"Vasocrine" Formation of Tumor Cell-lined Vascular Spaces: Implications for Rational Design of Antiangiogenic Therapies

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ABSTRACT

Here we report that B16F10 murine melanoma cells mimic endothelial cell behavior and the angiogenic process in vitro and in vivo. Cord formation in vitro by tumor cells is stimulated by hypoxia and vascular endothelial growth factor (VEGF) and inhibited by antibodies against VEGF and the VEGF KDR receptor (VEGFR receptor 2). We define regulation of tumor cell-derived vascular space formation by these vasoactive compounds as "vasocrine" stimulation. ICRF 159 (Razozone; NSC 129943) prevents tumor cell but not endothelial cell cord formation in vitro, and the antiangiogenic drug TNP-470 (NSC 642492) inhibits endothelial but not tumor cell cord formation in vitro. Both drugs inhibit formation of blood-filled vascular spaces in vivo. These results bear on the anticipated action of ICRF 159 in human clinical trials and novel strategies for targeting tumor blood supplies.

INTRODUCTION

The dependence of tumors on the proliferation of blood vessels to sustain growth and metastasis was noted more than 20 years ago (1). This observation led to the hypothesis that some forms of cancer treatment could be based on inhibition of the tumor blood supply (reviewed recently in Refs. 2 and 3). Predictably, tumor angiogenesis is a complex process regulated by redundant cascades of both inducers and inhibitors of endothelial cell growth and migration. Recent reports that blood vessels in tumors may not be entirely lined by endothelium add to the complexity. Some tumors contain vascular channels lined by tumor (not endothelial) cells termed "mimicry" (4, 5) or endothelial cells in conjunction with tumor cells termed "mosaicism" (6) or both (7, 8). In this regard, aberrant blood vessels were noted more than 50 years ago (9), and numerous other reports have documented such structures in human and rodent tumors throughout the following years (Ref. 10 and the references therein). In the present study, we demonstrate that murine B16F10 melanoma cells respond to vasoactive signals classically associated with effects on endothelial cells and angiogenesis with behaviors that resemble those also observed in relation to the effects of these factors on endothelial cells. We have defined this phenomenon as a "vasocrine" response on the part of the tumor cells. Moreover, we show for the first time that drugs with distinct effects on tumor and endothelial cells can affect a tumor blood supply comprised of both endothelial and tumor cell-lined spaces. These results may well impact the rational design of antiangiogenic drugs.

MATERIALS AND METHODS

In Vitro Cord-forming Activity. HUVECs (Clonetics, San Diego, CA) were maintained in EGM-2 medium (Clonetics) containing (as supplied by the manufacturer) 2% FBS, heparin, Long R insulin-like growth factor, ascorbic acid, human epidermal growth factor, VEGF, human fibroblast growth factor B, and hydrocortisone. B16F10 cells (DTP Drug Repository, Frederick, MD) were maintained in RPMI 1640 containing 10% fetal bovine serum and 1% glutamine. Minimal medium was DMEM with no additions unless otherwise indicated. TNP-470 and ICRF 159 were obtained from the DTP Drug Repository (Rockville, MD). Aliquots of stock solutions (10 mM in DMSO, stored at -70°C) were used once and then discarded. Matrigel (50% in media; Collaborative Biomedical Products, Bedford, MA) was added to each well of a 24-well plate on ice and allowed to gel for 1–2 h at room temperature. B16F10 cells or HUVECs were added to the Matrigel-coated wells at 6 x 10^4 cells/well for B16F10 cells or 3 x 10^4 cells/well for HUVECs in a final volume of 0.5 ml. The addition of reagents, as noted in the figure legends, was made to the cells immediately before the cells were plated. Twenty-four h later, pictures were taken of each well using a Leica DM IRB inverted microscope (Rockville, MD), and the treatment effect was assessed by measuring the length of the cords and the number of junctions formed using the Bioquant Image Analysis System (R&M Biometrics, Nashville, TN). Experiments were performed at least twice, and the data were pooled. The number of points/treatment group ranged from 6–16. Data are plotted ± SE.

