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Velayos, Fernando S.

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ABSTRACT

Angiogenesis is a complex process and is important in a variety of physiologic and pathologic conditions. Although tumors secrete several angiogenic factors, vascular endothelial growth factor, VEGF, is believed to be the main angiogenic factor in this process. The importance of VEGF in this process is supported by its presence in primary tumor specimens, as well as in urine and serum samples from cancer patients. More direct evidence for its functional importance is provided by experiments using human cancer cell lines transplanted into SCID or nude mice that demonstrated inhibition of tumor growth and metastasis by neutralizing anti-VEGF antibody. As a result, strategies designed to inhibit angiogenesis and inhibit VEGF have been examined with increased interest as a potential anti-cancer treatment.

We sought to determine if this well-supported functional importance of VEGF in tumor growth and metastasis could be extended to two VEGF-producing breast cancer cell lines, MDA-435 and MCF-7, not previously studied. In addition, we examined a prostate cancer cell line, DU-145, previously determined to be inhibited by anti-VEGF antibody, in a model which more closely resembles a clinical scenario where such a therapeutic strategy might be used. Our results were unexpected. The MDA-435 was inhibited by neutralizing anti-VEGF antibody, suggesting that VEGF is the main angiogenic factor in this cell line, however, neither the MCF-7 cell line, nor the previously sensitive DU-145 cell line were inhibited by neutralization of VEGF in the microenvironment.

A model accounting for these results is described. More importantly, these three cell lines with three different responses to neutralization of VEGF in the microenvironment may represent a functional heterogeneity of angiogenic pathways previously not described. They represent examples of types of potential treatment failures that must be identified in patients as anti-angiogenesis therapies enter clinical trials. More human cancer cell lines must be tested in order to determine if these results are unique to these non-VEGF responsive cancer cell lines or if this heterogeneity is widely found in human cancers.

INTRODUCTION

BACKGROUND

Angiogenesis, the formation of new blood vessels from pre-existing vasculature,[1] is a complex process that is important in a variety of physiologic and pathologic conditions. Tumor growth and metastasis are examples of pathologic conditions which are angiogenesis-dependent.[2] In 1971, Judah Folkman first suggested that the growth of a tumor could be arrested by inhibiting its blood supply.[3] He isolated a soluble factor, which he termed "tumor angiogenesis factor" (TAF), that was mitogenic *in vivo* for the capillary endothelium in a dorsal sac assay.[3] He suggested that "blockade of this factor (inhibition of angiogenesis) might arrest solid tumors at a diameter of a few millimeters."[3,4]

Since then, the field of angiogenesis research has expanded dramatically. Most of our current understanding, especially how angiogenic molecules operate and mediate angiogenesis, has been elucidated within the past 10 years.[5] A major milestone occurred in 1983 when the first angiogenic molecule, basic fibroblast growth factor (bFGF), was purified by heparin affinity chromatography.[5] Subsequently, over 40 such molecules have been identified.[2] These data, along with identification of angiogenesis inhibitors, has allowed for more sophisticated scientific inquiry and understanding of how these molecules induce and regulate this process. As a result of this increased understanding, investigation of therapeutic interventions seeking to manipulate angiogenesis has begun in many fields. In the field of wound healing and peptic ulcer disease, Szabo et al.[6] have experimentally demonstrated that administration of acid resistant basic fibroblast growth factor accelerates healing of duodenal ulcers, when compared to rodents treated with cimetidine. In the area of wound healing and perforated tympanic membrane repair, work carried out by Kato et al. has resulted in initiation of clinical trials using bFGF for this purpose.[7] Besides investigating ways of inducing of angiogenesis, recognition of the potential therapeutic

effects of controlling unabated capillary growth has also led to a search for safe and effective angiogenesis inhibitors.[8]

Folkman and Klagsburn introduced the concept of angiogenic diseases in 1987.[9] Angiogenic diseases are those in which abnormal capillary growth is a principal feature, or those in which normal regulatory processes for capillary growth fail. There are several examples, including rheumatoid arthritis,[10] psoriasis,[11] hemangiomas,[12] and approximately 20 eye diseases.[5] Ricketts et.al.[12] demonstrated successful therapeutic use of an angiogenesis inhibitor, interferon-2α, in patients with complex hemangiomas. Similar therapeutic approaches are under investigation for treating epithelial solid tumors. Several of these experimental anti-angiogenesis agents are either currently under study in animal models, or in clinical trials.

In 1993, 22 years after Folkman's proposal, Kim et.al.[13] demonstrated that blockade of an angiogenic factor with a monoclonal antibody resulted in inhibition of tumor growth in an animal model. Subsequent studies proved that this blockade inhibited metastases as well.[14,15] The angiogenic factor that was blocked is not called "tumor angiogenesis factor," but vascular endothelial growth factor (VEGF). It has been shown to be an important mediator of tumor-induced angiogenesis.[9]

It is useful to discuss VEGF and this research project within the larger context of what is known or unknown about angiogenesis. VEGF is only one of several angiogenic factors secreted by tumors. Release of this soluble factor is important, but it is only one of several components in the angiogenic cascade. Much is still not known, but it is clear that this process is more complex than a cause and effect relationship between tumor cells simply releasing a soluble angiogenic molecule, inducing the migration of endothelial cells to form new blood vessels, and nourishing a tumor and allowing it to grow.[5] Other components are increasingly being recognized as important in the regulation and acquisition of the angiogenic phenotype. Some of these factors include heparin and heparinases,[16] metalloproteases,[17] plasminogen activators and inhibitors,[18,19] angiogenesis inhibitors,[20]

tumor suppressors,[21] oncogenes,[22] integrins,[23] endothelial cells receptors,[24] matrix bound angiogenic factors,[25] and the paracrine effects of endothelial cells and the stroma on the tumor itself.[26]

It is clear that many questions remain about the interrelationships among the above factors and their differential effect on the ability of a tumor to grow and acquire an "angiogenic phenotype" *in vivo*.[5] Conversely, many questions have been answered in the area of identification, purification, and sequencing of soluble angiogenic molecules produced by tumors.[2] Since tumor release of an angiogenic molecule is the likely first step of the cascade, investigation of these soluble molecules has provided not only additional insight regarding their particular role in the overall angiogenic process, but has also provided an entry point in which to begin to answer some of the more complex issues described above. Targeting an important soluble angiogenic factor produced by tumors, as Folkman described and as Kim demonstrated, is a rational and a potentially effective anti-cancer strategy. The decision to target VEGF is based on increasing evidence that it is one of the more important soluble angiogenic factors secreted by human tumors. This conclusion is a result of data from both *in vivo* rodent models of human tumors[13-15] as well as evidence from clinical tumor specimens.[27-31]

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF), AN IMPORTANT MEDIATOR OF TUMOR INDUCED ANGIOGENESIS

The importance of VEGF in tumor-induced angiogenesis is supported by several different lines of evidence. Other putative tumor-associated factors have demonstrable angiogenic activity *in vitro* . Some of these factors include epidermal growth factor, transforming growth factors α and β, angiogenin, tumor necrosis factor α, acidic and basic fibroblast factors, and platelet-derived endothelial cell growth factor.[33] With the exception of FGF, PD-ECGF, and possibly EGF, these other factors have no direct mitogenic effect on endothelial cells. Their angiogenic effects are likely to be mediated indirectly.[33]

Furthermore, although FGF and PD-ECGF are proposed to be major mediators of angiogenesis, neither is a secreted protein. Therefore, each is unlikely to function as direct stimulators of angiogenesis except following cell injury or release from the matrix, where bFGF is stored.[25] In contrast, VEGF is: 1) secreted; 2) an endothelial cell mitogen;[5] 3) stored in the matrix to augment angiogenesis;[25] and 4) it increases the permeability of vessels 50,000 fold above that of histamine.[34] All of these are important properties in the pathophysiology of tumor angiogenesis.

