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Control of tumor growth in animals by infusion of an angiogenesis inhibitor

(cartilage/neovascularization/antiangiogenesis/B16 melanoma/V2 carcinoma)

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ABSTRACT Angiogenesis and tumor growth were inhibited in two different animal models by regional infusion of a partially purified cartilage extract. In rabbits bearing corneal implants of V2 carcinoma and receiving the inhibitor, vascular growth rates were <3% of those in control animals receiving either Ringer's solution or bovine trypsin inhibitor (Trasylol). Subconjunctival B16 melanoma implants in mice receiving the inhibitor weighed <2.5% of implants in mice receiving Ringer's solution, Trasylol, or albumin. Histologic study of major organs and standard blood tests revealed no toxic effects in any of the animals. The inhibitor did not retard the growth of either tumor cell type in tissue culture at concentrations as high as 1 mg/ml. These results suggest that the cartilage factor does not interfere with the growth of the tumor cell population directly but that it prevents tumor growth by inhibiting angiogenesis.

The capacity to induce the growth of new capillaries in the host is a characteristic common to most solid malignant tumors. This phenomenon has been called "tumor angiogenesis." It has been hypothesized (1, 2) that until a tumor nodule is penetrated by new capillaries the limitations imposed by diffusion of oxygen and nutrients prevent it from growing beyond a few millimeters in diameter. This hypothesis implies that inhibition of angiogenesis, by whatever means, might control tumor growth. For this reason, "antiangiogenesis" was proposed as a potential therapeutic approach (3).

Indirect evidence for this hypothesis was drawn from experiments with tumors in isolated perfused organs (4), tumor spheroids grown in soft agar (2), and tumor implants floating in the anterior chamber of the rabbit eye (5).

Sorgente *et al.* (6) showed that when cartilage was extracted with 1 M guanidine it lost its resistance to vascular invasion. Eisenstein *et al.* (7) reported that a 1 M guanidine extract of cartilage inhibited the growth of aortic endothelial cells in culture. Brem and Folkman (8) showed that an implant of cartilage placed adjacent to a V2 carcinoma in the rabbit cornea inhibited growth of new blood vessels toward the tumor. Langer *et al.* (9) isolated a partially purified factor from cartilage that, when administered locally by a sustained-release polymer (10), inhibited angiogenesis induced by V2 carcinoma implanted in the rabbit cornea.

We now show that this angiogenesis inhibitor limits tumor growth by stopping neovascularization when the inhibitor is infused regionally in either mice or rabbits. Furthermore, the inhibitor appears to have no toxic effect on tumor cells and no short-term toxicity for the host.

In one set of experiments, the tumor (V2 carcinoma) was separated from the nearest vascular bed by a relatively large distance (approximately 1.0 mm) by implanting the tumor in the rabbit cornea. This system permitted daily measurements of vessel growth rate and demonstrated that vascular proliferation was inhibited and, subsequently, tumor growth was controlled.

In a second set of experiments, the tumor (B16 melanoma) was implanted directly onto a dense capillary bed in the mouse conjunctiva. This contiguous relationship between tumor and vascular bed more closely resembles most human and animal tumors. In this experimental design, tumor growth was measured directly and the vascular response was determined histologically. Regional infusion of the angiogenesis inhibitor again prevented neovascularization and markedly limited tumor growth.

MATERIALS AND METHODS

Rabbit V2 Carcinoma Tests. *Animals.* Male New Zealand white rabbits weighing 1.8–2.3 kg were used.

Tumor. V2 carcinoma (11) was maintained by serial intramuscular and subcutaneous injection (8). Tumors were harvested before they reached a diameter of 2.0 cm, and 1.5 × 1.5 mm pieces were implanted into the cornea of the rabbit 1.0 mm central to the limbus as described (8, 9).

