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13. ABSTRACT ***(Maximum 200 Words)***

Incontrovertible evidence now exists that the growth of both primary and metastatic breast cancer depends on new blood vessel growth, or angiogenesis. Therefore, inhibition of blood vessel ingrowth into breast tumors should be therapeutically useful. We have detected an angiogenic factor, termed angiogenin (Ang), in human breast cancer cells and are developing inhibitors of its functions. To evaluate efficacy of these inhibitors, preclinical orthotopic models of human breast cancer in female athymic mice are in place in the laboratory. In Year 3 of Department of Defense funding we have demonstrated statistically that Ang antagonists, which include the antisense agent JF2S as well as a neutralizing monoclonal antibody (mAb) 26-2F, are capable of inhibiting the establishment of breast cancer cells injected subcutaneously into the mammary fat pad (mfp). Further, both of these classes of inhibitors can interfere with and, in certain cases, completely prevent the formation of macro- and microscopic lung metastasis in a model in which primary human breast cancer tumors are vigorously growing in the mfp. These therapeutic agents along with small molecule drugs and humanized antibodies under development should therefore be useful clinically for breast cancer therapy and prevention.

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James W. Fett 9/29/99

PI - Signature Date

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INTRODUCTION

Background. Carcinoma of the breast is now the most commonly diagnosed cancer among women in the United States and is second only to lung cancer in female cancer-related deaths. It is estimated that over 176,000 new cases will be diagnosed and approximately 43,000 individuals will die from this disease during 1998 (1). While local noninvasive breast cancer in many cases is curable, a sizeable proportion of these patients experience relapse. Furthermore, metastatic disease to distant sites is at present generally considered incurable with treatment aimed at increasing survival while maintaining a reasonable quality of life. Thus, more effective therapeutic maneuvers for the treatment or prevention of advanced, metastatic breast cancer are urgently needed.

Ideally, therapeutic goals for those patients with advanced breast cancer include prevention of development of metastasis where it has not occurred and eradicating occult or detectable metastatic lesions already present - both in conjunction with eliminating the primary cancer. Alternatively, in cases where complete cure or prevention of metastasis is not possible, managing patients in a state of metastatic "stasis" would also be a desirable outcome. We, as others, believe that novel therapeutic strategies that include an *antiangiogenic* component offer realistic hope of achieving these goals in the near future.

The critical contribution of angiogenesis - the development of a hemovascular network - to the growth of solid tumors is now undisputed (2). Angiogenesis also is a prerequisite for the development of metastasis since it provides the means whereby tumor cells disseminate from the original primary tumor, traverse through the circulation, and establish at distant sites (reviewed in ref. 3). Therefore, interference with the process of tumor-induced angiogenesis should be an effective therapy for both primary and metastatic cancers. Our own work along with the recent reports that drugs (4) as well as antibodies to a secreted angiogenesis factor (5) suppress the growth and metastatic spread of tumors in animals now lend solid experimental support to this proposition. Indeed, at least nine antiangiogenic agents are in clinical trials for treatment of several angiogenesis-dependent diseases, including cancer (M. J. Folkman, personal communication).

Importantly, the degree of angiogenesis in early-stage breast cancer correlates positively with metastatic recurrence and survival (6). Hence, interference with the angiogenic process in breast cancer should have profound therapeutic consequences.

Angiogenin. The recognized importance of neovascularization in cancer has prompted intensive efforts toward identifying the chemical mediators involved in this process. We have since 1985 studied the structure/function relationships of a potent tumor-associated mediator of the angiogenic process that we named angiogenin (Ang) (7). It has subsequently been extensively characterized both structurally and functionally. The 3-dimensional structure of Ang is now known (8) and is providing a basis for rational design of potential therapeutic inhibitors. Several antagonists of Ang's activity are available to us and are being evaluated as potential cancer therapeutics. Since when we initiated these studies in the mid-1980's reproducible models for the study of human tumor metastasis in mice were not available, we developed a subcutaneous (sc) tumor model in athymic mice which mimics metastasis to investigate the effect of Ang antagonists on the establishment and growth of human tumor xenografts. This model (termed sc prophylactic), in which a small number of tumor cells are injected, approximates in its early stage metastatic disease where a limited number of tumor cells "seed" to a site and establish and grow only if, among other criteria, they receive an adequate supply of blood vessels from the host. In initial studies we demonstrated that prophylactic treatment with the neutralizing antiAng monoclonal antibody (mAb) 26-2F (the antibody is described in ref. 9), which is not cytotoxic to tumor cells *in vitro*, in all cases delayed the appearance of HT-29 colon adenocarcinoma tumors and, strikingly, completely

