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13. ABSTRACT *(Maximum 200 Words)*

Incontrovertible evidence now exists that the growth of 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 mediator, named angiogenin (Ang), in human breast cancer cells and are developing inhibitors of its functions. In order to evaluate whether these inhibitors may be useful for treating breast cancer, we have during the grant period developed mouse models for both primary and metastatic growth of human breast cancer cells. Using these models we have shown that Ang antagonists (including monoclonal antibodies and antisense reagents) potently interfere with the establishment and metastatic spread of breast cancer cells. Further generation drugs, which include small molecule inhibitors, humanized antibodies and peptide mimetic drugs, are under development for eventual testing. In addition, multimodal therapies combining antiAng agents with conventional chemotherapeutic drugs will be evaluated in the future. Importantly, interest in this therapeutic approach has generated an intent to form a biotechnology company dedicated to translating these preclinical findings into treatment and prevention trials in patients.

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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 180,000 new cases will be diagnosed and approximately 41,000 individuals will die from this disease during 2000 (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 twenty antiangiogenic agents are in clinical trials for treatment of several angiogenesis-dependent diseases, including cancer (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

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 x 10^4 cells/mouse were mixed with the Ang antisense JF2S (a phosphorothioate oligonucleotide which targets the AUG start site region of the angiogenin gene, ref. 12) 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 Spontaneous Orthotopic Mouse Model for Breast Cancer Metastasis. In Year 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 (MDA-MB-435) 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 have completed an additional 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*.

Preclinical Mouse Model for Experimental Breast Cancer Metastasis. 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 during Year 4 developed a model of experimental metastases (in contrast to the spontaneous model discussed above) using MDA-MB-231 cells. In this case the tumor cells are injected intravenously (iv) into the tail vein of a mouse. This results in metastases in the lung. In experiments of this type depicted in Figure 2, MDA-MB-431 cells were injected into the tail vein followed by an initial ip administration of antisense JF2S or controls on Day 0. Follow up treatments were given ip for 73-75 days. At the termination of the experiment mice were sacrificed and lungs were examined macroscopically for the presence of observable metastasis. As can be seen in Figure 2, whereas a vast majority of PBS control-treated mice develop disseminated lung lesions, antisense JF2S is able to protect 35-40% of treated mice from forming metastasis. Sense control JF1S produces a negligible effect on metastasis. Using the same model system mAb 26-2F is also able to prevent the formation of metastasis in approximately 40% of the mice (Figure 3). **Thus, Ang antagonists are capable of interfering with the systemic establishment of distant metastasis by either preventing tumor cell attachment in the lung or preventing the development of the vasculature required for growth of metastatic tumors, or both.**

Drug Development.

i) *Chimerized and humanized antibodies*. As was reported previously, we have developed a chimerized version of the Ang neutralizing mAb 26-2F (13, see Addendum). 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 fully humanized versions of mAb 26-2F for ultimate clinical use. 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 (14) and human Ang (15) 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, collected a complete data set for the complex at 2.3 Å. In Year 4, upon further refinement, a new data set was collected at 2.0 Å which allows for visualization of all molecular entities. Using molecular replacement techniques the complete 3-dimensional structural of this complex has been solved, a diagrammatic representation is presented in Figure 4. Defining the critical contact regions between antigen and antibody should greatly facilitate the molecular engineering of humanized [complementarity-determining region (CDR)-grafted] antibodies. This work will be continued by us. Additionally, the completed x-ray structure will allow for development of CDR-based peptide mimetics. This latter strategy has lead to drugs with considerable potency (16).

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. Although, we had planned to initiate testing of these small molecule inhibitors in Year 4, progress on this front has not proceeded to a stage where this is feasible. Nevertheless, we plan on evaluating these reagents in our various preclinical mouse models in the future as they become available.

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.

Our intent is to use multimodal treatments in order to increase the efficacy of Ang antagonists in both the spontaneous and experimental models of breast cancer metastasis discussed above. This has not been possible until now due to the unanticipated length of time it has taken us to develop and validate relevant and reproducible mouse models for human breast cancer. In our opinion and those of colleagues in the field, a major drawback in this area is in the production of suitable cell lines and model systems which are less time consuming and are reproducible mimics of human breast cancer metastatic disease. These systems will be invaluable for both the study of the mechanisms and molecules contributing to the processes of metastasis and also for testing of novel therapies for the treatment and prevention of metastasis. We believe, however, that we have now achieved success in establishing relatively reproducible spontaneous and experimental metastasis models. Excitingly, Ang antagonists that have been tested demonstrate dramatic, although not complete, anti-metastatic effects. We are, therefore, now in a position to perform multi-agent therapy studies in order to potentially increase efficacy and further define clinical indications of our antiAng strategies for patient use. Additional funding sources will thus be sought to ensure the completion of this important aspect of our program.

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-dependent (MCF-7) and -independent (MDA-MB-435) breast tumors

- Using the above model system, demonstrated that the Ang antisense agent JF2S reproducibly and in a statistically significantly manner 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 MDA-MB-435L2 tumor cells growing in the mammary fat pad

- Using the above model system, demonstrated that both antisense JF2S and an antiAng neutralizing monoclonal antibody, mAb 26-2F, can dramatically interfere the formation of lung metastasis

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

- Established an experimental metastatic model in female athymic mice for metastatic spread to the lung of estrogen-independent MDA-MB-231 tumor cells injected iv into the tail vein in order to further determine at which stage of the metastatic cascade Ang inhibitors are effective.

- Using the above model system, demonstrated that both antisense JF2S and an antiAng neutralizing monoclonal antibody, mAb 26-2F, can dramatically interfere the formation of lung metastasis in this setting

- Ongoing drug development has generated a chimeric antibody for therapy. Additionally, the 3-dimensional structure of the complex between the Fab fragment of mAb 26-2F and Ang has been solved toward constructing humanized antibodies and CDR-based peptide mimetics

- Determined that the cytotoxic drug cyclophosphamide inhibits the growth of primary breast tumor cells growing orthotopically. With this information, combination studies in which antiAng agents together with cyclophosphamide (and other relevant breast cancer chemotherapeutic drugs) will be tested to potentially enhance antimetastatic effects of Ang antagonists

- IMPORTANTLY, this work supported by the Dept. of Defense has generated the interest of scientific/business individuals to the extent that a biotechnology company is being formed to translate these preclinical findings into patient care

REPORTABLE OUTCOMES: Sept. 1997 - Aug. 2000

Manuscripts and Abstracts

Piccoli, R., Olson, K.A., Vallee, B.L., and Fett, J.W. Chimeric Anti-Angiogenin Antibody cAb 26-2F Inhibits the Formation of Human Breast Cancer Xenografts in Athymic Mice. *Proc. Natl. Acad. Sci U.S.A.* **95**, 4579-4583, 1998.

