iridectomy scissors in sterile saline at room temperature. Intraocular implants were then performed in either of two ways.[4]

Vascular iris implants: A small (2 mm) peripheral corneal incision was made and a small fragment of freshly minced tumor inserted with fine forceps. Care was taken to deposit the implant directly on the iris at a distance from the incision, which then closed spontaneously (Fig. 1).

Avascular anterior chamber implants: A fresh tumor fragment was inserted into the anterior chamber via a central corneal incision. A coagulum routinely formed around the implant within the 1st few hours and subsequently retracted, tethering it to the inner surface of the corneal dome. These implants characteristically grew as small ovoid masses but remained suspended in the anterior chamber at a distance from the iris (Fig. 1).

All procedures were performed under sterile conditions. No antibiotics or special eye care were given and intraocular infection was not observed.

Measurement of Tumor Growth.—Tumor implants were examined daily by two observers with the aid of a Zeiss slit lamp stereomicroscope (Carl Zeiss, Inc., New York), at × 10–40 magnification. *En face* measurements of major and minor radii (X, Y) were obtained with an ocular micrometer at × 10 magnification (measurement error ± 0.1 mm). The third dimension (Z) of iris implants (thickness) and anterior chamber implants (radius) was estimated by tangential viewing and later corroborated by measurement of histologic sections of tumors at various stages of growth (applying + 20% shrinkage factor for fixation-dehydration artifact).

Vascular iris implants appeared to grow as hemispheroids; therefore, daily volumes were estimated by the formula $V = (4/3)\ \pi\ (X)\ (Y)\ (Z)/2$. Avascular anterior chamber implants appeared to grow as spheroids; their volumes were estimated by the formula $V = (4/3)\pi\ (X)\ (Y)\ (Z)$.

Cumulative growth curves were then constructed from calculated daily tumor volumes plotted on a semilogarithmic scale. The rate of change of volume during different phases of growth was estimated by the formula, Slope = $\log_{10}V_2 - \log_{10}V_1/t_2 - t_1$.

Demonstration of Vascularity.—The apparent onset of vascularization of small iris implants was documented in vivo by use of intravenous Fluorescite[5] (0.5 cc/kg). Perfused tumors rapidly developed a green-yellow coloration when viewed under cobalt blue slit lamp illumination and thus were easily differentiated from prevascular iris implants or avascular anterior chamber implants which remained gray or pearly white, respectively. The reliability of this test of vascularity was verified by histologic sampling at various stages of tumor growth.

Histologic Studies.—Specimens of iris tumor implants of various ages were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. The smaller avascular anterior chamber implants were fixed whole by immersion in 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 4 hr, washed overnight in caco-

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[2] Veterinary sodium pentobarbital (65 mg/ml), Samuel Perkins Co., Boston, Mass.

[3] Xylocaine hydrochloride (lidocaine), Astra Pharmaceutical Products, Inc., Worcester, Mass.

[4] We thank Dr. H. Zauberman (Retina Foundation, Boston) for his assistance in developing these techniques.

[5] Fluorescein (5%) in sterile aqueous solution, Moore/Kirk Laboratories, Inc., Hillside, N. J.

dylate buffer, postfixed in 1% osmium tetroxide, dehydrated, and embedded in Epon. 1-μ thick Epon sections were then stained with 1% toluidine blue.

In some experiments, 0.4 μCi methyl-thymidine-^{3}H (New England Nuclear Corp., Boston, Mass., specific activity 2 Ci/mM) in 0.2 cc of normal saline at 37°C was injected into the anterior chamber, 45 min before sacrifice. Radioautographs of histologic sections were then made according to standard techniques (8).

RESULTS

Vascular Iris Implants.—Daily slit lamp observations of tumor implants placed directly on the surface of the iris revealed a consistent pattern in all stock and experimental animals. These are represented schematically in Fig. 1.

Day 1: Iris implant appears as a small, white sliver surrounded by a haze of fibrin. A mild iritis is present near the corneal incision which has sealed.

Day 2: First evidence of growth is seen as a thin, gray cobblestoned coating covering the implant surface and adjacent iris. Iritis has resolved.

Days 3–5: Implant continues to grow slowly as a thin, gray plaque. An intense, localized proliferation of small iris vessels has developed around its periphery. Fluorescein testing demonstrates increased perfusion of this area of the iris, but the implant plaque remains unperfused.

Days 5–8: Vessels are visible within the implant, which has increased significantly in size, forming a thick, pink hemispherical mass. The surrounding iris develops corrugations and large vessels connect the implantation site with the limbus. Fluorescein testing readily demonstrates perfusion of the growing iris tumor.

Days 8–12: Tumor size increases exponentially. The perilimbal plexus becomes engorged and long (2–3 mm) vascular loops grow into that portion of the cornea overlying the iris tumor. A dense network of engorged, serpiginous vessels is evident over the entire iris.

Days 12–14: Progressive growth results in a distortion of the pupil and gradually the entire anterior chamber is filled by the vascular tumor mass.

Days 15–16: A dense, circumferential outgrowth of corneal vessels has developed, and corneal edema obscures the anterior chamber. The globe protrudes from the orbit and unless the experiment is terminated, spontaneous rupture occurs.