prevented their establishment in 25% of the mice (10). In subsequent studies adjustment of dosages and duration of administered antagonists enabled us to prevent completely the appearance of tumors in up to 60% of the mice using either of two mAbs or an Ang-binding protein with no observable adverse effects on the animals (11). Importantly, histological examination by our collaborator Dr. Marc E. Key, Vice President of Dako Corp., revealed a statistically significant decrease in the vascular density of those tumors that did develop in the Ang antagonist-treated mice. Thus, the availability of functional Ang is critical for the establishment of these tumors suggesting a therapeutically useful approach to the treatment of Ang-dependent malignancy. We have to date demonstrated that Ang antagonists are effective in inhibiting the growth of human colon, prostate, lung, brain, fibroblast and melanocyte tumors in preclinical mouse models.

Thus, with this extensive background and experience we were well positioned to extend our antiAng therapeutic strategies to the study of human breast carcinoma. A report of our most recent work supported by the Department of Defense on breast cancer follows.

BODY

I. Progress Report: Year 3 (Sept. 1, 1998 - Sept. 1, 1999)

Specific Aim 1: Antitumor effects of individual Ang antagonists

Preclinical Mouse Model (sc prophylactic) for Primary Breast Cancer Growth. In the MDA-MB-435 model which was optimized in Year 2, 4×10^4 cells/mouse were mixed with the Ang antisense JF2S and injected sc into the surgically exposed mammary fat pad (mfp) of female athymic mice. Follow-up injections of Ang antagonist were then given locally for 49 days. In two experiments we were able to completely prevent the establishment of tumors with treatment with antisense JF2S. Treatment in the same model with sense control JF1S, scrambled control JF14S or PBS as diluent control had no effect on the appearance of tumors with all treated mice developing palpable tumors by day 49. In year 3 we repeated these experiments so that appropriate statistical analyses could be performed. Table 1 summarizes the results of these four experiments. In all experiments **treatment with antisense JF2S prevented completely the formation of tumors** after injection with MDA-MB-435 breast tumor cells into the mfp, while all mice treated with either sense control JF1S, scrambled control JF14S or PBS developed palpable tumors. Using the Fisher's exact test, $p < 0.0001$ for the prevention of tumor formation by treatment with Ang antisense JF2S in comparison with treatment with the diluent control PBS. We are currently defining a dose response relationship for the antisense JF2S in this model so that we can use effectively measure additive or synergistic effects in planned combinatorial experiments which are components of Specific Aim 2.

Preclinical Mouse Model for Breast Cancer Metastasis. In Years 1 and 2 we were able to develop a reproducible model of distant breast cancer metastases from a primary tumor orthotopically placed in the mfp. Using a newly available cell line developed by Dr. Janet Price at the M. D. Anderson Cancer Center we were able to obtain reproducible lung metastases, as indicated by macroscopic observation and, for quantitative purposes, lung weights, in control mice 11 weeks after tumor cell injection. This is a full 8-10 weeks sooner than observed previously in the field using the parental cell line. Excitingly, in an initial experiment completed at the end of Year 2 we were able to protect 3 out of 7 mice treated with Ang antisense JF2S from the development of lung metastases. In Year 3 we repeated this experiment twice to confirm these results and allow for statistical analyses. As seen in Table 2, the percentage of JF2S-treated mice that developed tumors was very similar in the three experiments with,

on average, 39% of the mice failing to develop lung metastases as the result of antisense treatment ($p < 0.001$ for prevention of metastases formation by treatment with antisense JF2S in comparison with the PBS). In contrast, the PBS-treated groups and essentially all of the sense and scrambled-treated groups exhibited macroscopically observable lung metastases ($p > 0.05$ for the number of mice with metastases in either of the control [S]ODN-treated groups compared with the PBS-treated group). In Year 2 we were additionally able to show a small protective effect of the Ang neutralizing mAb 26-2F in the above MDA-MB-435L2 model, with 2 out of 8 mice treated with the mAb showing no metastases. In Year 3 we doubled the dose of mAb 26-2F and found an increased level of protection, with 6 out of 10 mice now free of metastases (Table 3). However, increasing the amount of mAb further by raising the number of weekly injections did not increase efficacy. **Thus, we have been able to demonstrate protection from macroscopically observable lung metastases formation in this orthotopic model of breast cancer using either an Ang-neutralizing mAb or an Ang antisense.**

