Prevention of Human Prostate Tumor Metastasis in Athymic Mice by Antisense Targeting of Human Angiogenin

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ABSTRACT

Purpose: Angiogenin is a potent positive mediator of neovascularization, a process required for both primary tumor growth and metastasis. In the present study, the effect of a fully phosphorothioated antisense oligodeoxynucleotide, designated JF2S, targeting the AUG translation initiation codon region of human angiogenin, on human prostate tumor development and metastasis in athymic mice was examined.

Experimental Design: JF2S was evaluated for its capacity to affect in vitro synthesis of angiogenin and subsequent tumorigenicity of transiently transfected prostate tumor cells in mice. In vivo treatment experiments were then conducted in which JF2S was used to prevent formation of tumors in an ectopic model and metastasis in an orthotopic model.

Results: Transient transfection of tumor cells with JF2S inhibited both angiogenin gene expression in vitro and tumorigenicity of these transfected cells in athymic mice. In therapy experiments, local treatment with JF2S completely protected mice from developing prostate tumors after s.c. injection of PC-3 human prostate tumor cells (P < 0.0001, survivor analysis). Most importantly, systemic prophylactic administration of JF2S prevented, in 47% of mice, formation of regional iliac lymph node micrometastases arising from primary tumors growing in the more natural orthotopic prostate setting (P = 0.0003, Fisher’s exact test). Furthermore, total protection from regional metastasis occurred in those mice in which JF2S treatment successfully diminished human angiogenin expression in vivo. Tumor-associated angiogenesis was also impaired by JF2S treatment. When therapy was delayed until all of the mice harbored primary tumors in the prostate, the incidence of regional metastasis was still significantly decreased (P < 0.005, survivor analysis).

Conclusions: These findings demonstrate that human prostate cancer establishment and spread in athymic mice is extremely susceptible to targeted disruption of tumor-derived human angiogenin gene expression. Therefore, angiogenin is a valid target against which to devise preventative strategies for prostate cancer metastasis.

INTRODUCTION

Carcinoma of the prostate is now the most frequently diagnosed and second leading cause of cancer deaths among men in the United States (1). Whereas disease localized to the gland can, in many cases, be eradicated by surgery or radiation therapy, the insidious nature of metastatic prostate cancer renders it incurable (2), with present therapies aimed at prolonging survival and maintaining quality of life. Conventional chemotherapy does not significantly affect the growth of metastases because, in part, of the low proliferation rate of androgen-independent metastatic prostate cancer cells (3). For these patients, new treatment strategies are urgently needed. In addition, more sophisticated and rigorous screening methods are resulting in earlier detection of prostate cancer. Therefore, equally critical challenges are to protect men who present with localized prostate cancer from suffering either local or systemic recurrence after initial therapy and to prevent disease progression in men who elect for a course of watchful waiting rather than surgical or radiological interventions.

Tumor-associated angiogenesis is under the control of both positive and negative regulators (4, 5). Because angiogenesis is required for primary and metastatic tumor growth (6), antiangiogenic therapies should be effective in combating carcinoma of the prostate. Indeed, the degree of blood vessel density in primary prostate tumors correlates with their pathological stage (i.e., Gleason score) and the incidence of metastases (7). Angiogenin is a Mr 14,000 protein isolated originally from medium conditioned by human colon adenocarcinoma cells based on its capacity to elicit neovascularization in now classic in vivo assays (8). Subsequently, it was shown to be expressed in a wide range of histologically distinct tumor types (9). In previous work, we demonstrated that angiogenin-neutralizing monoclonal antibodies and the angiogenin-binding protein actin protected up to ~65% of athymic mice from forming tumors after s.c. injection of human colon adenocarcinoma cells (10, 11). In these earlier studies the therapeutic effects observed were shown to most likely occur by reducing vasculature in animals treated with angiogenin antagonists.

In the current study we investigated whether antisense targeting of human angiogenin expression could be useful as an...
alternate means of cancer therapy or prevention. As opposed to the above mentioned studies in which the antitumor effect was achieved by binding of neutralizing antagonists to the angiogenin protein, an antisense approach was taken to determine whether decreasing the synthesis of angiogenin by tumor cells also resulted in antitumor activity. Through one of several possible mechanisms a complementary ODN sequence will interact with the mRNA of a target gene through Watson-Crick base pairing impeding subsequent protein translation (12). Thus, antisense reagents are well suited for target validation and functional genomic studies, and, importantly, they have recently entered the clinic as therapeutics for a variety of indications including cancer (13). We show here that down-regulating the synthesis of human angiogenin by an antisense ODN targeting the AUG translational start site region results in striking inhibition of both establishment and metastatic dissemination of human prostate tumors in mice.

