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A Strategy to Discover Circulating Angiogenesis Inhibitors Generated by Human Tumors

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Abstract

The phenomenon of inhibition of tumor growth by tumor mass has been studied in many experimental animal systems and has been observed in several clinical scenarios. Not until the recent discovery of angiostatin, a circulating angiogenesis inhibitor generated in the presence of a murine Lewis lung tumor, has a satisfactory mechanism been proposed to explain this phenomenon. Thus far, no other animal or human tumors are known to generate angiostatin. In this study, we utilized a mouse corneal neovascularization model to detect circulating inhibitors of angiogenesis generated by three human tumors grown in immunodecient mice: (a) the PC-3 human prostate carcinoma; (b) the CCL188 human colon carcinoma; and (c) the UBC urinary bladder carcinoma. Mice bearing these three primary tumors demonstrated significant inhibition of angiogenesis in the cornea induced by a pellet containing basic fibroblast growth factor. Corneas of mice bearing s.c. prostate and colon carcinomas showed signiﬁcant inhibition of vessel length, clock-hours of neovascularization, and vessel density. However, corneas of mice bearing s.c. bladder carcinomas demonstrated signiﬁcant inhibition of vessel density only. Three colon carcinomas (clone A, CX-1, and MIP101), the MDA-MB-435S breast carcinoma, the MM-AN melanoma, and the JE-3 choriocarcinoma did not signiﬁcantly inhibit corneal neovascularization.

Introduction

We have described previously a murine Lewis lung carcinoma model in which the presence of a primary s.c. tumor inhibits growth of its lung metastases (1). We have shown that the serum and urine of these tumor-bearing animals contain angiostatin, a novel inhibitor of angiogenesis. We hypothesize that the primary Lewis lung tumor sequesters the angiogenic peptide vascular endothelial growth factor into the circulation in excess of its generation of the angiogenic inhibitor angiostatin. Preliminary data suggest that angiostatin is not synthesized by the tumor cell per se but is generated by enzymatic cleavage of angiostatin from host plasminogen, possibly by an enzyme produced by the tumor cell. Because the circulating half-life of angiostatin is signiﬁcantly longer than that of vascular endothelial growth factor (1), tumor cells at a distant metastasis cannot induce neovascularization, and their growth is suppressed. In this way, the presence of the primary tumor can suppress angiogenesis induced at a distant site in the animal. We have shown that mice bearing the primary Lewis lung tumor exhibit complete inhibition of corneal neovascularization induced by a pellet of bFGF3 implanted in the cornea (1). We have also found signiﬁcant inhibition of angiogenesis using the same mouse corneal micropocket assay when mice are treated systemically with puriﬁed angiostatin. Whether other murine or human tumors generate angiostatin or other inhibitors of angiogenesis remains unknown. This study utilizes a reproduceable mouse corneal neovascularization model to discover circulating angiogenesis inhibitors generated by several human tumors.

Materials and Methods

Animals. Male and female 6–8-week-old SCID mice were obtained from the Massachusetts General Hospital (Boston, MA). All mice were acclimated in a barrier care facility, caged in groups of ﬁve or less, and fed a diet of animal chow and water ad libitum. Animals were anesthetized in a methoxyﬂurane (Pitman-Moore, Mundelein, IL) chamber before all procedures, and studies were conducted according to a protocol approved by the Animal Ethics Committee of Children’s Hospital (Boston, MA).

Cell Culture. The human prostate carcinoma cell line (PC-3) was a gift of Dr. M. Shanum (University of California, San Francisco, CA) and was maintained as monolayers in a 1:1 mixture of DMEM and Ham’s F-12 medium supplemented with 10% heat-inactivated FCS (JRH Biosciences, Lenexa, KS), 2 mM L-glutamine, 100 units/ml penicillin, and 100 jig/ml streptomycin. The human colon carcinoma cell lines (CCL188, clone A, CX-1, and MIP101) were a gift of Dr. J. M. Jessup (Deaconess Hospital, Boston, MA) and were cultured as monolayers in RPMI 1640 with 10% heat-inactivated (56°C for 20 min) FCS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, and antibiotics. The human MDA-MB-435S breast carcinoma cell line was a gift of Dr. B. Zetter (Children’s Hospital, Boston, MA) and was cultured in DMEM with 10% FCS, 2 mM L-glutamine, and antibiotics. The human UBC urinary bladder carcinoma cell line was a gift of Dr. R. Montesano (University of Geneva, Geneva, Switzerland) and was grown as monolayers in DMEM with 10% FCS, 2 mM L-glutamine, and antibiotics. The human melanoma (MM-AN) was a gift of Dr. H. R. Byers (Boston University, Boston, MA) and was cultured in DMEM supplemented with 10% bovine calf serum, 2 mM L-glutamine, and antibiotics. The human choriocarcinoma (JE-3) was obtained from American Type Culture Collection (Rockville, MD) and was grown in DMEM with 10% FCS, 2 mM L-glutamine, and antibiotics. All cell lines were cultured in an atmosphere of 10% CO2, except the colon carcinoma lines, which were placed at 5% CO2. Media and bovine calf serum were obtained from JRH Biosciences. Stock solutions of L-glutamine, antibiotics, and trypsin-EDTA (0.05% trypsin-0.53 mM EDTA) were obtained from Life Technologies (Grand Island, NY).