In order, however, to assure ourselves that our macroscopic observations indicating complete protection of metastatic formation was indeed correct, we decided to quantitate metastasis *microscopically* in addition to macroscopic observation and lung weight quantitations. Although the latter techniques are certainly accepted in the field for lung metastasis quantitation in these types of models, we wished to determine if the mice that appeared to be metastases-free by macroscopic observation were, in fact, free of micrometastases. In addition, we wanted to further decrease the time needed further for completion of experiments to increase our productivity. In initial experiments in Year 3 we determined, by sacrificing mice at different times after tumor cell injection, that while only 5% of the mice exhibited macroscopically observable metastases at sacrifice on day 42, microscopically observable metastases were present in 50% of the mice at this time. We are currently conducting experiments to determine the time needed for essentially 100% of diluent control mice to develop micrometastases. Nevertheless, we have completed one experiment to test the efficacy of Ang antisense JF2S in preventing micrometastases. In Table 4 the proportion of mice with 0, 1 to 10 or greater than 10 metastatic lesions is compared for PBS- and Ang antisense-treated mice at sacrifice on day 58. Strikingly, 54% of the mice treated with Ang antisense JF2S showed no evidence of lung micrometastases in comparison with 10% of the PBS-treated mice ($p < 0.001$). In addition, of those mice who did demonstrate micrometastases, the percentage of mice with more than 10 metastases was quite different between the JF2S- and PBS-treatment groups (17 and 68%, respectively, $p < 0.001$). **Thus, we have shown that Ang antisense JF2S can reduce, and in over 50% of the mice, prevent the formation of lung micrometastases in the presence of an orthotopically-growing primary breast tumor.**

Oligonucleotides are occasionally noted for exhibiting biological effects unrelated to direct antisense mechanisms. We therefore investigated during Year 3 whether Ang antisense JF2S can reduce the amount of Ang produced *in vitro* by the cells, MDA-MB-435L2, which are now used in our metastasis model, and if this reduction in Ang synthesis would then lead to changes in *in vivo* tumor growth. Using Lipofectin as the necessary carrier for such *in vitro* transfections, MDA-MB-435L2 cells were transfected with either sense control JF1S or antisense JF2S. As shown in Figure 1A, when the tissue culture supernatant was collected 48 hours later and assayed by ELISA, the concentration of secreted Ang was reduced to 62% of that produced by the control Lipofectin-treated cells. The sense JF1S [S]ODN caused only a slight decrease in secreted Ang (92% of the amount produced by the control Lipofectin-treated cells). These same tumor cells were then harvested and injected orthotopically into the mfp of female athymic mice. Thirty-four days later the mice were sacrificed and the tumors when present were removed and weighed. Tumors were found on all mice injected with the control Lipofectin or sense JF1S-transfected cells (Figure 1B). In contrast, only 62% of the mice injected with the Ang antisense-transfected cells developed tumors. Additionally, the average weight of those tumors that did

develop from the antisense JF2S-treated cells was only 59% of the average weight of the tumors produced by the control Lipofectin-treated cells (Figure 1C). The tumors produced by the sense control JF1S-transfected cells were actually slightly heavier than the those resulting from injection of the control Lipofectin-treated cells. Thus, the Ang antisense JF2S can be shown to specifically reduce the production of Ang *in vitro* in the MDA-MB-435L2 cell line and prevent or retard subsequent primary tumor growth by these cells. It therefore follows that the antimetastatic activity produced by JF2S is also a direct result of interference with Ang synthesis in these tumor cells *in vivo*.

Mechanistically it would be of interest to determine the stage of the metastatic cascade that the above Ang antagonists inhibit. To this end we have begun developing a model of experimental metastases (in contrast to the spontaneous model discussed above) using MDA-MB-435L2 cells. In this case the tumor cells are introduced directly into the vasculature by means of injection under the splenic capsule. These types of models are noted for their production of metastases in the liver, a common site of metastases in human breast cancer. To our knowledge this has not been attempted with this cell line previously. In the initial experiment of this type two groups of female athymic mice were injected under the splenic capsule with MDA-MB-435L2 cells. In one group the spleen was removed 5 minutes after cell injection and in the other the spleen was left in place for the duration of the experiment. Six weeks later the mice were sacrificed. All mice in both groups harbored liver metastases. Since some of the mice in the group in which the spleen was not removed also showed tumors in the spleen, the spleen will be removed in future experiments in the interest of reducing variability in the mice in terms of tumor burden. Experiments are underway to test the Ang neutralizing mAb 26-2F and Ang antisense JF2S in this experimental model of metastases.