A histologic section of a well-developed vascularized iris tumor (day 8) is illustrated in Fig. 2. The iris and ciliary body have been infiltrated by the growing tumor. The tumor consists of anaplastic epithelial cells and contains many small blood vessels (Fig. 3).

The microscopic structure of an early implant (day 3) is seen in Fig. 4. A thin plaque of tumor cells is seen on the surface of the iris. The cells appear well-preserved and mitotic figures are seen. Small vessels are present in the subjacent iris, but the tumor is not yet vascularized.

When calculated daily tumor volumes were plotted on a semilogarithmic scale, all iris implants appeared to follow a characteristic growth curve (Fig. 5).

Its sigmoid shape correlates with three distinct phases of tumor development.

Phase I.—

Prevascular (days 1–5): The small implant grows slowly (slope *a*) as a thin plaque, dependent upon diffusion for nutrient supply and catabolite removal. A

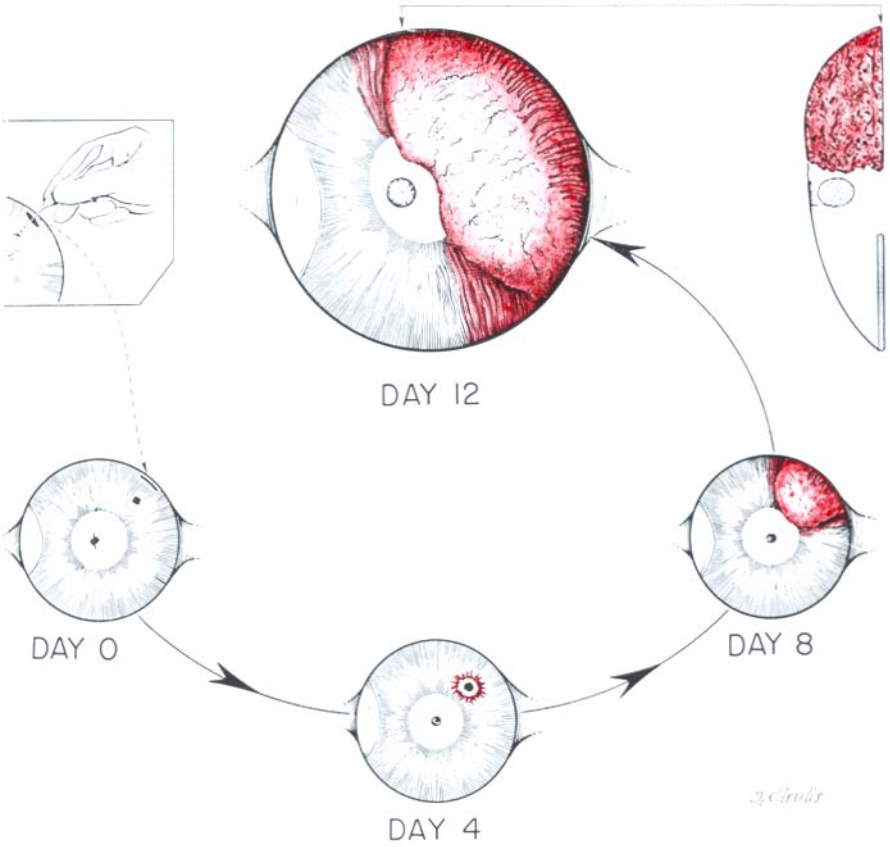


FIG. 1. The patterns of development of two simultaneous implants of Brown-Pearce tumor in the rabbit eye. The anterior chamber implant remains avascular and stops expanding at a small size, while the iris implant vascularizes and grows progressively. Inset demonstrates technique of placing a small tumor fragment on iris through a peripheral corneal incision.

neovascular response is being elicited from adjacent host tissues but the implant is not yet perfused (negative fluorescein test).

Phase II.—

Vascular (days 6–11): An abrupt change in growth rate (slope *b*) is noted once vascularization has occurred (positive fluorescein test). The perfused tumor then begins to grow exponentially, doubling its volume every 11.5 hr on the average (10 experiments).

Phase III.—

Late growth (days 12–14): As the volume of tumor approaches that available in the anterior chamber, growth slows (slope *c*).

Slopes *a*, *b*, and *c*, for 10 iris implants, are tabulated in Table I. The rate of growth during the prevascular phase (slope *a*) is approximately one-tenth that during the vascular phase (slope *b*). The primary inflection of the growth curve (*a* to *b*) is seen to coincide in each case with the change from "negative" to "positive" in the fluorescein test, i.e., with vascularization of the implant.

Volume doubling times, $(t_D) = \log_{10} 2/\text{slope}$, are summarized in Table II. Tumor volume doubled every 0.48 days (11.5 hr) during the vascular phase, compared with every 4.95 days before vascularization (mean values for 10 experiments).

At 14 days, vascular iris implants had increased an average of more than 15,000 times their initial volume, in a series of 10 experiments (Table II). Mean final volume was 331 mm^3, which approaches the total volume available in the anterior chamber of the rabbit eye.