Olson, K.A. and Fett, J.W. Inhibition of Tumor Growth and Metastasis by Angiogenin Antisense Therapy. *Proc. Am. Assoc. Cancer Res.* **39**, 665A, 1998.

Olson, K.A., Key, M.E. and Fett, J.W. Inhibition of Breast Cancer Establishment and Metastasis in Mice by Angiogenin Antagonists. Submitted.

Crystal Structure of Human Angiogenin Complexed to the Fab Fragment of its Neutralizing monoclonal antibody 26-2F at 2.0-Å Resolution. In preparation.

Lectures (and associated abstracts/posters)

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.

Lecture and Poster - "Inhibition of Breast Cancer Development and Metastasis in Mice by Angiogenin Antagonists". Presenter: James W. Fett. Co-author on abstract: Karen A. Olson, Ph.D. Venue: "DoD Era of Hope Meeting", Atlanta, Georgia, June 8-11, 2000.

Patent

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

CONCLUSIONS

During 4 years of Department of Defense support we have validated our contention that antagonists of Ang are effective against establishment of both primary breast cancer cells implanted in the mfp and against formation of metastasis originating from such primary tumor growth and from intravenous introduction of tumor cells. 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 relevant metastatic models for evaluation of antiAng metastatic effects. We have now succeeded in developing both spontaneous and experimental models of human breast cancer metastasis in mice. Using these models we have shown that both the antisense JF2S drug as well as the mAb 26-2F potently inhibit the formation of disseminated lung metastasis. 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. We further intend to attempt to increase efficacy in these models with combination treatments incorporating appropriate chemotherapeutic drugs.

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 as well as CDR-based peptide mimetic drugs. 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. This work will continue.

Importantly, the research support of the Dept. of Defense along with the resultant preclinical results obtained have culminated in the intent to form a startup biotechnology venture whose mission will be to expedite translation of our strategies for the prevention and/or treatment of breast cancer metastasis into the clinical. Thus, we believe that the trust in and financial support of our breast cancer research program by the American taxpayer and Dept. of Defense was well justified. We will continue our efforts toward the ultimate eradication of this dreaded disease.

PERSONNEL RECEIVING PAY FROM RESEARCH EFFORT: Sept. 1997 - Aug. 2000

Name	Dates	% Effort
James W. Fett, Ph.D. (PI)	9/01/97-8/31/00	20
Karen A. Olson, Ph.D.	9/01/97-8/31/00	30
Justin Steele, B.S.	9/97/97-5/30/00	70
Nicole L. Steffen, B.S.	5/31/98-6/30/00	70
Najat J. Ziyadeh, B.S.	11/99; 12/99; 06/00	100

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APPENDICES: i) Illustrations (4 Tables and 4 Figures)

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ii) 1 Reprint - 3 Pages

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 x 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 were injected into the surgically exposed mfp of athymic mice on day 0 (4×10^6 /mouse). The next day treatment was 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 was 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 were injected into the surgically exposed mfp of athymic mice on day 0 (4 x 10^6/mouse). The next day treatment was 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 were injected into the surgically exposed mfp of athymic mice on day 0 (2 x 10^6 /mouse). The next day treatment was begun with PBS (diluent control) or Ang antisense JF2S (600 μg/mouse) given ip. This treatment was 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.

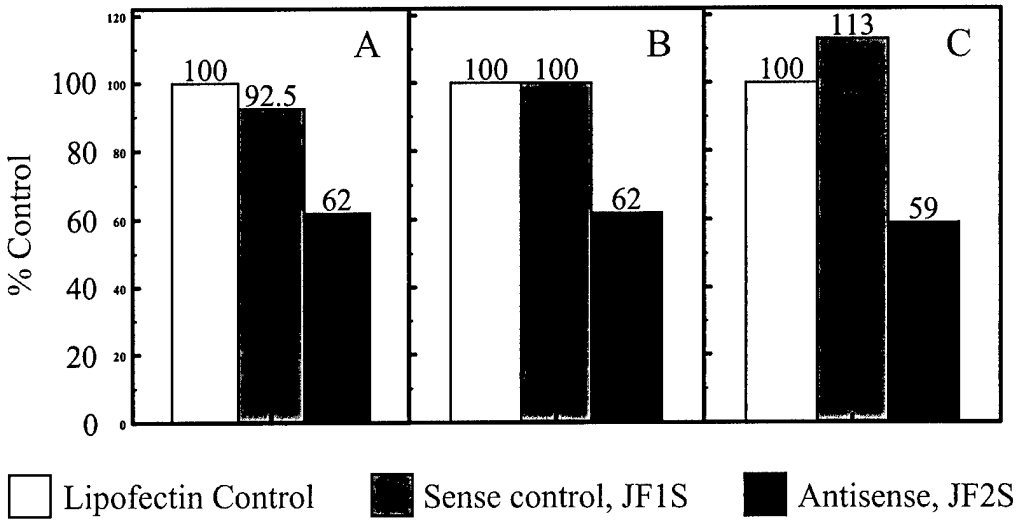

Figure 1. *Ex vivo* transfection of MDA-MB-435L2 cells inhibits Ang secretion *in vitro* and subsequent tumorigenicity in mice. MDA-MB-435L2 cells were treated with either Lipofectin alone or Lipofectin together with 1 μM of antisense JF2S or sense control JF1S *in vitro*. After 48 hours the supernatants 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 panel 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).