Drug Development.

i) *Chimerized and humanized antibodies*. As was reported last year, we have developed a chimerized version of the Ang neutralizing mAb 26-2F (12). This form has been shown to be active in neutralizing Ang activity in our tumor models. However, to reduce even further the potential for immunogenicity, we are in the process of producing a fully humanized version of mAb 26-2F. Toward this end we have been collaborating with Dr. K. R. Acharya at the University of Bath, UK, an expert in x-ray crystallographic techniques and protein modeling. Dr. Acharya, in continuing collaboration with this Center has solved the crystal structures of both bovine (13) and human Ang (14) to 1.5 and 2.0 Å, respectively. As an initial step toward humanization, Fab fragments of mAb 26-2F have been supplied to Dr. Acharya and crystals of the Fab-human Ang complex have been successfully produced. In year 3 Dr. Acharya, working at the synchroton at Daresbury, UK and the EMBL outstation at Hamburg, Germany, has collected a complete data set for the complex at 2.3 Å and has solved the structure using the molecular replacement technique. This 3-dimensional structural analysis will be invaluable for designing humanized antibodies as well as CDR-based peptides and mimetics. We are currently planning for the return of Dr. Renata Piccoli, with whom we made the chimerized version of mAb 26-2F, to our laboratory for the purpose of producing the humanized form of this antibody.

iii) *Small Molecule Inhibitors.* We have demonstrated previously that the ribonucleolytic activity of Ang, although extremely weak against conventional substrates, is nonetheless essential for the biological action of the protein. Therefore, molecules that are potent inhibitors of this activity might have considerable potential as anti-cancer agents. Dr. Robert Shapiro, a colleague at the Center and collaborator on this project, is spearheading efforts to develop small molecule inhibitors of the enzymatic activity of Ang to be used by us therapeutically. Using an initial kinetic approach he has identified two lead inhibitory compounds, 5'-diphosphoadenosine 2'-monophosphate (ppA-2'-p) and its 2'-deoxyuridine derivative (dUppA-2'-p). Although neither ppA-2'-p nor dUppA-2'-p binds sufficiently tightly to Ang be useful as a drug, the interactions are strong enough to allow structural studies to be performed. Interactions between Ang and these identified inhibitors are being solved both in solution by NMR

analysis (being performed by Dr. Shapiro's collaborator Dr. Feng Ni at the National Research Council of Canada) and in the crystal state (being performed by Dr. Acharya). Progress on both fronts is being made. These data together with computer-aided analysis using Quanta (Molecular Simulations) software will then allow for rational design and synthesis of inhibitors strong enough (estimated Ki values of 1-10 nM at physiological pH) to be used in mouse experiments. Thus, we are on schedule to commence therapy trials with small molecule inhibitors during Year 4.

Specific Aim 2: Combined effects of antiAng agents and cytotoxic drugs

In our proposed Statement of Work we planned to investigate the combined effects of Ang antagonists with cytotoxic drugs commonly used in the treatment of breast cancer. For these studies we will investigate the effects of combination treatments in the orthotopic setting, since as discussed above we have already been able to achieve 100% protection of primary tumor establishment using an antiAng antagonist alone (Specific Aim 1). Therefore, as a first step we set out to determine the appropriate doses of two drugs, doxorubicin and cyclophosphamide, which would affect the growth rate of MDA-MB-435L2 cells in the sc prophylactic model which serves as our "screening mode" assay as identified in the "Statement of Work". Tumor cells were injected orthotopically into the mfp on day 0. Doxorubicin (10 mg/kg body weight), cyclophosphamide (150 mg/kg body weight) or normal saline as diluent control was given ip on day 48 when the tumors had an average volume of 258 mm^3. No difference was observed by day 60 in the growth rate of the tumors in the doxorubicin-treated mice in comparison with the growth rate of the tumors in the saline control mice. In subsequent experiments we will use different dose protocols to achieve an effective anti-tumor range for this drug. However, by day 60 tumor size in the cyclophosphamide-treated mice was only 29% of that observed in the saline-treated mice. Therefore, cyclophosphamide will be used as our initial chemotherapeutic drug for combination studies. As mentioned above, we are currently performing dose-response studies of JF2S in the prophylactic screening assay. When completed, suboptimal doses of JF2S (as well as mAb 26-2F) will be used together with cyclophosphamide in order to observe any additive or synergistic therapeutic effects. If observed in the screening sc model, this combinatorial therapeutic strategy will be extended for use in metastasis trials in order to potentially improve upon the already evident antimetastatic effects of administration of individual Ang antagonists.