MATERIALS AND METHODS

Mice, Cells, and Supplies. Outbred male athymic mice (Crl: nu/nu) were obtained at 5 weeks of age from the isolator-bred colony of Charles River Laboratories (Wilmington, MA). They were maintained under strict temperature- and humidity-controlled specific pathogen-free conditions and used for experiments when 6–7 weeks of age. All of the procedures were performed in accordance with approved animal protocols following guidelines established by the Harvard Medical Area Standing Committee on Animals. The PC-3 androgen-insensitive human prostate tumor cell line was obtained from the American Type Culture Collection (Manassas, VA). PC-3M cells, a variant of PC-3 selected for their increased metastatic potential (14), were supplied by Dr. Isaiah Fidler (University of Texas M. D. Anderson Cancer Center, Houston, TX). PC-3 cells were propagated in Ham’s F12K medium and 7% fetal bovine serum, whereas PC-3M cells were maintained in Eagle’s MEM containing sodium pyruvate and nonessential amino acids supplemented with 2% MEM Eagle vitamin mixture and 10% fetal bovine serum. Both growth mediums were additionally supplemented with 2 mm L-glutamine, 100 µg/ml gentamicin sulfate, and 0.5 µg/ml fungizone. Cultures were free of Mycoplasma as determined using the Gen-Probe Rapid Detection System (Gen-Probe, San Diego, CA). Lipofectin cationic lipid, OptiMEM, DNase I, and TRizol reagent were from Life Technologies, Inc. (Rockville, MD). Matrigel basement membrane component was obtained from Becton Dickinson (Bedford, MA).

Oligonucleotides. Custom-synthesized fully phosphorothioated ODNs were obtained from either Promega (Madison, WI) or Boston BioSystems (Bedford, MA). Their sequences are as follows: antisense complementary to the AUG translational start site region of human angiogenin (15): 5’-GCC CAT CAC CAT CTC TTC-3’ (JF2S); sense control: 5’-GAA GAG ATG GTG ATG GGC-3’ (JF1S); and scrambled sequence control: 5’-CCT AGC CTC ACT TCT CCA-3’ (JF14S). JF2S is not expected to affect the expression of murine angiogenin because of several base mismatches that occur in the corresponding AUG region of the murine angiogenin sequence (16). [S]ODNs were manufactured according to cGMP specifications.

Fig. 1 Ex vivo transfection of prostate tumor cells inhibits angiogenin secretion in vitro and subsequent tumorigenicity in mice. PC-3 (A1) or PC-3M (B1) cells were treated with either Lipofectin alone or Lipofectin together with 1 µM of antisense JF2S or sense control JF1S as described in “Material and Methods.” The results for sense JF1S- (■) or antisense JF2S- (●) transfected cells are presented relative to those of Lipofectin-treated cells (100%; □). A1 and B1, In Vitro, the amount of angiogenin secreted by [S]ODN-transfected PC-3 (A1) and PC-3M (B1) cells was determined by ELISA and normalized to cell number; the SE of triplicate determinations of these amounts for Lipofectin-, JF1S-, and JF2S-transfected cells was 9, 10, and 7%, respectively, for PC-3 cells and 10, 5, and 2%, respectively, for PC-3M cells. A1 and B1, In Vivo, the above transiently transfected cells were subsequently injected s.c. into athymic mice (five mice per treatment group). The average weight of PC-3 and PC-3M tumors that appeared after injection of these ex vivo-transfected cells are shown (A1 (PC-3); n = 5 for Lipofectin and JF1S treatment groups, n = 1 for the JF2S treatment group; and B1 (PC-3M); n = 5 for Lipofectin and JF1S treatment groups, n = 3 for the JF2S treatment group). The SEs of the average weights of the PC-3 tumors in the Lipofectin and JF15 groups were 26 and 26%, respectively; only one tumor arose in the JF2S group. The SEs for the weights of the PC-3M tumors were 20, 12, and 12% in the Lipofectin, JF1S, and JF2S groups, respectively, A2 and B2, photographs of excised PC-3 (A2) and PC-3M (B2) tumors of which the average weights are reported in A1 and B1 (In Vivo). Tumors resulting from injection of cells treated ex vivo with Lipofectin (top row), Lipofectin plus either sense JF1S (middle row), or antisense JF2S (bottom row) are shown.