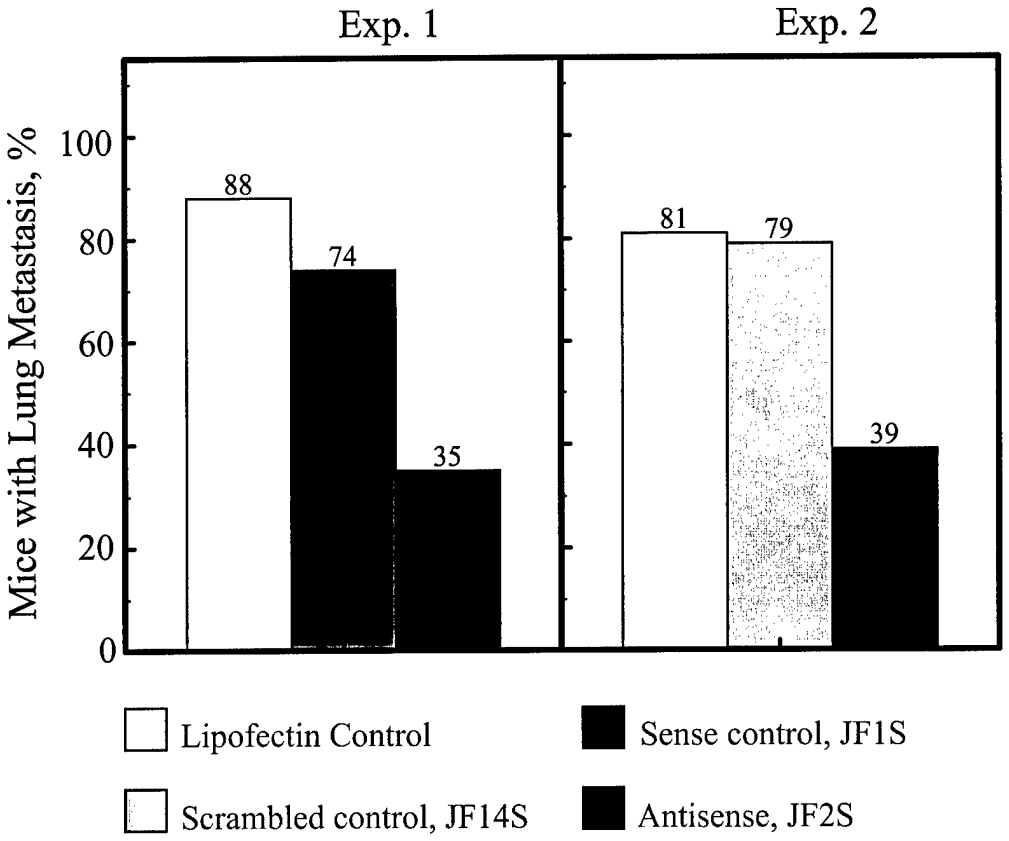

Figure 2. Treatment of MDA-MB-231 breast tumor cells *in vivo* with Ang antisense JF2S inhibits the formation of lung metastasis. MDA-MB-231 cells (4 x 10^5/mouse) were injected iv into the tail veins of female athymic mice; the mice were then injected ip with either PBS (diluent control, white bars) or Ang sense control JF1S (400 μg/mouse, dark gray bar, Experiment 1), Ang scrambled control JF14S (400 μg/mouse, light gray bar, Experiment 2) or Ang antisense JF2S (400 μg/mouse, black bars). Mice were then treated ip 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 sacrifice on day 73-75. At sacrifice, the lungs were examined using a dissecting microscope for the presence of metastases. In experiment 1, n=17 for the PBS and antisense treatment groups and n=19 for the sense control treatment group; in experiment 2, n=16, 19, and 18 for the PBS, scrambled control and antisense treatment groups, respectively.

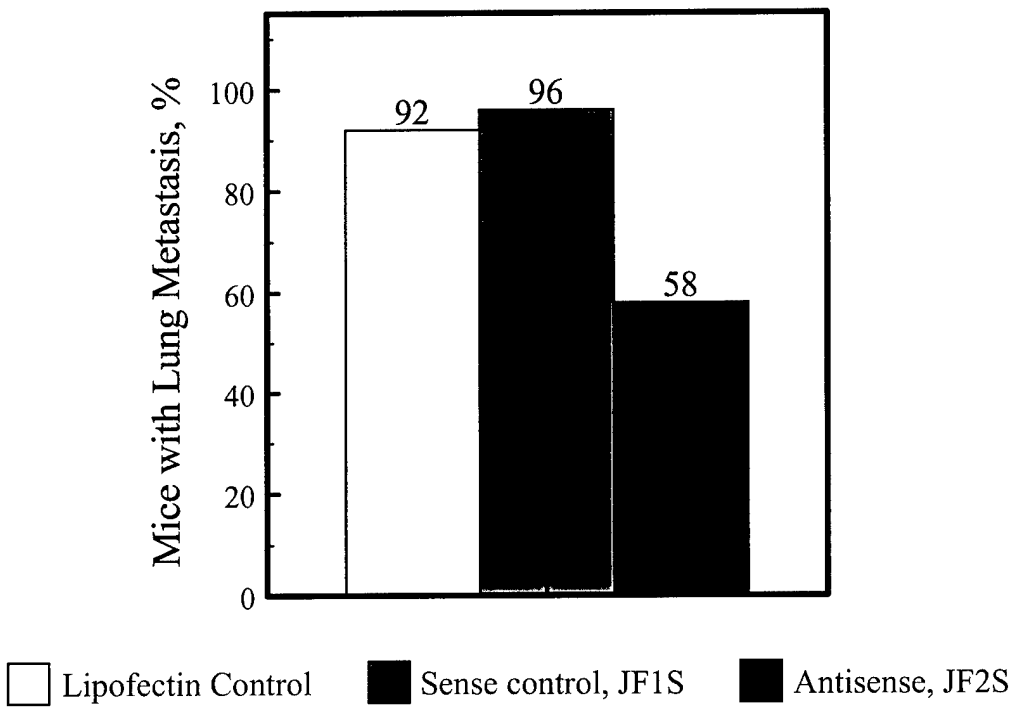

Figure 3. Treatment of MDA-MB-231 breast tumor cells *in vivo* with Ang mAb 26-2F inhibits the formation of lung metastasis. MDA-MB-231 cells (4×10^5/mouse) were injected iv into the tail veins of female athymic mice; the mice were then injected ip with either PBS (diluent control, white bar) or immunoglobulin control MOPC 31C (300 μg/mouse, gray bar) or mAb 26-2F (300 μg/mouse, black bar). Mice were then treated ip 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 sacrifice on day 73-75. At sacrifice, the lungs were examined using a dissecting microscope for the presence of metastases. n=12, 27 and 26 for the PBS, immunoglobulin control MOPC 31C and mAb 26-2F, respectively.

