INHIBITION OF TUMOR ANGIOGENESIS MEDIATED BY CARTILAGE*

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We have previously suggested that solid tumor growth is not continuous, but may be separated into two stages, avascular and vascular (1, 2). In the avascular phase, spheroidal tumors cannot generally exceed a diameter of 1–2 mm or a population of more than 10^6 cells (3, 4). Further growth occurs after new capillaries have been elicited from the host, and have penetrated the tumor. Tumors elicit these new capillaries from the host by releasing a diffusible material, which we have termed tumor-angiogenesis-factor (TAF),[1] which is mitogenic to capillary endothelial cells (5–8).

Under the usual conditions of transplanting experimental tumors, the avascular phase is brief; i.e. 3–5 days (5). However, under special conditions, the avascular phase can be prolonged and tumors then become dormant. For example, when tumors are suspended in the anterior chamber of the rabbit eye, new vessels cannot reach them, and the tumors stop growing at about 1 mm diameter, although they remain viable (3).

We have suggested that if a means could be found to inhibit TAF, or block its stimulatory effect upon capillaries, tumors might be held in the avascular phase (9, 10). We now show that cartilage from newborn rabbits strongly inhibits capillary proliferation induced by tumors. The data suggests that this inhibition may be mediated by a diffusible factor, thereby preventing these tumors from progressing to the vascular phase.

Materials and Methods

This phenomenon was demonstrated in the rabbit cornea and additional supportive experiments were carried out in the chorioallantoic membrane (CAM) of the chick embryo. In the rabbit, V2 carcinoma was implanted in a corneal pocket, and a tiny piece of cartilage was placed between the tumor and the limbal edge of the cornea. Appropriate control tissues were substituted for the cartilage in the eyes of other rabbits. In the chick embryo, tumor implants or crude fractions of TAF were placed on the CAM, and cartilage was implanted nearby.

CORNEAL IMPLANTS

Cartilage. 70 neonatal rabbits were used in this study to obtain cartilage for 113 implantations into the rabbit cornea. New Zealand white rabbits were killed on the day of birth by cervical disloca-

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[1] *Abbreviations used in this paper:* CAM, chorioallantoic membrane; TAF, tumor-angiogenesis-factor.

tion. The skin was washed in a solution of distilled water containing 0.1% (wt/vol) hexachlorophene, 70% isopropyl alcohol, and 0.1% eosin Y dye and allowed to dry. Both scapulae were removed by aseptic technique and placed in Ringer's solution. Connective tissue and brown fat were dissected from the cartilages. The protruding tip of cartilage (4 x 2 mm) farthest from the bone was used for implantation (Fig. 1). This portion of cartilage was cut into smaller pieces, approximately 1.0 x 1.5 mm, with a scalpel. As a control, some of these cartilage fragments were lyophilized for 2 days, boiled for 10 min in distilled water, and then reconstituted in Ringer's solution for at least 30 min at room temperature before implantation. After reconstitution these cartilages had a consistency similar to untreated cartilage.

Neonatal Cornea. As an additional control, pieces of cornea were removed from the same neonatal rabbits that donated the cartilage. The cornea was excised from the limbal edge with a scalpel. Each cornea, which was approximately 1.2 mm in diameter, was then used directly as an implant instead of cartilage. 13 corneas were used.

Neonatal Bone. Fragments of neonatal scapular bone (1 x 1.5 mm) were implanted into two rabbit corneas.

Tumor. A stock of V2 rabbit carcinoma was maintained by serial intramuscular and subcutaneous injection in adult NZW rabbits. Tumors were harvested before they reached 2 cm diameter. Nonnecrotic, well-vascularized portions of growing tumors were excised aseptically and cut into 1.5 x 1.5 mm pieces in Ringer's solution and implanted into the cornea within 1 h.

Intracorneal Grafting Technique. Male NZW rabbits weighing 4–5 lbs were anesthetized with intravenous pentobarbital (25 mg/kg) and 2% xylocaine solution was applied to the cornea. The eye was proptosed and rinsed intermittently with Ringer's solution to prevent drying. The adult rabbit cornea has a diameter of approximately 12 mm. An intracorneal pocket was made by a technique that we have previously described (11). Briefly, an incision approximately 0.15 mm deep and 1.5 mm long was made in the center of the cornea with a no. 11 scalpel blade, using aseptic technique. A 5 mm-long pocket was formed within the corneal stroma by inserting a 1.5 mm wide, malleable iris spatula. In the majority of animals, the end of the corneal pocket was extended to within 1 mm of the corneal-scleral junction. In a smaller series of 22 rabbits implanted with tumor alone, pockets were placed at greater distances—2–6 mm from the corneal-scleral junction—by starting the incision away from the center.