Infusion system. Rabbits were infused through the right common carotid artery at a rate of 14.4 ml per day with the infusion system described by Conn and Langer (12). In this way, the inhibitor was delivered preferentially to the right eye; the left eye received inhibitor at a lower effective dose (Fig. 1).

Measurements. Rabbit corneas were examined with a Zeiss slit-lamp microscope at ×6 to ×10 magnification. The length of the longest vessel (accuracy, ±0.1 mm), measured from the limbus, was recorded.

Mouse Melanoma Experiments. *Animals.* Male C57BL/6J mice weighing 25–30 g were used.

Tumor. B16 melanoma was obtained from Arthur D. Little Company and serially passaged in mice as subcutaneous axillary implants. Fragments (1.0 × 1.0 mm) of tumor were excised 14 days after the subcutaneous implantation, kept in lactated Ringer's solution for <35 min, and implanted onto the conjunctiva as described below.

Mice were anesthetized by intraperitoneal injection of 3.6% chloral hydrate at 0.1 ml/10 g of body weight. Ether inhalation anesthesia was used as necessary for supplementation.

The eye was proptosed and held in position with a small hemostat at the lateral canthus. Under ×15 magnification, an incision was made into the bulbar conjunctiva at the limbus superiorly by using a glass micropipette (Pyrex capillary tubes,

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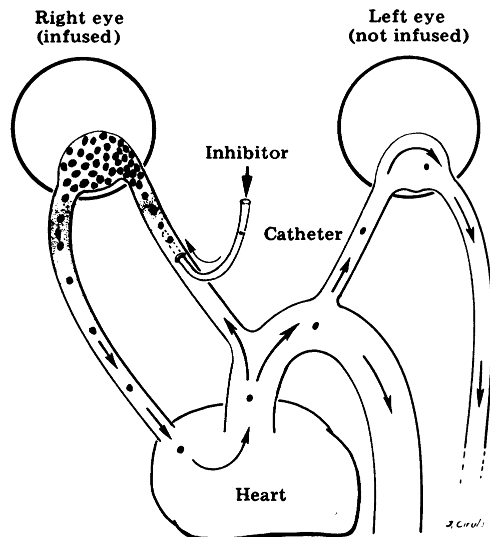

FIG. 1. Diagram of infusions through the right common carotid artery in both rabbits and mice. Technetium pertechnate scans demonstrated that the concentration reaching the right eye was 15- to 60-fold greater than that reaching the left eye.

1.0 mm outside diameter, 0.2 mm wall width; Fredrick Dimmock, Inc., Millville, NJ) with a sharp beveled edge approximately 70 μm in diameter. With microsurgical forceps, this incision was widened and a subconjunctival pocket was developed peripheral to the limbus, 2.0 mm in length and 1.0 mm in width. The tumor implant was then placed into the pocket. Tumor in a subconjunctival pocket rests in a vascular bed, in contrast to the rabbit corneal pocket where the tumor lies remote from the vascular bed.

Infusion system. Mice were infused at a rate of 0.96 ml/24 hr with a system modified from the technique of Brown and Goffinet (13). By positioning a silicone catheter in the right common carotid artery, the infusion system was thus arranged to deliver high concentrations of inhibitor to the right eye and orbital tissues and low concentrations to the left eye and orbital tissues (Fig. 1). The other end of the catheter was tunneled subcutaneously and brought out through the flank and taped to the tail and to a rubber band swivel apparatus. Animals were individually housed and had access to food and water ad lib. The fluid was delivered by a Harvard infusion pump (Harvard Apparatus Co., Millis, MA) that was modified to hold 1.0-ml syringes.

Measurements. Tumors were measured (animals under ether anesthesia) starting on day 5 after implantation. A Nikon stereomicroscope was used with an accuracy of ±0.1 mm. Mice were sacrificed on day 7 and the tumors were excised and weighed. Measurements were made without knowledge of how the animals had been treated.