For confocal microscopy, B16F10 melanoma cells were grown on a coverslip coated with Matrigel in a 35-mm culture dish. After 24 h, the cells were stained with 2 mM DiC06 (Molecular Probes, Eugene, OR) for 30 min. Stained cultures were sectioned optically using a Zeiss confocal microscope (model 410; Carl Zeiss, Inc., Thornwood, NY) with a 40 x 1.2NA Apochromat objectives. Fluorescence was excited with a 488 nm band of argon/krypton laser, and emitted fluorescence was detected using a 510–525 nm band pass filter.

Cell Viability. Cells (2500 in 0.1 ml media) were placed in each well of a 96-well plate 24 h before treatment. On the day of treatment, test samples (10 μl) were added to the appropriate wells, and the cells were incubated for 24 h at 37°C in a humidified CO2 incubator. Cell viability was determined using WST-1 (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer’s instructions.

Effects of Hypoxia. For cord forming assays, Matrigel plates prepared as described above were placed either directly into a 37°C humidified CO2 incubator (normoxic conditions) or into a modular incubator chamber (Billups Rothenberg, Del Mar, CA) and flushed for 20 min with a mixture of 1% O2, 5% CO2, and 94% N2 (hypoxic conditions). The chamber was then placed into a 37°C incubator, and both plates were incubated overnight. For determination of VEGFR-2 tyrosine phosphorylation, 1 x 10^7 cells were plated into 100-mm cell culture dishes 24 h before treatment. The plates were then placed under hypoxia. At the indicated time points, the cells were washed with ice-cold PBS and lysed with 50 mM Tris-HCl (pH 7.4) containing 1% NP40, 150 mM NaCl, 2 mM sodium orthovanadate, and complete protease inhibitor mixture (Roche Molecular Biochemicals). The lysates were centrifuged at 4°C for 10 min at 10,000 x g, and the supernatant was used for Western blotting or immunoprecipitation. Immunoprecipitations were performed by using 0.6 μg of cell lysate incubated overnight at 4°C with 4 μg of rabbit polyclonal anti-VEGF-R2 (N-931; Santa Cruz Biotechnology, Santa Cruz, CA). Resulting

Received 11/11/02; accepted 3/31/03.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Funded in whole or in part with federal funds from the National Cancer Institute, NIH, under Contract No. NO1-CO-56000.

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3 The abbreviations used are: HUVEC, human umbilical vein endothelial cell; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; DTP, Developmental Therapeutics Program; PECAM-1, platelet/endothelial cell adhesion molecule 1; mVEGF, murine VEGF.
immune complexes were pelleted using 30 μl of 50% GammaBind G-Sepharose slurry (Amersham Pharmacia Biotech, Piscataway, NJ), washed three times with lysis buffer, and resuspended in 2× Laemmli buffer. Samples were then subjected to Western blot analysis. For Western blot, protein samples (50 μg) in Laemmli buffer were resolved by 8% SDS-PAGE. After transfer to nitrocellulose membranes, blots were probed with anti-VEGF-R2 (0.5 μg/ml in 5% milk) or monoclonal anti-phosphotyrosine antibodies (1:2,000 dilution in 1% BSA; Sigma, St. Louis, MO). After incubation with 1:10,000 dilution horseradish peroxidase-labeled antirabbit IgG (Amersham Pharmacia Biotech) or 1:10,000 dilution antismouse IgG (Amersham Pharmacia Biotech), proteins were visualized using an enhanced chemiluminescence system (ECL; Amersham Pharmacia Biotech).

VEGF ELISA. The levels of VEGF protein in cell-free supernatants were assayed using a commercially available kit (R&D Quantikine; R&D Systems, Inc., Minneapolis, MN). A standard curve was generated using recombinant VEGF provided in the kit. Samples were assayed in duplicate, and results are from two independent experiments.