Avascular Anterior Chamber Implants.—

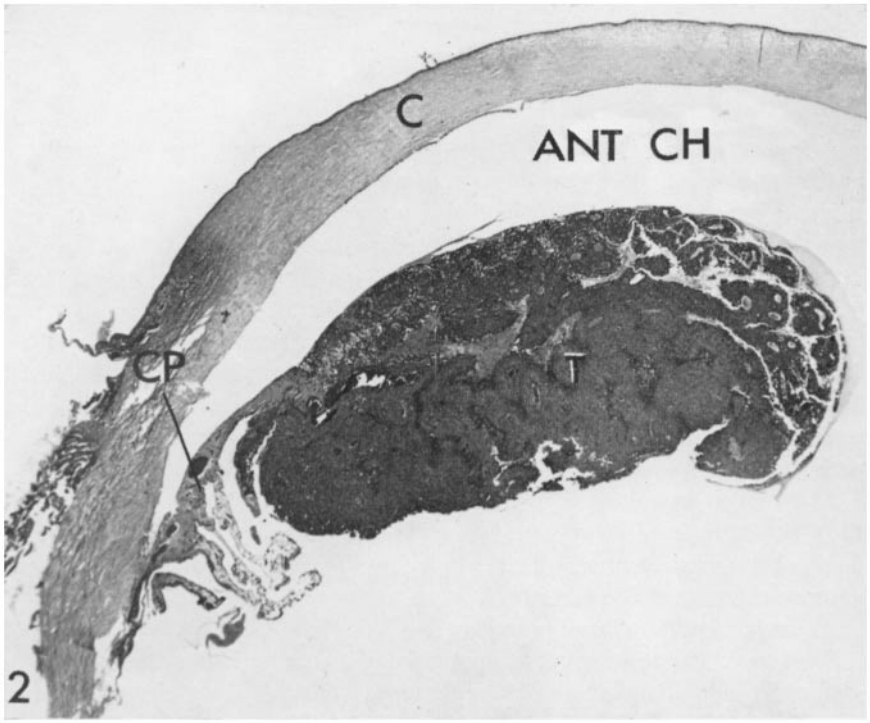
Short term: The relative growth potential of avascular implants was initially studied by paired implantation of two comparable small pieces of tumor: in one eye, directly on the iris, and in the other, suspended in the anterior chamber. In every instance, iris implants vascularized and grew progressively as described above, forcing termination of the experiment within 14–16 days. In contrast, all anterior chamber implants failed to vascularize, remaining as small, pearly white masses unperfused on fluorescein testing.

Daily measurements often proved technically difficult during the 1st few days because of adherent fibrin strands. However, when early observations were possible, two phases of growth were evident. During the 1st 6–8 days, avascular implants tended to increase slightly in size, becoming ovoid in shape and covered with a gray cobblestoned cortex (as seen under × 40 magnification); a dormant period then followed during which no significant further increase in size was noted (Fig. 1).

A microscopic cross section of a typical avascular tumor (BP No. 11L) early in this dormant period (day 7), is seen in Fig. 6 A. A cortex of histologically well-preserved tumor cells surrounds a core of more vacuolated cells, some of which appear to be degenerated or frankly necrotic (Fig. 6 B). Mitotic figures are found in the outer layer. No vessels are present. The in vivo diameter was 0.8 mm, corresponding to a calculated volume of 0.27 mm^3, and had not increased during the 48 hr preceding sacrifice.

At 14 days, avascular tumors had increased an average of four times their initial volume in a series of 10 experiments. Mean final volume was 0.56 mm^3 ($\pm$0.24 SD).

This striking contrast between avascular and vascular tumor growth potentials is demonstrated graphically in Fig. 7. The mean daily volumes ($\pm$SD) of 10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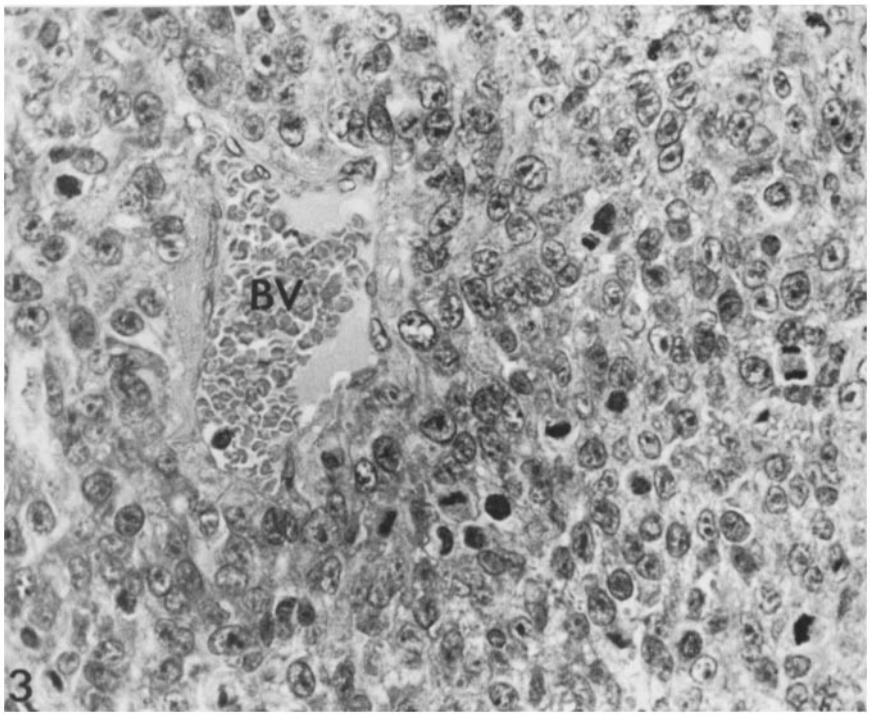
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avascular anterior chamber implants are plotted on a linear scale over a 14 day period. No implant attained a volume greater than 1 mm^3 during this time interval. A typical iris implant growth curve (BP No. 29R) is superimposed for comparison. Its early growth, during the prevascular phase (days 1–5), falls within the range of the avascular implants. However, once it has vascularized (positive fluorescein test on day 6), growth becomes exponential, enabling it to attain a final volume of 394 mm^3 (day 14), approximately 16,000 times its initial volume.