Preparation of Angiogenesis Inhibitor. The angiogenesis inhibitor was prepared by a modification of our earlier method (9). Cartilage was excised from veal scapular bones within 48 hr after slaughter and all connective tissue was carefully removed. Cartilage slices (2 kg) were then extracted in 20 liters of 1.0 M guanidine·HCl/0.02 M sodium malate, pH 6, for 24 hr at 25°C. This solution was filtered and then concentrated to 500 ml in a Millipore ultrafiltration unit containing a PTGC cassette (cutoff, M_r 10,000). The retentate was dialyzed against water for 12 hr at 4°C and then desalted by using the same Millipore unit. The solution was judged to be effectively desalted when a drop of it added to a drop of 1% $AgNO_3$ gave no precipitate. It was then lyophilized, subjected to trypsin-Sepharose affinity chromatography, and sterilized as described (9).

Infusion Solutions. Angiogenesis inhibitor, Trasylol (a bovine trypsin inhibitor, Sigma), and bovine serum albumin (Sigma) were dissolved in lactated Ringer's solution (Abbott) and heparin was added to a concentration of 6 units/ml. Protein contents of all solutions as determined by the Lowry method (14) were identical.

In each set of tests, three experimental animals and up to 95 controls were used. The number of experimental animals was limited because of the scarcity of the inhibitor. To obtain 1 mg of the partially purified angiogenesis inhibitor, 500 g of cartilage was required.

The doses and patterns of administration used in both sets of experiments were determined by preliminary screening experiments. Because of the limited amount of inhibitor available, our experiments were designed to determine whether we could affect the vascular and tumor growth rates rather than to determine median lethal doses or to obtain a dose-response curve.

Mice received approximately 100 times the dose of inhibitor given to rabbits on a weight basis. This was because mouse infusions were started after the rabbit study was complete, and our first goal was to establish the toxic or lethal concentration for the inhibitor. Our original dose of 4 mg/day caused no host toxicity, yet angiogenesis inhibition was so striking (see *Results*) that we continued the series at this level.

Histology and Blood Tests. Rabbits and mice were sacrificed by using anesthesia at a lethal level, and the eyes including cornea and sclera were removed along with other major organs and fixed in 10% buffered formalin. Histologic sections were embedded in paraffin or Epon and were stained with hematoxylin/eosin or methylene blue. Blood was drawn from rabbits before sacrifice, and standard determinations of liver and renal functions, electrolyte balance, and hematologic functions were performed.

Cell Culture. Pieces of V2 carcinoma removed during tumor passage were minced and incubated in trypsin/EDTA solution (GIBCO, calcium- and magnesium-free) for 30 min at 37°C and washed with Eagle's medium (Flow Laboratories, Rockville, MD) supplemented with 10% calf serum (GIBCO), penicillin (50 units/ml), and streptomycin (50 μg/ml). The cells were plated in 60-mm culture dishes and allowed to grow to confluence.

Pieces of B16 melanoma removed during tumor passage were treated in the same way and also were grown to confluence.

B16 melanoma and V2 carcinoma cells were trypsinized from confluent plates and placed in the wells of a 24-well plate (Falcon, no. 3001) at a density of 5000 cells per well as determined by a Coulter particle counter (Coulter Electronics, model 2F, Hialeah, Florida). To ensure satisfactory plating, after 8 hr the cells were incubated in Dulbecco's medium with 10% calf serum containing Trasylol, bovine serum albumin, or angiogenesis inhibitor at protein concentrations from 1 μg/ml to 4 mg/ml. The controls consisted of cells incubated in medium with 10% serum alone. The cells were counted by observing five high-power fields across the center of each well by phase-contrast microscopy on days 1, 2, and 3 after plating. At the end of day 3, the cells were fixed in methanol and stained with 1% crystal violet. The cells in each well were counted again in five fields with the aid of a grid.