Pieces of cartilage or appropriate controls were implanted first in the distal end of the pocket. The tumor was then implanted in the pocket just behind the cartilage, as in Fig. 3. The implants always remained in their original position and the intracorneal pockets sealed spontaneously. The implantation procedure lasted 5–10 min. Three rabbits with V2 carcinoma and cartilage developed an eye infection and were therefore sacrificed during the first week and not included in this study.

Stereomicroscopic Observations. Each cornea was examined every other day with the aid of a Zeiss slit-lamp stereomicroscope at 6–40 × magnificantion (Carl Zeiss, Inc., New York). The rabbits were not anesthetized. Measurements of the growth rate of new vessels and tumor diameter were made with an ocular micrometer at 10 × magnification (measurement accuracy ± 0.1 mm). During each observation the following measurements were made: total number of vessels in the cornea, maximum vessel length, average vessel length, distance between cartilage and nearest vessel, distance between tumor and nearest vessel, distance between tumor edge and limbus, and tumor size (length and width). Although the cartilage-to-limbus distance and cartilage size remained constant, they were recorded at each observation to confirm accuracy. More than 36,000 measurements were made over a period of 1 yr. Color photographs were taken to document major changes.

Vascular Injections with Colloidal Carbon. Before sacrifice, rabbits were anesthetized and given injections of colloidal carbon (Gunther-Wagner, Pelikan ink, Hanover, Germany) through both carotid arteries by a technique previously described (5). The carbon completely filled new vessels within the cornea.

Histology. Rabbits were sacrificed, and the entire cornea was excised with its attached sclera. Those specimens containing colloidal carbon were photographed immediately. All specimens were immersed in 10% buffered formalin. Paraffin sections were stained with hematoxylin and eosin.

CAM IMPLANTS

The CAM of 7-day old, embryonated white Leghorn eggs were exposed by using the false air sac technique (12). A field of neovascularization was produced by implanting 1-mm fragments of Walker

carcinosarcoma on 8-day old CAM in 39 eggs. In 11 eggs, a crude preparation of TAF was used to induce neovascularization. This was derived from Walker carcinosarcoma cells in culture, as previously described (1). Approximately 30 μg of protein containing TAF activity were placed on a piece of Millipore filter which was then implanted on the CAM (Fig. 7). Neonatal cartilage was implanted 1–2 mm from the source of neovascularization. In 11 eggs Walker tumor and boiled cartilage were implanted in a similar manner.

Control experiments were carried out in 71 eggs. Neonatal cartilage and lyophilized boiled cartilage were placed alone on the CAM.

Results

CORNEAL IMPLANTS

Tumor Alone. V2 carcinoma alone was implanted into 39 corneas. In every case, the tumor grew slowly as a thin intracorneal plate and remained avascular until one edge grew within 2.5 ± 0.5 mm of the limbus (Fig. 3). At that point pre-existing vessels in the limbus began to proliferate. New capillaries began to grow into the avascular cornea toward the tumor in 2–5 days. By the end of the first week, up to 30 new capillaries were advancing toward the tumor. The initial growth rate of the new capillaries averaged 0.22 ± 0.12 mm/day, but by the second week the rate had increased to 0.48 ± 0.16 mm/day (Table I). Within 3 wk after the onset of neovascularization, all of the tumors were large, exophytic masses, greater than 1 cm in diameter, which enveloped the entire eye (Fig. 2 F). At this time the capillaries were growing at a rate of 0.61 ± 0.14 mm per day. All of these tumors became vascularized and none regressed (Table II).

Tumor With Neonatal Cartilage. Neonatal cartilage was implanted between the V2 carcinoma and the limbus of the eye in 53 corneas. The initial tumor-limbal distances were similar to those implanted with tumor alone. The time of onset of neovascularization was similar to that in the corneas containing only tumor. The first difference in corneas containing cartilage was observed by the end of the first week: the density of vessel growth was less; only approximately five new capillaries were observed to be advancing toward the tumor. By the second week, another major difference was observed: as the vessel tips grew closer to the cartilage, vessel growth diminished (Fig. 2). The capillaries grew very slowly, averaging only 0.12 ± 0.16 mm/day (Fig. 4, Table I). In some corneas, the vessels stopped advancing and actually regressed; in others the vessel tips appeared to form clover-leaf patterns, with oscillating movements. In still others, vessels advanced slowly, so that the average growth rate of all vessels remained at 0.18 mm/day or less (Table I, Fig. 4).