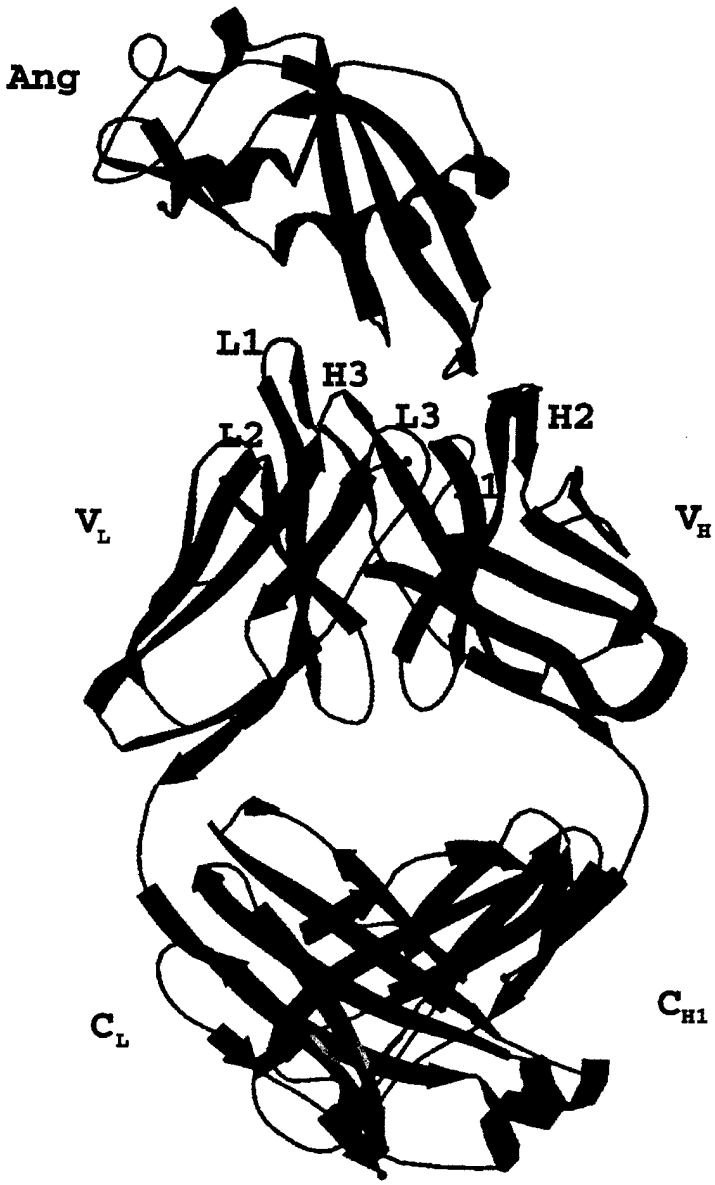

Figure 4. Crystal structure of the complex of Ang with mAb 26-2F Fab

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Chimeric anti-angiogenin antibody cAb 26–2F inhibits the formation of human breast cancer xenografts in athymic mice

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Contributed by Bert L. Vallee, January 26, 1998

ABSTRACT **Angiogenin (Ang), an inducer of neovascularization, is secreted by several types of human tumor cells and appears critical for their growth. The murine anti-Ang monoclonal antibody (mAb) 26–2F neutralizes the activities of Ang and dramatically prevents the establishment and metastatic dissemination of human tumor cell xenografts in athymic mice. However, for use clinically, the well-documented problem of the human anti-globulin antibody response known to occur with murine antibodies requires resolution. As a result, chimeric as well as totally humanized antibodies are currently being evaluated as therapeutic agents for the treatment of several pathological conditions, including malignancy. Therefore, we have constructed a chimeric mouse/human antibody based on the structure of mAb 26–2F. Complementary DNAs from the light and heavy chain variable regions of mAb 26–2F were cloned, sequenced, and genetically engineered by PCR for subcloning into expression vectors that contain human constant region sequences. Transfection of these vectors into nonproducing mouse myeloma cells resulted in the secretion of fully assembled tetrameric molecules. The chimeric antibody (cAb 26–2F) binds to Ang and inhibits its ribonucleolytic and angiogenic activities as potently as mAb 26–2F. Furthermore, the capacities of cAb 26–2F and its murine counterpart to suppress the formation of human breast cancer tumors in athymic mice are indistinguishable. Thus cAb 26–2F, with its retained neutralization capability and likely decreased immunogenicity, may be of use clinically for the treatment of human cancer and related disorders where pathological angiogenesis is a component.**

Angiogenesis, a multifaceted process by which new blood vessels form, occurs in many physiological and pathological situations, including cancer. Indeed, the critical contribution of angiogenesis to the growth, invasiveness, and metastatic dissemination of tumor cells is now well documented (reviewed in refs. 1 and 2). Mediators that affect angiogenesis are thus appropriate molecular targets against which to direct anticancer therapeutic strategies. One of these, angiogenin (Ang), a unique member of the ribonuclease superfamily of proteins, is a potent inducer of neovascularization and is serving as the focus of ongoing investigations into its structure/function relationships and clinical applications (reviewed in ref. 3).

Because Ang was originally isolated from medium conditioned by a human tumor cell line (4) and subsequently shown to be expressed by several histologically distinct types of human tumors (5), inhibitors of its functions have been developed to evaluate their antitumor effects. One of these, the murine monoclonal antibody (mAb) 26–2F, neutralizes the ribonucleolytic, angiogenic, and mitogenic activities of human Ang (6, 7). It is an IgG1κ with a binding affinity of 1.6 nM that recognizes a discontinuous epitope in Ang involving Trp-89 and residues in the segment 38–41, located in two adjacent loops of the Ang 3-dimensional structure (6, 8). Although not directly cytotoxic to tumor cells *in vitro*, mAb 26–2F is extremely effective in interfering with their establishment and metastatic spread in athymic mice (9–11). Thus, Ang antagonists should be of major clinical utility for the treatment of cancer.

The use of murine antibodies in patients is problematic, owing to their decreased serum half-lives and induction of human anti-mouse antibody immune responses, directed mainly against mouse Ig constant (C) regions (12–15). The latter is of particular concern in the case of antiangiogenesis therapies, where chronic administration of therapeutic agents may be required. To minimize this problem, chimeric antibodies have been genetically engineered in which murine heavy (H) and light (L) chain variable (V) domains are combined with human C regions, thereby replacing ≈70% of the murine antibody molecule with human sequences (16, 17). Several of these chimerized antibodies are under evaluation in patients for a variety of diseases (18–20). Therefore, as a first step toward producing an anti-Ang antibody amenable to clinical testing, a mouse/human chimeric analogue of mAb 26–2F has been constructed. Here we describe the cloning and sequencing of the V_L and V_H domains of mAb 26–2F and their expression together with human C regions as a fully assembled chimeric mAb (cAb 26–2F). cAb 26–2F is very similar if not identical to its murine counterpart in binding affinity, Ang neutralization capacity, and, importantly, in its antitumor activity against human breast cancer xenografts in athymic mice.

MATERIALS AND METHODS

Mice. Female athymic mice were obtained at 5 weeks of age from the isolator bred colony of Charles River Breeding Laboratories and maintained under specific pathogen-free conditions in a temperature- and humidity-controlled environment. Experiments were begun 1 week later.