3The abbreviations used are: ODN, oligodeoxynucleotide; [S]ODN(s), fully phosphorothioated ODN(s); RT-PCR, reverse-transcription PCR; VPF/VEGF, vascular permeability factor/vascular endothelial cell growth factor.
Prevention of Metastasis by an Antisense [S]ODN protocol, day 0: 400 One hour later treatments with PBS or [S]ODNs were initiated. [S]ODNs were then administered 6×/week for weeks 2 and 3 (50 μg/dose), and 4×/week for weeks 4–7 (50 μg/dose). Mice were examined 2×/week for the presence of palpable tumors. n = 32, 20, 20, and 10 for the PBS (−−), antisense JF2S (−−), sense JF15 (−−), and scrambled JF14S (−−) groups, respectively. Presented are combined data from four separate experiments. After cessation of treatment on day 49 JF2S-treated tumor-free mice were kept for an additional 16 days (experiment 1), 227 days (experiment 2), 90 days (experiment 3), or 21 days (experiment 4) with no evidence of observable tumors at necropsy.

Table 1 Effect of antisense JF2S on blood vessel pattern in orthotopic PC-3M primary tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of mice</th>
<th>Blood vessels</th>
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<tr>
<td></td>
<td></td>
<td>Total(SE)</td>
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<tr>
<td>PBS</td>
<td>10</td>
<td>20.8 (4.2)</td>
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<tr>
<td>JF2S</td>
<td>11</td>
<td>26.5 (4.4)</td>
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Athymic mice were injected orthotopically with PC-3M tumor cells (3.75 × 10⁵) on day 0. Treatment by i.p. injection with JF2S or PBS as diluent control was begun 1 h after cell injection as described in Fig. 3. At sacrifice on day 9 the tumors were removed, fixed, processed, and embedded. Cross-sections of the entire tumor were then stained with an antibody to factor VIII to detect blood vessels as described in “Materials and Methods.”

Average number of blood vessels in a cross-section of the entire tumor.

Average number of blood vessels in the periphery (defined as one high-powered field from the capsule toward the interior of the tumor) of the cross-section divided by the average number of blood vessels in the central area not included in the periphery.

"In Vitro" Cytotoxicity. Direct killing of tumors cells by [S]ODNs was assessed using a [³H]thymidine uptake assay (10).

Transient Transfections in Vitro and Subsequent Tumorigenicity. PC-3 or PC-3M cells were seeded in triplicate into 35-mm diameter tissue culture dishes (2.5 × 10⁵ cells/3 ml growth medium) and incubated overnight. They were then transiently transfected according to protocols supplied with the Lipofectin reagent. Briefly, cells were washed once in HBSS and once with OptiMEM. Solutions (1 ml) of OptiMEM (no treatment), antisense JF2S (0.1, 1.0, or 2.0 μM), or sense control JF15 (1.0 μM) together with Lipofectin (5 μg) or Lipofectin alone in OptiMEM were then added to appropriate dishes in triplicate and incubated overnight. Transfections were repeated the next day. After an additional 24-h incubation supernatants were removed and human angiogenin concentrations were determined by ELISA (17). For normalization, cells were detached with trypsin-EDTA, and cell number was determined with a Model ZF Coulter counter (Coulter, Miami, FL).

For tumorigenicity studies, cells treated as above with 1 μM of antisense JF2S or sense control JF15, or with Lipofectin alone were trypsinized and washed with serum-containing growth medium and HBSS. Ex vivo-treated viable cells (1.25 × 10⁵ or 2.5 × 10⁵ cells/mouse for PC-3 or PC-3M cells, respectively) were then mixed with a 1:2 proportion by volume of Matrigel to cells and injected s.c. into the left shoulder of athymic mice. After 25 or 17 days (for PC-3 or PC-3M-injected cells, respectively) mice were sacrificed and tumors excised, weighed, and photographed.