RESULTS

Rabbit Infusions. *Vascular Growth.* Three days after new blood vessels began growing toward the tumor, infusion of the cartilage-derived inhibitor was started in three rabbits. Controls consisted of 21 corneas infused with Ringer's solution, 4 infused with Trasylol, and the 3 untreated eyes of the experimental animals. In the control animals, the mean corneal vessel growth

rates were 0.32 mm/day (Ringer's), 0.35 mm/day (untreated eyes in experimental group), and 0.46 mm/day (Trasylol) over the 6-day infusion period. In contrast, the average growth rate of vessels in the right corneas of the animals receiving the cartilage-derived inhibitor was 0.01 mm/day (Fig. 2).

The angiogenesis inhibitor affected the density of vessels as well as their rate of growth. Although all controls showed an increase in vessel number from approximately 4 to >100 vessels per cornea during the 6-day infusion period, 2 of the 3 treated corneas showed no increase in vessel density. The third cornea showed only a small increase in vessel density and a 0.1-mm increase in length. Yet, even this cornea was markedly less vascularized than the most mildly vascularized cornea of the controls (Fig. 3).

For 2 days, the rabbits were infused at a slightly higher level of inhibitor (3 mg/day) to determine if this increased dose would inhibit vascular growth in the contralateral eye (Fig. 2). We were not able to observe any quantitative differences compared to controls.

Tumor growth. All 28 controls developed large three-dimensional vascularized tumors. By contrast, the tumors in the treated eyes grew much more slowly and did not become vascularized during the infusion. New vessels resumed growth toward the tumor implant after the inhibitor infusion was discontinued (although in some cases a lag phase was observed) (Fig. 2). When the vessels reached the edge of the tumor, tumor growth also resumed. However, in two of the three treated animals, the tumors eventually regressed after vascularization.

Toxic effects. Gross and microscopic findings in all organs were within normal limits in the treated animals as well as in control animals with tumor and control animals without tumor. Also, leukocyte count, hematocrit value, and results of tests of liver and kidney function were within normal limits (15, 16). Alkaline phosphatase activity of the treated animals was below normal values (16) (and that of tumor-bearing and non-tumor-bearing controls) by a factor of 3.

Mouse Infusions. *Tumor growth (B16 melanoma).* Accurate measurements of tumor growth were attainable only after day 4 following implantation. Twenty-four hours after im-

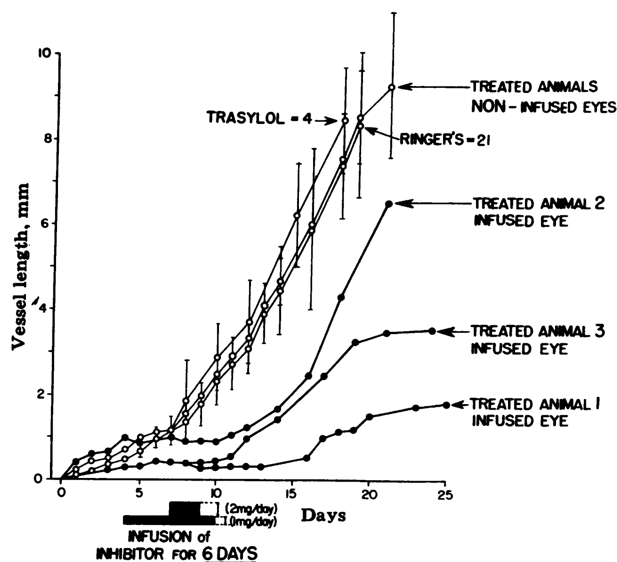

FIG. 2. Inhibitor and control solutions were infused through the right carotid artery in rabbits carrying V2 carcinoma in each cornea. Rabbits were sacrificed when tumors became large, protruding masses. The vessel length at this time averaged 9 mm. Results are shown as mean ± SD.