KEY RESEARCH ACCOMPLISHMENTS

- Established a sc prophylactic "screening mode" orthotopic assay in female athymic mice for evaluation of the capacity of Ang antagonists to inhibit the establishment of human primary estrogen-independent breast tumors
- Using the above model system, demonstrated that the Ang antisense agent JF2S reproducibly and in a statistically significantly fashion can completely protect mice from tumor establishment
- Determined that the mechanism by which this antitumor effect occurs is most likely via a specific antisense inhibition of Ang synthesis by breast cancer tumor cells
- Established a spontaneous orthotopic, metastatic model in female athymic mice for metastatic spread to the lung of estrogen-independent tumor cells growing in the mammary fat pad

- Using the above model system, demonstrated that both JF2S and an antiAng neutralizing monoclonal antibody, mAb 26-2F, can interfere and in certain cases completely prevent the formation of lung metastasis

- Determined that this antimetastatic effect occurs both at the level of macroscopic and microscopic lesions

- Initiated studies using an experimental metastasis model (in which breast tumor cells are directly introduced into the circulation via injection into the splenic capsule with subsequent metastasis to the liver) in order to determine at which stage of the metastatic cascade Ang inhibitors are effective.

- Determined that the cytotoxic drug cyclophosphamide can inhibit the growth of primary breast tumor cells growing orthotopically

- With the above information, will initiate combination studies in which antiAng agents together with cyclophosphamide will be used to potentially enhance anti-breast cancer effects of both primary and metastatic tumor growth

- Ongoing drug development has generated a chimeric antibody for therapy to be extended into the construction of a completely humanized antibody counterpart, as well as identified lead small molecule Ang antagonists to be used therapeutically

REPORTABLE OUTCOMES - 1999

Lecture - "Angiogenin: Opportunities for Research and Clinical Studies". Presenter: James W. Fett, Ph.D. Venue: "Tools for Drug Discovery Symposium: the Angiogenesis Model", Philadelphia, PA, April 9, 1999.

Lecture and Abstract - "Angiogenin - Therapeutic Opportunities". Presenter: James W. Fett. Co-author on abstract: Karen A. Olson, Ph.D. Venue: "Fifth International Meeting on Ribonucleases", Warrenton, Virginia, May 12-16, 1999.

Patent - "Chimeric and Humanized Antibodies to Angiogenin". Inventor: James W. Fett, Ph.D. Filed: April 5, 1999.

CONCLUSIONS

During the third year of Department of Defense support we have extended and confirmed the therapeutic studies which in Year 2 indicated that antagonists Ang were effective against establishment of both primary breast cancer cells implanted in the mfp and against formation of metastasis originating from such primary tumor growth. We have now repeated these studies sufficiently to where statistical significance is observed. Remarkably, complete protection from primary tumor development has been achieved by treatment with the Ang antisense phosphorothioate JF2S. Currently, dose response studies are underway with JF2S in this assay in order to define suboptimal concentrations to be used in

combination with cyclophosphamide for evaluation of additivity/synergy in both the sc and metastatic model systems.

We believe that the major indication for antiAng therapy will be for the treatment and/or prevention of breast cancer metastasis, the form of disease that kills patients. As a result, a major effort has been placed on establishing in the laboratory a relevant metastatic model for evaluation of antiAng metastatic effects. We have now succeeded by using a recently available cell line, MDA-MB-435L2, selected for this purpose. During the third year of funding we have not only demonstrated that the formation of macroscopic lung metastatic lesions can be inhibited by treatment with Ang antagonists, but that interference with micrometastatic disease can also be achieved. Thus, we are encouraged that by implementing our antiAng strategy clinically, prevention and/or therapy of both micro- and macroscopic breast cancer metastasis may be achieved. Importantly, we have also substantially reduced the turnaround time of the metastasis assay thus increasing productivity and reducing the use of reagents and mice.

