Prolonged Tumor Dormancy by Prevention of Neovascularization in the Vitreous

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INTRODUCTION

Capillary proliferation is important in the pathogenesis of both neoplasia (1, 10, 16, 21) and diabetic retinopathy (2, 33, 38). For tumors, growth occurs in 2 phases: the avascular phase, in which growth is limited by diffusion of nutrients, followed by the vascular phase, in which rapid growth is associated with neovascularization (19). A potent signal for capillary proliferation diffuses from the tumor (26), stimulating capillaries at distances as far as 2- to 5 mm from the neoplasm (12, 24, 25). A wide variety of animal and human tumors contain this biochemical message, called TAF4 (23, 37).

We report here that the vitreous chamber of the eye provides a unique model to study the in vivo behavior of tumors in the avascular phase. The vitreous, the space between the lens and the retina, consists of collagen, glycosaminoglycans, and proteins similar to the ground substance of connective tissue or the interstitial compartment of other tissues (5). The vitreous normally lacks blood vessels. In diabetes, however, blindness can result from the proliferation of retinal capillaries into the substance of the vitreous (14).

Because of the possibility that a diffusible vasoformative substance, similar to TAF, mediates this process, we implanted tumors into the vitreous at various distances from the retina. Tumor angiogenesis, unexpectedly, proceeded differently than in previous experimental sites. In the vitreous, tumors remained in a prolonged avascular state despite proximity to retinal capillaries; they became vascularized only when contiguous with the retinal surface.

SUMMARY

Tumors release a diffusible substance that stimulates neovascularization. To study the neovascularization that occurs in diabetic retinopathy, we implanted V2 carcinomas and mouse ependymoblastomas into the vitreous of experimental animals. In the vitreous, unlike previous sites, the tumors failed to stimulate neovascularization. They grew for weeks as small, unvascularized, three-dimensional aggregates of cells. Explosive growth into a large, vascularized mass occurred when the avascular tumors reached the retinal surface. The vitreous proved to be a valuable model for observing the in vivo growth of small, solid tumors. Xenografts survived for months without evidence of immune rejection. The consequence of the prolonged avascular state is the restriction of tumor size. The normal vitreous may act to inhibit capillary proliferation. An understanding of the mechanism for maintaining the avascular state may lead to therapeutic blockade of neovascularization. This would be important in the management of diabetic retinopathy and neoplasia.

MATERIALS AND METHODS

Tumor Stock. Two long-term lines of experimental tumors, the rabbit V2 carcinoma (30) and the mouse ependymoblastoma (40), were transferred s.c. in homologous animals every 2 to 3 weeks. The vascular edge of a palpable 1- to 2-cm nodule was passed through a cytosieve and the cells were suspended in 2 ml of cooled lactated Ringer solution. Viability exceeded 90% by trypan blue exclusion.

Host Animals. Tumor cells were transplanted to the vitreous of either the rabbit or the dog. The New Zealand White rabbit was used because (a) the V2 carcinoma is homologous to it, (b) the rabbit’s retinal vessels lie in direct contact with the vitreous gel, free from surrounding glial tissue, and (c) the vessels are restricted to a limited zone of the retinal surface, so that comparisons could be made between vascularized and nonvascularized areas (13). The retina of the dog more closely resembles that of the human because it is completely vascularized and has an inner limiting membrane between the vitreous and the retinal vessels. The retinal vessels of young puppies, however, can proliferate into the vitreous in response to an experimental stimulus (hyperoxia) (32).

Intravitreal Implantation. General anesthesia was induced with i.v. pentobarbital (Pitman-Moore, Inc., Washington Crossing, N. J.) for adult albino rabbits, and i.m. fentanyl-droperidol (Veterinary Laboratories, Inc., Lenexa, Kans.) for adult and neonatal dogs. The pupils were dilated with tropicamide (Alcon Laboratories, Inc., Fort Worth, Texas). After topical anesthesia, a 27-gauge needle was passed through the superior-temporal sclera, 4 mm posterior to the limbus. The needle tip could be advanced to any point between the lens and the retina with the guidance of an indirect ophthalmoscope. Facility with this instrument

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4 The abbreviation used is: TAF, tumor angiogenesis factor.

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was learned rapidly; a standard, readily available, 20-diopter convex lens and a light source was used (35). Fifty μl, containing 10⁴ to 10⁵ cells, were injected gently. Each inoculation took less than 1 min and was atraumatic; the vitreous remained transparent. Contact with the lens or retina was avoided to prevent a cataract or retinal tear.

Ophthalmoscopy. Serial observations were made with the indirect ophthalmoscope, supplemented by slit-lamp photography and fluorescein angiography. Tumor size was estimated by comparison with injected microspheres of known diameters (45 ± 5, 97 ± 7, and 325 ± 9 μm, aluminum microspheres from the Particle Information Service, Grant’s Pass, Ore.).

