A corneal micropocket assay for angiogenesis in the rat eye. GEORGE A. FOURNIER, GERARD A. LUTTI, SANDRA WATT, ALLAN FENSELAU, AND ARNALL PATZ.

A corneal micropocket assay for angiogenesis in the rat eye is described in detail. With our test system, a partially purified, low molecular-weight endothelial cell growth stimulatory factor isolated from the Walker 256 rat carcinosarcoma is demonstrated to have potent angiogenic activity in vivo. The advantages and applications of our rat corneal assay are discussed.

The exponential growth of solid tumors has been shown to be highly dependent on their ability to induce the formation of new vascular channels from contiguous host tissues.1 Solid tumors elaborate a diffusible tumor angiogenic factor (TAF) that is responsible for tumor-directed growth of new blood vessels from adjacent capillaries.2

The relationship between tumor and host vasculature has been studied in several in vitro and in vivo test systems. The major assays for angiogenesis in vivo are neovascularization of the chicken embryo chorioallantoic membrane1 and the neovascular responses induced by intracorneal implantation of test substances in the rabbit.1 The disadvantages of these assays are that they usually involve xenogeneic donor tissue and are carried out in genetically poorly characterized host animals. The rat dorsal air sac assay2 is of limited value because large quantities of material containing TAF activity are required and continued observation of the progression of the neovascular response cannot be performed. A mouse corneal assay has been described that obviates these problems3 but appears to require very intricate and time-consuming microsurgery. Assays measuring aortic endothelial cell proliferation in vitro4 are not entirely satisfactory models for angiogenesis because they ignore endothelial cell migration, a mechanism in angiogenesis. In addition, such assays differ from the in vivo phenomenon, where host capillary endothelium is the target of TAF.

An assay for angiogenesis in the rat has several advantages: it permits the utilization of knowledge of inbred rat lines and makes use of the extensive data available for the Walker 256 rat carcinosarcoma, a potent source of TAF.1 The rat can safely be rendered immune deficient by x-irradiation5 so that inflammation-induced neovascularization is prevented. In contrast, the rabbit sustains a high level of mortality from such manipulation. Combined treatment with x-irradiation and repeated injection of antineutrophil serum in rats has been shown to eliminate infiltration of injured corneas by neutrophils,6 which have been implicated in the induction of neovascularization.6

The corneal assay is an excellent test system for studying angiogenesis reactions, since the cornea provides a transparent avascular substratum in which vessels originate from a distance, the corneoscleral limbus, thus permitting continued measurement of new blood vessel growth. We have developed a corneal micropocket assay in the rat as a test system for angiogenesis. The current study deals with vascular events after corneal implantation of slow-release pellets containing a partially purified endothelial growth stimulatory factor of rat origin.

Materials and methods

Animals. Male retired breeder Sprague-Dawley rats (Sprague-Dawley Labs, Madison, Wis.) weighing 300 to 500 gm were used for all experiments.

Test substances in slow-release polymers. We have tested three substances that were isolated from the Walker 256 rat carcinosarcoma maintained in Sprague-Dawley rats in the manner outlined by Fenselau et al.7:

1. Crude ethanol extract of Walker 256 tumor homogenate.
2. Partially purified growth stimulatory factor for cultured fetal bovine aortic endothelial cells, which has been demonstrated to be an acid-stable, low molecular-weight substance (fraction C1).
3. An additional column fraction derived from Walker 256 tumor extracts, which is inactive when tested for endothelial cell growth stimulatory activity in vitro (fraction B).

The test substances were handled doubly masked.

The lyophilized substances were mixed in one drop of 10% Elvax 40 (an ethylene-vinyl acetate copolymer, 40% vinyl acetate by weight) in methylene chloride. The resulting mixture was vacuum dried in a glass Petri dish and cut into 30 pellets, which were sized to 0.2 mm3 with a No. 11 scalpel blade and iris forceps. All procedures were performed with aseptic technique.