We have also pursued our drug development program to the stage where construction of humanized versions of the murine neutralizing monoclonal antibody, 26-2F, can soon begin. (A chimerized antibody has already been prepared). The structure of the complex between the Fab fragment of mAb 26-2F and Ang has been solved by our collaborator, Dr. Acharya. Critical contact points between the Ang protein and the antigen-combining site of mAb 26-2F are now identified. These determinations will facilitate the design of humanized counterparts of the mouse antibody. This work will be facilitated by the arrival, during Year 4, of Dr. Piccoli from Italy who was instrumental in constructing the chimeric counterpart of mAb 26-2F during Year 2. Additionally, progress is continuing to be made on the design of small molecule inhibitors of Ang. Structures of complexes between Ang and lead drug candidates are being investigated both in solution (using NMR) and in the crystal state (using x-ray crystallographic techniques). The data generated will be used in conjunction with state-of-the-art computer modeling to rationally design inhibitors of sufficient strength to be tested therapeutically during Year 4 as planned.

Thus, we continue to validate the proposition that the use of antagonists directed against Ang, either alone or in combination therapies, should be effective clinically for the prevention and/or treatment of metastatic breast cancer.

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APPENDICES (illustrations - 4 Tables and 1 Figure)

Pages 13-18

Table 1. Prevention of MDA-MB-435 primary tumor growth in athymic mice by treatment with Ang antisense JF2S [a]

Treatment	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Total
PBS	8/8 [b] (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)	32/32 (100%)
Sense control, JF1S	8/8 (100%)	8/8 (100%)	8/8 (100%)	8/8 (100%)	32/32 (100%)
Scrambled control, JF14S		14/14 (100%)		10/10 (100%)	24/24 (100%)
Ang antisense, JF2S	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/8 (0%)	0/32 [c] (0%)

[a]Mixtures of cells (4×10^4 cells/mouse) with either PBS, Ang sense control JF1S (400 μg/mouse), Ang scrambled control JF14S (400 μg/mouse) or Ang antisense JF2S (400 μg/mouse) were injected into the surgically exposed mfp on day 0. Mice were then treated sc with one half the day 0 dose of the same material 6 times per week for the first two weeks, followed by the same dose given 4 times per week until day 49. [b]Number of tumor-bearing mice on day 49/total number of mice in the treatment group; expressed as a percentage in parentheses below the fraction. [c] By Fisher's exact test, $p < 0.0001$ for prevention of MDA-MB-435 tumor formation by treatment with Ang antisense JF2S in comparison with PBS.

Table 2. Treatment of MDA-MB-435L2 lung metastasis with Ang antisense JF2S: Prevention of formation of macrometastases [a]

Treatment	Exp. 1	Exp. 2	Exp. 3	Total
PBS	8/8 [b] (100%)	10/10 (100%)	6/6 (100%)	24/24 (100%)
Sense control, JF1S	8/8 (100%)	7/8 (88%)	10/11 (91%)	25/27 (93%)
Scrambled control, JF14S		11/12 (92%)	18/19 (95%)	29/31 (94%)
Ang antisense, JF2S	4/7 (57%)	10/15 (67%)	8/14 (57%)	22/36 [c] (61%)

[a]MDA-MB-435 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (4 x 10^6/mouse). The next day treatment is begun with either PBS (diluent control), Ang sense control JF1S (300 μg/mouse), scrambled control JF14S (300 μg/mouse, Exp. 2 and 3) or Ang antisense JF2S (300 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice on day 77. [b]Number of mice in which macroscopically observable metastases was present in the lungs at sacrifice/total number of mice in the treatment group; expressed as a percentage in parentheses below this fraction. [c]By Fisher's exact test, $p < 0.001$ for prevention of MDA-MB-435L2 lung metastases by treatment with Ang antisense JF2S in comparison with PBS.