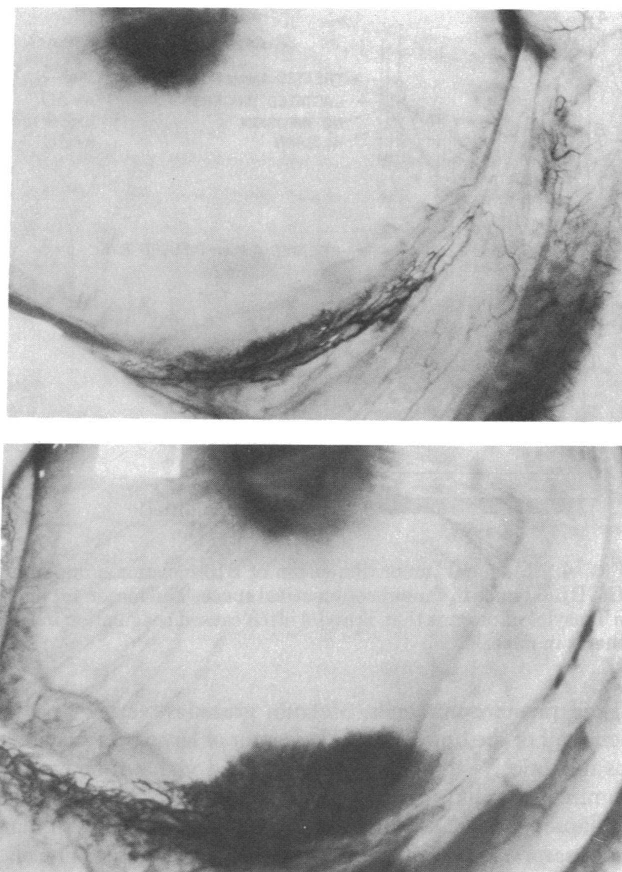

FIG. 3. Corneas in treated (*Upper*) and untreated (*Lower*) rabbits after 6-day infusion period (day 10). Note the much larger and broader vascular front in the untreated cornea. Before the infusion started (day 4), the vessels were shorter and less dense in the untreated cornea than in the treated cornea. (×10.)

plantation, little evidence of either the implant or of the procedure itself was evident. Between days 3 and 4 a small flat black plaque appeared in the area of the subconjunctival pocket. The tumor cells rapidly grew into a three-dimensional mass. In control animals, focal hemorrhages occurred on the tumor surface, and large dilated vessels entered its base on day 5 to day 6. This was not observed in treated animals. Measurement of tumor length was the only serial determination possible. Tumor width and height could not be accurately quantitated until after removal because the tumor grew behind the orbital margin and the conjunctival reflection.

The rate of tumor growth was similar in all of the control animals. In non-infused mice, the tumor length increased at a rate of 0.69 mm/day (Fig. 4). In mice infused with lactated Ringer's, the rate was 0.64 mm/day. In mice infused with albumin or Trasylol, tumors grew at rates of 0.60 and 0.65 mm/day, respectively.

In contrast, the tumors in the right eyes of the animals infused with the cartilage-derived inhibitor showed growth rates of only 0.06 mm/day. The left eyes of these animals served as internal controls and had tumor growth rates similar to those of the other controls—i.e., 0.74 mm/day (Fig. 4).

Because the length of the tumor does not accurately reflect its three-dimensional growth, all animals were sacrificed on day 7 and the tumors were weighed. Control tumors weighed an average of 7.60 mg and no statistically significant difference in tumor weights was observed between any of the control groups. By contrast, the average tumor weight in the treated eyes was 0.18 mg, <2.5% that of controls (Fig. 5 *a* and *b*).