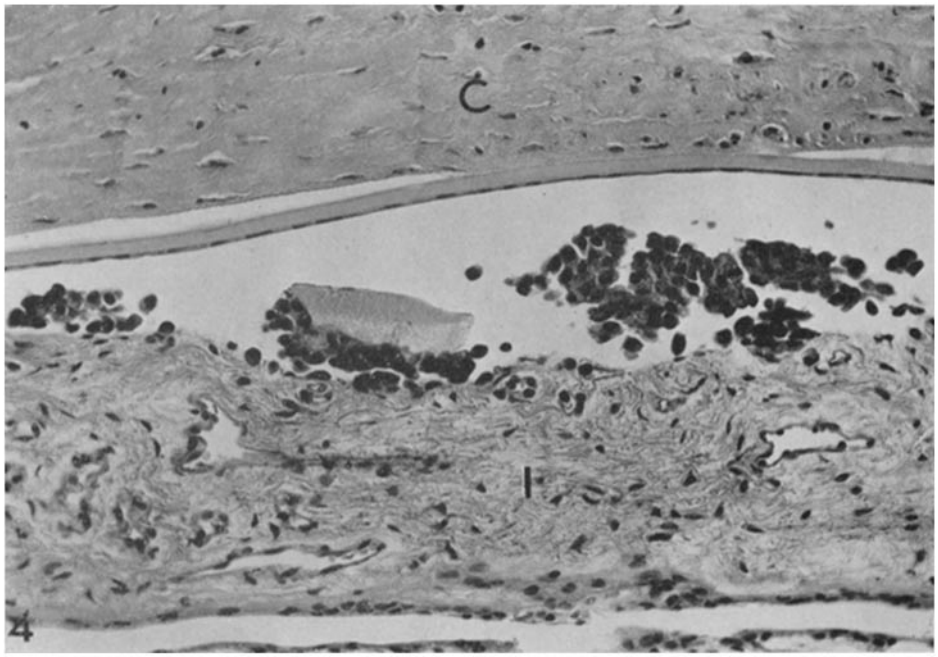


FIG. 4. Section through a "prevascular" tumor implant, showing a plaque of well-preserved tumor cells on the surface of the iris. *C*, cornea; *I*, iris. Approx. × 250.

Avascular Anterior Chamber Implants.—

Long term: In order to study the behavior of avascular tumors over a longer period, "solitary" anterior chamber implants were made. However, even in this situation, rapidly growing, vascular iris tumors developed, presumably from cells shed during the initial procedure or subsequently from the viable but

FIG. 2. Light micrograph of a large vascularized tumor 8 days after implantation on the iris. The tumor (*T*) has completely replaced the iris proper and invaded the ciliary body. *ANT. CH*, anterior chamber; *C*, cornea; *CP*, ciliary processes. Stained with hemotoxylin and eosin. × 15.

FIG. 3. A higher magnification of the vascularized tumor. The epithelial tumor cells are anaplastic and exhibit numerous mitoses. *BV*, blood vessel. Approx. × 400.

relatively dormant avascular implant. Therefore, certain implants were sterilely removed after 14–21 days' observation and reimplanted in the anterior chambers of fresh animals. In this manner, avascular tumors were followed for greater than 6 wk.

A direct comparison of final to initial volumes in these "second generation" implants is probably not valid because of the artifact of surgical reimplantation.

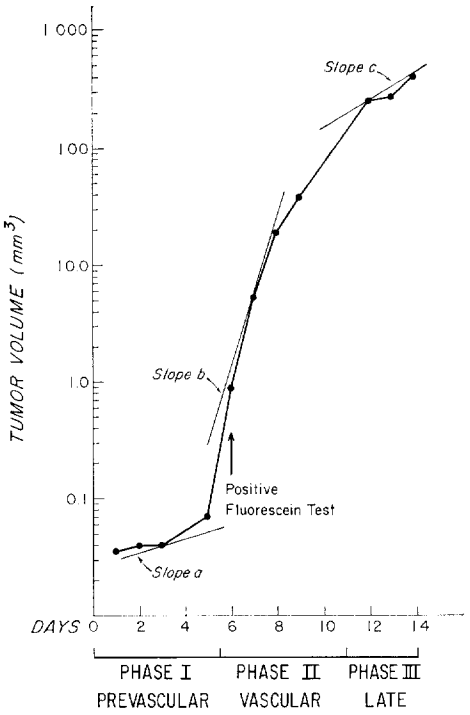


FIG. 5. The characteristic growth curve of an iris implant (BP No. 29R) plotted on a semi-logarithmic scale. Positive fluorescein test on day 6 represents earliest evidence of perfusion of the tumor, and coincides with the beginning of exponential volume increase. Slopes "*a*," "*b*," and "*c*," corresponding to prevascular, vascular, and late phases of growth, are indicated.