Implantation technique. Rats were anesthetized intramuscularly with a 50:50 solution of Rompun and ketamine hydrochloride (0.1 ml/100 gm). Eyes...
Fig. 1. Maximum capillary length (mm) at various days after surgery for: partially purified endothelial cell growth stimulatory factor, fraction C, (0.11 O.D. 260/pellet) (○), crude ethanol extract of Walker 256 rat tumor homogenate (0.16 O.D. 260/pellet) (●), and a fraction from tumor homogenate that is inactive when tested for endothelial cell stimulatory activity in vitro, fraction B (0.11 O.D. 260/pellet) (□). With Wilcoxon rank sum analysis, all pairs were significantly different (p < 0.01), except for the crude ethanol extract and inactive substance at day 16 (p = 0.22). Error bars = 1 S.D.

were irrigated with Ringer's lactate, propapsed with a dental dam, and topically anesthetized with 0.5% proparacaine hydrochloride solution. With a cataract knife (E-63; Storz, St. Louis, Mo.), a 0.3 mm transverse incision was made centrally, penetrating about halfway through the corneal stroma. The corneal micropocket was formed with a dull keratome blade (No. OP-600, Mueller, Chicago, Ill.) and extended to 1 mm from the corneoscleral limbus. The micropocket was enlarged to a final size of 0.3 by 1.5 mm by insertion of fine forceps. An Elvax pellet that had previously been placed in sterile Ringer's lactate was deposited at the base of each pocket with fine forceps. The micropocket was closed by sliding the forceps over the corneal epithelium overlying the pocket with the side of the forceps. The eye was irrigated with antibiotics (Neosporin ophthalmic solution; Burroughs Wellcome Co., N.C.). All procedures were performed aseptically.

**Stereomicroscopic observations.** Rats were anesthetized with Rompun and ketamine hydrochloride as described above. Eyes with corneal implants were examined at 4-day intervals with a Zeiss slit-lamp stereomicroscope (Carl Zeiss, Inc., New York) at 25× and 40×. Maximum vessel length and pellet limbus distance were measured during each observation at 40× with an ocular micrometer. The number of blood vessels and presence of corneal edema were also recorded. Serial diagrams of the vascular reactions were made, and photographs were taken to document major changes.

**Histologic studies.** Rats were sacrificed at day 16 after surgery. Eyes were enucleated and immersed in 10% neutral-buffered formalin. Paraffin sections were stained with hematoxylin and eosin.

**Results.** Elvax pellets containing the crude ethanol extract of Walker 256 rat tumor homogenate (4.9 O.D. 260/30 pellets) were implanted in 27 corneas. New vascular channels penetrated the cornea centripetally from the adjacent corneoscleral limbus and proceeded towards the pellet (Fig. 2, b). The pattern of corneal neovascularization was similar to that described for intracorneal implantation of tumor tissue in rabbit and mouse eyes: capillary loops developed perpendicular to limbal vessels and progressively enlarged with the appearance of secondary vascular branches that grew toward the polymer. By day 16, vessels had stopped advancing and began regressing (Fig. 1).

Seventeen corneas implanted with pellets containing the partially purified endothelial growth stimulatory factor (3.4 O.D. 260/30 pellets) demonstrated a similar pattern of neovascularization. A substantial front of capillaries had grown into the avascular cornea by day 4, and in 16 cases the front reached the pellet by day 8 (Figs. 2, c, and 3, c). These corneas displayed a greater maximum capillary length than that in corneas implanted with polymer containing crude ethanol extract of tumor homogenate (Fig. 1). The data were analyzed with the Wilcoxon rank sum test and the difference between the two was found to be significant at all four points plotted (p < 0.01). Capillary regression was not observed in corneas containing the partially purified factor.