Histologic study of tumors in control animals showed densely

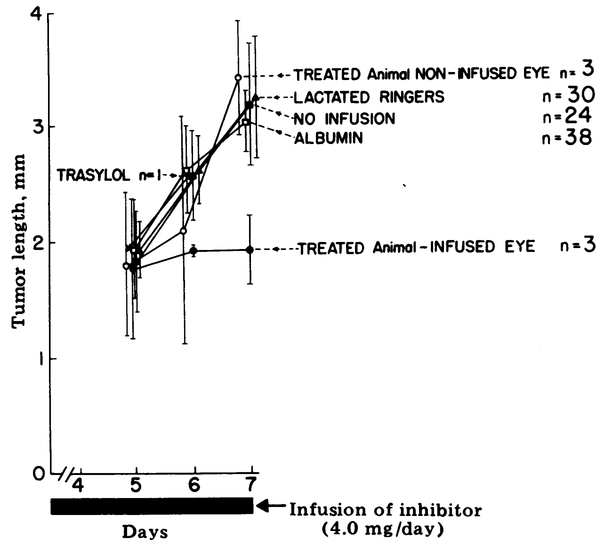

FIG. 4. Maximal tumor dimension of B16 melanoma implants in C57BL/6J mice in the subconjunctival space. The reason for only one Trasylol infusion is that Trasylol often caused coagulation in the catheter in mice.

packed pleomorphic cells. Melanin granules were found in about half of the tumor cells. The center of large tumor masses was necrotic. Within the densely packed viable tumor cells, prominent capillary blood vessels were present. They showed a collapsed lumen and were lined with endothelial cells similar in appearance to proliferating endothelium (Fig. 5*c*). The endothelial cell density in these vessels was higher than the density of the flat endothelium in adjacent tissue not exposed to tumor.

In contrast, animals treated with the angiogenesis inhibitor showed only a few melanin-producing tumor cells which did not form a solid tumor mass but were scattered as individual pleomorphic, often spindle-shaped, cells within a rather loose, edematous stroma. Spaces between the tumor cells were wide and contained extravasated erythrocytes in some areas and necrotic tumor cells in others. The newly branching tumor vessels and active, dense, endothelial cells observed in the controls were not noted in the treated animals. Instead, adjacent tissues showed dilated vessels with flat, sparse, quiescent endothelium. Occasionally, individual tumor cells were adjacent to these vessels but no directional growth of vascular tissue into the tumor implant was detectable (Fig. 5*d*).

Toxic effects. The infusion apparatus did not appear to affect tumor growth rates or the general well-being of the animal. Animals infused with the inhibitor did not demonstrate any signs of illness. They ate and drank normally throughout the infusion and were normally active. Histological features of the major organs were within normal limits. In two of the three treated animals, a widened femoral epiphyseal plate with increased numbers of chondrocytes was observed.

Cell Culture. B16 melanoma cells incubated with angiogenesis inhibitor, Trasylol, or bovine serum albumin at concentrations of 1–1000 μg/ml grew at approximately the same rate as controls (Fig. 6). At 4 mg/ml, all three solutions caused slight inhibition. The inhibitor caused no reduction in the growth rate of V2 carcinoma cells at concentrations as high as 1 mg/ml. At 4 mg/ml, the inhibitor caused a 10% reduction in growth rate of V2 carcinoma cells compared to controls. In all cases, including those in which cell growth rates were less than in controls, the tumor cells proliferated and appeared healthy.