However, tumors followed for 16, 21, 34, and 44 days retained their ovoid shape and gray cobblestoned surface, but failed to increase significantly in size. Their final volumes averaged less than 0.5 mm^3. Avascularity remained apparent on slit lamp examination and was readily verified by fluorescein testing.

Microscopic examination of cross sections of 16-, 34-, and 44-day old tumors confirmed their avascularity. These tumors consisted of a central core of necrotic cells and debris surrounded by layers of well-preserved tumor cells (Fig. 8), in which occasional mitotic figures were seen (Fig. 9). In those experiments in

TABLE I

Rates of Growth and Time of Vascularization of 10 Iris Tumors

Experiment No.	Growth curve slopes* (Prevascular) *a*	(Vascular) *b*	(Late) *c*	Primary inflection (*a* to *b*)	Vascularization‡
				day	*day*
1	0.04	0.49	0.23	5–7	7
2	0.05	0.68	0.14	5–6	6
3	0.08	0.58	—	5–6	6
4	0.03	0.96	0.14	6–7	7
5	0.04	0.87	0.25	6–7	7
6	0.16	0.54	0.13	4–6	6
7	0.08	0.51	0.21	6–7	7
8	0.19	0.70	0.15	5–6	6
9	0.06	0.64	0.12	5–6	6
10	0.12	0.56	0.12	5–7	7
Mean ± SD =	0.085 ± 0.055	0.65 ± 0.155	0.16 ± 0.051		

* Estimated from semilogarithmic plots (see text).

‡ "Positive" fluorescein test or gross appearance of tumor vessels.

TABLE II

Volume Doubling Times and Final Volumes of 10 Iris Tumors

Experiment No.	Volume doubling time* Prevascular	Vascular	Late	Final volume‡	Final volume/initial volume§
		days		*mm³*	
1	7.50	0.61	1.31	224	9,333
2	6.00	0.44	2.15	306	6,008
3	3.76	0.52	—	350	1,555
4	10.0	0.31	2.15	230	38,333
5	7.5	0.35	1.20	162	16,164
6	1.9	0.56	2.32	400	33,333
7	3.76	0.59	1.43	400	1,481
8	1.58	0.43	2.00	368	12,270
9	5.01	0.47	2.50	394	15,760
10	2.50	0.54	2.51	480	4,800
Mean ± SD =	4.95 ±2.00	0.48 ±.019	1.95 ±0.176	331 ±153	15,438 ±3,546

* Calculated time interval for doubling of tumor volume during prevascular, vascular, and late phases of growth, based on average rates of change of volume (slopes *a*, *b*, *c*) during each interval ($t_D = \log_{10} 2/\text{slope}$).

‡ Calculated tumor volume on day 14.

§ Initial volume of implants averaged less than 0.10 mm³.

which thymidine-^{3}H had been injected into the anterior chamber 45 min before sacrifice, radioautographs of growth-arrested avascular tumors demonstrated labeled nuclei in the peripheral cell layers (Fig. 10).

The malignant growth potential of dormant-appearing 16- and 34-day old tumors was demonstrated by reimplantation directly on the iris in fresh animals.

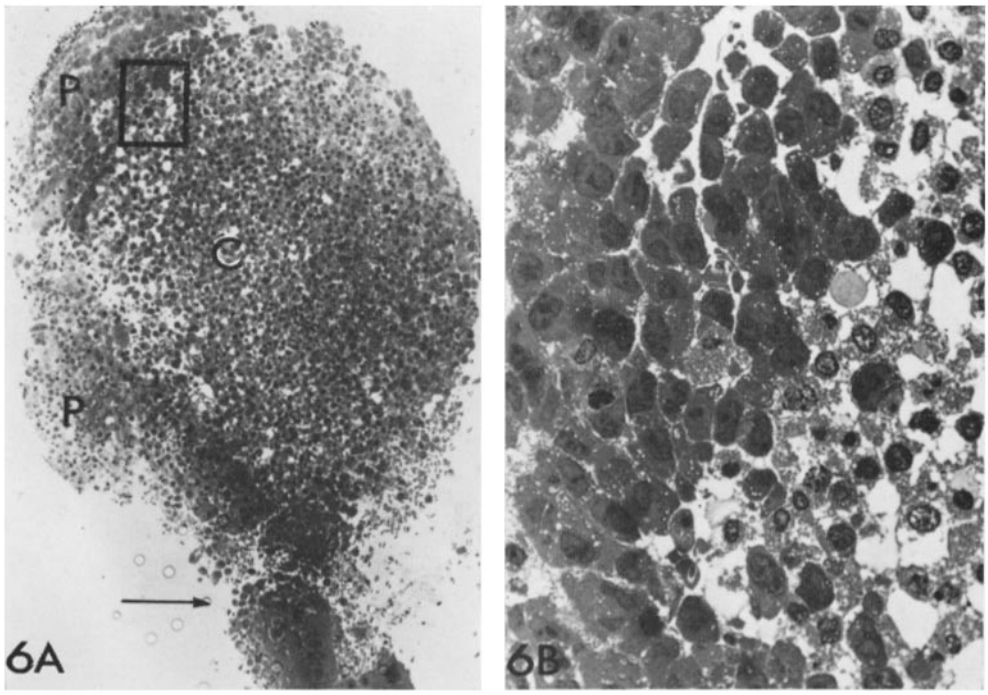


FIG. 6. (A) Cross section through an avascular anterior chamber tumor 7 days after implantation. The tumor consists of a solid mass of cells which appear vacuolated and occasionally necrotic in the center (*C*) but well-preserved in periphery (*P*). The *arrow* points to the coagulum stalk tethering the tumor to the corneal endothelium. 1 μ thick Epon section stained with toluidine blue. × 110. (B) Higher magnification of the area outlined in Fig. 6 A showing transition between vacuolated and intact cells.