The presence VEGF has been demonstrated via immunohistochemistry and *in situ* hybridization in a variety of surgical specimens of different cancers, including colon,[27] breast,[28] lung, [29] vulvar,[30] ovarian,[31] and gastric[32] carcinomas. In addition, VEGF levels are higher in the serum and urine of patients with some of these cancers compared to controls. In fact, 70% of individuals with solid tumors have elevated levels of VEGF, providing additional evidence to suggest that VEGF may play an important role.[35]

White[36] cites two lines of direct evidence to demonstrate the importance of VEGF expression in tumor growth: first, Chinese hamster ovary cells (CHO) that do not produce VEGF are not tumorigenic in nude mice. However, when they are induced to express VEGF, they become tumorigenic.[37] Second, use of a neutralizing anti-VEGF monoclonal antibody to eliminate VEGF from the environment of VEGF expressing tumor cells, prevents neovascularization and inhibits tumor growth and metastasis.[13-15] These data taken together, imply that VEGF can mediate conversion of a tumor to an unrestricted, angiogenic phenotype which can readily metastasize to distant sites.

SIGNIFICANCE AND PURPOSE

The purpose of the project is to address two issues: 1) determine whether the inhibitory effect on tumor growth and metastasis by anti-VEGF antibody seen in several different human carcinoma cell lines transplanted into SCID mice, can be extended to two different breast carcinoma cell lines; 2) whether the inhibitory effect of anti-VEGF

antibody in a VEGF-sensitive prostate carcinoma cell line, is maintained when systemic antibody administration is begun after the primary tumor is resected.

Current research has not yet addressed the role of VEGF in breast cancer. Although VEGF expression and VEGF receptor expression are upregulated in clinical specimens of breast cancer,[38,39] this data only provides indirect evidence for the importance of VEGF in breast cancer. It does not conclusively prove that VEGF is important in either tumor growth or metastasis. In order to begin to understand its role, the approach used in this project was to eliminate VEGF from the environment of VEGF-secreting breast cancer cells inoculated subcutaneously in SCID mice using a monoclonal anti-VEGF antibody. Given the promising results from neutralizing VEGF in other cell lines,[13-15] combined with the significant impact of breast cancer in our society, determining the importance of VEGF in this disease may provide new therapeutic options and help guide future research efforts in this disease.

The second purpose of this project is to determine the ability of anti-VEGF antibody to inhibit tumor recurrence and growth of micrometastases after surgical resection of primary tumors inoculated in SCID mice. The cell line used, DU-145, is a prostate carcinoma cell previously shown to be inhibited by anti-VEGF antibody. Our experimental design models a clinical scenario in which this antibody could be used: after removal of the primary tumor, when micrometastases have already been established. In nearly 50% of all cancer patients, and almost 30% of breast cancer patients, surgical resection of the primary neoplasm is not curative because metastasis has already occurred.[40] Often metastases are too small to be detected (<5mm in diameter) when the primary neoplasm is surgically resected with a curative intent.[40]

It is intuitive that if the antibody is effective in inhibiting tumor growth and metastasis when there is a large tumor burden, then it is likely that it will inhibit micrometastases when the primary tumor is resected. Clinical experience and experimental data, however, indicate that this is not always the case.[41,42] In some instances, removal

of a primary tumor can have significant systemic effects on cancer cells which may impact on metastases and recurrence.[41,42]

There is a rich history of clinical and scientific observation dating back to at least the beginning of this century noting that one tumor can inhibit the growth of another or inhibit its own metastases.[41] Angiostatin, a recently discovered protein provides the first molecular evidence of this phenomenon.[41] In 1994, O'Reilly[41] demonstrated that angiostatin inhibited the growth of metastases in a Lewis lung carcinoma model. Only when the tumor was removed, did metastases neovascularize and grow. In addition, the urine and serum from tumor-bearing mice, but not controls, specifically inhibited endothelial cell proliferation. Although angiostatin has not been discovered outside of this model, the rapid growth of metastases after surgical resection of the primary tumor has been anecdotally observed clinically.[40]

In addition to rapid growth of metastases already present, surgical removal of a primary tumor may seed the circulation with cancer cells and increase the likelihood of new metastases or tumor recurrence.[42] Data obtained after removal of breast cancer, for example, demonstrated intraoperative shedding of tumor cells, even though the boundaries of the cancerous lesion are never violated.[42] Although the majority of the circulating tumor cells are rapidly destroyed in the bloodstream within 24 hours, removal of a tumor and resultant angiogenic wound repair creates a growth factor-stimulated microenvironment. These growth factors can increase the survival of cancer cells, increasing the likelihood that they will implant either locally or at distant sites. Given that one likely setting in which this antibody would be administered is after a cancer has been resected, administration of anti-VEGF antibody after removal of the primary tumor is an important next step in determining the ability of this antibody to inhibit tumor recurrence and growth of remaining micrometastases.

HYPOTHESES

1. Neutralization of VEGF with an anti-VEGF antibody will inhibit tumor growth and metastasis compared to controls in two VEGF-secreting breast cancer cell lines, MDA-435 and MCF-7, inoculated into SCID mice
2. In mice inoculated with the prostate cancer cell line DU-145, previously shown to respond to anti-VEGF therapy administered up to 6 weeks after tumor inoculation, surgical removal of the primary tumor followed by systemic administration of anti-VEGF antibody will inhibit tumor recurrence and metastases compared to untreated mice.

MATERIALS AND METHODS

Cell Lines

Breast carcinoma cell line MCF-7 and prostate carcinoma cell line DU-145, transfected with the luciferase expression vector pRKluc, were a gift from Dr. Ostap Melnyk, University of California, San Francisco. Breast carcinoma cell line MDA-435, transfected with the luciferase expression vector pRKluc, was a gift from Dr. David Schott, California Pacific Medical Center. The pRKluc expression vector contains a CMV-driven promoter, pRK5. All cell lines were cultured in a humidified environment containing 5% CO_2 at 37° C. The MCF-7 cell line was maintained in Eagle's MEM medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and insulin (10 μg/ml). The MDA-435 cell line was maintained in Leibowitz's L-15 medium (Gibco BRL Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The DU-145 cell line was maintained in Eagle's MEM medium, supplemented in 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml).

Antibodies

The following murine monoclonal antibodies were used: antibody A6.4.1, a neutralizing anti-VEGF antibody raised against recombinant VEGF; [33] and 6E10 control antibody, an irrelevant antibody of the same isotype (IgG_1) directed against the viral protein gp120. Both antibodies were a gift from Dr. K. Jin Kim (Genentech, South San Francisco, CA)

VEGF ELISA

MDA-435, MCF-7, and DU-145 cells were seeded separately ($1x10^5$) in 1.0 ml of complete culture media (DME H-16/F-12 supplemented with 10% fetal calf serum) on 24-

well tissue culture plates. Conditioned media was collected after seven days. ELISA was performed as previously described.[37] The limit of assay sensitivity is 0.2 ng/ml.[15]

Study 1: Breast Cancer Cell Line Tumorigenesis and Metastasis Assay

Six-week-old female ICR *scid/scid* (Taconic, Germantown, NY) mice weighing 20-30 grams were maintained in a pathogen free environment throughout the experiment. All mice were caged in groups of six and fed a diet of animal chow and water *ad libitum.* Tumor cells were dissociated from subconfluent culture plates with trypsin (3 minutes), washed, resuspended in sterile PBS, and diluted in a 1:1 ratio in Matrigel for a total volume of injection of 0.1 ml. Initial concentration of tumor cells was adjusted so that mice were injected subcutaneously with $5x10^6$ MDA-435 or MCF-7 tumor cells in the right flank. Treatment was initiated 1 week after tumor cell inoculation. Anti-VEGF antibody A4.6.1 was injected intraperitoneally at a dose of 100 μg twice weekly in a total volume of 0.1 ml. In similar *in vivo* models,[13] this dose produced maximal tumor inhibition. Intraperitoneal injection of 0.1 ml PBS served as the control. Each group comprised 6 mice. Tumor sizes were determined twice weekly in three dimensions. Tumor volume was calculated using a standard formula *width x length x height.* Animals were euthanized at 6 weeks. Primary tumors were excised, necropsies were performed, and lungs were harvested. Luciferase assay was performed as described below.