In Vivo Treatment with Antisense. For ectopic studies, untransfected PC-3 cells in HBSS were mixed with PBS vehicle control or [S]ODNs and immediately injected together with a 1:2 proportion by volume of Matrigel to other components s.c. into the left shoulder of athymic mice (1 × 10⁴ viable cells/mouse; 100 μl). Follow-up local s.c. injections of [S]ODNs were then administered for 49 days. Mice were examined twice/week for the presence of palpable tumors. Four separate experiments were performed, two with [S]ODNs from Promega and two with [S]ODNs from Boston BioSystems (experimental results were indistinguishable between the two sources).

For orthotopic metastasis studies, untransfected PC-3M cells were harvested and injected (3.75 × 10⁶ viable cells in 10 μl of HBSS/mouse) into a surgically exposed prostate lobe of an
athymic mouse as described (18). In one series of experiments, treatment (i.p.) was begun 1 h later with either PBS or [S]ODNs from Boston BioSystems. At sacrifice on days 37–41 primary tumors were excised and weighed and blood was drawn. The two regional iliac lymph nodes from each mouse were removed and preserved in phosphate-buffered formalin. The tissues were subsequently dehydrated, embedded in paraffin, cut into 4-mm sections onto slides, stained with H&E, and examined microscopically in a blinded fashion for the presence of micrometastasis.

In a separate set of metastasis experiments, treatment was initiated either 1 h after tumor cell injection (day 0) or delayed until days 14 or 28. Separate groups of 4–10 untreated mice that received tumor cells on day 0 were sacrificed on days 14 or 28, commensurate with treatment initiation, to determine the average primary tumor sizes at these time points.

Analysis of in Vivo Human Angiogenin mRNA and Protein Levels. Primary PC-3M tumors growing in the prostate were excised, minced, and total RNA and protein were extracted using TRIzol. RNA concentrations were determined by absorbance at 260 nm, and purity was assessed by gel electrophoresis. Total RNA (1 μg) was treated with DNase I, and RT-PCR was immediately performed using the SuperScript One-Step RT-PCR System (Life Technologies, Inc.). Primers specific for human angiogenin were: forward, 5'-GCT GGT GCT GTG TGG CT-3' and reverse, 5'-GCT GTT CTT GGG TCT A-3', resulting in a product of 592 bp. Primers for human β-actin were: forward, 5'-ACA ATG AGC TGC GTG TGG CT-3' and reverse, 5'-TCT CCT TAA TGT CAC GCA CGA-3', resulting in a product of 372 bp. PCR products were electrophoresed on precast agarose gels containing ethidium bromide.

Protein extracts, dialyzed against 0.15 M NaCl before analysis, and mouse serum were analyzed for angiogenin concentrations by ELISA (15) except that the AmpliQ substrate system (Dako Corp., Carpinteria, CA) was substituted for p-nitrophenyl phosphate to increase sensitivity. The ELISA detects human but not murine angiogenin (19).

Quantitation of Tumor Blood Vessel Density. Primary PC-3M tumors were examined for blood vessel content by factor VIII staining as described (11).

Statistics. Estimates of survivor functions, obtained by the Kaplan-Meier product limit method, and Mantel-Cox tests of equality of survivor functions were computed for experiments using s.c. injected PC-3 cells. Survival analysis measures time-to-response, which in these investigations is the time-to-appearance of a palpable tumor. For the orthotopic PC-3M model, Fisher’s exact test (2-tailed) was used to compare differences in the number of mice harboring iliac lymph node metastasis in the separate treatment groups. Differences between the treatment groups in the number of blood vessels in primary tumors as well as the amount of angiogenin protein in primary tumor extracts and mouse sera were analyzed using the Wilcoxon rank-sum test with correction for ties. The Statistical Analysis System version 8.0 (SAS Institute, Cary, NC) was used for all of the data analysis. A P < 0.05 was considered significant for all of the tests.

RESULTS

In Vitro Cytotoxicity. No differences between [3H]thymidine incorporation were seen between untreated- and JF2S-treated (up to 5 μM in concentration) PC-3 or PC-3M cells (data not shown), indicating that the antisense agent does not directly kill tumor cells.