In Vitro Treatment Study. Female C57BL/6 mice, 4–6 weeks of age, were obtained from the National Cancer Institute Animal Repository (Frederick, MD) and housed in sterile microisolator cages in an Association for Assessment and Accreditation of Laboratory Animal Care-approved facility under USDA animal care and use guidelines. All compounds used in these studies for treating mice were obtained from the DTP Drug Repository (Rockville, MD). B16F10 melanoma cells (1 × 10⁶ cells in 0.1 ml 0.9% saline; Ref. 11) were implanted s.c. on the lateral wall just caudal to the axillary region. Treatment began one day after tumor cell injection. Treatments included control (10% DMSO in Klucel (DTP, Division of Cancer Treatment and Diagnosis, National Cancer Institute Drug Repository, Rockville, MD)), i.p., every day for 10 treatments; TNF-γ (3% ethanol in saline), 30 mg/kg, s.c., every other day for 6 treatments; and ICRF 159 (10% DMSO in Klucel), 30 mg/kg i.p., every day for 10 treatments. Animals were sacrificed on day 13. The percentage of treated tumor mass/control tumor mass (%T/C) was calculated by dividing the median tumor weight of the treated group by the median tumor weight of the control group and multiplying by 100. To confirm the antitumor effects of the drugs, these experiments were repeated six times with similar outcomes. Student’s t test comparison of the treated and control groups indicate a statistically significant reduction in tumor weight for the ICRF 159-treated group (P = 0.01) and TNF-γ-treated group (P = 0.02).

Patency. Donor RBCs obtained from strain-matched mice were rendered fluorescent by incubating them with PKH-26 (Sigma) following the manufacturer’s instructions. Thirty s after the RBCs were injected into the tail vein, the mice were sacrificed, and the tumor was excised immediately. The excised tumors were fixed, cryoprotected in 30% sucrose, placed in embedding medium and Accreditation of Laboratory Animal Care-approved facility under USDA animal care and use guidelines. All compounds used in these studies for treating mice were obtained from the DTP Drug Repository (Rockville, MD), B16F10 melanoma cells (1 × 10⁶ cells 0.1 ml 0.9% saline; Ref. 11) were implanted s.c. on the lateral wall just caudal to the axillary region. Treatment began one day after tumor cell injection. Treatments included control (10% DMSO in Klucel (DTP, Division of Cancer Treatment and Diagnosis, National Cancer Institute Drug Repository, Rockville, MD)), i.p., every day for 10 treatments; TNF-γ (3% ethanol in saline), 30 mg/kg, s.c., every other day for 6 treatments; and ICRF 159 (10% DMSO in Klucel), 30 mg/kg i.p., every day for 10 treatments. Animals were sacrificed on day 13. The percentage of treated tumor mass/control tumor mass (%T/C) was calculated by dividing the median tumor weight of the treated group by the median tumor weight of the control group and multiplying by 100. To confirm the antitumor effects of the drugs, these experiments were repeated six times with similar outcomes. Student’s t test comparison of the treated and control groups indicate a statistically significant reduction in tumor weight for the ICRF 159-treated group (P = 0.01) and TNF-γ-treated group (P = 0.02).

Transmission Electron Microscopy. Tissue preparation was as described previously (12). Thick plastic sections (0.5 μm) were stained with 0.5% Toluidine Blue-O in 1% sodium borate buffer; thin plastic sections (60 nm) were stained with uranyl acetate and lead citrate and photographed with an electron microscope (Hitachi H7000; Hitachi, Tokyo, Japan) at 75 kV.

Microscopic Analysis. Tumors from each of the treatment groups (six from the control group and three from the TNF-γ and ICRF 159 treatment groups) were collected and prepared for histological evaluation by embedding in paraffin. Sections (5 μm) were cut from four different levels, stained with H&E, and examined with a Zeiss Axiohot 2 microscope. Slides were scanned (DianoScan T1200; Agfa, Ridgefield Park, NJ) at resolution of 1200 ppi, and images were analyzed with Image Pro Plus 3 for Windows 95/NT. The entire area of the section and total area of blood in tissue in and outside the vessels were calculated for each of the sections based on color segmentation. The respective areas were expressed as ratios and presented as percentage of blood area of the total area of the section. Statistical analysis was performed with the Welch modified two-sample test.