Study 2: Surgical Resection and Tumorigenesis/Metastasis Assay

Six week old female ICR *scid/scid* (Taconic, Germantown, NY) mice weighing 20-30 grams were maintained in a pathogen free environment throughout the experiment. All mice were caged in groups of six and fed a diet of animal chow and water *ad libitum.* Mice were injected subcutaneously with $5x10^6$ DU-145 tumor cells in the same manner as described above. Tumors were allowed to grow and measured weekly in three dimensions. Tumor volume was calculated using a standard formula *width x length x*

height. Our goal was to resect the primary tumors after micrometastases were present. A group of 24 "sentinel" mice were designated for that purpose and also served to monitor metastases after removal of the primary tumor. These mice received only surgical resection of their primary tumor along with the treated and control groups. Three to four sentinel mice were euthanized approximately every two weeks. Primary tumors were excised, necropsies were performed, and lungs, left femurs (opposite side of tumor) , brains. and spleens were harvested. Luciferase assay was performed as described below.

On day 35, micrometastases had been established in the "sentinel" mice. Primary tumors were excised on day 36 post-inoculation. Briefly, mice were anesthetized with 1% inhalational isofluorane delivered by facemask. Aseptic technique, anesthetic, and surgical protocols were followed in accordance with the UCSF Animal Care and Use Manual.[63] Loose cutaneous tissue near the tumor was grasped with fine forceps and a 1-1.5 cm linear incision was made near the tumor mass with fine point scissors. The tumor was freed from the adjacent tissue and via blunt dissection following naturally formed tissue planes. Care was taken to not violate the boundaries of the tumor tissue itself. The tumor was removed after separation from its main blood supply. Minimal hemorrhage was noted. Wound edges were approximated and sutured with one Michel suture clip (Fine Science Tools, Foster City, CA). If a few animals, a slightly larger incision was made and required two suture clips. Incision to suture time was between 5 and 10 minutes per mouse and all procedures were without bleeding or surgical complications.

After surgery, the isofluorane facemask was removed and the mice were placed in a cage, under a heat lamp, during the entire post-operative recovery period. The mice regained consciousness within 3-5 minutes and did not demonstrate respiratory distress. Mice were observed for at least 30 minutes in the post-operative period. All mice survived the procedure and were fully active by the end of the observational period. Mice were closely monitored for the next 48 hours for signs of distress, shock, and changes in feeding, water uptake, and urination. Wound scars were monitored for the next week for

pus, necrosis, or healing abnormalities. Sutures were removed 1 week after tumor resection. All wounds had appropriate scar formation. Intraperitoneal anti-VEGF or control antibody treatment was begun at this time. All mice survived until euthanized.

Anti-VEGF antibody was administered intraperitoneally at a dose of 100 μg twice weekly in a total volume of 0.1 ml. Intraperitoneal injection of 100 μg 6E10 antibody in a volume of 0.1ml served as the control. The anti-VEGF group comprised 11 mice. The 6E10 control group compromised 10 mice.

Mice were visually inspected and manually palpated for local recurrence on a weekly basis. Any recurrence of tumor growth was measured in three dimensions and its volume was calculated as described above. All mice with recurrences were euthanized at day 82 of the experiment. The remaining mice continued recurrence free until day 109, when the experiment was terminated, when these mice were euthanized. Mice in both the recurrence and nonrecurrence groups had necropsies performed, and brain, left femur, lung, and spleen were harvested. Mice with recurrences had their tumors excised. Mice without recurrences had the wound area carefully palpated manually and visually inspected to ensure that the site was free of recurrence. Harvested organs were assayed for luciferase activity as described below.

Luciferase Assay

Organs removed at necropsy were assayed for luciferase activity, which is indicative of the presence of luciferase expressing tumor cells in the organ. First, tissues were pulverized into a fine powder and 1x luciferase lysis buffer (Sigma Chemical Company, St. Louis, MO) was added at a ratio of 2μl lysis buffer:1 μg tissue. The tissue/lysis buffer mixture was incubated for 2 hours at 4° C and centrifuged at 4800rpm for 25 minutes at 4° C. 100 μl of luciferase substrate (Sigma Chemical Company, St. Louis, MO) was added to 20 μl of tissue supernatant in a cuvette and loaded into the luminometer (Turner Designs, Sunnyvale, CA) immediately after gentle mixing.

Background was determined by measuring the activity of 100 μl of luciferase substrate without the tissue supernatant. Tumor tissue supernatant served as the positive control. Spleen tissue supernatant served as the negative control.[62]

RESULTS

MDA-435, MCF-7, and DU-145 secrete VEGF

VEGF secretion in both MDA-435 and MCF-7 was confirmed by ELISA of conditioned media of MDA-435 and MCF-7 cultures, respectively. MDA-435 secretes 4.1 ng/ml and MCF-7 secretes 0.4 ng/ml. Effect of anti-VEGF antibody on proliferation *in vitro* was not tested. Level of DU-145 was reported by Ostap Melnyk, M.D.(UCSF) to be 11.1 ng/ml, obtained by ELISA of conditioned media.

Study 1:Inhibition of tumor growth of MDA-435 breast carcinoma by anti-VEGF antibody

The effect of inhibiting VEGF on *in vivo* tumor growth and dissemination of MDA-435 was studied. Two groups of six ICR/SCID mice were each inoculated with $5x10^6$ cells, injected subcutaneously in the right flank. Antibody treatment with A4.6.1, a VEGF neutralizing antibody with antitumorigenic activity *in vivo*, was begun one week after tumor inoculation. The dose used was 100 μg biweekly which has been shown to produce maximal tumor inhibition.[13] Control groups were treated with saline alone.

Primary tumor size was measured biweekly after each injection, and is represented in Figure 1a. The inhibitory effect of the antibody on tumor growth was almost immediate and was sustained throughout the duration of the experiment. The saline treated control group demonstrated very rapid, linear growth during this same time period. The VEGF treated tumors only grew an average of 32 mm^3 over the time course of the experiment while the control group grew 490 mm^3. The differences in tumor sizes became statistically significant ($p<0.05$) at day 16 (day 9 of treatment) and remained significant through the end of the experiment. At the end of the experiment (day 44), the mean tumor size for the control group was 570.3 mm^3 while the anti-VEGF treatment group was 151.8 mm^3 ($p=0.0006$).

Study 1: Effect of anti-VEGF antibody on tumor metastases of MDA-435 is indeterminate due to absent luciferase expression

Mice inoculated with MDA-435 breast carcinoma were euthanized after 44 days, their primary tumors were excised, and their lungs were harvested. No gross metastatic tumors were present at necropsy. Whole organ lysates for luciferase activity were prepared and the results are presented in Figure 2a. None of the lysates had luciferase activity at levels above that of background. The anti-VEGF group had a mean luciferase activity of 0.009 ± 0.011 Relative Light Forming Units (RFLU), while in the treatment group it was 0.011 ± 0.003 RLFU. The MDA-435 tumor was noted to have a low luciferase activity normalized to tumor mass compared to the MCF-7 cell line. After correction for mass, the production of luciferase was only 3.5% that of MCF-7 on a per gram basis (*data not shown*).

To correct for the loss of luciferase activity in MDA-435, I transfected this cell line with the luciferase expression vector, pRKluc, containing a CMV-driven expression vector, pRK. Several high expressing luciferase clones were obtained both by electroporation and liposome technique. Two of these clones were recently tested in SCID mice and show a good growth rate after 6 weeks, retained expression of luciferase expression, and consistent metastases to the lungs and brain as determined by detection of luciferase. As a result, the role of VEGF on metastases in this breast cancer line is being reevaluated.

Study 1: Neutralizing anti-VEGF antibody does not inhibit primary tumor growth of MCF-7 breast carcinoma

The effect of inhibiting VEGF on *in vivo* tumor growth and dissemination in MCF-7 was studied. Two groups of six ICR/SCID mice were each inoculated with 5×10^6 cells,

injected subcutaneously in the right flank. Antibody treatment with A4.6.1, was begun on day 7 and was carried out in a similar fashion as was done for MDA-435. Primary tumor sizes measured bi-weekly are depicted in Figure 1b.