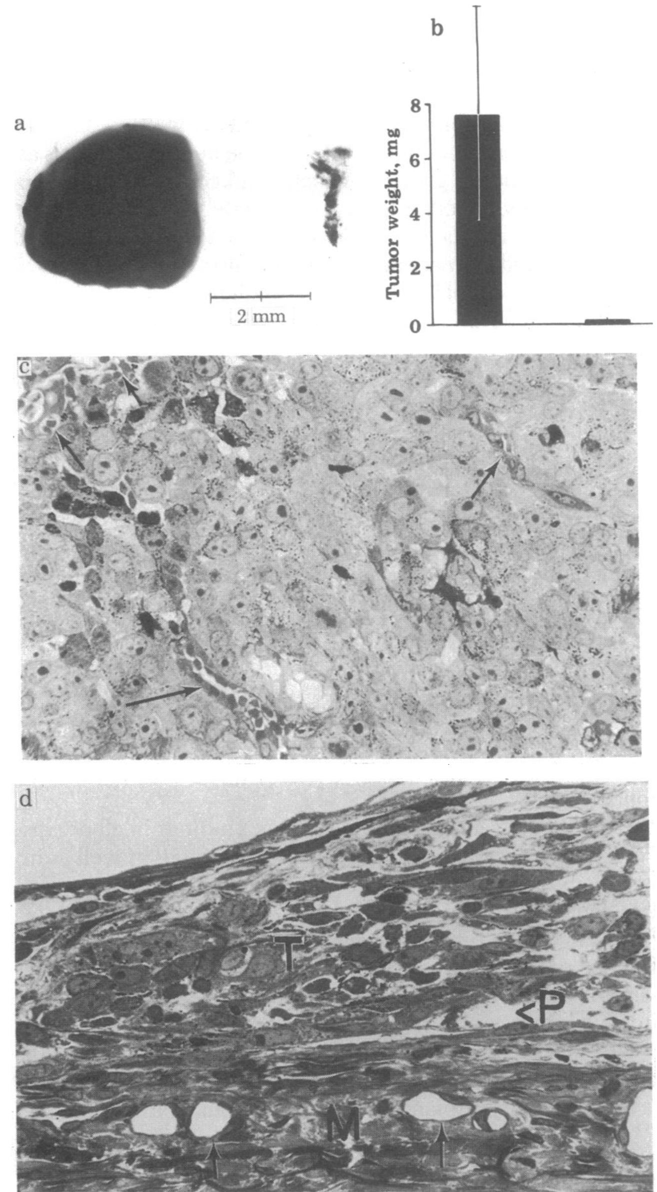

FIG. 5. (*a*) Excised B16 melanoma implants from untreated and treated mice at day 7. (*b*) Weights (mean ± SD) of excised B16 melanoma implants from untreated (*Left*) and treated (*Right*) mice at day 7. The untreated group includes all 15 controls—Ringer's solution, albumin, Trasylol, and the noninfused eyes of the treated animals—run in the same series as the treated tumors. (*c*) Section of B16 melanoma from untreated mouse, day 7. (×160.) The tumor contains densely packed melanoma cells interspersed with new capillary blood vessels (arrows) that penetrate throughout the tumor mass. Most tumor cells appear healthy. (*d*) Section through the whole tumor mass (T) of B16 melanoma from treated mouse, day 7. (×160.) The tumor implant has not been penetrated by new vessels and appears essentially avascular. There are only sparse melanoma cells. The vessels (arrows) in the contiguous vascular bed of muscle (M) are somewhat dilated and contain flat, nonactive, endothelial cells and there is no evidence of capillary proliferation or neovascularization. The edge of the avascular tumor is at plane P.

DISCUSSION

This study demonstrates that regional infusion of an angiogenesis inhibitor derived from cartilage will stop tumor vascularization and prevent subsequent tumor growth in two different species without toxicity to the animal host. This potential therapeutic approach appears to have no toxic effect on tumor cells *in vitro*. Furthermore, the inhibitor is a natural tissue product.