Growth of Tumors in Vivo. Tumor cells were harvested from subconfluent cultures with trypsin-EDTA (1 min), washed in medium, and resuspended in 0.96% PBS. The ﬁnal concentration was adjusted to 1 X 107 cells/ml. For the corneal micropocket assay, SCID mice were given s.c. injections of 1 X 106 cells in 0.1 ml of PBS in the proximal dorsa in the midline.

Mouse Corneal Micropocket Assay. Primary s.c. tumors were allowed to grow in SCID mice until they reached at least 500 mm3, requiring 4 weeks or longer after injection of tumor cells. Corneal pockets were created with a modiﬁed von Graefe cataract knife (as modiﬁed from Muthukkaruppan and Auerbach; Ref. 2) in the eyes of mice bearing primary s.c. tumors and control...
mice without tumors as described. Into each pocket, a 0.34 × 0.34 mm sucrose–aluminum sulfate (Buhk Meditec, Copenhagen, Denmark) pellet coated with hydron polymer type NCC (IFN Sciences, New Brunswick, NJ) containing 80–100 ng of bFGF (Takeda Pharmaceuticals, Tokyo, Japan) was implanted. The pellets were positioned 1.0 mm from the corneal limbus and erythromycin ophthalmic ointment (E. Fougera, Melville, NY) was applied to each operated eye. The corneas of all mice were routinely examined by slit-lamp biomicroscopy on postoperative days 5 through 7 after pellet implantation. Vessel length and clock-hours of neovascularization were measured on the sixth postoperative day when all corneas were photographed. Freshly prepared bFGF pellets were used for each experiment consisting of non-tumor-bearing control animals and animals bearing primary s.c. tumors of each type studied.

**Computer-assisted Corneal Vessel Density Determination.** For each cornea examined, the area of new vessel growth was delineated on a color photographic print. The outlined area was digitized using a Hewlett Packard ScanJet IIcx to produce a 300-dot-per-inch, extra heavily sharpened, 256 grayscale image. The captured image was converted to its negative image to enhance contrast of the vessels against the background. The image was saved in tagged image file format and ported to the Image/pcp image analysis program (Ver. 1.56b32; NIH, Bethesda, MD). A grayscale threshold value for conversion of the image to black and white was determined by the image analyzer by comparing the computer image to the original photograph and approximating the best vessel density match to the original image. A sharpening filter was used twice to enhance the contrast of adjacent pixels, and a smoothing filter was used to eliminate extraneous noise in the image. To quantitate the total number of pixels in each scanned area, the image was converted into a binary form, and the pixel count of the binary image with a value of 0 (corresponding to white) was added to the pixel count with a value of 256 (corresponding to black). The percentage of area corresponding to vessels was obtained by dividing the pixel count with a value of 0 by the total pixel count. At least three separate corneas from control mice or mice bearing each tumor type were analyzed in triplicate. The original color photographs were coded to allow a masked analysis by the investigator. Inhibition of vessel density in corneas of tumor-bearing animals was determined as a percentage of control values obtained from non-tumor-bearing animals.

**Results and Discussion**

Nine different human tumor cell lines were grown as primary s.c. tumors in male SCID mice (Table 1). Tumors grew to a size of at least 500 mm³ within 4 weeks after implantation of tumor cells. At this time, hydron pellets containing 80–100 ng of bFGF were implanted into the corneas of tumor-bearing and control mice. Six days after pellet implantation, maximal neovascularization induced by the bFGF pellet was observed in the corneas of all control mice not bearing primary tumors (Fig. 1, left panels). In these control corneas (n = 26), new vessels crossed the cornea from the limbus toward the pellet within 6 days after pellet implantation, occasionally penetrating into the pellet. In contrast, mice bearing s.c. primary PC-3 human prostate carcinomas exhibited the most dramatic inhibition of bFGF-induced corneal neovascularization of all the tumors studied (Fig. 1). As compared to control corneas (n = 10), vessel length, circumferential clock-hours, and density of the corneal vessels were inhibited by 80, 53, and 73%, respectively, in the corneas (n = 11) of these tumor-bearing animals (Fig. 2).

Similarly, mice bearing s.c. primary CCL188 human colon carcinomas and UBC urinary bladder carcinomas demonstrated significant inhibition of corneal neovascularization induced by a bFGF pellet (Fig. 1). Corneas of mice with primary colon carcinomas (n = 12) showed 31% inhibition of vessel length, 29% inhibition of clock-hours, and 63% inhibition of density of new corneal vessels when compared to control corneas (n = 11; Fig. 2). The neovascular response in the corneas (n = 3) of mice bearing primary urinary bladder carcinomas was inhibited most dramatically when density of new vessels was quantitated compared to that of control corneas (n = 5; 68% inhibition) but not when vessel length or clock-hours were measured (2 and −13% inhibition, respectively; Fig. 2).