Tumor growth was modest in both groups until day 16 of the experiment, when there was a rapid increase in the growth rate in both the treated and control groups. This rapid growth rate was sustained until the end of the experiment. The tumor size and growth rate of both the treated and control groups were similar for the duration of the experiment. At the end of the experiment, mean tumor size in the anti-VEGF treated group was 853.4 ± 91.0 mm^3, while in the control tumor group, the mean tumor size was 792.4 ± 250.0 mm^3 ($p=0.8232$) .

Study 1: Neutralizing anti-VEGF antibody does not inhibit metastasis of MCF-7 breast carcinoma

Mice inoculated with MCF-7 breast carcinoma were euthanized after 6 weeks, their primary tumors were excised, and their lungs were harvested. No gross metastatic tumors were present at necropsy. Whole organ lysates for luciferase activity were prepared and is presented in Figure 2b. Unlike the MDA-435 tumors, there was considerable luciferase activity in lung whole organ lysates of MCF-7 injected animals. The degree of metastases detected in the lungs was comparable in the anti-VEGF treated group and control group. Mean luciferase activity in lungs of the anti-VEGF group of animals was 0.297 ± 0.120 RLFU, while in the control group, it was 0.307 ± 0.114 RLFU ($p=0.95$).

Study 2: Neutralizing anti-VEGF antibody administered after removal of primary tumor does not inhibit tumor recurrence in DU-145 prostate carcinoma

The efficacy of anti-VEGF antibody on inhibition of *in vivo* tumor recurrence after primary tumor resection was studied in DU-145 prostate carcinoma. Previously, marked

inhibition of tumor growth and metastasis by anti-VEGF antibody was observed.[15] In the experiments reported here, primary tumors were resected 36 days after tumor inoculation. Decision to resect the tumors was made by evaluating luciferase activity in the lungs and brains of the "sentinel" mice (group of mice used to monitor micrometastases) and their ability to survive surgery. Three of 4 sentinel mice with intact, untreated, tumors had micrometastases either in the brain or the femur contralateral to the tumor at this time (*Figure 6*). It was decided to resect the primary tumors, because previous experience[15] had demonstrated that delaying the surgery any further might allow additional mice to begin losing weight, possibly decreasing the survival rate after surgery. All of the mice (100%) survived the surgery.

Antibody treatment with A4.6.1 was begun one week after primary tumors were resected. Staples were removed at that time and demonstrated healed wounds. Tumor recurrences first appeared 5 weeks after the primary tumor was removed and were detected as late as 8 weeks after primary tumor removal. Tumor recurrences appeared in both the treated and control groups and continued to grow in the anti-VEGF treated group despite inhibitory doses of anti-VEGF antibody therapy. A total of 5 of 10 control mice had recurrences, while a total of 4 of 11 anti-VEGF antibody treated mice had tumor recurrences ($p=0.55$). All recurrences occurred in the area beneath and adjacent the surgical scar, nearest the right hind limb. None of the recurrences occurred at the union of the wound margin, nor eroded through the skin.

Figure 4 retrospectively examines the mean size of tumors pre-operatively in mice with recurrences and compares their sizes in the treated and control groups. Figure 4a demonstrates that the size of the primary tumor in the treated and control groups was not statistically significant at the time of resection. Besides not statistically inhibiting the number of recurrences, systemic anti-VEGF antibody administration did not statistically inhibit the size of the tumor recurrences. Although there was a small sample size ($N=2$), the recurrence tumors in the anti-VEGF treated group were larger than the controls (*Figure*

4b). The mean size of a recurrence tumor in the anti-VEGF treated group was 1199.4 ± 430 mm^3. The control group recurrence tumors were smaller, with a mean volume of 487.3 ± 225.9 mm^3. The mean recurrence volume of the tumors between the treated and control groups was not statistically significant (*p=0.200*).

Retrospective analysis shown in figure 3 demonstrates that mice with recurrences typically had larger primary tumors and more rapidly growing primary tumors at the time of resection. The difference in tumor size between the recurrence and nonrecurrence primary tumors was statistically significant beginning at day 29 post-inoculation (*p=0.0209*) and remained so through day 36 (*p=0.0047*), when the primary tumors were resected. The mean primary tumor size at resection in mice with recurrences was 868.9 ± 90.2 mm^3. In mice without recurrences, mean primary tumor size at resection was 486.1 ± 76.4 mm^3. The difference in tumor size between the anti-VEGF treated and control animal groups was not statistically significant.

Study 2: Effect of neutralizing anti-VEGF antibody on micrometastases in mice with and without tumor recurrence after removal of primary tumor.

Mice with recurrences were uniformly euthanized at day 82 after initial tumor inoculation. At necropsy, lungs, brain, spleen, and the femur contralateral to the tumor were harvested. Spleen and femur whole organ lysates had low luciferase activity, near background levels (*Figure 5*). The brain had slightly more luciferase activity, although it was low and also near background in both the anti-VEGF and control groups. The lungs had the highest luciferase activity of all of the organ lysates. The luciferase activity was higher in the treated group, with a mean luciferase activity of 0.312 ± 0.299 RLFU compared to 0.047 ± 0.021 RLFU in the control group. This difference was not statistically significant(*p=0.163)* . The anti-VEGF treated group in this analysis was comprised of only two mice, one without luciferase activity above background in the lungs and one with activity.

The other two of the four mice in the anti-VEGF treatment group were not included in this analysis because they had cancer dissemination in the bloodstream, as evidenced by extraordinarily high luciferase expression in all organs, including the spleen. The spleen is typically negative for luciferase activity except when there are disseminated luciferase expressing cells in the bloodstream. The luciferase levels in organ lysates of these two mice were up to 10,000 times higher than the organs of the other mice (*data not shown*). Since these mice did not allow for accurate measurement of metastases because they also had freely disseminated cancer cells in the bloodstream of that organ, they were excluded from the above analysis.

All mice without tumor recurrences were euthanized at day 109. Brain, femurs, lungs, and tumors were harvested, and assayed for luciferase. As can be seen in Figure 5b, the effect of the antibody on micrometastases is indeterminate because luciferase detection is at background in all three organs, for both the treated and the control groups. Examination of Figure 6 shows the progression of metastases during the time frame of the experiment. Mice with no recurrence, receiving no treatment, showed an elevation in metastases in brain and femur homogenates after surgery, but then luciferase activity fell to background levels by day 109.

DISCUSSION

Vascular endothelial growth factor (VEGF) is believed to be an important mediator of tumor-induced angiogenesis. VEGF expression has been demonstrated in a variety of primary epithelial tumors.[26-32] Elevated levels of VEGF have been found in the urine and serum of cancer patients.[35] In addition, previous *in vivo* experiments in which VEGF was neutralized with a monoclonal antibody showed profound inhibition of primary tumor growth and metastasis in several different human cancer cell lines.[13-15,43,44] VEGF has several biochemical properties which make it an ideal angiogenic factor and principal mediator of tumor-induced angiogenesis. These properties include its solubility, so that it can diffuse and act in a paracrine fashion on endothelial cells; its ability to induce permeability,[25] important in producing extravasation of fibrinogen and proteolytic factors to facilitate endothelial cell migration and metastatic seeding of the circulation;[45] and its endothelial cell-specific mitogenicity which is essential in inducing endothelial cell proliferation. Moreover, there is increasing evidence that it can activate additional pathways believed to be important in angiogenesis.[17-19, 23] Given that it is likely an important mediator of tumor growth and metastasis, VEGF has also been closely examined as a potential anti-cancer target.[13-15,43,44.]

Our data suggest that secretion of VEGF may not be predictive for whether it is the primary angiogenic factor in a given cancer cell line. Other non-VEGF mediated pathway(s) may also be important. Specifically, our data demonstrate three VEGF-secreting cancer cell lines with three different responses to neutralizing doses of anti-VEGF antibody. Growth of one breast cancer cell line, MDA-435, was inhibited by anti-VEGF antibody. This is consistent with similar experiments carried out in different human cancer cell lines transplanted in SCID and nude mice. Growth of another breast cancer cell line, MCF-7, was not inhibited by anti-VEGF antibody. Although this cell line secretes less VEGF than MDA-435, other reports have shown less inhibition of growth with decreasing

levels of VEGF secretion, but never a lack of inhibition in a VEGF secreting cell line. This lack of response to anti-VEGF antibody by a VEGF secreting cell lines has not been previously reported. Finally, we have obtained preliminary evidence that the clinical setting in which anti-VEGF antibodies are administered may be critical in determining the responsiveness to this form of therapy. The prostate cancer cell line, DU-145, although initially shown to be inhibited by anti-VEGF antibody when administration was begun up to six weeks after initial tumor inoculation, was refractory to treatment when injections of the antibody were begun after surgical removal of the tumor.