Transient Transfection of Tumor Cells with Antisense JF2S Inhibits the Production of Angiogenin in Vitro and Decreases Subsequent Tumorigenicity in Mice. Before initiating in vivo treatment experiments, the capacity of antisense JF2S to reduce in vitro angiogenin production by PC-3 and PC-3M cells was evaluated. In three separate experiments JF2S, at doses of 0.1, 1.0, and 2.0 μM, in the presence of carrier Lipofectin decreased angiogenin secretion to an average of 58, 22, and 31% for PC-3 cells and 79, 44, and 44% for PC-3M cells, in comparison to the amount secreted by untreated cells.

We then determined whether JF2S-transfected tumor cells would have a decreased capacity to form s.c. tumors in mice. As depicted in Fig. 1, in comparison with control Lipofectin-treated cells, transfection of both PC-3 and PC-3M cells with JF2S, under conditions that inhibited the secretion of angiogenin protein in culture to 35% (Fig. 1, A1, In Vitro) and 52% (Fig. 1, B1, In Vitro), respectively, led to a marked reduction in the average weight of those tumors that appeared [to 42% (Fig. 1, A1, In Vivo) and 44% (Fig. 1, B1, In Vivo), respectively]. More interestingly, whereas tumors developed in all of the mice injected with either Lipofectin (Fig. 1, A2 and B2, top rows) or sense control JF1S (Fig. 1, A2 and B2, middle rows) -treated PC-3 (Fig. 1, A2) or PC-3M (Fig. 1, B2) cells, observable tumors did not form in four of five (PC-3) and two of five (PC-3M) mice injected with JF2S-transfected tumor cells (Fig. 1, A2 and B2, bottom rows). Of some note was that whereas control JF1S transfection also decreased PC-3 and PC-3M angiogenin production in vitro, albeit less dramatically (Fig. 1, A1 and B1, In Vitro), this treatment did not translate into appreciable reductions in tumor size in vivo after injection of the transfected cells (Fig. 1, A1 and B1, In Vivo; and Fig. 1, A2 and B2, middle rows). Furthermore, control [S]ODNs did not exhibit antitumor effects in any of the therapeutic experiments described below.

In Vivo Treatment with Antisense JF2S Completely Prevents the Appearance of PC-3 Tumors in an Ectopic Model. Because the initial experiments described above demonstrated that JF2S tumor cell transfection effectively reduced the amount of angiogenin to a level where subsequent tumorigenicity was impaired, studies of in vivo JF2S administration as a prostate cancer preventative against untransfected tumor cells were begun. PC-3 cells were mixed with PBS, JF2S, or [S]ODNs controls and injected s.c. into athymic mice. To mimic a metastatic event where a few tumor cells localize and begin to grow, the cell dose chosen was the lowest one that reproducibly resulted in tumor growth in 100% of PBS-treated control mice. Local s.c. treatment was continued for 49 days. Strikingly, in four experiments summarized in Fig. 2, administration of JF2S...
completed prevented the formation of PC-3 tumors, whereas tumors appeared in all of the mice after treatment with sense JF1S, scrambled JF14S control [S]ODNs, or PBS as diluent control (P < 0.0001 for treatment with JF2S in comparison with either of these three controls). Furthermore, tumors did not arise, up to 33 weeks after therapy in the case of experiment 2, in any of the JF2S-treated mice after treatments were ceased. However, JF2S did not affect the growth of PC-3 cells when treatment was delayed until after tumors had become palpable (data not shown).

Prophylactic Treatment with Antisense JF2S Interferes with the Formation of Regional Metastasis in an Orthotopic Model. To investigate whether JF2S delivered systemically can inhibit the development of spontaneous metastasis, we used an orthotopic model of prostate cancer in which a primary tumor serves as a reservoir to constantly release tumor cells. Metastatic PC-3M cells were injected into one lobe of a surgically exposed prostate gland followed by i.p. treatment with PBS, JF2S, sense control JF1S, or scrambled control JF14S beginning on day 0. At the time of sacrifice, the average weight of all of the primary tumors within an individual experiment was approximately the same regardless of treatment regimen. This was not surprising, because this model was designed to produce a very aggressive primary tumor that grows quickly and yields metastases in a relatively short time. In the three separate experiments performed, all of the mice treated with PBS developed micrometastases in at least one iliac lymph node as did mice treated with control sense and scrambled [S]ODNs (Fig. 3). In contrast, 47% of the mice treated with JF2S were protected from development of iliac lymph node metastases (range: 36–55% protection; P = 0.0003, 0.00005, and 0.0011 in comparison with treatment with PBS, JF1S, and JF14S, respectively). Reducing the dose of JF2S used in Fig. 3 to daily doses of 50 μg resulted in only 20% of the mice being protected from metastases (n = 30; P = 0.033 in comparison with the first dose regimen). Increasing the daily dose of JF2S to 500 μg failed to improve efficacy (38% of mice protected; n = 16; P = 0.758 in comparison with the first dose regimen).