Monoclonal Igs. The mAb 26–2F (6) was purified from ascites fluid by affinity chromatography using GammaBind Plus Sepharose (Pharmacia). Ascites fluid (80 ml) was diluted 1:1 with PBS, centrifuged, and the supernatant filtered through a glass fiber filter and 0.2 μm cellulose nitrate filter. After a

Abbreviations: Ang, angiogenin; cAb, chimeric antibody; CAM, chorioallantoic membrane; H and L, Ig heavy and light chains, respectively; V and C, Ig variable and constant regions, respectively.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF039414 (V_L) and AF039415 (V_H)].

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further dilution with PBS to 400 ml, the antibodies were adsorbed onto the gel, washed with PBS, and eluted with 0.1 M glycine·HCl into tubes containing an appropriate amount of 1 M Tris·HCl for neutralization. Following dialysis against 0.9% NaCl, the antibodies were quantified by enzyme-linked immunoadsorbent assay (ELISA), and stored at −70°C. The chimerized analogue of mAb 26–2F (cAb 26–2F, see below) obtained from each of the transfectoma cell types was purified from ascites fluid as described above. MOPC 31C, a nonspecific IgG1κ-secreting mouse hybridoma (CCL 130, American Type Culture Collection) was propagated, and IgG purified from ascites as described (9).

Cell Lines. The murine nonproducing myeloma cell lines P3X63-Ag8.653 (P3X) (CRL 1580) and Sp2/0 (CRL 1581) were obtained from the American Type Culture Collection. The estrogen-sensitive MCF-7 and estrogen-insensitive MDA-MB-435 human breast cancer cell lines were supplied by Marc E. Lippman (Georgetown University Medical Center) and Isaiah J. Fidler (University of Texas M.D. Anderson Cancer Center), respectively. We have determined that both cell lines secrete Ang *in vitro*. All cells were maintained in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, and antibiotics (growth medium).

Isolation of mAb 26–2F V_L and V_H Region cDNAs. Polyadenylated RNA was prepared from mAb 26–2F-producing hybridoma cells using the PoliATtract System 1000 mRNA isolation kit (Promega). The procedure followed for V_L and V_H cDNA isolation was essentially that of Coloma *et al.* (21) with minor modifications. For V_H first-strand cDNA synthesis, the reaction used the C_H1 antisense primer MγC.C_H1 AS and avian myeloblastosis virus reverse transcriptase (Promega). V_H cDNA amplification was performed using MγC.C_H1 AS as the antisense primer and a set of three universal sense primers complementary to the N termini of most V_H leader sequences (MHALT1.RV, MHALT2.RV, and MHALT3.RV). The V_L domain-encoding cDNA was obtained using the Pharmacia Mouse ScFv Module/Recombinant Phage Antibody system. V_L cDNA amplification was performed using *Taq* DNA polymerase (Promega), the C region MC_k AS.XBA antisense primer, and five universal sense primers complementary to the N terminus of V_L leader sequences (MLALT1.RV, MLALT2.RV, MLALT3.RV, MLALT4.RV, and MLALT.5).

PCR amplifications of both V_H and V_L cDNAs were carried out for 30 cycles in a MicroCycler thermal controller (Eppendorf) under the following conditions: 1 min denaturing (94°C), 2 min annealing (55°C), 2 min extension (72°C) followed by a final extension step of 7 min (72°C). The products were analyzed by electrophoresis in a 1.5% TAE agarose gel stained with ethidium bromide. The amplified cDNAs were then electrophoresed on a 2% low melting agarose gel in 0.5× TAE and eluted using a Magic PCR Preps DNA Purification kit (Promega).

Subcloning and Sequencing. Each V domain-encoding cDNA was ligated into a pT7Blue T vector (Novagen) using T4–DNA ligase (Promega). The ligation mixture was used for transformation of NovaBlue competent cells (Novagen). Plasmid DNA minipreps were analyzed by 1.5% agarose gel electrophoresis after digestion with appropriate restriction enzymes. Several clones containing inserts of the expected size were sequenced in both directions using a Sequenase 2.0 sequencing kit (United States Biochemicals).

V Domain cDNA Engineering. To clone V_L and V_H cDNAs into their appropriate expression vectors, they were each subjected to further PCR reactions using the following primers: H chain sense primer: MHALT2.RV (21) hybridizing to the N terminus of the H chain leader sequence and containing the *Eco*RV restriction site for cloning into the H chain expression vector. H chain antisense primer (H-P2 antisense): CTAGCTAGCTGAGGAGACGGTGACTGAGGTTCCT hybridizing to the J region and containing a *Nhe*I site for cloning into the C_H1 region of the H chain expression vector. L chain sense primer (L-P2 sense): GGGGATATCCACCATGGAGACAGACACACTCCTGCTATGGGTCCTGCT corresponding to oligonucleotide MLALT1.RV (21), containing a 10 nucleotide extension at the 3′ end and hybridizing to the N terminus of the L chain leader sequence. An *Eco*RV site is present for cloning in the L chain expression vector. L chain antisense primer (L-P2 antisense): AGCCGTCGACTTACGTTTCAGCTCCAGCTTGGTCCCAG hybridizing to the J region and containing a splicing signal sequence as well as a *Sal*I site for cloning into the intronic sequence of the L chain expression vector.

The amplified products were gel purified and cloned into pT7Blue T vectors for sequencing as described above. For both modified V_L and V_H domains, the cDNAs from two identical clones were excised with either *Eco*RV and *Sal*I (for V_L) or with *Eco*RV and *Nhe*I (for V_H) for cloning into expression vectors.

Construction of Chimeric Genes. The L and H chain expression vectors (pAG4622 and pAH4604, respectively) were constructed (21) and kindly provided by Sherie L. Morrison (University of California, Los Angeles). The pAG4622 vector contains the genomic sequence encoding the C-region domain of the human κ L chain and the *gpt* (22) selectable marker. The pAH4604 vector contains the *his*D (23) selectable marker in addition to sequences encoding the human H chain γ1 C-region domain. The promoter region in each vector is derived from the anti-dansyl mAb 27–44 (21). For each V_L and V_H domain, cDNA fragments obtained from two identical clones were appropriately digested and ligated into their respective expression vectors. The ligated products were used to transform HB101 competent cells (Promega) and the recombinant vectors were isolated using the Wizard Plus Maxipreps DNA purification system (Promega). Prior to transfection, they were linearized with the *Pvu*I isoschizomer BspCI restriction enzyme (Stratagene) and gel purified.

Transfection and Selection. The chimeric H and L chain expression plasmids were cotransfected into SP2/0 or P3X nonproducing myeloma cells by electroporation as described (21). Following transfection, the cells were kept on ice for 10 min, diluted in growth medium, and placed into 96-well tissue culture plates (1×10^4 cells per well). The cells were refed 48 hr later with growth medium containing histidinol (Sigma) at a final concentration of 5 or 10 mM for SP2/0 or P3X cells, respectively. After ≈14 days, supernatants from growing colonies were screened by ELISA for the presence of chimeric antibodies.