Inhibition of MDA-435 by anti-VEGF Antibody

Growth of the MDA-435 breast carcinoma was inhibited significantly (73%) by neutralizing VEGF from the microenvironment. In over 6 weeks of the experiment, the tumor grew only by a mean volume of 32 mm^3, compared to a mean increase in volume of 490 mm^3 in the control group. This suggests that the presence of VEGF is important in the ability of MDA-435 to acquire the angiogenic phenotype and is consistent with the results of others demonstrating that VEGF is a significant mediator of tumor-induced angiogenesis.[13-15,37,43,44]

Since this cell line showed profound inhibition of tumor growth, we also hypothesized that metastases would be inhibited as well. In the time frame of this experiment, gross metastases did not occur. At this early stage, inhibition of metastases is a result of the effect on primary tumor microvessels preventing tumor entry into the circulation.[14] As shown in Figure 2a, the luciferase activity in lung homogenates of both treated and control groups were at background levels. The lack of luciferase expression in the lung, however, is likely to be misleading. Luciferase expression in the MDA-435 tumor cell line was much lower than in either the MCF-7 or DU-145 tumor cell lines. This indicates that luciferase expression is very low in this tumor and therefore not appropriate for interpreting these results at this time. This cell line was recently transfected again and

several clones demonstrate high luciferase expression. As a result, the effect of neutralizing anti-VEGF antibody on metastases in this breast cancer line is being reevaluated.

Lack of inhibition of MCF-7 by anti-VEGF Antibody

Unlike the MDA-435 breast cancer cell line, the growth of MCF-7 in SCID mice was not inhibited by neutralizing anti-VEGF antibody, nor was there inhibition of lung metastases. The antibody does not appear to inhibit the primary angiogenic process in the tumor, thereby allowing tumor cells to enter the circulation and disseminate.

One explanation for this phenomenon is that neovascularization within the tumor enlarges the surface area of vascular endothelium for potential escape of tumor cells into the circulation.[5] These leaky new vessels provide the entry point for tumor cells to enter the circulation and implant in distant organs. Another, is that the tumor cell already carries the angiogenic-inducing phenotype when shed from the primary breast cancer, increasing its ability to induce new blood vessel formation and likelihood for successful growth at a metastatic site than would a nonangiogenic inducing cell.[5] Specimens were obtained from the MDA-435 and MCF-7 tumors for microvessel count and will be analyzed at a later time. Microvessel count is a measure of tumor angiogenesis and also correlates with degree of metastasis,[29,48,49] Our prediction is that there will not be a difference in microvessel density between the treated and control groups in the MCF-7 cell line, whereas the MDA-435 breast cancer line will have reduced microvessel density in the treated group.

There are two broad categories of possible explanations for the inability of VEGF neutralizing antibody to inhibit tumor growth or metastasis in the MCF-7 breast carcinoma while profoundly inhibiting tumor growth in the MDA-435 breast carcinoma line. The first, is that angiogenesis in MCF-7 breast carcinoma is not mediated by VEGF. The second, is that lack of inhibition may be the result of the antibody not fully inhibiting VEGF secretion in the microenvironment.

With regard to the first possible explanation, the mechanism of MCF-7 induced angiogenesis is unknown. This study represents the first observation of failure of anti-VEGF antibody to inhibit angiogenesis in a VEGF-secreting cell line. This may represent publication bias, whereby only those studies with positive (i.e. a difference between treatment and control) are both likely to be submitted and published, or it may be a truly novel observation. Regardless, it functionally demonstrates that the role of VEGF may be different in different cancers, even within the same organ. Up until now, neutralization of VEGF has always been associated with inhibition of angiogenesis. In addition, two cell lines from the same organ have not been studied previously.

Factor or factors involved in this proposed VEGF-independent angiogenic pathway are still to be determined. Basic FGF is an important endothelial cell mitogen and is found in many tumors and in the urine of cancer patients.[51] PDGF-A, which is structurally similar to VEGF, has been shown, along with VEGF to be correlated with angiogenic and metastatic factors in breast cancer. [52]

The above hypotheses could be tested in several ways. First, more complete *in vitro* screening of angiogenic factors expressed by tumor cell lines such as VEGF, bFGF, and PDGF, using ELISA or Western blotting, as well as *in vivo* determination of these factors in tumors by immunohistochemistry and *in situ* hybridization, would identify which angiogenic factors are expressed in the MCF-7 and the MDA-435 tumors. Next, the functional role of the particular angiogenic molecules which are highly expressed in MCF-7 can be examined by use of a monoclonal inhibitory antibody against that factor. The effect on tumor growth and metastasis could then be determined.

The second possible explanation for the unresponsiveness of MCF-7 to inhibiting doses of anti-VEGF antibody is that the A4.6.1 antibody may not completely neutralize VEGF. The dose of antibody used, 100 μg biweekly, is sufficient to neutralize tumor-secreted VEGF-mediated angiogenesis.[13] Although an *in vivo* dose-response curve examining inhibition of angiogenesis was not done for the MCF-7 cell line, Kim

demonstrated the maximal inhibition of VEGF-mediated angiogenesis with this antibody in a variety of cell lines is between 50-100 μg.[13] No additional inhibition was seen at doses up to 400 μg biweekly. In fact, 100 μg biweekly has become a standard dose for subsequent experiments with significant inhibition of angiogenesis achievable at this dose.[13-15, 43]

One possibility is that tumor secreted VEGF is not fully neutralized. Upregulation of VEGF expression does occur in response to a variety of stimuli, such as hypoxia.[36] Low oxygen tension is most commonly found at the center of a tumor.[36] This possibility can be examined *in vitro* by exposing MCF-7 cells to hypoxic and normoxic growth conditions, collecting the media, and comparing VEGF expression via ELISA or Western blot. Low VEGF expressing cells like MCF-7 are known to be more sensitive to hypoxia than higher expressing cells like MDA-435. These lower expressing cells can dramatically upregulate the expression of VEGF.[36] In contrast, constitutively higher VEGF-expressing lines, like MDA-435, do not upregulate dramatically to hypoxia because either they are near their maximal VEGF expression or they are less sensitive to hypoxic stimuli. It is unlikely, however, that the upregulation of VEGF in MCF-7 expression would be higher than the high VEGF-expressing cells which are profoundly inhibited by anti-VEGF antibody.

The second possibility is that alternative VEGF species, such as VEGF-B and VEGF-C, are not recognized by the A4.6.1 antibody. VEGF-B and VEGF-C were discovered in the past year. Recognition of these other species by the A4.6.1 antibody has not been investigated. It is unclear if either VEGF-B or C have a role in tumorigenesis. VEGF-B is located primarily in the heart, skeletal muscle, and pancreas. It has a high affinity for heparin so that it is primarily bound to matrix.[56] VEGF-C is important in formation of the venous and lymphatic vasculature, but its role other than in development is unknown. VEGF-C binds to Flt-4 receptor in the lymphatics, but can also bind to the KDR VEGF receptor on endothelial cells.[55] The VEGF-B receptor is not known. Detection of

VEGF-B and VEGF-C expression in any of the three cancer cell lines by ELISA or Western blotting might provide additional insight on their role in our observed results.