Corneas of mice with four other human tumors, including JE-3 choriocarcinoma, MM-AN melanoma, clone A colon carcinoma, and CX-1 colon carcinoma, demonstrated minimal inhibition of vessel length and clock-hours (Fig. 2). Vessel length was inhibited by 0, 18, 0, and 4%, respectively, whereas clock-hours were inhibited by −10, 7, −6, and −6%, respectively, for these four tumors. Inhibition of vessel density as a percentage of control was 34, 19, 31, and 29%, respectively, in corneas of mice bearing these four human tumors (Fig. 2). Whether these changes in vessel density ≤35% reflect the presence of a circulating angiogenesis inhibitor remains unclear. Although we measured only the three parameters of vessel length, circumferential clock-hours, and vessel density, it is intriguing to speculate on their correlation with discrete events involved in corneal neovascularization (10). Perhaps those corneas in which only vessel density is affected (but not vessel length or clock-hours) are associated with an angiogenesis inhibitor that acts more potently on vascular sprouting than endothelial cell proliferation or migration. In contrast, those corneas in which all three measured parameters are affected may be associated with inhibition of endothelial cell proliferation, migration, and sprouting.

Not all human tumors grown in mice are associated with inhibition of angiogenesis in the cornea. Mice bearing s.c. primary MDA-MB-435S human breast carcinomas did not exhibit any significant inhibition of bFGF-induced corneal neovascularization (5 and 10% inhibition of vessel length and clock-hours, respectively; Figs. 1 and 2). In fact, vessel density in corneas of mice bearing the MDA-MB breast carcinoma was increased by 30% compared to that of control corneas (Fig. 2). This lack of inhibition of new vessel growth in the cornea was seen in both male and female SCID mice bearing this human breast carcinoma (data not shown). A similar finding was obtained when the MIP101 colon carcinoma was studied in the same manner (5, 0, and −3% inhibition of vessel length, clock-hours, and vessel density, respectively; Fig. 2).

Taken together, these data show the utility of a reproducible in vivo mouse corneal neovascularization model to discover potential circulating angiogenesis inhibitors generated by human tumors grown in mice. We have shown that the presence of three primary s.c. human tumors in mice, PC-3 prostate carcinoma, CCL188 colon carcinoma, and UBC urinary bladder carcinoma, is associated with significant inhibition of bFGF-induced angiogenesis at a distant site in the cornea. Analogous to angiostatin and its generation by murine Lewis lung carcinomas in vivo (1), these tumors could secrete a human antiangiogenic molecule into the circulation that is active against endothelial cells in the mouse, or the tumor cells could generate an angiogenesis inhibitor by acting on a host murine molecule. Of note, our assay may fail to detect those human tumors grown in mice that secrete an angiogenic inhibitor that is
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Fig. 1. Inhibition of bFGF-induced corneal neovascularization in mice bearing primary s.c. tumors of human prostate carcinoma (PC-3), human colon carcinoma (CCL188), and human urinary bladder carcinoma (UBC) but not in mice bearing human breast carcinomas (MDA-MB-435S). Hydron pellets containing sucrose aluminum sulfate and 80 ng of bFGF were implanted in the corneas of control SCID mice (left panels) and mice bearing primary tumors of at least 500 mm³ (right panels). Fresh pellets were used for each experiment shown in each row. Corneas were photographed on the sixth postoperative day by slit-lamp photomicroscopy. Arrowheads, locations of the pellets (P).

Fig. 2. Quantitation of inhibition of bFGF-induced corneal neovascularization in SCID mice bearing nine different primary s.c. human tumors. Inhibition is determined as percentage of control measurements of vessel length, circumferential clock-hours of neovascularization, and vessel density made on the sixth postoperative day. Control animals were age-matched mice without primary tumors. Negative values for inhibition are synonymous with stimulation. Ca., carcinoma.

species specific. We are currently analyzing serum and urine of tumor-bearing animals for inhibitory activity against endothelial cell proliferation in preparation for purification. Previous experiments utilizing the prostate carcinoma cell line provide evidence to support the presence of a circulating angiogenesis inhibitor in these tumor-bearing mice. Working with a subline of the PC-3 prostate carcinoma, Ware and DeLong (11) reported an increased number of lymph node and lung metastases in animals after resection of the primary tumors, whereas tumor-bearing animals demonstrated a restricted number of metastases. This finding is another example of the phenomenon of inhibition of tumor growth by tumor mass, which can be partly explained by the generation of an angiogenesis inhibitor by the primary tumor that inhibits growth in a secondary metastasis (1). We have also assayed conditioned media from cultured tumor cells for antiangiogenic activity. Conditioned media of UBC urinary bladder carcinoma cells significantly inhibits DNA
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The synthesis of capillary endothelial cells. These biochemical data correlate with our in vivo corneal data on inhibition of angiogenesis in the presence of certain human tumors and emphasize the importance of analyzing other human tumors with this reproducible assay.

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References


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