Two selected stable transfectants, P4–5 and S13–1, obtained from the transfection of P3X or SP2/0 cells, respectively, were subcloned twice by limiting dilution. To obtain sufficient material for further analysis, cAb 26–2F from each cell source was purified from ascites fluid as described above.

Immunoassays. *Screening ELISA.* Chimeric antibody producing transfectomas were detected by a modification of the screening ELISA described in Fett *et al.* (6). Briefly, affinity-purified goat anti-human IgG Fc (γ-chain specific) and goat anti-human κ chain (each at 10 μg/ml, Organon Teknika–Cappel), or human Ang (1 μg/ml) was coated onto 96-well plates. Following blocking of the wells with 0.5% ovalbumin, 50 μl of culture supernatant diluted 1:1 with 0.25% ovalbumin was added. After a 2-hr incubation at room temperature, the plates were washed and alkaline phosphatase-labeled goat anti-human IgG (Kirkegaard & Perry Laboratories) was added to each well, followed 1 hr later by addition of *p*-nitrophenyl phosphate to the washed plates. The reaction was stopped with 3 N NaOH and absorptivities were measured on a Dynatech MR600 ELISA plate reader at 405 nm with a turbidity reference of 630 nm.

Radioimmunoassay for binding affinity. A competition radioimmunoassay for binding affinity (6) with the following

modifications was used to determine IC_{50}s, the concentration of unlabeled Ang at which the binding of its iodinated derivative is decreased by 50%. Plates were coated (10 μg/ml in borate coating buffer, 50 μl per well) with either goat anti-mouse IgG Fc (γ-chain specific, Organon Teknika–Cappel) for capture of mAb 26–2F or goat anti-human IgG Fc (see above) for capture of the chimeric antibody. Radioactivity was determined using a Micromedic 4/600plus Gamma Counter.

Concentration Determinations. Ig concentrations were determined spectroscopically, assuming that a 1 mg/ml solution has an absorbance of 1.43 at 280 nm.

tRNA Assay. Formation of perchloric acid soluble fragments from yeast tRNA was measured as described (24).

Angiogenesis Assay. The chicken chorioallantoic membrane (CAM) assay was used according to Fett *et al.* (6).

Western Blot Analysis. The general procedures for SDS/10% PAGE, transfer, and Western blotting have been described (25). Samples were boiled in a buffer containing 5% 2-mercaptoethanol before loading onto the gel. For detection of human components, goat anti-human IgG Fc and κ chain antibodies were used. Ig chains were visualized with alkaline phosphatase-labeled rabbit anti-goat IgG and nitroblue tetrazolium as substrate.

Antitumor Activity *in Vitro*. Direct cytotoxicity of cAb 26–2F toward MDA-MB-435 and MCF-7 cells was examined using a described [^{3}H]thymidine assay (9).

Antitumor Activity *in Vivo*. This was assessed by a modification of the orthotopic model of human breast cancer tumor growth in athymic mice described by Price *et al.* (26). Tumor cells (MDA-MB-435 or MCF-7) were harvested by standard trypsinization procedures, washed in Hanks' buffered salt solution, and counted by trypan blue exclusion hemacytometry. Viable cells (MDA-MB-435, 5×10^5 in 10 μl, or MCF-7, 1×10^6 in 20 μl) were injected into the surgically exposed mammary fat pad using a manual repeating dispenser (Hamilton). For MCF-7 cells a pellet of 17β-estradiol (0.72 mg per pellet, 60-day release; Innovative Research of America) was placed 1 cm from the site of tumor cell injection as the source of standard estrogen supplementation. The incision was closed with an autoclip and local subcutaneous treatment was begun within 30 min as described in the legend to Fig. 4. Tumor growth was monitored by caliper measurements.

RESULTS AND DISCUSSION

Isolation of cDNAs Encoding mAb 26–2F V Domains. Polyadenylated RNA was isolated from mAb 26–2F-producing hybridoma cells. cDNA sequences encoding the mAb 26–2F variable domains (V_L and V_H) were amplified by PCR using gene-specific primers designed to hybridize to the leader sequence of each domain (5′ primers) and to the C region N-terminal coding sequences positioned immediately downstream of the V–J region (3′ primers). Using this strategy, no amino acid substitutions that could effect chimeric antibody activity are introduced into the V_L or V_H cDNAs.

V_L and V_H amplified cDNAs were then cloned into pT7Blue T vectors and recombinant plasmids, isolated from independent clones, were sequenced. For each type of cDNA, at least two clones were identical. The nucleotide and deduced amino acid sequences for the V_L and V_H domains of mAb 26–2F are shown in Fig. 1. According to the classification of Kabat *et al.* (27), the DNA sequences encode V_H IIID and V_k III V regions, each including three complementarity-determining regions and four framework regions. The deduced amino acid sequence of the first 16 N-terminal amino acids of each V domain is identical to that obtained by Edman degradation of the protein (data not shown).

Construction and Expression of Chimeric Antibody Genes. V_H and V_L cDNAs were modified at their 3′ end by removing the N-terminal sequence of the murine C region and adding a