Lack of Inhibition of DU-145 by anti-VEGF Antibody after Resection of Primary Tumor

Although the DU-145 prostate cancer cell line was previously shown to be sensitive to anti-VEGF antibody, in this experiment it was insensitive to VEGF inhibition after tumor resection. Factors which may be important and associated in accounting for recurrence and metastasis in spite of anti-VEGF antibody include the effects of the surgical procedure,[42,57] wound healing and subsequent angiogenesis,[40,42] and less likely, anesthesia.[40]

The end points of this phase of the project were to examine the effects of anti-VEGF antibody on local recurrence and metastases. Local recurrence was defined as regrowth of tumor at or near the site of surgical resection. The most surprising result was not only that the anti-VEGF group had recurrences, but had approximately the same number of recurrences as the control group (5 of 10, control; 4 of 11, treated). A larger sample size, of course, would strengthen this conclusion statistically. In general, proposed mechanisms for tumor recurrence vary widely. Most of the mechanistic hypotheses of recurrence address mechanical factors for metastasis, and have not examined the molecular mechanism as closely. Theories on recurrence postulate that it is due to residual disease left at the primary site.[40] Others cite intraoperative tumor shedding of cells which has been best described in breast cancer.[57] Finally, increased survival time of disseminated cells and the immunosuppressive effects of anesthesia may also play a role.[42] Regardless of the mechanism, recurrence occurs in both groups. The effects of surgery on recurrence in this experiment appears to be equal. In addition, differential surgical techniques do not appear to play a factor in our results.

Recurrences in our treated and control animals arose between weeks 5-8 after resection. Immediately in the post-surgical period, wound healing, an angiogenic process, is activated. This process is activated for a short period of time and then quickly shut off.[5] After wound repair, the microvasculature returns to its unstimulated, non-angiogenic environment.[5] There are many angiogenic factors which are involved in wound healing, utilizing non-VEGF mediated pathways, which may account for failure of the anti-VEGF antibody to prevent initial growth of any residual tumor foci. These factors decline quickly, however, so we hypothesize that there must be a permanent change induced by wounding, which accounts for the sustained unresponsiveness of recurrence tumors to inhibitory doses of anti-VEGF antibody.

Angiogenic factors are typically expressed at maximal levels in the acute wound healing phase, and drop rapidly thereafter. [58] Although bFGF is considered to be the principal wound angiogenic factor,[58] VEGF produced by keratinocytes is important in wound repair as well.[59] Soluble angiogenic factors such as bFGF, VEGF, TNF-α, and others are found in high concentrations in the wound and wound fluid, but rapidly decline as the wound heals and scars. bFGF can be stored in endothelial cells and released by surgical trauma.[58] In addition, platelet degranulation, macrophages, degradation of matrix components, migrating keratinocytes, and other cells contribute other angiogenic cytokines.

We hypothesize that this highly angiogenic environment in the wound can stimulate these foci of residual tumor cells and micrometastases, and aid in their growth.[42] An example of how this occurs is illustrated in a study by Bogden et.al.[60] In that study, DU-145 tumors implanted either at a wound site or on the distant, contralateral side were seven times greater in size in the wounded animals than in non-wounded ones.[60] This short-lived angiogenic environment, however, does not appear to explain how the unresponsiveness to inhibitory doses of anti-VEGF antibody can be sustained in these previously sensitive DU-145 prostate cancer cells. This environment may have been vital in promoting a recurrence

and initial unresponsiveness to anti-VEGF antibody, but cannot account for how this unresponsiveness was maintained.

Recurrences in our experiment did not begin to appear until week 5, and wounds at 1 week appeared healed, which is consistent with the short-lived angiogenic response to wound-healing. It also makes it unlikely that continuous angiogenic stimulation from the wound is a mechanism responsible for our results. Chronic stimulation of the residual foci of tumor by mouse VEGF, which is unrecognized by the A4.6.1 antibody or any other angiogenic factor is unlikely for two reasons. First, wound healing is tightly regulated and is turned on only for a few days, then quickly shut off. The homeostatic baseline for blood vessels is a quiescent microvasculature or non-angiogenic state. In addition, the effects of a continually activated wound could be readily identified visually in the form of a chronic, nonhealing wound. All wounds were healed by one week and remained intact for the entire experimental period. The results of this experiment would indicate that wounding or some other factor in the perioperative period causes a permanent change in the DU-145 cell line.

In order to explain the sustained unresponsiveness of the DU-145 cell line after primary tumor resection, we hypothesize that there may be a permanent change in the DU-145 cells. This possibility was both unexpected and interesting because in previous experiments, DU-145 tumor cells required VEGF expression for the angiogenic phenotype as a *de novo* tumor, however, in this experiment, the same cell line appeared to not require VEGF when they represented residual tumor cells in a wound. In order to verify that our results are not due to a change in the intrinsic sensitivity of DU-145 to VEGF, the resection experiment will be repeated with an additional control, whereby the primary tumor is not resected, but systemic antibody is administered.

The mechanism by which recurrence and anti-VEGF unresponsiveness occurs is not known. The observed change in VEGF responsiveness is a novel finding It underscores the complexity of angiogenesis, and is a model for one type of treatment failure which might occur with anti-VEGF therapy. It is unknown if this observation is

unique to this cell line or if it can be applied to other VEGF-secreting, VEGF-antibody responsive cell lines. It does lead, however, to a variety of additional experiments in order to verify these results and better understand the possible mechanisms involved.

By repeating Bogden's experiments and then adding systemic administration of anti-VEGF antibody to the model, we can examine the effects of wounding on angiogenesis and metastasis. If wounding truly causes a permanent switch in phenotype, then the injected cells should not be inhibited by anti-VEGF antibody. The antibody could be administered at 1 week, 4 weeks and 6 weeks. In addition, monoclonal antibodies against bFGF or other wound healing growth factor can be administered to see if one can suppress the angiogenic process. Finally, a further control would be to resect the tumor recurrence using the same model as described in this project, then reinoculate mice with these tumor cells, and determine if these mice are resistant to anti-VEGF therapy. These experiments would definitively answer whether there is truly a permanent change in phenotype, or simply a reversible remodeling due to latent wound stimuli which alters these cancer cells.

Evaluation of metastases was another important end point in determining the efficacy of anti-VEGF treatment. Mice with recurrences were euthanized and their organs were evaluated for metastases. All mice, even with small recurrences were evaluated for metastatic disease. Examination of several organs shows that the anti-VEGF group had higher levels of metastatic disease in the lung than the control groups. Although not statistically significant, the graph suggests that neutralizing VEGF may have induced angiogenesis. There currently does not exist any known mechanism by which this can occur. The more likely explanation is our small sample size. Although there are not enough mice to accurately match mice for tumor size, a way to approach the sample size problem is to inoculate a new set of control mice and remove their primary tumors. Any mice with recurrences would be allowed to grow to the tumor size of the metastatic, anti-

VEGF treated group. Their organs could be evaluated for metastases and compared to the high metastases group.

Mice without recurrence were evaluated for the effects of anti-VEGF antibody on micrometastases. Mice without recurrences typically had slower growth curves and smaller tumors at the time of resection. Both of these factors imply that these tumors may have been less angiogenic. Microvessel density, a measure of angiogenesis, is known to correlate with likelihood of metastases, but its association with tumor recurrence has not been studied. The next step in this experiment is to determine if microvessel density, a marker of angiogenesis, also correlates with local recurrence.

Figure 6 shows that micrometastases were apparently lost in both the treatment and control groups. Resection of tumors occurred between weeks 5 and 6, when regular monitoring of metastases in mice demonstrated three of four mice with metastatic disease. A previous experiment in this cell line demonstrated that this is a critical period during which luciferase expression in the lungs increases considerably. We may have resected the tumors a week prematurely.

At the time of tumor resection, metastases were microscopic. What is interesting is that there is an initial increase in metastases in the brain and femur after tumor resection. Figure 6 tracks metastases in four different organs of untreated mice. The rise in luciferase detection in the brain and femur is likely due to the systemic cytokines and other yet undefined stimuli as a result of tumor resection and the resultant angiogenic wound healing process. Lack of metastases in the spleen, a highly vascular organ, indicates that the metastases are truly in the organ and not in the vasculature. This is confirmed by the findings in the lung. In addition, lack of new metastatic disease in the lungs after resection, indicates that the surgery most likely did not seed new metastases, but rather stimulated any metastases which were present. It may be that there are metastases in the lungs but that the vector is lost. This is not likely given the high expression of luciferase in the tumor. Delaying resection of the primary tumor by at least a week and more frequent post-

operative monitoring of metastases may further elucidate the mechanism for metastases in the SCID mouse model.