In 17 of 18 corneas implanted with polymer containing the inactive fraction (3.4 O.D. 260/30 pellets), maximum capillary length was less than that in corneas containing crude ethanol extract, with mean vessel length not greater than 0.17 mm. In one third of the cases no neovascularization penetrated the cornea (Fig. 2, a). With Wilcoxon rank sum analyses, all pairs were significantly different (p < 0.01) except at 16 days (p = 0.22). Blank Elvax pellets in eight out of eight cases produced
Fig. 2. Stereomicroscopic photographs of corneas containing Elvax pellets impregnated with test substances. *P*, Elvax pellet; *L*, corneoscleral limbus. Days refer to time after implantation of the pellets. *a*, Fraction B from tumor homogenate, which is inactive when tested for endothelial cell growth stimulatory activity in vitro (12 days). No neovascularization has penetrated the cornea (0.11 O.D. 260/pellet). *b*, Crude ethanol extract of Walker 256 rat tumor homogenate (9 days). A few capillaries reached the pellet (0.16 O.D. 260/pellet). *c*, Partially purified endothelial cell growth stimulatory factor or fraction C (8 days). A substantial front of capillaries developed and arborized in the vicinity of the pellet (0.11 O.D. 260/pellet).

Fig. 3. Histologic photographs of the cornea at 16 days after implantation of Elvax pellets impregnated with test substances. Polymer dissolves in fixative leaving the pellet space (*P*). Corneoscleral limbus is marked (*L*). Sections were stained with hematoxylin and eosin. The inflammatory response in these corneas was graded modest to no response. *a*, Inactive substance or fraction B. Negative response. (×50.) *b*, Crude ethanol extract. (×50.) *c*, Partially purified angiogenic substance, fraction C. Numerous capillaries are present (arrows) around the pellet space. (×50.)

Minimal or no neovascular response, with vessels not growing more than 0.15 mm.

The neovascular response observed was not associated with inflammation by clinical criteria, since no corneal edema was appreciated on stereomicroscopic examination. In some of the histologic sections there was a modest degree of inflammation characterized by an occasional lymphocyte and macrophage and rare polymorphonuclear leukocyte. The inflammatory response was comparable in the three experimental groups (Fig. 3) and could not be correlated with the amount of neovascularization.

Discussion. The rat corneal micropocket assay
provides a new experimental model for the study of angiogenesis. Our test system has numerous advantages: it permits assessment and comparison of the angiogenic activity of substances isolated from non-neoplastic rat tissue and rat tumors such as the Walker 256 carcinosarcoma in an allogeneic in vivo system. This is important because a significant inflammatory response is induced by xenogeneic donor tissue in the rabbit corneal micropocket assay. The paucity of histologic inflammation in our experiments is probably related to the fact that test substances are of Sprague-Dawley origin. However, the scope of substances testable in our assay can be expanded to include xenogeneic material, since the rat can safely be rendered immune deficient by x-irradiation. The rat corneal micropocket assay has the additional advantages of being facile and economical.

These experiments demonstrate that both the crude ethanol extract and partially purified endothelial growth stimulatory factor from Walker 256 tumor homogenate have angiogenic activity in vivo. In addition, the partially purified factor is more potent than the crude ethanol extract since it effected a significantly higher capillary growth rate (0.21 vs. 0.08 mm/day, respectively, for the initial 4-day interval) at slightly lower dosages (0.11 O.D. 260/pellet vs. 0.16 O.D. 260/pellet). The fact that our low molecular-weight substance derived from Walker 256 tumor is angiogenic agrees with the findings of Weiss et al. However, we have demonstrated this in the animal of origin for this tumor-derived substance.

The absence of capillary regression before day 16 in corneas implanted with partially purified factor may mean that the capillaries have matured faster than those in corneas containing crude ethanol extract. However, since a continued stimulus is required for the maintenance of blood vessels in this system, this finding may simply indicate that the partially purified substance is more slowly released from the polymer than the ethanol extracted material.

In addition to being implicated in tumor growth, angiogenic substances play a prominent pathophysiological role in numerous ocular diseases, including corneal graft rejection, diabetic retinopathy, and retrolental fibroplasia. Our rat corneal micropocket assay could quicken the purification and characterization of these angiogenic substances as well as the naturally occurring inhibitors of angiogenesis. With increased understanding of neovascularization and its control, effective modalities for treating cancer and the neovascular opthalmopathies can be formulated.

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Key words: angiogenesis, rat corneal micropocket assay, corneal neovascularization, tumor angiogenic factor

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Polymethylmethacrylate intraocular lenses were left in 10% NaOH or 10% KOH for various periods of time. Contact angles were unaltered and electron spectroscopy