Effect of Antisense JF2S Treatment on Target Angiogenin Levels in Vivo. As an indicator of whether angiogenin levels were affected in vivo by administration of JF2S, representative PC-3M primary tumors were excised at the termination of one of the experiments described in Fig. 3, and total RNA and protein were extracted. RT-PCR analysis indicated that the expression of human angiogenin was indeed depressed in tumors in which regional metastases were absent in contrast to tumors derived from PBS-treated, metastasis-harboring controls (Fig. 4A). Interestingly, angiogenin mRNA levels from tumors derived from JF2S-treated mice in which metastasis developed remained near those of controls. Human angiogenin protein concentrations in tumor extracts showed a similar pattern (Fig. 4B). Whereas the amount of angiogenin per gram of tissue extracted from primary tumors of either PBS-treated mice or those mice treated with JF2S in which metastasis was detected was essentially the same [73 ± 18 (SE) versus 80 ± 24 pg/g of tissue, respectively; P = 0.448], the amount of angiogenin obtained from tumors of mice successfully protected from metastasis by JF2S was significantly less than either of the two former groups (22 ± 1 pg/g of tissue; P = 0.0017 and 0.0055, respectively). Serum samples collected at sacrifice were also examined for human angiogenin concentrations by ELISA (Fig. 4C). Once again, whereas angiogenin concentrations in the sera of PBS-treated mice or those mice with metastasis that were treated with JF2S were not significantly different (339 ± 30 versus 308 ± 62 pg/ml, respectively; P = 0.656), the level of angiogenin in sera of metastasis-free mice given JF2S was 6-fold less (51 ± 19 pg/ml) than that found in either the PBS control (P = 0.00003) or JF2S-treated mice with metastasis (P = 0.00099).

Effects of Antisense JF2S on Vasculature. Primary orthotopic PC-3M tumors were excised on either day 9 or day 41 after tumor cell injection and treatment initiation on day 0 using the protocol detailed in Fig. 3 and stained with an antibody to factor VIII. The tumors taken from mice sacrificed on day 9 showed no significant difference as determined by the Wilcoxon rank-sum test in the total number of blood vessels between PBS- and JF2S-treated mice (Table 1). Interestingly, however, there was a significant difference between the ratios of the number of vessels in the peripheral area near the capsule to the number in the central region of the tumor for the control versus antisense treatment groups (P = 0.0447; approximately one-half the
number of central vessels present in PBS control tumors were observed in tumors excised from JF2S-treated mice (data not shown). Within the JF2S treatment group sacrificed on day 9 were, of course, those mice in which JF2S treatment would eventually prevent metastasis (on average, 47%) and those mice in which metastasis would still occur. Unfortunately, we were not able to differentiate between these two groups, because no metastasis was evident in any of the iliac lymph nodes at this early time.

In the larger tumors from the mice sacrificed on day 41, there was once again no significant difference in the total number of blood vessels between control PBS- and JF2S-treated mice (with or without iliac lymph node metastasis; data not shown). However, a comparison of the distribution of vessels in these tumors, as was performed for the smaller tumors above, was not possible because of the large amount of central necrosis.