Summary

Angiogenesis is a complex process and is important in a variety of physiologic and pathologic conditions. Although tumors secrete several angiogenic factors, vascular endothelial growth factor, VEGF, is believed to be the main angiogenic factor in this process. The importance of VEGF in this process is supported by its presence in primary tumor specimens, as well as in urine and serum samples from cancer patients. More direct evidence for its functional importance is provided by experiments using human cancer cell lines transplanted into SCID or nude mice that demonstrated inhibition of tumor growth and metastasis by neutralizing VEGF antibody. As a result, strategies designed to inhibit angiogenesis and inhibit VEGF have been examined with increased interest as potential anti-cancer strategies.

We sought to determine if this well-supported functional importance for VEGF in tumor growth and metastasis could be extended to two breast cancer lines not previously studied. In addition, we also examined a previously studied prostate cancer cell line determined to be sensitive to anti-VEGF antibody, in a model which more closely resembled a clinical scenario where such an therapeutic strategy might be used. Our results were unexpected. Although one cell line, MDA-435, responded to neutralizing anti-VEGF antibody, suggesting that VEGF is the main angiogenic factor in acquisition of an angiogenic phenotype in this cell line, neither the MCF-7 cell line, nor the previously sensitive DU-145 cell line responded to neutralization of VEGF from the microenvironment.

Possible explanations for these results were described above. More importantly, these three cell lines with three different responses to neutralization of VEGF from the microenvironment may represent a heterogeneity of angiogenic pathways. More cancer cell

lines must be tested in order to determine if these results are unique to these two non-VEGF responsive cancer cell lines or if this heterogeneity is widely found in human cancers.

As researchers are examining the potential role of anti-VEGF and anti-angiogenesis therapeutic modalities against cancer, these data suggest that it will be important to identify which patients, which types of cancers, and which clinical scenarios would most benefit from this type of treatment for it to be a viable strategy. Tumors are known to make several angiogenic factors, so that some researchers have proposed that "angiogenic profiles" of cancers would help guide such treatment. In these three examples, however, although only VEGF expression was screened for, all three of these cancer cell lines secreted VEGF, but they were not all responsive to neutralizing anti-VEGF antibody. Future studies, of course, must screen these cell lines *in vitro* and *in vivo* for other angiogenic factors, however a functional screening might be more informative as to which angiogenic factor is the primary factor. Examination of DU-145 provides an example for this need: this cell line was previously responsive to anti-VEGF therapy under one set of conditions in a prior experiment, but not responsive when the primary tumor was removed. We have begun to examine the role of PET scanning as a screening test for selecting responsive tumors in animal models by investigating distinguishing differences in glucose utilization, permeability, and other parameters which may distinguish VEGF-responsive from non-responsive tumors.

Much more understanding is required regarding how the different angiogenic factors, tumor, and host environment interact in acquisition of the angiogenic phenotype. VEGF is clearly an important angiogenic factor, however, our results indicate that other non-VEGF pathways may have a more significant importance than currently believed. Investigation and publication of the existence of these exceptions should be encouraged. This not only will improve our understanding of the complex angiogenic process, but as anti-angiogenic agents are entering clinical trials, it will provide more sensitive ways of identifying which patients are more likely to benefit from such therapy. It may also provide

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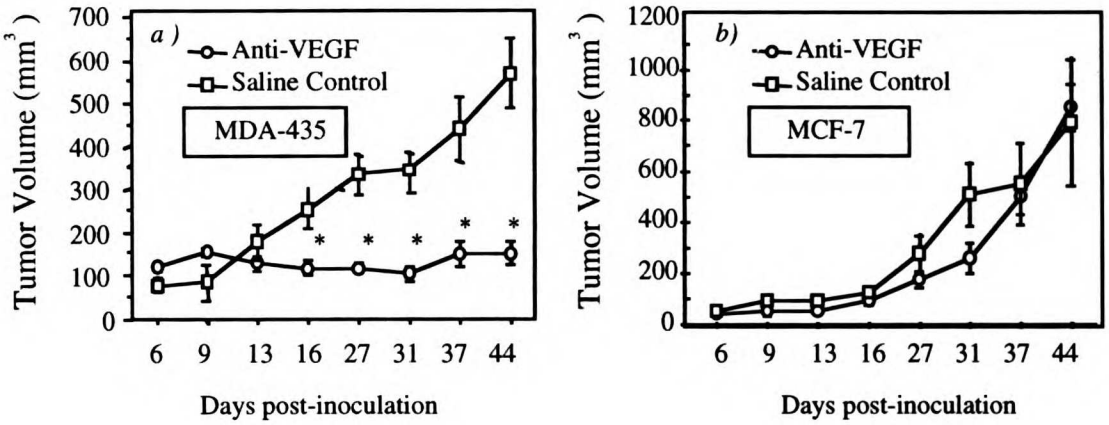

Figure 1. Growth of MDA-435 primary breast carcinoma *(a)*, but not MCF-7 breast carcinoma *(b)* is inhibited by systemic administration of anti-VEGF antibody. Treatment was initiated 7 days after tumor inoculation.. MDA-435 and MCF-7, *N=6* animals per group. Error bars indicate standard error of the mean. (*)Statistically significant difference in tumor size ($p<0.05$).

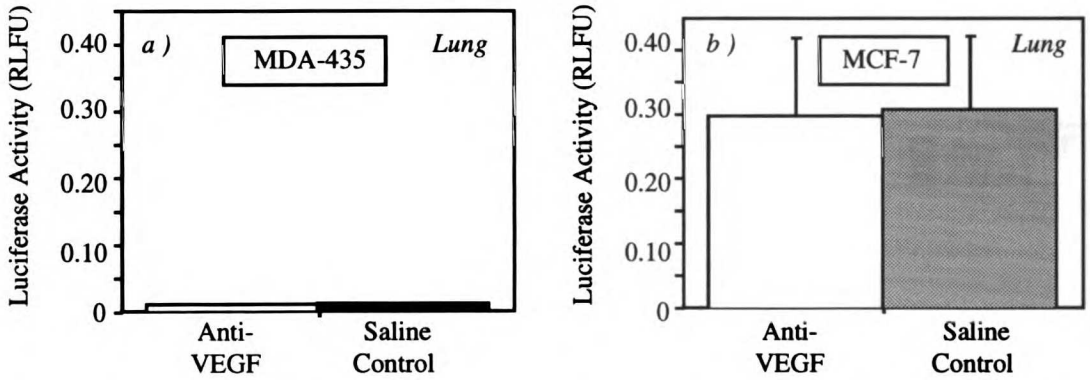

Figure 2. Quantification of metastases in lung homogenates using a luciferase detection model. Quantification of lung metastases in ICR *scid/scid* female mice inoculated with MDA-435 breast carcinoma transfected with luciferase expression vector *(a),* reveals luciferase activity is at background levels for both the treatment and the control groups. Luciferase activity in lungs of ICR *scid/scid* female mice inoculated with MCF-7 breast carcinoma transfected with luciferase expression vector *(b),* is elevated and equal for both groups, and demonstrates in this cell line metastatic disease in the lungs which is not inhibited by anti-VEGF antibody administration. MDA-435 and MCF-7, *N=6* per group. Error bars indicate standard error of the mean.