In 53 corneas containing tumor with cartilage, 15 tumors failed to vascularize during the 5–16 wk of observation (Table II). These nonvascularized tumors ap-

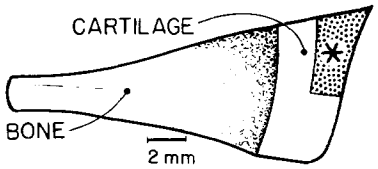

FIG. 1. Neonatal rabbit scapula. Shaded area containing (*) indicates zone of cartilage used in this study.

TABLE I

Mean Rate of Vessel Growth in Cornea (mm/day)

	Weeks									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
V2 carcinoma	0.22 ± 0.12 n = 22	0.48 ± 0.16 n = 20	0.61 ± 0.14 n = 7							
V2 carcinoma + neonatal cornea	0.20 ± 0.06 n = 11	0.39 ± 0.14 n = 11	0.58 ± 0.06 n = 6							
V2 carcinoma + boiled cartilage	0.17 ± 0.07 n = 7	0.37 ± 0.16 n = 7	0.55 ± 0.11 n = 5							
V2 carcinoma + cartilage	0.16 ± 0.08 n = 53	0.12 ± 0.16 n = 52	0.15 ± 0.15 n = 45	0.18 ± 0.20 n = 34	0.08 ± 0.13 n = 20	0.08 ± 0.13 n = 16	0.08 ± 0.12 n = 9	0.08 ± 0.10 n = 6	0.07 ± 0.08 n = 4	−0.09 n = 1

Mean rates and standard deviations of vessel growth. Rates were determined by weekly differences in maximum vessel length. Rates were calculated only after the onset of neovascularization, thus in all cases the tumor was close enough to the limbus to induce angiogenesis. n = number of corneas in each category.