After an initial prevascular phase, a vascular iris tumor developed, which grew rapidly, filling the anterior chamber. The histologic appearance of these reimplanted tumors was typical of any vascularized iris implant (Fig. 11).

DISCUSSION

These experiments provide in vivo evidence that prevention of neovascularization can block the growth of a solid tumor at an early stage. It appears that vascularization permits an implant to enter exponential growth, while avascularity forces it to remain dormant at a small size.

Avascular carcinomas were created by anterior chamber implantation in a susceptible rabbit strain. Unable to elicit new capillary ingrowth, these small spherical tumors appeared to survive by simple diffusion, suspended in the aqueous humor. After a brief period, each ceased growing at a diameter less than 1 mm and then remained dormant for as long as 6 wk. That these avascular spheroids were dormant, rather than necrotic, was demonstrated by (*a*) his-

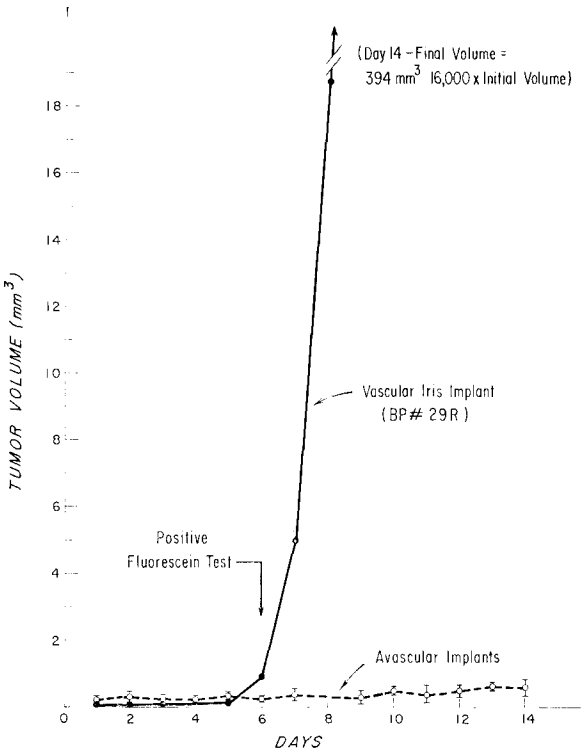


FIG. 7. A typical iris implant growth curve (BP No. 29R) and the mean daily volumes of 10 avascular anterior chamber implants are plotted on a linear scale for comparison. Positive fluorescein test on day 6 indicates time of vascularization of iris tumor.

tologic sections that showed intact cells with thymidine-^{3}H-labeled nuclei and mitotic figures in the periphery of the mass, and (*b*) their ability to grow rapidly when transplanted to a site where vascularization could occur.

Control studies in which comparable small implants were placed directly on the iris revealed that each passed through a prevascular growth phase early in its course. This period was objectively defined by testing for perfusion with intravenous fluorescein. A sharp inflection in the growth curve of each iris implant correlated with the time of vascularization. Volume doubling times in the vascular phase averaged 11.5 hr, compared with 4.95 days in the prevascular

phase. In contrast, the growth curves of avascular anterior chamber tumors reached a plateau within the range of early prevascular iris implants and remained there as long as neovascularization was prevented.

The fact that neovascularization is a necessary condition for malignant growth was again demonstrated when dormant avascular tumors were placed in