RESULTS

Vasoactive Signals Cause B16F10 Melanoma Cells to Mimic Endothelial Cell Behavior in Vitro. HUVECs plated on Matrigel in growth factor-containing medium elongated and anastomosed into a network of typical cord-like structures by 24 h in culture (Fig. 1a). Optical sectioning with confocal microscopy was used to determine three-dimensional structure. The presence of spaces or lumens within some HUVEC cords was observed, and a typical example is shown in Fig. 1c. B16F10 melanoma cells also formed cords in three-dimensional cultures containing Matrigel. In growth factor-containing medium, B16F10 cells began to align within 4 h (data not shown). By 24 h, these patterns evolved into the honeycomb-like structure typified in Fig. 1b that consisted of multilobular clusters of cells connected by cord-like structures. Analysis by optical sectioning with a confocal microscope demonstrated that these “melanoma cords” also contained lumen-like spaces (Fig. 1d). Cord formation by tumor cells under these conditions was not a single cell line phenomenon because human breast (MDA-MB 231), ovarian (SKOV-3), and Kaposi’s sarcoma (KS Y-1) tumor cell lines also formed cords under these conditions (data not shown).

Cord junctions and length were determined in cell culture medium with or without growth factors. B16F10 melanoma cell cord formation required contact with a reconstituted basement membrane (Matrigel) as well as growth factors (Fig. 1e). Contact with Matrigel in minimal medium (medium lacking growth factors) caused the melanoma cells to form small clusters with short discontinuous cords. Melanoma cells on Matrigel were exposed to minimal medium containing only mVEGF to test the effects of this vasoactive agent in the absence of other growth factors. Significant reorganization of the cells into the mature cords began to occur when 10 ng/ml exogenous mVEGF in minimal medium was added to the wells (Fig. 1e). Cord formation induced by mVEGF was complete by 30 ng/ml mVEGF.

Hypoxia can drive reorganization of HUVECs into cord-like networks through autocrine stimulation of these cells by VEGF (13). Hypoxia also can stimulate VEGF production and cord formation by B16F10 melanoma cells (Fig. 1e). Under normoxic conditions, cells placed in minimal medium on Matrigel for 24 h formed clumps of cells with short processes as described in the preceding paragraph (VEGF production < 31 pg/ml from 2–12 h). Parallel cultures grown for 24 h under hypoxic conditions increased VEGF production (31–185 pg/ml over 12 h) and displayed cord formation that was disrupted by antibodies against mVEGF but not by an irrelevant antibody of the same isotype (Fig. 1e). Based on these data, VEGF appears to contribute to hypoxia-induced cord formation of B16F10 tumor cells.

VEGFR-2 appears to mediate the major actions of VEGF on endothelial cells (Ref. 14 and the references therein). We therefore investigated whether VEGFR-2 is expressed on B16F10 cells in culture and whether it is affected by hypoxia. We found that B16F10 melanoma cells do express VEGFR-2 and that hypoxia increases phosphorylation without increasing protein content (Fig. 2a). Hypoxia induced a 2-fold increase in VEGFR-2 phosphorylation by 4 h that was sustained for at least 8 h (Fig. 2b). To determine whether
activation of VEGFR-2 was directly involved in mediating B16F10 cord formation, anti-VEGFR-2 antibody was added to the cultures before being placed under hypoxic conditions. Cord formation induced by hypoxia was disrupted by the antibody against the VEGFR-2 but not by an irrelevant antibody of the same isotype (Fig. 2c). Thus, B16F10 melanoma cells respond to tumor cell-secreted VEGF to promote elongation and cord formation by activating VEGFR-2.

Based on these in vitro data, we propose the existence of cellular mechanisms that we call vasocrine regulation. In this scheme (Fig. 3), tumor cells may secrete angiogenic factors, which can cause proliferation of endothelial cells in the familiar ways described previously to promote angiogenesis, the proliferation of new blood vessels by endothelial cells. However, we propose here that tumor cells may respond to these factors in a vasocrine fashion, whereby the tumor cells themselves assume certain of the properties of blood vessels, thus causing some tumor cells to function like endothelial cells. If this idea is correct, drugs may be imagined that affect tumor blood flow by altering the tumor cell contribution to the tumor’s vascular space as well as affecting endothelial cells directly.