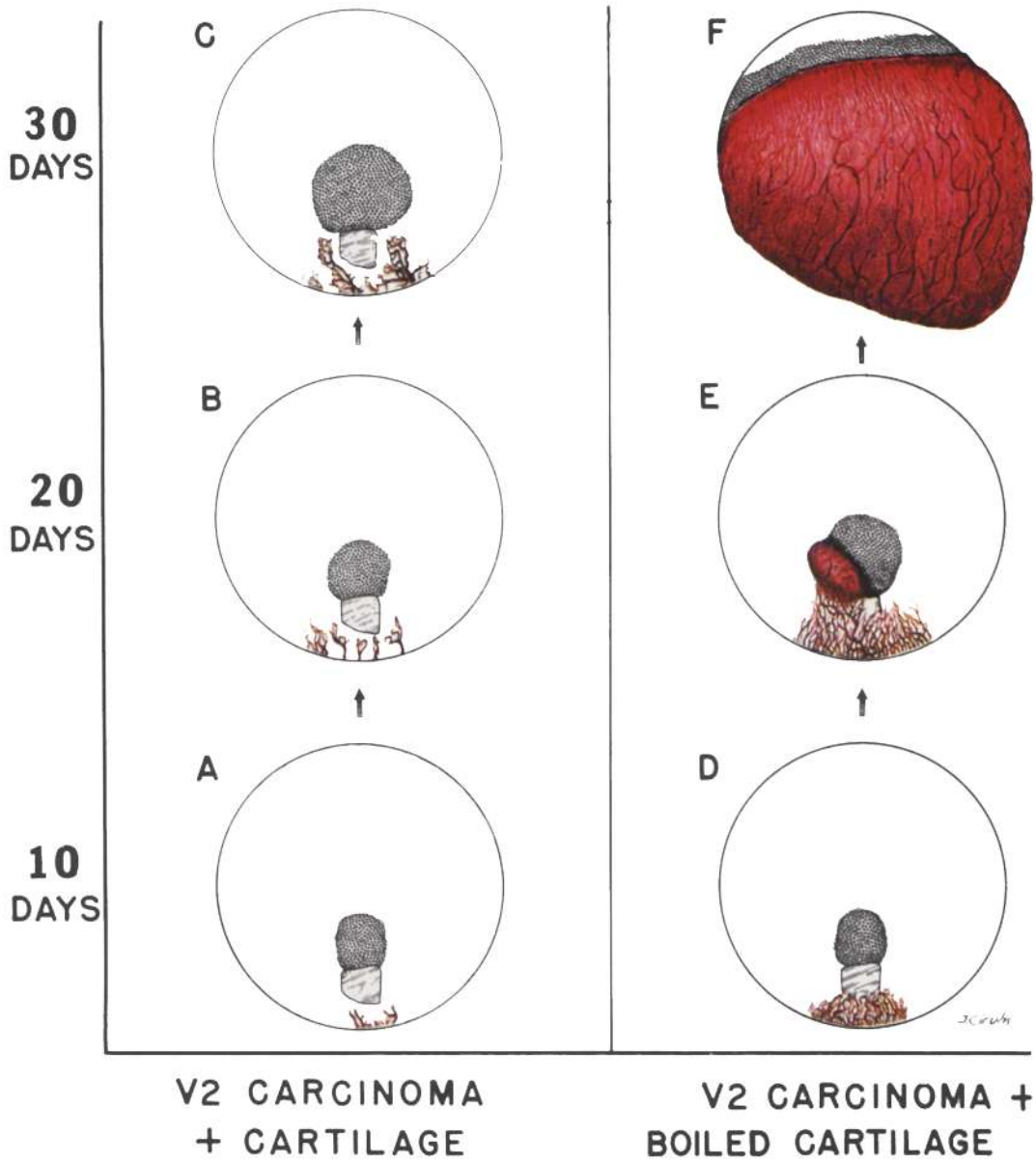

FIG. 2. Representative corneas after implantation of tumor with active cartilage or with boiled cartilage. This diagram is drawn to scale by tracing color photographs of the actual specimens. The diameter of the cornea is 12 mm. When cartilage is implanted with tumor, the vessels are inhibited from reaching the tumor (A, B, C). With inactive cartilage, the vessels enter the tumor by the 15th day and rapid tumor growth follows (E), leading to a large exophytic mass (F). A similar result (D, E, F) is obtained when the boiled cartilage is replaced by neonatal cornea or when tumor alone is implanted.

TABLE II

Prevention of the Vascular Phase of V2 Carcinoma

Corneal implant	No. of tumors	No. remaining avascular	% remaining avascular
V2 carcinoma + cartilage	53	15	28
V2 carcinoma + boiled cartilage	7	0	0
V2 carcinoma +neonatal cornea	11	0	
V2 carcinoma	39	0	

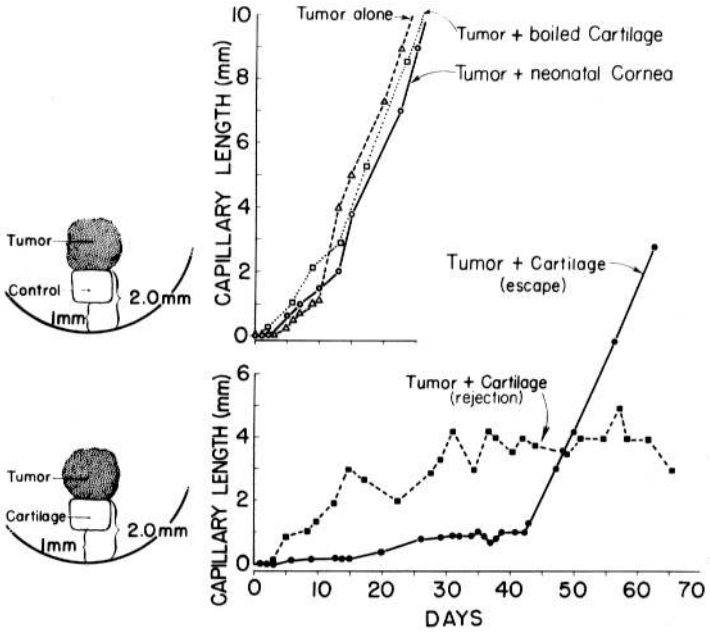

FIG. 3. Inhibition of capillary growth by cartilage. In the upper graph the maximum capillary length at any given time is the same in the corneas implanted with boiled cartilage, neonatal cornea or with no added tissue. The lower graph demonstrates the inhibition of capillary growth when active cartilage is implanted with tumor. There are two patterns of response to cartilage represented by these two typical cases. In both cases there is significant inhibition of capillary growth for at least 40 days. Eventually 72% of the tumors escape the cartilage inhibition and enter the rapid vascular growth phase. In 28% of the experiments, although vessels were present in the cornea, the tumors remained avascular and appeared to be rejected.

peared to undergo immunological rejection. Our impression of immunologic rejection was supported by histologic sections (Fig. 5) of corneas from two animals, and by re-challenge with fresh V2 carcinoma in three animals. The V2 re-challenge was implanted either in the opposite sector of the cornea, or intramuscularly, and the tumor was then rapidly rejected. We have maintained the V2 carcinoma for over 4 yr in our laboratory and in that time it has never regressed spontaneously when implanted intramuscularly or intracorneally.