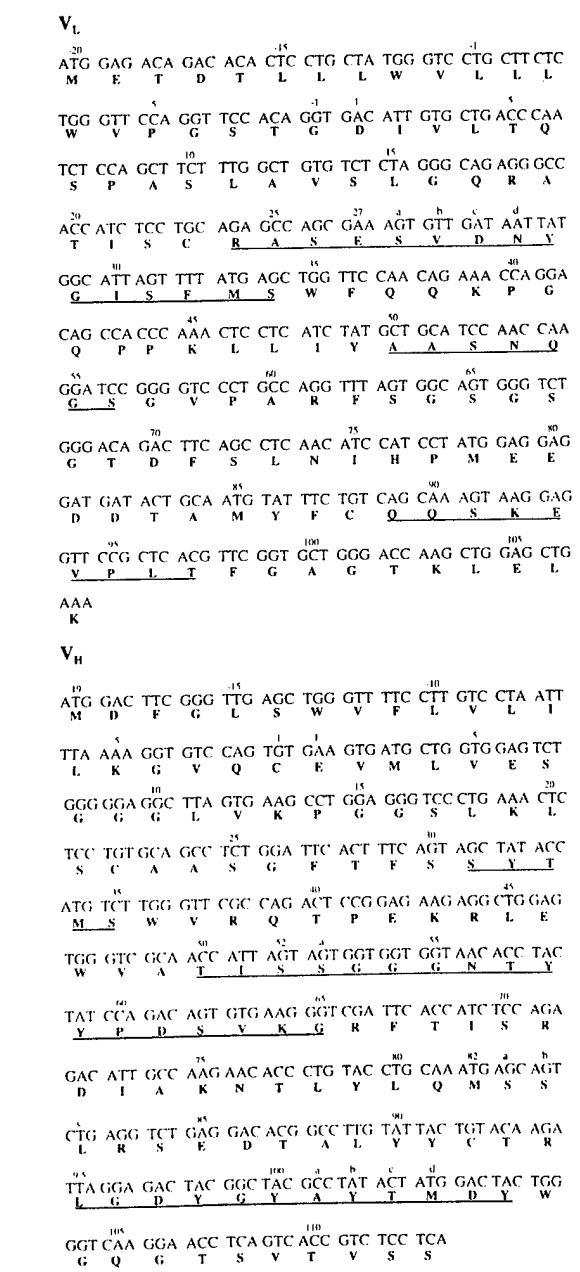

FIG. 1. Nucleotide and deduced amino acid sequences for mAb 26–2F V_L (*Upper*) and V_H (*Lower*) domains. The sequences were interpreted according to Kabat *et al.* (27). Underlined amino acids comprise the three complementarity-determining regions. Portions of the leader sequence are not necessarily correct because they correspond to the PCR primers.

splicing signal sequence at the V_L 3′ end. The resulting V_H and V_L cDNA-containing plasmids, prepared from independent clones, were digested with *Eco*RV and *Xba*I, gel purified, and amplified by PCR using primers complementary to the signal peptides (sense primers) and to the 3′ end (antisense primers) of each V_H and V_L domain. The gel-purified PCR products were cloned into pT7Blue T vectors and independent clones were sequenced. The sequence analyses confirmed that the expected DNA assembly had been achieved. For each modified V_H and V_L domain, the cDNA from two identical independent clones were excised with either *Eco*RV and *Sal*I (for V_L) or *Eco*RV and *Nhe*I (for V_H) and gel purified. The V_L and V_H cDNA products were ligated into their respective expression vectors. Several clones, isolated from HB101 competent cell transformation, were analyzed with appropriate restriction

enzymes. Recombinant vectors were isolated in duplicate from two distinct clones, each of which derived from independent V_L- or V_H-containing plasmid clones. Prior to transfection, the recombinant vectors were linearized with *Pvu*I and gel purified.

Combinations of chimeric H and L chain-containing vectors were cotransfected into either P3X or SP2/0 cells by electroporation. Cells were grown in 96-well plates and selected for the presence of the *his*D marker by including histidinol in the growth medium. Transfection efficiencies for both cell lines under these conditions were greater than 1 in 10^4. At approximately 2 weeks after transfection supernatants from surviving cells were assayed by ELISA. These indicated that the vast majority of transfectomas produced human Ig chimeras that bound to Ang; cells secreting only chimeric L chain genes were detected in a small percentage of wells. Two chimeric antibody producing master wells designated S13–1 and P4–5, obtained from the transfection of SP2/0 and P3X cells, respectively, were selected as stable transfectants and subcloned twice by limiting dilution.

Purification and Structural Characterization of cAb 26–2F. S13–1 or P4–5 transfectoma cells were injected into pristane-primed athymic mice to generate ascites fluid. Antibody was then subsequently isolated by protein G-Sepharose affinity chromatography. The total yield of purified cAb 26–2F from either transfectoma source was ≈3 mg per mouse.

Purified S13–1- and P4–5-derived chimeric antibodies were first subjected to 10 cycles of Edman sequence analysis. L and H chain N-terminal amino acids of both chimeric antibodies were identical (data not shown) and correspond to those of the original mAb 26–2F.

Western blot analysis using reagents specific for human κ and γ1 C region determinants showed that cAb 26–2F from either transfectoma cell source contained reduced chimeric L and H chains of the expected molecular weights (≈25,000 and 55,000, respectively) (Fig. 2). Under nonreducing conditions, cAb 26–2F derived from either S13–1 or P4–5 migrated to a position corresponding to 160,000 daltons (data not shown), thus indicating that the chimeric L and H chains were correctly assembled into complete H_2L_2 molecules.

The IC_{50}s for S13–1- and P4–5-derived cAb 26–2F are 2.1×10^{-9} M and 2.4×10^{-9} M, respectively, values that are essentially indistinguishable, within the error of the assay, to that obtained for mAb 26–2F (1.6×10^{-9} M).

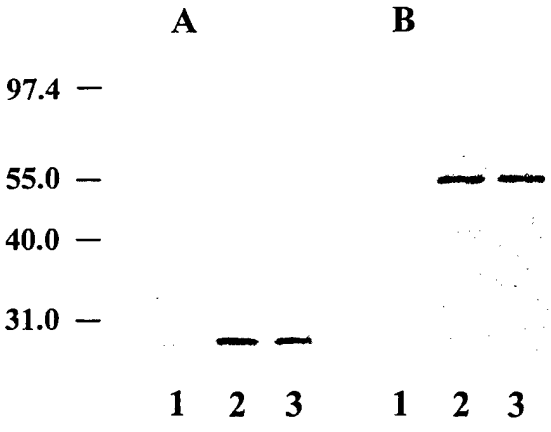

FIG. 2. Western blot analysis of cAb 26–2F. Reduced proteins (400 ng) were separated by SDS/10% PAGE and transferred to nitrocellulose sheets. These were incubated with either goat anti-human κ chain (*A*) or goat anti-human IgG Fc-specific (*B*) antibodies followed by treatment with alkaline phosphatase-labeled rabbit anti-goat IgG and nitroblue tetrazolium. Lane 1, mAb 26–2F; lane 2, cAb 26–2F from S13–1; lane 3, cAb 26–2F from P4–5. Molecular weight standards ($\times 10^{-3}$) are at left.

Table 1. Effect of cAb 26-2F derived from S13-1 or P4-5 myeloma cells on the activity of human Ang in the CAM assay

	mAb							
Group	Ang	26-2F	S13-1	P4-5	MOPC 31C	Assay results*	$P^\dagger$	Status
I	+	−	−	−	−	25/45 (56)	0.0009	Active
II	+	+	−	−	−	10/45 (22)	0.9556	Inactive
III	+	−	+	−	−	11/46 (24)	0.8038	Inactive
IV	+	−	−	+	−	11/45 (24)	0.7594	Inactive
V	+	−	−	−	+	26/45 (58)	0.0004	Active
VI	−	+	−	−	−	9/42 (21)	0.9718	Inactive
VII	−	−	+	−	−	7/45 (16)	0.4492	Inactive
VIII	−	−	−	+	−	13/42 (31)	0.3258	Inactive
IX	−	−	−	−	+	15/45 (33)	0.2154	Inactive

Combined data represent three sets of assays. Each individual assay employed between 15 and 19 eggs. Amount applied per egg was 10 ng of Ang and 100 ng of IgGs.