Drug Effects on Cord Formation by Endothelial or Melanoma Cells in Vitro. TNP-470 (NSC 642492), a synthetic derivative of fumagillin (15), and ICRF 159 (Razoxane, NSC 129943; Ref. 16) represent drugs reported to affect tumor vasculature. TNP-470 is known to potently inhibit endothelial cell proliferation (17), whereas ICRF 159 in very early studies was found to “normalize” (tumor cell-derived vasculature becomes less leaky) tumor blood vessels (18), although the cellular and molecular basis for this phenomenon has not been understood. TNP-470 (100 nM) inhibited the morphological transformation of HUVECs on Matrigel (Fig. 4, compare c with a), but HUVEC cord formation was unaffected by the same or higher concentrations of mVEGF. Additionally, some plates also in cell culture medium without any additives were placed under hypoxic conditions with or without 10 μg/ml anti-mouse VEGF or an irrelevant antibody of the same isotype. Unless otherwise noted, magnification was ×10.
Melanoma Cells Form Functional Blood-carrying Channels in Tumors. The formation of blood-carrying channels by B16F10 tumor cells was examined in s.c. implanted B16F10 tumors. In this model, melanosomes are easily seen in the cytoplasm and constitute a marker for the melanocytic nature of the cells. In Fig. 5a, RBCs, other blood cells, and free tumor cells are seen in a channel-like structure directly in contact with melanosome-containing tumor cells. Clefts are often seen in these channels (Fig. 5b and c). These results are consistent with prior reports that other blood cells have been observed to be in direct contact with tumor cells in other rodent melanoma models (20).

Because a functional blood channel would be one directly connected to the circulatory system, providing a route through which blood might actually circulate, we assessed whether spaces that contained the RBCs were obviously connected to the central circulation and whether they could be bordered by endothelial cells or also by tumor cells. Donor RBCs labeled with the red fluorescent dye PKH-26 were infused into the tail vein of C57BL/6 mice bearing day 13 B16F10 tumors. Mice were sacrificed after 30 s, and the tumors were excised and processed for microscopy. In a bright-field section, RBCs are seen in a cylindrical vessel as well as in an irregularly shaped space (Fig. 5d, yellow and white arrows, respectively). Unlike capillaries rimmed by endothelial cells (Fig. 5e, yellow arrow), the edges of the irregularly shaped RBC-containing space did not react with antibodies against PECAM-1 (CD31), an endothelial cell marker (Ref. 21; Fig. 5e, white arrow). Thus, red fluorescence-labeled blood cells appeared essentially simultaneously in both PECAM-1-positive and -negative spaces, providing evidence of patency, i.e., a direct connection of both endothelial and tumor cell-lined spaces to the central circulation into which the labeled RBCs were introduced. This indicates that at least some tumor cell-lined blood channels are functional blood vessels in these B16F10 tumors and not merely extravascular blood pools.

**Drug Effects on Blood-containing Channels Lined by Endothelial or Melanoma Cells.** ICRF 159 and TNP-470 were tested in mice bearing B16F10 tumors. Tumor measurements and tumor specimens were obtained at 13 days. Both ICRF 159 and TNP-470 slowed tumor growth [percentage of treated tumor mass/control tumor mass (%T/C) was 34% and 56%, respectively, at day 13]. Tumors from drug- and vehicle-treated mice were examined histologically, and representative tumors from day 13 are shown (Fig. 6, a–f). A control tumor contains numerous vascular channels and pools of blood (Fig. 6, a and d). The blood pools are diminished in both TNP-470 (Fig. 6, b and e) and ICRF 159 (Fig. 6, c and f)-treated tumors. The surface area of blood (intra- and extravascular) in the tumor sections was 5.9 ± 1.0, 2.8 ± 0.3, and 1.2 ± 0.2% in control, TNP-470-, and ICRF 159-treated tumors, respectively (P = 0.0019 for control versus TNP-470, P = 0.0021 for control versus ICRF 159, and P = 0.0023 for TNP-470 versus ICRF 159). The decline was not due to tumor sampling size because similar results were obtained with size-matched tumors.