The remaining 38 tumors implanted intracorneally together with cartilage

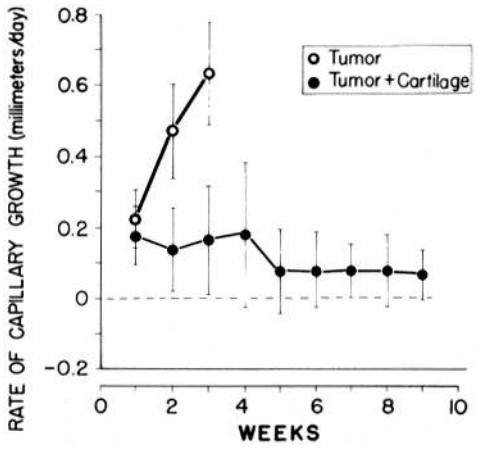

FIG. 4. Rate of capillary growth with and without cartilage. Note that some vessels exposed to cartilage actually regressed at the 4th to 10th wk.

eventually became vascularized, although this was delayed by an average of 3 wk compared to controls. This vascularization resulted from the steady growth of the tumor plate in two dimensions within the corneal lamellae. Vessels were then elicited at sites of the limbus remote from the cartilage. Once the tumor was vascularized, rapid exponential growth ensued, and the cartilage was completely covered by tumor (Fig. 6).

Tumor With Boiled Cartilage. In all seven corneas that contained lyophilized-boiled cartilage and implants of V2 carcinoma, there was no suppression of capillary proliferation: tumor vascularization proceeded at the same rapid rate as when tumor alone was implanted (Fig. 2, 3; Table I).

Tumor With Neonatal Cornea. Pieces of neonatal rabbit cornea were implanted with V2 carcinoma in 13 corneal pockets. The neonatal cornea served as another control of an avascular, viable tissue occupying the same space in the tumor pocket as would be occupied by cartilage. In all cases there was normal tumor neovascularization; vessels grew at the same rapid rate as when tumor alone was implanted (Fig. 3, Table I). All tumors grew rapidly after vascularization and became exophytic (Table II).

Neonatal Cartilage Alone. Neonatal cartilage was implanted alone in 38 corneal pockets. When the protruding tip of cartilage most distal from the bone was used, there was no reaction in the cornea in 14 out of 20 implants. However, when sections of cartilage contiguous to bone were used, 11 out of 18 implants induced a significant vascular response of 1 mm long capillaries which advanced to the edge of the pocket. For this reason, the tip of cartilage farthest from the bone was used in all experiments where cartilage was combined with tumor (Fig. 1).

Neonatal Bone Alone. Bone from the neonatal rabbit scapula was implanted in two corneal pockets, and in both cases there was a significant vascular response. New capillaries grew a distance of 1 mm to reach the edge of the corneal pocket.