*Results are expressed as the ratio of positive to total surviving eggs; the percentage of positive eggs is given in parentheses.

†Significance was calculated from χ^2 values of data recorded at 48 ± 2 hr based on comparison with water controls tested simultaneously (10 positive eggs/46 total surviving eggs, 22% positive). To be designated active samples must have a value of $P < 0.05$.

Functional Characterization of cAb 26–2F. A comparison of the capacity of cAb 26–2F with its murine counterpart to inhibit the angiogenic activity of Ang on the CAM is shown in Table 1. Statistical analysis by the χ^2 test indicates that cAb 26–2F purified from either S13–1 (group III, $P = 0.8038$) or P4–5 (group IV, $P = 0.7594$) is as potent as mAb 26–2F (group II, $P = 0.9556$) in inhibiting the biologic activity of an equimolar amount of Ang, which alone is highly active (group I, $P = 0.0009$). The control MOPC 31C is not inhibitory (group V, $P = 0.0004$). The Igs alone are inactive on the CAM (groups VI–IX, $P > 0.05$).

To this point, the structural and functional data taken together indicated that, as expected, the chimeric antibodies derived from transfection of either SP2/0 or P3X myeloma cells were identical. However, in the course of these studies it was observed that cells derived from clone S13–1 proliferated at a greater rate and, in general, maintained a higher percentage of viable cells in culture. In addition, S13–1 adapted easily to growth in protein-free medium, whereas P4–5 cells died under these conditions, an important consideration when large-scale production necessary for clinical trials is contemplated. For the above reasons, the remaining data to be

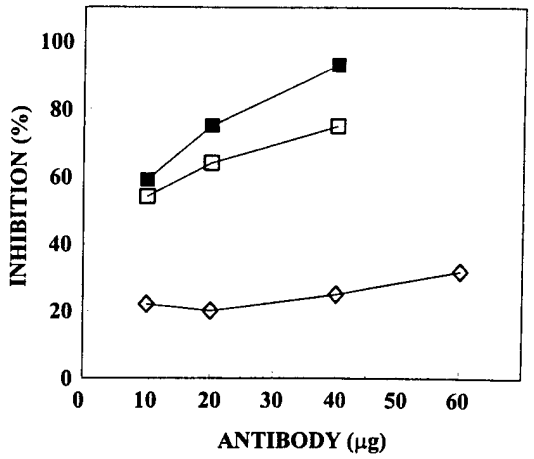

FIG. 3. Inhibition of the ribonucleolytic activity of Ang by mAb 26–2F (■), cAb 26–2F (□), or control MOPC 31C (◇). Ang was preincubated with the indicated amounts of Igs and assays were performed in 33 mM Hepes/33 mM NaCl, pH 6.8, at 37°C according to Shapiro *et al.* (24).

reported were generated with cAb 26–2F derived solely from clone S13–1, which will serve as the future source of the chimeric antibody.

The capacity of cAb 26–2F to inhibit tRNA degradation by Ang was determined by measuring the rate of formation of perchloric acid-soluble fragments. The inhibition curves obtained with mAb 26–2F, cAb 26–2F, and the control MOPC 31C are shown in Fig. 3. At 10 μg, the two antibodies are equally inhibitory, whereas at higher concentrations cAb 26–2F is only slightly less active.

The antitumor activity of cAb 26–2F was subsequently examined using modifications of an orthotopic tumor cell model (26). The results depicted in Fig. 4 indicate that cAb 26–2F is as effective as mAb 26–2F in preventing the formation of tumors of human breast cancer origin. Whereas all PBS- and control MOPC 31C-treated mice develop MDA-MB-435 (Fig. 4*A*) or MCF-7 (Fig. 4*B*) tumors by days 17 and 28, respectively, the chimeric and murine antibodies completely prevent the appearance of tumors in ≈40% (MDA-MB-435) and ≈50% (MCF-7) of the treated mice. Because cAb 26–2F does not interfere with thymidine uptake and, by inference, killing of tumor cells *in vitro* (data not shown), the antitumor effects observed most likely result from the inactivation of tumor-secreted Ang and subsequent interruption of the angiogenic process.

In summary, we have constructed a recombinant chimeric mouse/human anti-Ang antibody, cAb 26–2F, in which the V_L and V_H regions of mAb 26–2F were inserted into expression vectors containing C regions of human κ chains and γ1 H chains. The resultant chimera retains the properties of the original mAb, including potent activity against human tumor cell xenografts. As a consequence, cAb 26–2F should provide a powerful immunotherapeutic for the treatment of human cancer and other conditions where inhibition of pathological angiogenesis is desired.

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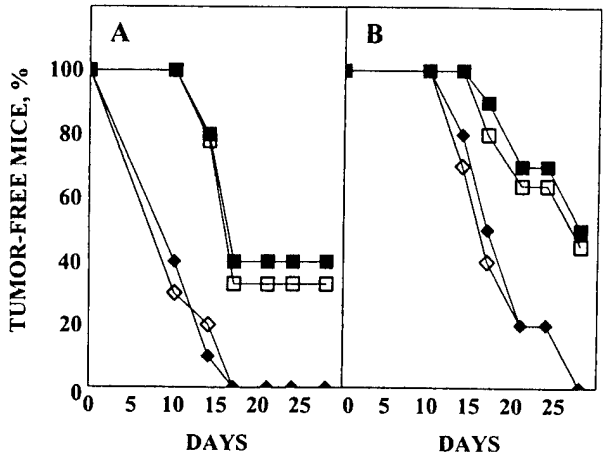

FIG. 4. Prevention of MDA-MB-435 (*A*) and MCF-7 (*B*) tumor formation by mAb 26–2F or cAb 26–2F. Tumor cells [5×10^5 (*A*) or 1×10^6 (*B*) per mouse] were injected into the surgically exposed mammary fat pad on day 0. For MCF-7 cells, a 17β-estradiol pellet was implanted in each mouse as a source of exogenous estrogen. Within 30 min of tumor cell injection the mice were treated with local subcutaneous injections of either PBS (♦) or Igs [mAb 26–2F (■), cAb 26–2F (□), MOPC 31C (◇); 240 μg/dose (*A*) and (*B*)]. Mice were then treated locally with 120 μg/dose (*A*) or 240 μg/dose (*B*) 6 times per week until sacrifice on day 28. $n = 10$ for all groups.

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