Similarly, both ICRF 159 and TNP-470 decreased the number of PECAM-1 positively stained structures in B16F10 tumors. Frozen sections from day 13 tumors from vehicle- or drug-treated mice were prepared and analyzed (Fig. 6, g–i). Sections were examined by bright-field microscopy to be certain that only tumor tissue, and not skin, muscle, or artifacts due to sectioning, would be measured (data not shown). Tiled images of the entire area of each section were recorded at an initial magnification of ×20. The total area of fluorescence-positive structures per total area examined and the average area of individual PECAM-1-positive structures were calculated.
culated for each section in each group, and the averages were obtained. ICRF 159 decreased the percentage of total area of PECAM-1-positive structures from 4.52% to 2.5% (P < 0.0001) or 45% compared with a 23% reduction by TNP-470 (P < 0.0003). Interestingly, ICRF 159 increased the average area of individual PECAM-1 structures by 63% over vehicle-treated mice (0.00044 to 0.00072, P = 0.016). TNP-470 did not significantly affect the area of individual PECAM-1 structures over vehicle-treated mice (0.00044 to 0.00034, P = 0.08). These results provide evidence that TNP-470, with primary effects on the endothelial cell compartment (Fig. 4, compare c with a), or ICRF 159, with primary effects on the tumor cell compartment (Fig. 4, compare f with b), can both influence the architecture of blood-carrying spaces in tumors.

DISCUSSION

Tumor angiogenesis refers to the formation of new blood vessels from preexisting vessels and involves endothelial cell growth, migration, and tube formation. Current antiangiogenic strategies target many of the steps and factors involved in that process. However, many papers through the years, as well as our present work, have reported that some tumors contain blood channels derived partly or entirely from tumor cells themselves [Refs. 4–9 and 18; references noted in McDonald et al. (10)].

How do these tumor cell-lined channels form? Vasculogenic mimicry proposes that the tumor cells can adopt endothelial-like phenotypes and form de novo tumor channels in the absence of endothelial cells (Ref. 22 and the references therein). Alternatively, shedding
endothelial cells may directly expose underlying tumor cells to blood (6). In the current study, we show that B16F10 melanoma cells mimic the physiological behavior of endothelial cells in vitro. Hypoxia stimulates endothelial cells in culture to produce VEGF, which drives the formation of endothelial cell cords (13). We show here that hypoxia stimulates B16F10 cells in culture to produce VEGF, which drives the formation of melanoma cell cords. We define the response of tumor cells to angiogenic factors leading to behavior (such as in vitro cord formation analogous to in vitro angiogenesis) as a vasocrine response of the tumor cells, as outlined in Fig. 3. In this model, at least some of the physiological signals that stimulate tumor angiogenesis could also stimulate tumor cell vascular space formation. Although we recognize that tumor cells have been known to express and respond to molecules also evoking responses in endothelial cells (23), the functional consequences of that response have not been thoroughly delineated. Our data support the idea that among the possible responses is the acquisition of functional behaviors on the part of tumor cells that are similar to those of endothelial cells. Aggressive melanoma cells that form blood-carrying channels in tumors also exhibit cord formation and express endothelial-like markers (4). Future studies will determine whether endothelial-associated markers are expressed in B16F10 melanoma cells undergoing cord formation.

Clearly, the overall contribution and significance of the tumor-lined blood channels have to be determined. We have presented data that address the functional significance of such structures in the B16F10 murine melanoma model. At least some of the RBC-containing channels are connected to the host vasculature because fluorescent RBCs injected into the tail vein simultaneously appeared in both PECAM-1-positive and -negative RBC-containing channels. Hence it appears that the RBCs in these channels are streaming and not merely extravasated pools of blood. These data are consistent with results reported by Warren and Shubik (20), who observed RBCs in tumor cell-lined clefts of melanoma transplants and thought that they were fed and drained by capillaries. Moreover, blood flow in tumor channels in an inflammatory breast cancer xenograft has recently been described (24). Taken together, the functionality of these structures means that they can contribute to nourishing a tumor and, as discussed further on, bear on the prognosis of disease progression.