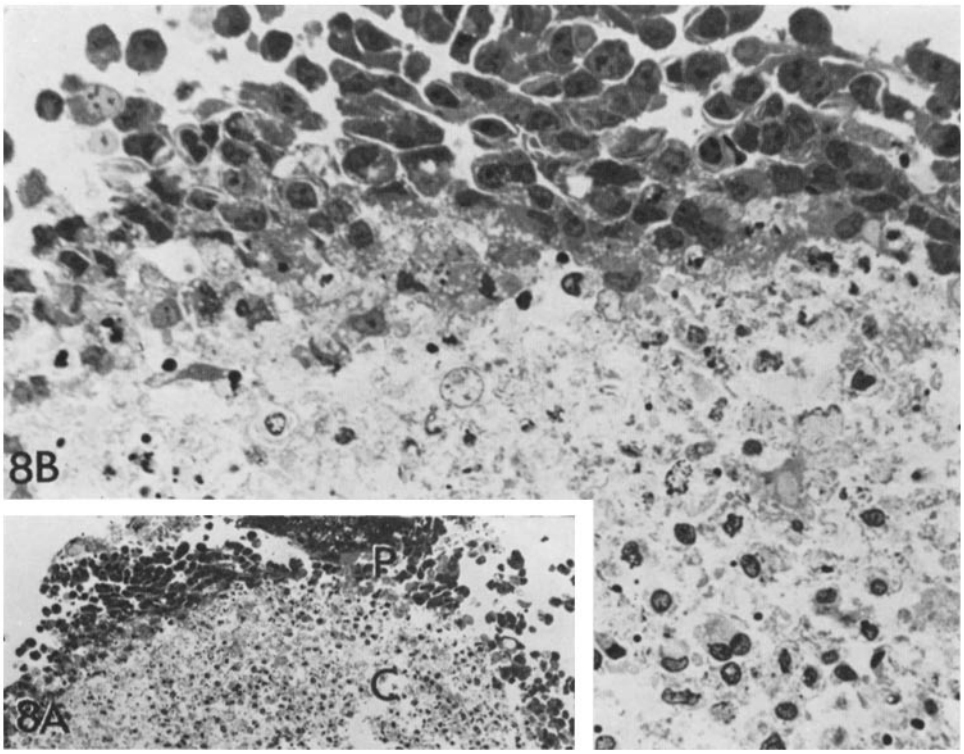
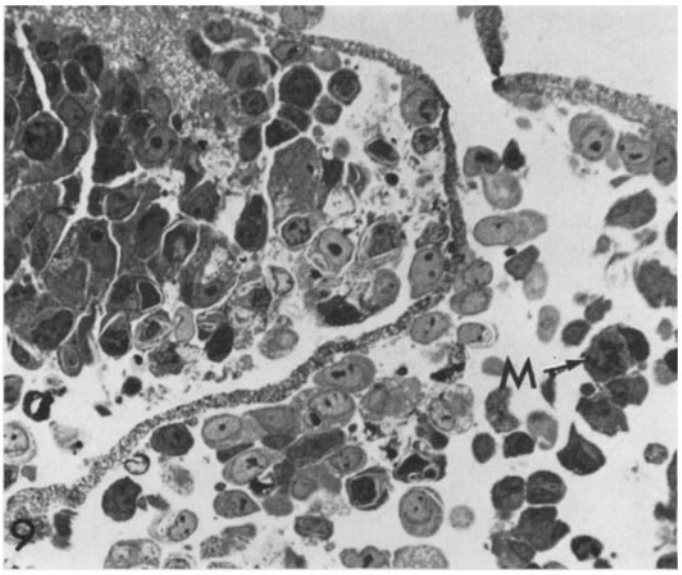


FIG. 8. (A) 1 μ thick Epon section of an avascular tumor 34 days after implantation into anterior chamber. Note the necrotic center (*C*) and cellular periphery (*P*). × 100. (B) Higher magnification of area outlined in Fig. 8 A, showing boundary between necrotic and intact cells. Stained with 1% toluidine blue. × 500.

contact with the iris. Vascularization was followed by growth to several thousand times initial volume within 10–14 days.

Histologic sections of avascular spheroids revealed several layers of intact tumor cells, containing mitotic figures and thymidine-^{3}H-labeled nuclei, covering a core which became more necrotic in older implants. This suggested that a balance of cell renewal and cell death might exist in these dormant tumors.

In the previous literature, "dormancy" has been used to refer to individual tumor cells which were thought to persist "symbiotically" for long periods, but



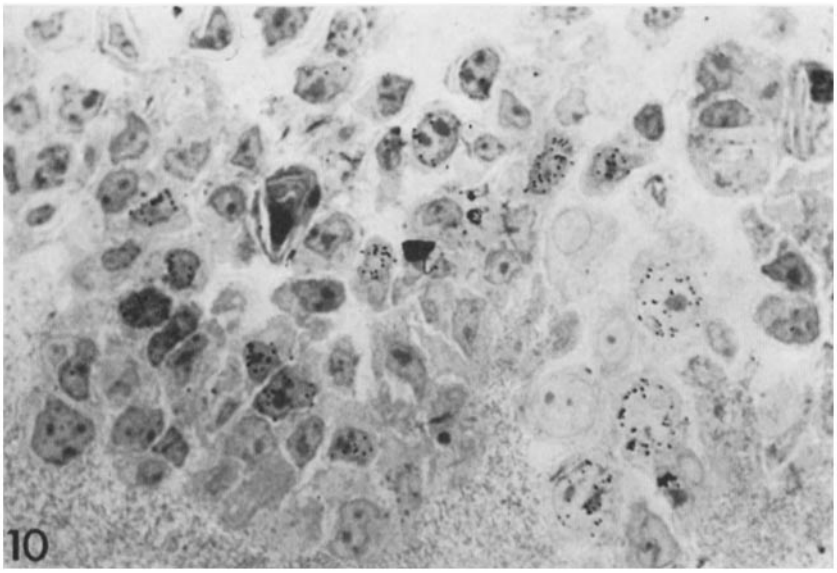


FIG. 9. 1 μ thick Epon section of tumor cells from the periphery of an avascular tumor 16 days after implantation. Note mitotic figure (*arrow*). × 400.