Histology. At the completion of the experiments, some animals received an intravitreal injection of [3H]thymidine (specific activity, 14 Ci/m mole; Schwarz/Mann, Orangeburg, N. Y.) for autoradiography (15); others received an intracarotid infusion of colloidal carbon (Guenther-Wagner, Hannover, Germany) to outline the microvasculature (25). All eyes were enucleated, fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin for routine histological examination.

RESULTS

Rabbit Carcinoma. Tumor growth was observed in 66 of 75 rabbit eyes. For as long as the tumors were within the vitreous, up to 100 days, they remained unvascularized. Proliferation of retinal vessels never occurred until the tumors were contiguous with the retina.

After the 1st week, the eyes contained generally 1 but as many as 12 tumors displaying 2 patterns of growth: (a) spheroidal nodules; these grew slowly, remained avascular, and achieved maximal diameters of 0.25 to 0.50 mm, (b) cylindrical stalks; these had similar cross-sectional diameters but grew as far as 3 to 7 mm along a path directed towards the retinal vessels on the optic disc. This path followed a posteriorly directed movement of fluid normally present in the rabbit vitreous (28). Eventually, many of the spheroidal tumors also produced a linear stalk directed towards the optic disc (Chart 1A and B).

Once the tumor stalks reached the retinal surface, the tumors became vascularized by proliferating retinal vessels (Chart 1C). The vascularized tumors entered a new, explosive phase of growth (Chart 2). Within 2 weeks, a large exophytic mass, representing approximately a 19,000-fold increase in volume, grew along the vascularized portion of the retina and protruded into the vitreous (Chart 1D). After local invasion into the retina and optic nerve, the tumors infiltrated the choroid and the sclera.

Exponential growth was also observed in 11 of 75 eyes that developed vascularized carcinomas at the injection site on the scleral surface. The intravitreal portion of these tumors, along the injection tracks, remained avascular with almost no change in size.

Ocular changes were absent in 20 eyes that received injections of controls: 0.9% NaCl solution, India ink, aluminum microspheres, fresh liver homogenates, or boiled V2 carcinomas. Liver homogenates and boiled tumors disappeared after a few weeks. The surrounding vitreous media remained transparent throughout the study, as it had in the eyes with the viable tumor. Retinal vascular changes were absent in all of the control eyes.

Histologically, the vascularized tumors contained anaplastic cells with mitotic figures as well as proliferating capillaries. By contrast, the unvascularized tumors showed an outer layer of 10 to 20 viable cells and an inner necrotic center (Fig. 1). Incorporation of [3H]thymidine was restricted to the tumor cells at the periphery of the tumor; the endothelial cells of the retina failed to incorporate [3H]thymidine in the eyes with the unvascularized tumor. These tumors and the surrounding vitreous were free of fibroblasts, leukocytes, or other cells from the host.

In another set of experiments, xenografts of rabbit V2 carcinoma were transplanted to the vitreous of the dog. During 6 months of observation, these tumors grew very slowly in 5 of 13 puppy and 3 of 4 adult eyes. The tumors remained close to the lens and did not approach the retinal surface.
surface; the retinal vessels appeared normal. Histologically, the tumors resembled the unvascularized nodules seen in the rabbit vitreous. Viable tumor cells were clumped together in small colonies; host cells or vascular elements were absent (Fig. 2).

Mouse Brain Tumor. The mouse ependymoblastoma formed spheroidal colonies in 8 of 12 rabbit eyes. These tumors were observed for more than 4 months. The tumors remained unvascularized and at a distance of greater than 2 mm from the retinal surface (Fig. 3A). As in the previous experiment with xenografts, there was no gross or microscopic evidence of an immune rejection. Histologically, the cells appeared viable, especially at the periphery of the colony (Fig. 3B).

**DISCUSSION**

These studies show that: (a) the vitreous provides a valuable model to investigate the growth of transplantable tumors; (b) this model displays the longest demonstration of in vivo avascular tumor growth; and (c) tumor angiogenesis proceeds differently in the vitreous than in previously studied models. This difference suggests that vitreous may act as an inhibitor of neovascularization.

The advantages of the vitreous over conventional in vivo and in vitro systems are: (a) the vitreous chamber is virtually acellular so that growth can be observed without contamination by host cells; (b) the clarity of the media permits immediate, direct observation of tumor colonies as small as 0.02 mm in diameter. Small tumors of this size might be useful in studies of early neoplastic events or micrometastases (34); (c) the viscosity of the vitreous gel and its collagenous matrix enable the tumor implants to remain in a relatively fixed position for repeated observations; (d) the techniques are simple. The vitreous contains its own nutrient media, obviating the complexities and artifacts inherent in tissue culture and in perfusion of organ cultures (20).