Endothelial cells attached to a gel composed of reconstituted basement membrane proteins (Matrigel) rapidly align and form three-dimensional tube-like structures, “cords” (25, 26). This behavior has been taken as evidence that endothelial cells cultured on Matrigel retain the information to mimic the angiogenic process in vitro (27). In some studies, tumor cells that did not form highly aggressive tumors and tumor cell-lined blood carrying channels did not form cords on Matrigel (4). On a practical level, inhibition of endothelial cell cord formation in culture is used to assay potential angiogenic stimulators or inhibitors. Cultured ulveal melanoma cells (4), as well as several ovarian and breast cancer cell lines (22), show cord-forming activities in cell culture. In the current study, we used cord formation of B16F10 tumor cells, analogous to that of endothelial cells, to assay two drugs for inhibition of this cord-forming activity. TNP-470, a synthetic derivative of the fungal antibiotic fumagillin (15), is an antiangiogenic agent and an inhibitor of endothelial cell cord formation (Ref. 17 and this work). However, TNP-470 did not inhibit cord formation by the B16F10 tumor cells.

We noted the work by Le Serve and Hellmann (18), who reported that ICRF 159, a member of the bisdioxopiperazine class of antitumor drugs, altered the abnormal character of tumor vessels. The abnormal character of tumor vessels is well known to extravasate macromolecules (28) and possibly tumor cells, thereby promoting metastatic disease (9, 29, 30). Defects in endothelial cell linings could contribute to tumor vessel leakiness as reported recently by Hashizume et al. (31); however, the authors could not discount the presence of other cell types in the composition of these leaky vessels. In fact, tumor cell-lined sinusoidal vascular channels are poorly formed and leaky (29). Because ICRF 159 made tumor vessels less leaky (18), we reasoned that this drug might affect some aspects of in vitro angiogenesis such as cord formation. We found that ICRF 159 only affected cord formation in vitro by the melanoma cells. Melanomas in humans (4, 7) and rodent models (20) reportedly form blood-containing channels lined by tumor cells. Thus, inhibition of cord-forming activity by ICRF 159 led us to examine the effect of this drug on the characteristics of tumor vessels in s.c. implants. ICRF 159 quantitatively decreased blood pools, and PECAM-1 positive structures in these tumors more than TNP-470.
Le Serve and Hellmann (18) further reported that “normalization” of blood vessels by ICRF 159 led to a decrease in metastases from a spontaneously metastasizing Lewis lung carcinoma. A preliminary experiment also using a spontaneously metastasizing Lewis lung carcinoma demonstrated a decrease in the number of lung lesions by ICRF 159, but not by TNP-470.4 Thus, our results both uphold and extend proposals that a drug might be used to alter tumor vasculature to prevent escape of tumor cells from abnormal tumor blood vessels (18) and improve delivery of therapeutics (18, 32). The clinical significance of these results is underscored because ICRF 159 (Razozone) was only recently reported to have minimal toxicity in a Phase II study in patients with renal cell carcinoma and to merit further clinical evaluation in combination with other antiangiogenic agents (33).

In summary, we have shown that B16F10 tumor cells respond to some of the physiological signals that regulate in vitro angiogenesis in a manner analogous to endothelial cells, e.g., cord-forming activity, and we have termed this vasocrine behavior. Although we recognize that the significance of the vasocrine response of tumor cells to clinical behavior remains to be evaluated further, the cord forming assays described here may be useful for identifying other possible distinct markers associated with tumor cell cord formation. These molecules might then be examined for correlations with tumor clinical behavior. Additionally, tumor cell cord-forming activity can be used to assay drugs for inhibitory activity. Because other tumor cell types form cords in vitro (4, 22), as well as contribute to blood vessel formation in tumors, this assay may have broader potential to predict drugs/agents that can normalize the tumor vasculature. The s.c. implants of B16F10 cells contain blood vessels lined by melanocytes, readily identified by the presence of melanosomes in the cytosol, in direct contact with RBCs. At least some of these vessels are also in direct contact with the tumor blood circulation because labeled RBCs appear simultaneously in both PECAM-1-stained and unstained structures, but not in the interstitial tumor tissue. Finally, we have shown that ICRF 159, a drug still being studied clinically, can decrease both blood pools and PECAM-1-positive structures in these tumors. Understanding the mechanism of action of ICRF 159 to normalize the vasculature promises to increase our understanding of factors contributing to metastasis as well as drug delivery and may lead to novel strategies for cancer therapy. The experimental infrastructure described herein presents a platform from which to launch those studies.

ACKNOWLEDGMENTS

The technical support of Dale Ruby is gratefully acknowledged. We thank Dr. Patricia Steeg for her interest and very helpful comments.

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