FIG. 10. Radioautograph of a 1 μ thick Epon section taken from the periphery of an avascular tumor 16 days after implantation and 45 min after injection of thymidine-^{3}H into the anterior chamber. Note the large number of labeled nuclei. × 400.

subsequently could be stimulated to malignant growth (9, 10). Arrest of the cell cycle or special immunologic characteristics have been suggested as possible mechanisms (11). The current studies provide evidence for a new concept of dormancy, the growth arrest of a population of neoplastic cells which does not become vascularized. Though a portion of its cells remain viable and mitotically active, total expansion of the avascular tumor mass ceases at a small size. Viewed in this light, dormancy need not be an attribute of a tumor cell per se, but rather that of a small mass of tumor cells which has failed to vascularize and therefore cannot express its potential for malignant growth.

In this way, spontaneous failure of tumor neovascularization could account for clinical instances of delayed metastasis or local recurrence. Similarly, in

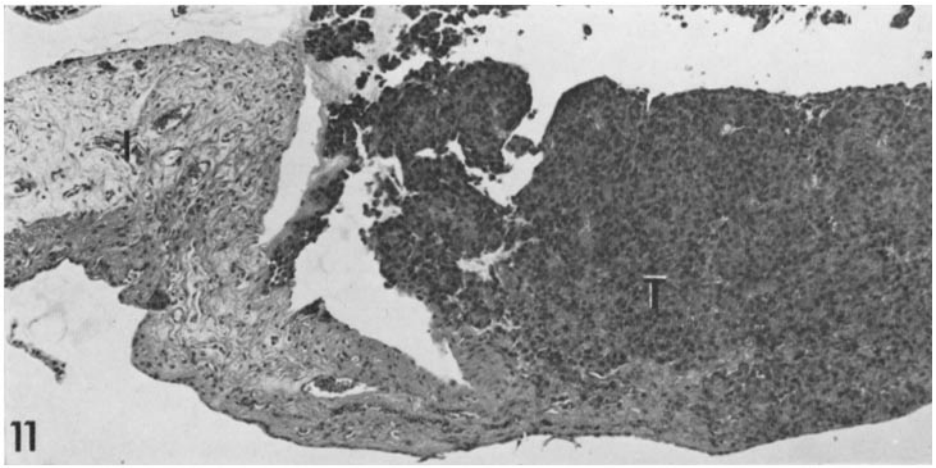


FIG. 11. Hematoxylin-eosin section from an iris (*I*) on which an avascular anterior chamber tumor was implanted. The tumor (*T*) became vascularized and grew rapidly. × 200.

those animal models in which surgical trauma, wound healing, or regeneration are the triggering events for activation of dormant tumor foci (10, 12), new vessel outgrowth may be playing an important role.

It is unlikely that the dormancy of avascular tumors observed in this study is the result of immunologic mechanisms. Cells shed from dormant anterior chamber spheroids were able to develop into large tumors if they became implanted on the iris and vascularized. Also avascular tumors transplanted into a fresh host showed no tendency to increase in size.

Similarly, this dormancy cannot be explained by some "growth inhibitor" in the aqueous humor. Iris implants were exposed, during their prevascular phase, to the same milieu as avascular tumors, yet remained capable of malignant growth. Also mitotic figures and thymidine-^{3}H-labeled tumor cells were found on the surface of dormant spheroids, in intimate contact with the aqueous humor.

The mechanism of this population dormancy, occurring when neovascularization has been prevented, is not elucidated by these experiments. However, recent studies by Folkman and Hochberg,[6] in this laboratory, suggest an explanation. Spheroidal colonies of malignant cells suspended in soft agar medium consistently stop expanding at a small size despite provision of fresh nutrient medium. Histologic sections of these growth-arrested spheres show a thin cortex of viable, mitotically active cells surrounding a necrotic core, similar to the avascular tumors described above. Radioactive thymidine labeling reveals a progressive decrease in the DNA-synthetic cell fraction as these colonies approach their maximum diameter. These authors propose that this reflects the accumulation of growth-inhibiting metabolites in a diffusion-limited spherical mass. These colonies thus appear to be an in vitro analogue for the small, dormant avascular tumors observed in the current study.

Population dormancy, therefore, appears to be the fate of avascular solid tumors. If neovascularization can be prevented, local malignant growth and perhaps distant metastasis (13) will not occur. These observations thus suggest that specific blockade of tumor-induced angiogenesis would be effective in controlling neoplastic growth.

SUMMARY

Dormant solid tumors were produced in vivo by prevention of neovascularization.

When small fragments of anaplastic Brown-Pearce carcinoma were implanted directly on the iris in susceptible rabbits, they always vascularized. A characteristic growth pattern, consisting of prevascular, vascular, and late phases, was observed, which terminated with destruction of the eye within 2 wk. The beginning of exponential volume increase was shown to coincide with vascularization of the implant, as demonstrated by perfusion with intravenous fluorescein and by histologic sections.

In contrast, implants placed in the anterior chamber, at a distance from the iris, did not become vascularized. After initial growth into spheroids, they remained arrested at a small size comparable to prevascular iris implants, for periods as long as 6 wk. Although dormant in terms of expansion, these avascular tumors contained a population of viable and mitotically active tumor cells. When reimplanted on the iris, vascularization was followed by rapid, invasive growth.

These observations suggest that neovascularization is a necessary condition for malignant growth of a solid tumor. When a small mass of tumor cells is prevented from eliciting new vessel ingrowth from surrounding host tissues, population dormancy results. These data suggest that the specific blockade of tumor-induced angiogenesis may be an effective means of controlling neoplastic growth.

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[6] Folkman, J., and M. Hochberg. Unpublished data.

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