The vitreous appears to be an immunologically privileged site since incompatible grafts survive over prolonged periods. Characteristics of vitreous that may account for its immune privileged status include avascularity and an intercellular matrix that prevents the host immune cells from attacking the transplant (5). Mouse brain tumors survived for over 4 months in the vitreous, compared to less than 3 weeks in the cornea (9), which is a previously described immune privileged site (6, 24).

There is not only a prolonged survival of transplantable tumors but also a prolonged survival of tumors in the avascular state. The consequence of prolonged growth in the avascular state is the restriction of tumor size. This principle is illustrated in Chart 2 by the difference in the growth rate of the unvascularized carcinomas in the vitreous and the vascularized carcinomas on the retinal surface. Growth of populations of cells living in 3-dimensional aggregates is limited by the diffusion of nutrients; cells at the surface have an abundant supply and replicate; cells in the center die of malnutrition. The balance between replication and necrosis accounts for tumor dormancy (22). When capillaries penetrate the dormant nodule, the perfusion of nutrients results in rapid growth. The same principle applies to the clinical presentation of human eye tumors, e.g., retinoblastoma. Large vascularized masses appear on the retinal surface, but intravitreal metastases are dormant nodules, remain avascular, and rarely exceed a diameter of 1 mm (18).

In the vitreous, tumor angiogenesis proceeds differently from previous sites where tumors consistently stimulate neovascularization at distances up to 2 to 4 mm (12, 24, 25). In the cornea, implants of V2 carcinoma 1 mm from the limbus attract new vessels within 4 days, and the tumors are vascularized after 7 days. In the anterior chamber, similar to the vitreous in its avascularity and paucity of cells, tumors stimulate vessels at a distance of several mm. By contrast, in the vitreous, tumors remain unvascularized for an average of 42 days (Chart 3). Even at a distance as close as 0.1 mm, blood vessels fail to proliferate toward the tumor. Vascularization occurs only when the growing edge of the tumor contacts the retinal surface.

The vitreous, therefore, may interfere with the transfer of a diffusible, vasoproliferative stimulus from the tumor to the host endothelial cells. It could exert this effect by 1 of 2 plausible mechanisms: (a) a direct inhibition of endothelial cell proliferation or (b) a limitation of the diffusion of TAF from the tumor to the retinal vessels. Vitreous contains huge aggregates of negatively charged protein-polysaccharides that can act as molecular sieves, trap large macromolecules, and block their diffusion (31, 36). Other explanations, such as direct toxicity to the tumor cells (4) or inability of proliferating capillaries to penetrate the vitreous are shown to be unlikely by the results of autoradiographic studies. Tumor cells in the vitreous within 0.1 mm of the retina incorporated [H]thymidine but endothelial cells of the retina did not. This implies that vitreous is not toxic to tumor cells and that the retinal vessels are not proliferating.

These studies support the concept that blockade of angiogenesis, i.e., “antiangiogenesis” could arrest tumor growth at a tiny size (16). Antiangiogenesis has been suggested as a potential therapeutic adjunct (17). Recently, an inhibitor of tumor angiogenesis has been demonstrated in neonatal rabbit cartilage (7, 8). Cartilage is a relatively avascular tissue that, like vitreous, consists almost entirely of an extracellular matrix composed of water, collagen, and protein-polysaccharide complexes (11, 39).

Why are these tissues avascular? Developmental studies of cartilage and vitreous in humans have shown that both tissues are vascularized in the embryo but that vessels

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**Chart 3. Prevention of vascularization in the vitreous**. V2 carcinomas, implanted in the rabbit cornea at 1 mm from the nearest vessels, stimulate the ingrowth of capillaries and are vascularized after 7 days. In the vitreous, these tumors fail to stimulate retinal neovascularization; vascularization occurs only when the tumors contact the retinal surface, e.g., in these experiments after 42 ± 22 days.
regress after birth (27, 29). If an inhibitor of capillary proliferation exists in cartilage or vitreous, its normal biological role could be the regulation of this vascular regression. This would be important in understanding the mechanism and possible control of retinal neovascularization in diabetes. Further experiments on the vitreous-vascular and vitreous-tumor interactions might provide additional clues leading to the blockade of neovascularization. Such blockade of neovascularization would have therapeutic implications in diabetic retinopathy and pathological corneal neovascularization as well as in neoplasia.

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REFERENCES

Fig. 1. A, diagonal section of an unvascularized V2 carcinoma, 100 days after transplantation to the rabbit vitreous, growing as a thin, linear stalk. H & E, × 64. B, histology of the same stalk. H & E, × 300.

Fig. 2. Histology of the V2 carcinoma, 5 months after transplantation, growing as an avascular spheroid in the dog vitreous. H & E, × 1,000.
Fig. 3. A, an avascular colony of mouse ependymoblastoma cells (upper left), 47 days after transplantation to the rabbit vitreous, approximately 2.5 mm from the retinal surface (lower right). H & E, × 40. B, histology of the periphery of the mouse tumor showing many small cells with nuclear pleomorphism and prominent chromatin. H & E, × 1,000.
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