Histology. Sections through corneas containing cartilage alone showed that

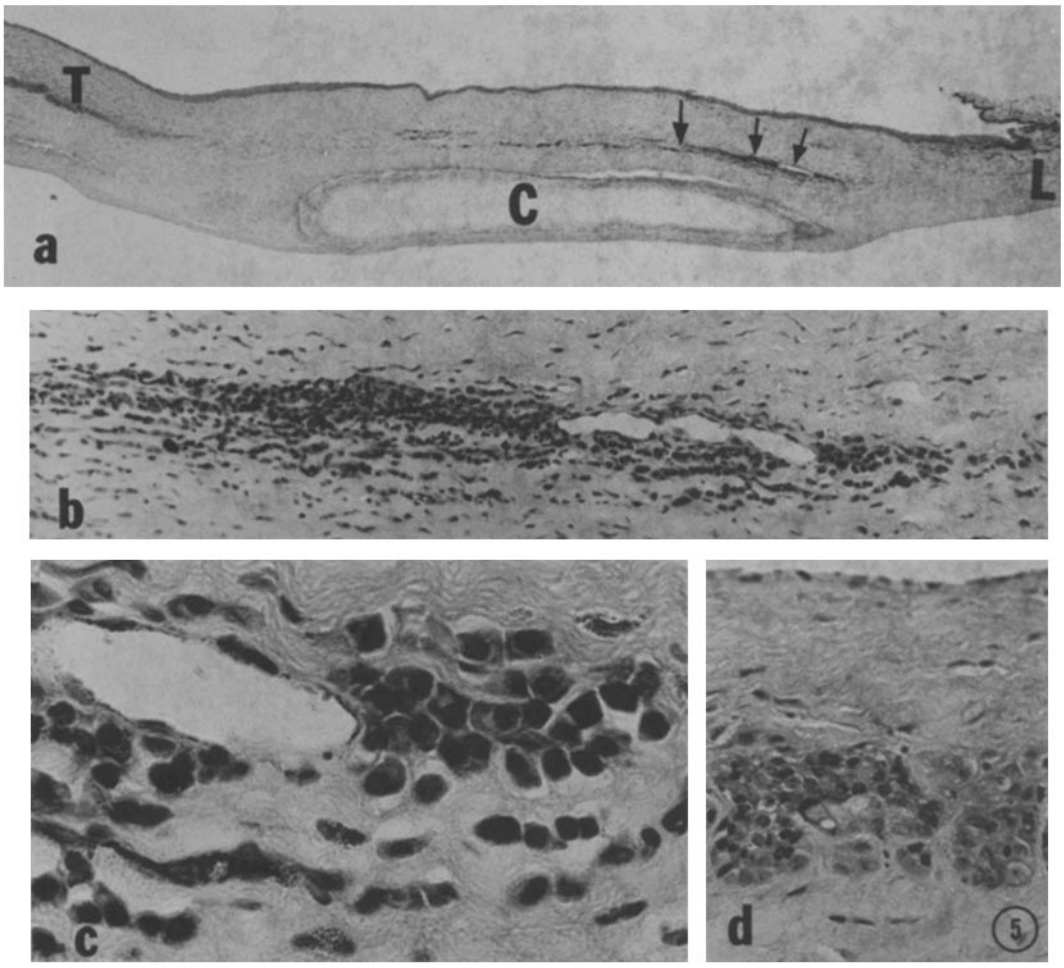

FIG. 5. (*a*) Vessels (arrows) stimulated from limbus (L) are anterior to cartilage (C) approaching V2 carcinoma (T) which was implanted 2 mm from the limbus. Hematoxylin & eosin; × 20. (*b*) Higher magnification (× 160) of area of intense perivascular infiltrate identified by arrows in (*a*). (*c*) Higher magnification (× 640) of infiltrate seen in (*b*). Note conspicuous plasma cells. (*d*) Higher magnification (× 250) of tumor (T) from (*a*).

the cartilage appeared viable throughout the duration of the experiment. The lacunae contained cells with healthy nuclei. The cartilage did not elicit an inflammatory reaction. Tumors alone, in the avascular phase, grew as a plate with healthy appearing cells, similar to the growth pattern previously reported (11). When the tumors were vascularized, they grew into large, nodular masses, occasionally with central necrosis.

When tumors were combined with cartilage, both tissues grew in contact with each other and there was no evidence that either tissue inhibited or destroyed the other (Fig. 6). Avascular tumors did not invade the cartilage.

In those tumors that failed to vascularize or regressed (Fig. 5 *a*), the striking findings were: (*a*) A band of blood vessels anterior to the cartilage approached

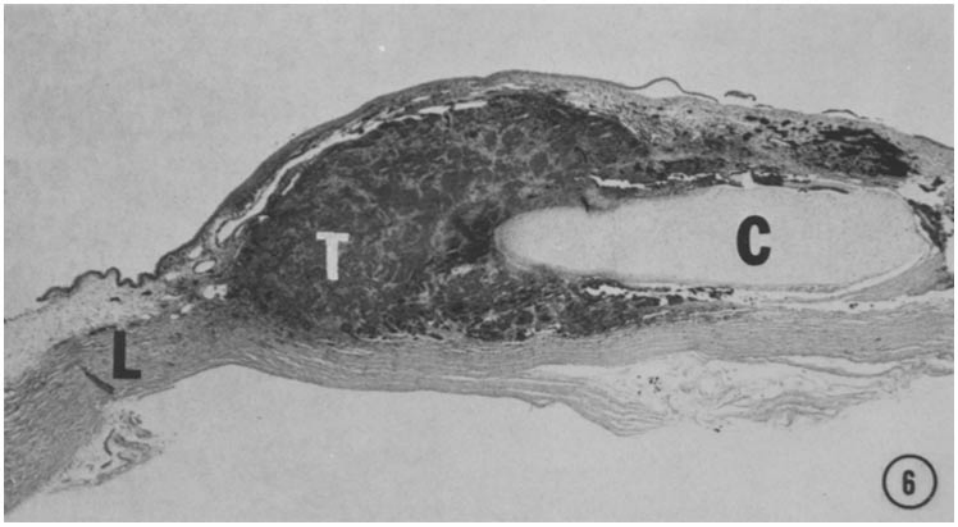

FIG. 6. Sagittal section of cornea containing vascularized V2 carcinoma (T) and cartilage (C), 60 days after implantation. Although cartilage inhibited neovascularization for over 40 days, once the tumor became vascularized, it grew rapidly into an exophytic mass covering the cornea. Even when vascularized, the tumor did not destroy the cartilage. L = limbus. Hematoxylin & eosin × 19.