Delaying Antisense JF2S Treatment until Primary Tumors Are Established also Significantly Interferes with Metastasis Formation. To determine whether the antimetastatic effect of JF2S could still be observed in mice with pre-established primary tumors, treatments were delayed until days 14 or 28 after PC-3M cell injection. Previous time course experiments had shown that in the orthotypic model all of the mice injected with 3.75 × 10⁶ viable cells harbored macroscopically observable prostate tumors on necropsy by day 14. On the day that delayed treatments were begun, a separate group of 4–10 untreated mice that had also been injected with cells on day 0 were sacrificed so that the average primary tumor size could be determined for that time point within an individual experiment. Additionally, in each experiment, a group of mice treated with JF2S or PBS beginning on day 0 was included. Untreated mice injected with tumor cells on day 0 and sacrificed on day 14 or day 28 harbored, on average, primary tumors weighing 46 and 167 mg, respectively. In these sets of mice there were no significant differences at each of these time points between the four experiments in terms of primary tumor size, thereby allowing the data to be pooled from four separate experiments. As shown in Fig. 5, 97% of PBS-treated mice contained metastatic iliac lymph nodes on days 37–41. Treatment on day 0 protected 39% of the mice from developing metastasis (P = 0.00004), whereas delaying treatment until day 14 or day 28 still resulted in significant protection in 34% (P = 0.0006) and 26% (P = 0.0041) of the mice, respectively. In fact, there was no significant difference in efficacy between treatment on day 14 or day 28 (P = 0.462) nor between treatment on day 0 and either of the delayed time points (P = 0.658 and 0.185, respectively).

**DISCUSSION**

The intractability of advanced, disseminated prostate cancer demands that new paradigms be developed for therapy and, ideally, prevention of this disease. As one such approach, antiangiogenesis therapies are now under critical examination for the clinical treatment of cancer (21), including that of the prostate (22, 23), and instances of successful outcomes have been reported (24, 25). The promise of such an approach is great. Importantly, antiangiogenesis agents in general may not cause toxicity nor induce resistance (26), properties typical of present-day chemotherapeutic drugs.

Herein, we demonstrate that interference with the tumor-associated expression of angiogenin can result in profound antiprostate cancer activity in mice, specifically as it relates to metastatic dissemination, without observable side effects. This was determined with the use of an antisense [S]ODN, JF2S, which was designed to recognize the AUG translational start site region of angiogenin. In initial experiments, JF2S treatment decreased the secretion of angiogenin by both PC-3 and PC-3M cells growing in culture. Tumorigenicity of these JF2S *ex vivo* treated cells was greatly impaired after their injection into athymic mice.

In subsequent *in vivo* treatment experiments, local administration of JF2S completely protected mice injected s.c. with untransfected PC-3 tumor cells from forming tumors. Most importantly, in an orthotypic model of human prostate cancer using the aggressive PC-3 variant cell line, the incidence of regional micrometastasis was significantly reduced by prophylactic, systemic administration of the antisense drug. A dramatic antimetastatic effect was also evident when treatment was delayed until primary tumors were already present in the prostate; this capacity of JF2S to prevent metastasis even in the presence of an actively growing primary tumor augurs well for the potential use of angiogenin antagonization in adjuvant therapy for human prostate cancer. The antimetastatic activity of JF2S did not result from merely a decreased primary tumor burden in antisense-treated mice in comparison with those treated with PBS, because: (a) as mentioned above, the average weight of the primary tumors in all of the treatment groups was essentially equal; and (b) within each experiment, for every JF2S-treated mouse that was free of regional metastasis a matched, in terms of primary tumor weight, PBS-treated mouse harboring lymph node metastases could be found. In addition, antimetastatic activity observed in the orthotypic model directly correlated with diminution of *in vivo* tumor-derived angiogenin. It then

![Fig. 5](https://clinicalcancerres.aacrjournals.org) Prevention of PC-3M prostate tumor metastasis by treatment with antisense JF2S after establishment of primary tumors. Tumor cells were injected and iliac lymph nodes harvested and examined as for Fig. 3. For the four separate experiments summarized, treatment with JF2S was begun on day 0 (▪; n = 46), on day 14 (▴; n = 38), or on day 28 (▲; n = 43). JF2S was administered as in Fig. 3 with the exception that in the latter two groups treatment initiation was delayed until 2 and 4 weeks, respectively, after initial tumor cell injection into the prostate. In each experiment additional mice were treated with PBS beginning on day 0 (□; n = 38).

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follows that if tumor-associated angiogenin levels can be sufficiently suppressed or its activities sufficiently inhibited, substantial antitumor effects should be realized. Interestingly, metastasis can be inhibited dramatically without completely silencing the expression of angiogenin, an observation also described in tumor studies where other proangiogenic factors were targeted (27, 28).