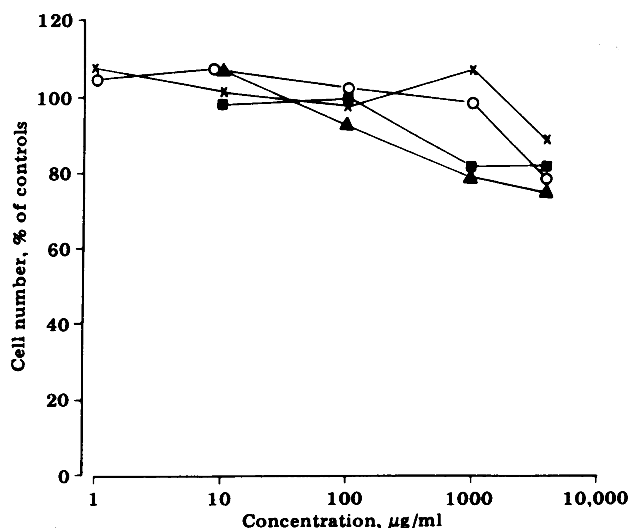

FIG. 6. Effect of the inhibitor, Trasylol, and albumin, on tumor cells in culture. Cells were fixed and counted 72 hr after exposure. X, Inhibitor and V2 carcinoma; ■, albumin and melanoma; O, inhibitor and melanoma; ▲, Trasylol and melanoma.

Implantation of V2 carcinoma in the rabbit cornea at a distance from the vascular bed permitted the quantitative measurement of inhibition of capillary proliferation and the subsequent limitation of tumor growth. The inhibition of the growth of B16 mouse melanoma further demonstrated that an aggressive solid tumor could be halted by an angiogenesis inhibitor, despite the fact that the tumor was implanted contiguous to a normal vascular bed.

The finding that inhibition of angiogenesis led to limitation of tumor growth was confirmed by including three different types of controls.

(*i*) To control for nonspecific inhibition due to infusion of protein in general or antitrypsin activity in particular, other protein solutions were infused including Trasylol, a bovine trypsin inhibitor. These infusions had no angiogenesis inhibitory effect.

(*ii*) To control for nonspecific inhibition of tumor growth due to "sickness" or debilitation of the animal, the left eyes of all treated animals received the inhibitor but at a substantially lower concentration than the right eye. If the animals were "sick," tumor suppression should have occurred in the left eye as well as the right eye. In fact, all tumors in the left eyes grew and became vascularized at the same rate as did tumors in untreated eyes.

(*iii*) To control for the possibility that the inhibited vessels might have stopped growing "spontaneously" or for some nonspecific reason, the behavior of the tumors in the treated eyes of the rabbits before and after infusion was studied carefully. Vessel growth essentially stopped in all eyes treated with the high dose of angiogenesis inhibitor; blood vessel growth always resumed when the angiogenesis inhibitor treatment was discontinued (Fig. 2).

These data suggest but do not prove that the inhibitor acts on growing new blood vessels rather than directly on the tumor itself. Before it becomes vascularized, the V2 carcinoma grows slowly in two dimensions within the collagen layers of the cornea. The treated tumor exhibited this same behavior under the stereomicroscope, suggesting that the V2 cells themselves were not affected by the inhibitor. In addition, histologic sections showed non-necrotic tumor cells present in the treated mice and rabbits. Furthermore, the angiogenesis inhibitor did not suppress the growth of B16 melanoma and V2 carcinoma cells *in vitro*.

One limitation of the tumor models used is that quantitative measurements of both tumor growth and angiogenesis cannot be made in the same animal system. In the mouse, the tumor is contiguous to the vascular bed and the new vessels are rapidly surrounded by growing tumor, so it is not possible to measure vessel length. However, tumor weight is an accurate measure of tumor growth and the melanoma is easily identified. By contrast, in the rabbit cornea, vessel length can be accurately measured because the tumor is separated from its vascular bed by a significant distance. However, measurement of tumor growth by weight is subject to large error, because the tumor infiltrates the cornea and the edge of the tumor is difficult to identify accurately.

Scarcity of the inhibitor precludes the possibility of testing it on tumors at more advanced stages of growth and vascularization. The point we wish to stress is that this angiogenesis inhibitor derived from cartilage may be considered as a prototype. It is possible that other angiogenesis inhibitors will be found or synthesized and that they may be even more potent (17). These results provide support for the concept of antiangiogenesis (3, 18, 19)—that control of tumor growth may be possible through control of neovascularization.

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