the tumor plate. (*b*) Near the tumor plate, these vessels exhibited intense perivascular mononuclear infiltrate with conspicuous plasma cells (Fig. 5 *b*). (*c*) The tumor plate showed foci of neutrophilic infiltrate and necrosis, with islands of still viable tumor (Fig. 5 *d*). (*d*) The perivascular infiltrate, located away from the cartilage, was surrounded by an area of condensation of the stromal cells of the cornea (Fig. 5 *c*). In these sections, the cartilage looked healthy throughout and was not associated with any inflammatory reaction.

CHORIOALLANTOIC MEMBRANE IMPLANTS

When fresh neonatal cartilage was implanted with Walker carcinosarcoma as a neovascularizing source, 23 out of 28 membranes showed an avascular zone of 1-2 mm diameter around the cartilage (Fig. 7). A similar zone was observed in 9 out of 11 membranes when cartilage was implanted with TAF fractions. When cartilage alone was implanted, 50 out of 56 implants remained unvascularized and free of vessels. However, when boiled cartilage alone was implanted, it became completely covered by vessels from the CAM in 13 out of 15 implants. There was no zone of inhibition in all 11 CAM's implanted with a neovascularizing source and boiled cartilage.

Discussion

These experiments show that cartilage inhibits capillary proliferation induced by tumor. This inhibitory effect appears to operate over short distances of up to 2.0 mm, and displays a gradient from cartilage source to the limbal edge of the

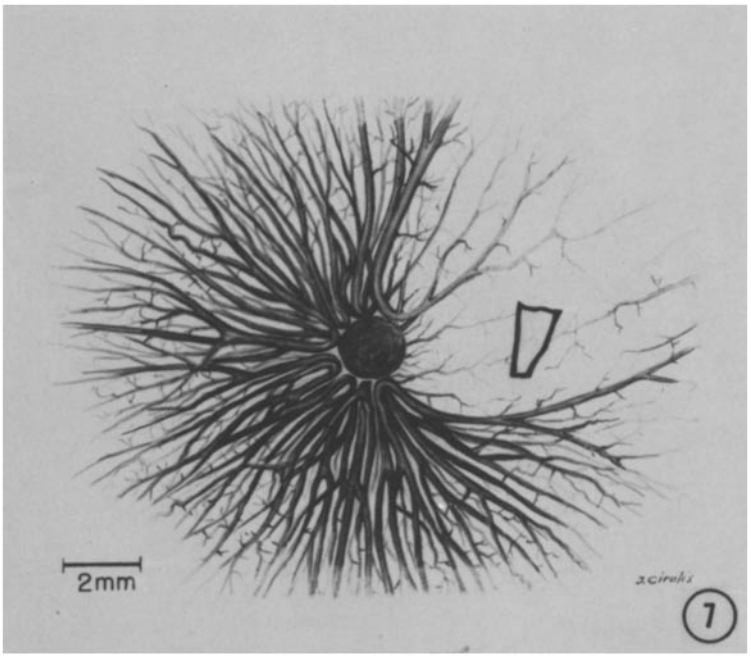

FIG. 7. Diagram of experiment in which TAF granules (circle in center) and a cartilage fragment (on right side) were implanted on CAM. The neovascularization produced by TAF was inhibited in the zone surrounding the cartilage.

cornea. The highest concentration of the inhibitor seems to be closest to the cartilage because the growth rate of proliferating capillaries slows as the capillaries approach the cartilage. This happens even though the advancing capillary tips are in proximity to the tumor implanted just beyond the cartilage. The inhibitor appears to be continuously produced by the cartilage, because the inhibitory effect on capillary growth lasted throughout the longest observation period; i.e. 4 mo, at which time the cartilage appeared viable by histology. Boiled neonatal cartilage and fresh neonatal cornea lack the capacity to inhibit capillary proliferation.