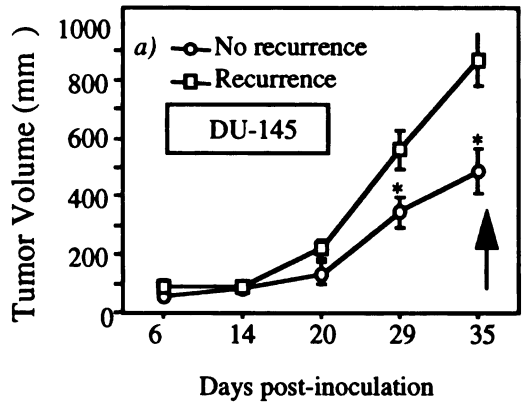

Figure 3. Retrospective comparison of primary tumor size and growth rate in DU-145 inoculated SCID mice with and without recurrence. Tumors were resected (*arrow*) at day 36, post-inoculation. Mice subsequently were classified to an anti-VEGF antibody treatment group or a control group. Mice were examined retrospectively at the end of experiment (day 109) and classified on the basis of recurrence. Mice with recurrences had larger and faster growing tumors at resection than mice that did not. Both recurrence and non-recurrence groups contain treated and control animals. For each yes/no category, there was no statistical difference between the treated and control groups. No recurrence, *N=9;* Yes recurrence, *N=10.* Error bars indicate standard error of the mean. (*) Statistically significant difference in tumor size ($p<0.05$) observed at day 29 and 35. ($p=0.0047$ at day 35; *recurrence vs. no recurrence*)

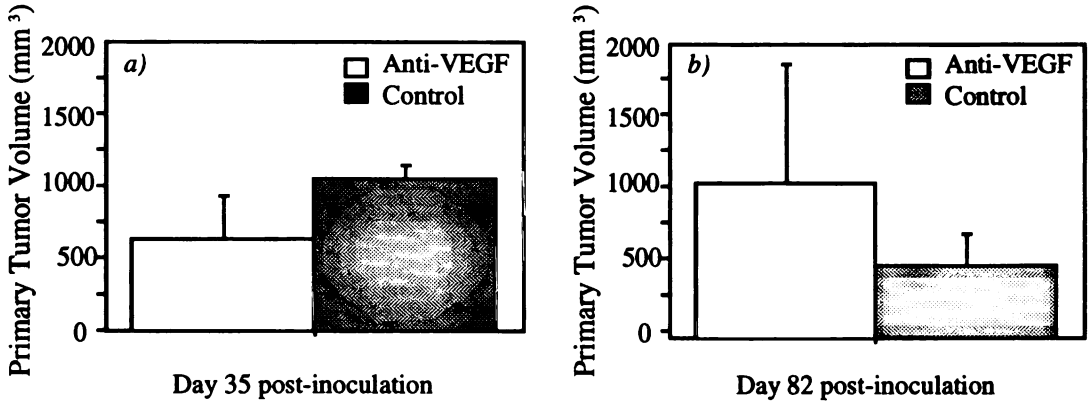

Figure 4. Comparison of primary tumor size at resection and recurrence tumor size between treatment and control groups. Anti-VEGF antibody treated mice had smaller primary tumor sizes compared to controls at resection *(a)*, however they were larger than controls at euthanasia *(b)*. Only mice with recurrences are represented in this analysis. Anti-VEGF, *N=4;* Control, *N=5.* Error bars indicate standard error of the mean. Differences in tumor volume were not statistically significant.

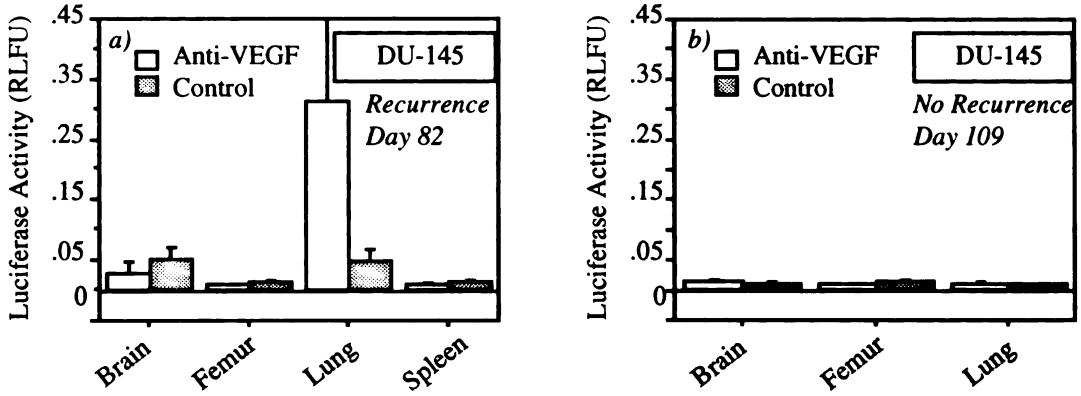

Figure 5. Metastases in whole organ homogenates in mice with and without tumor recurrence. Mice with recurrences were euthanized at day 82 (*a*) post-inoculation. There is no statistical difference between the treated and control groups. Two mice in the anti-VEGF treated groups were cachectic and were therefore excluded from this analysis. Mice without recurrence were euthanized at day 109 (*b*) and have luciferase levels at background. Recurrence: Anti-VEGF, *N=2,* Control, *N= 5;* No recurrence: Anti-VEGF, *N=6,* Control, *N=5.* Error bars indicate standard error of the mean.

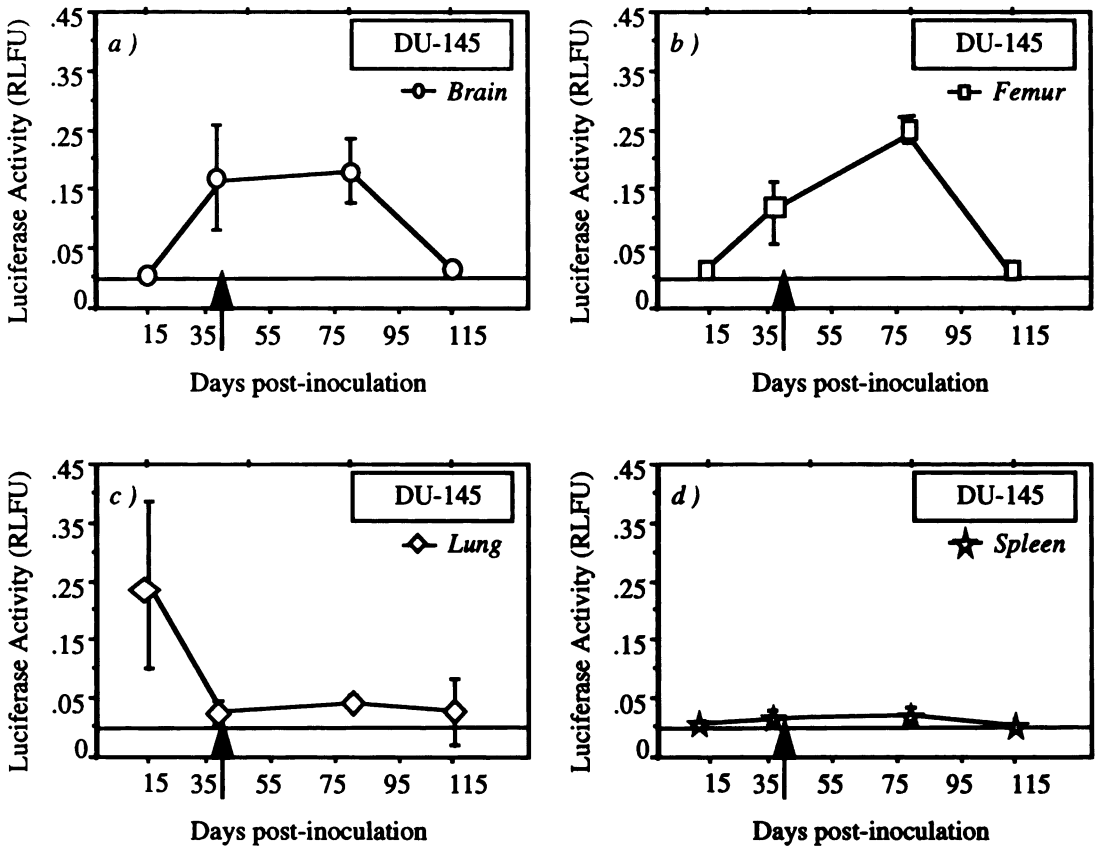

Figure 6. Quantification of metastases in four organ homogenates before and after primary tumor resection. Mice with no treatment were euthanized at the indicated time points after inoculation with $5X10^6$ DU-145 carcinoma cells. Brain, femur, lung, and spleen were harvested at each time point and assayed for the presence of metastatic disease via a luciferase assay of whole organ homogenates. Primary tumors were resected at day 36 (*arrow*) after primary tumor inoculation and subsequent measurements were performed on mice without tumor recurrence. Mice used for the following time points: Day 14, *N=3*; Day 35, *N=4*; Day 77, *N=3*; Day 109, *N=3*. Error bars represent standard errors of the mean.

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