Table 3. Treatment of MDA-MB-435L2 lung metastasis with Ang neutralizing mAb 26-2F: Prevention of formation of macrometastases [a]

Treatment	Exp. 1	Exp. 2
PBS	8/8 [b] (100%)	7/7 (100%)
mAb 26-2F, Protocol 1	6/8 (75%)	7/9 (78%)
mAb 26-2F. Protocol 2		4/10 (40%)
mAb 26-2F, Protocol 3		3/8 (38%)

[a]MDA-MB-435L2 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (4 x 10^6/mouse). The next day treatment is begun with either PBS (diluent control, 100 µl/mouse, 4 times per week), mAb 26-2F (Protocol 1:120 µg/mouse, 4 times per week; Protocol 2: 240 µg/mouse, 4 times per week; Protocol 3: 240 µg/mouse, 6 times per week) given ip. [b]Number of mice in which macroscopically observable metastasis was present in the lungs at sacrifice/total number of mice in the treatment group; expressed as a percentage in parentheses below this fraction.

Table 4. Treatment of MDA-MB-435L2 lung metastasis with Ang antisense JF2S: Prevention of formation of micrometastases [a]

Treatment	Number of lung micrometastases/mouse		
	0	1-10	>10
PBS	2/21 [b] (10%)	6/21 (28%)	13/21 (62%)
Ang antisense, JF2S	14/26 [c] (54%)	10/26 (38%)	2/28 [d] (8%)

[a]MDA-MB-435L2 breast tumor cells are injected into the surgically exposed mfp of athymic mice on day 0 (2×10^6/mouse). The next day treatment is begun with PBS (diluent control) or Ang antisense JF2S (600 μg/mouse) given ip. This treatment is continued 4 times per week until sacrifice. [b]Number of mice in which microscopically observable metastasis was present in the lungs at sacrifice/total number of mice in the treatment group; expressed as a percentage in parentheses below this fraction. [c]By Fisher's exact test, $p < 0.001$ for the prevention of lung micrometastases by treatment with Ang antisense JF2S in comparison with PBS. [d] $p < 0.001$ by Fisher's exact test for the appearance of more than 10 metastases/lung in the Ang antisense-treated group in comparison with those mice treated with PBS.

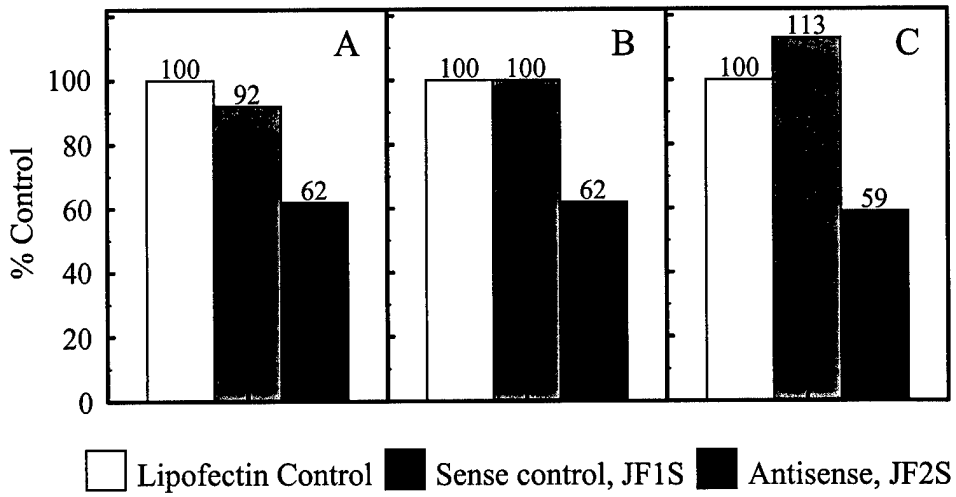

Figure 1. *Ex vivo* transfection of MDA-MB-435L2 cells inhibits Ang secretion *in vitro* and subsequent tumorigenicity in mice. MDA-MB-435L2 cells were treated with either Lipofectin alone or Lipofectin together with 1 μM of antisense JF2S or sense control JF1S *in vitro*. After 48 hours the supernatants was harvested and the amount of Ang secreted was determined by ELISA and normalized to cell number (A). These cells were then injected orthotopically into the mfp of female athymic mice. Thirty-four days later the mice were sacrificed and the tumors when present were removed and weighed. The average number of tumors per *ex vivo* treatment group and the average size of those tumors that did appear are shown in panels B and C, respectively. In all panels the results for sense JF1S- (gray bars) or antisense JF2S- (black bars) transfected cells are present relative to that of Lipofectin-treated cells (100%, white bars).