The cartilage implant delayed tumor vascularization in all cases, and prevented vascularization completely in 28% of tumors, but the remaining tumors did eventually become vascularized. One explanation for this is that the growing tumor plate acts as a source of increasing concentration of TAF, while the nongrowing cartilage becomes a source of a fixed concentration of inhibitor of capillary growth. Eventually, the TAF diffusing through the cornea would reach a level sufficient to override the inhibitor effect. Also, because avascular tumors in the cornea generally grow in two dimensions between the corneal lamellae, the tumor can grow away from the small zone of inhibition around the cartilage, and induce vessel growth from a remote region of limbus.

These experiments suggest that the cartilage inhibitor does not antagonize TAF. It apparently inhibits capillary proliferation in a more direct way. If TAF were inhibited, its activity should be diminished immediately upon release from the tumor, and the onset of neovascularization should be greatly delayed. There was no significant difference, how-

ever, in the first appearance of vessels between tumor implanted with boiled cartilage and tumor implanted with viable cartilage.

We have isolated an extract of the active cartilage that can inhibit capillary proliferation induced by tumors. The cartilage was minced into a solution of lactated Ringer's with 28 mM Hepes buffer, adjusted to pH 7.4 with NaOH, and then rocked gently at 4°C for 8 h. The cartilage cells and debris were then centrifuged at 4,000 *g* for 1 h and the supernate dialyzed exhaustively against distilled water for 2 days and then lyophilized. This extract has produced a zone of capillary inhibition in 28 out of 31 cases on the CAM when implanted with Walker carcinosarcoma or TAF. Work is in progress on this inhibitory factor's effect in the cornea and on the characterization of the active fraction.

Mature cartilage is relatively avascular tissue that contains very few cells and consists almost entirely of an extracellular matrix composed of water, collagen, and protein polysaccharide complexes (13). It has not been established whether the avascularity of cartilage is due to a physical or chemical block (14). In mice, carcinomas induced in the ear by chemical carcinogens never infiltrate the cartilage, but grow instead in the tissue surrounding the cartilage (15). Cartilage-derived tumors, chondrosarcomas, are among the least vascular neoplasms (16).

Developmental studies (17, 18) on cartilage in humans have shown that embryonic cartilage is vascularized, but that the blood vessels disappear in the early neonatal period. This suggests the possibility that a factor capable of inhibiting vessels might exist in neonatal cartilage.

This investigation was begun following a discussion with Dr. Harold Slavkin, who had observed that cartilage placed on the chorioallantoic membrane did not become vascularized.[2] When we reproduced this experiment, we also saw that neonatal cartilage did not become vascularized from the CAM vessels. Recently, Eisenstein et al. have reported a similar finding (19). When we extended these experiments and placed the cartilage in a field of neovascularization induced by tumor or TAF, a zone of inhibition of capillary proliferation appeared around the cartilage (Fig. 7). The disadvantage of the CAM soon became apparent: an inhibitory effect could not be quantitated. Measurements of capillary growth rate were not possible. The great advantage of studying the cartilage inhibitor in the rabbit cornea was that accurate measurements of capillary growth rate could be made because the tumor, cartilage and new vessels were all arranged linearly.

In summary, the inhibitory effect of cartilage in the cornea operated in two ways: (*a*) the rate of capillary growth was significantly reduced, and (*b*) 28% of the tumors never entered the vascular phase. By contrast, when the cartilage was heat inactivated or replaced by neonatal cornea of the same size, or when tumor was implanted alone: (*a*) the rate of capillary growth was never reduced and (*b*) all tumors became vascularized.

This is the first time, to our knowledge, that a diffusible material from normal tissue has been shown to inhibit capillary proliferation induced by tumors. The

[2] Slavkin, H. C. 1972. Department of Biochemistry, University of Southern California, personal communication.

material appears to produce no inflammation in the cornea. When this inhibitory factor is further purified and characterized, it may prove useful as a therapeutic means of maintaining tumor dormancy by "antiangiogenesis" (9, 10).

Summary

Capillary proliferation induced by tumor is shown to be inhibited by neonatal scapular cartilage. Using the rabbit cornea as an assay, the cartilage implant decreased the rate of capillary growth, induced by tumor, by an average of 75%. Vascularization was prevented completely in 28% of tumors. The inhibitory effect of small cartilage implants operates over distances of up to 2.0 mm and displays a gradient from the cartilage source. The experiments suggest that the cartilage inhibitor does not antagonize tumor angiogenesis factor, but appears to inhibit capillary proliferation directly. The inhibitory material does not elicit an inflammatory response in either the rabbit cornea or in the chick chorioallantoic membrane. Thus with further purification, it may prove useful as a means of maintaining tumor dormancy by "antiangiogenesis."

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