JF2S exerts its antitumor effects by inhibiting the expression of human angiogenin mainly through a sequence-specific antisense mechanism of action, as shown in Figs. 2 and 3 in which the administration of two control [S]ODNs did not inhibit either s.c. tumor establishment or formation of metastasis. Additional support for this sequence-specific mechanism includes the observations that JF2S did not inhibit expression by PC-3 and PC-3M cells of the β-actin housekeeping gene by Northern analysis (data not shown) and that sequences such as G quartets, palindromes, and 5′-CpG-3′, known to cause nonantisense biological effects in vivo (29), are not present in any of the [S]ODNs used.

Because direct tumor cell killing by JF2S was not demonstrated, it is most probable that the antiprostate tumor effects observed in response to treatment with JF2S are achieved through decreased expression of tumor-associated angiogenin with concomitant interference with the angiogenic process. In the ectopic model, the decrease in tumor-secreted angiogenin may, for example, result in the formation of avascular tumor masses, which could then be eliminated by host immune defenses, including natural killer cells known to exist in athymic mice (30). This effect on tumor growth must occur at an early stage of tumor establishment and growth, because in this model treatment of established tumors had no effect on their rate of growth. In the orthotopic model, we demonstrated that JF2S was indeed capable of reducing the de novo production of central blood vessels in primary tumors, which could thereby reduce hematogenous dissemination of tumor cells to lymph nodes. From this it can be postulated that JF2S treatment did not affect tumor cell co-option of preexisting vasculature, which could occur mainly in the tumor periphery near the capsule (e.g., Ref. 31), but instead reduced the development of new blood vessels, true angiogenesis, in the tumor interior. Thus, the available data indicate that one way in which JF2S may affect metastatic dissemination is by reduction of early angiogenesis within the primary tumor site. Unknown at present is whether the decrease in angiogenin levels could in addition interfere with dissemination to regional lymph nodes via lymphatic vessels.

Ectopic tumor models, used as the traditional in vivo method of assessing cancer therapies (32), are relatively easy to perform and can be informative but suffer from the fact that tumors are challenged to grow in unnatural environments, usually s.c., and rarely give rise to metastasis (33). Whereas at present no model currently available reproduces all of the aspects characteristic of human prostate cancer, i.e., slow doubling time, initial androgen dependence with eventual progression to androgen independence, prostate-specific antigen production, and metastasis to lymph nodes and bone (34), the orthotopic model used here simulates a natural course of prostate cancer in which an androgen-insensitive primary tumor growing in the prostate subsequently disseminates to the regional lymph nodes, a major site of metastatic spread in prostate cancer patients (35).

The ability of these cells to metastasize can be attributed, at least in part, to permissive interactions between tumor cells and their natural prostatic tissue milieu (36). Importantly, evaluating cancer therapies or preventative strategies in this setting rather than solely in an ectopic environment may well be more predictive of ultimate clinical outcome (37, 38).

The control of both normal and neoplastic prostate development is influenced by numerous regulatory proteins, including those that participate in angiogenesis (39, 40). Cells of the PC-3 lineage, for example, produce multiple proangiogenic factors, including angiogenin, as we have shown, as well as VPF/VEGF, basic fibroblast growth factor, and interleukin 8 (41, 42). Therefore, it may be somewhat surprising that targeting just one of these, namely angiogenin, results in such dramatic effects on prostate tumor cell establishment and metastatic spread. However, although intense study has been devoted to prostate physiology and pathology in recent years, the contribution of growth factors, in general, and angiogenic factors, in particular, to these processes have not been defined satisfactorily. For example, the prototypic angiogenic factor VPF/VEGF, whereas present in clinical prostate cancer specimens (43), has not been shown to necessarily correlate with invasion, metastasis, or aggressiveness of prostate cancer (43, 44). Additionally, in one study the concentrations of VPF/VEGF or basic fibroblast growth factor did not correlate with the increased levels of endothelial cell stimulatory activity detected in urine samples from prostate cancer patients (45). These observations taken together imply other angiogenic mediators, which may include angiogenin, as potentially more relevant to the angiogenic phenotype of carcinoma of the prostate. Although studies similar to those cited above have not been undertaken for angiogenin as yet, the data presented in this report suggest that angiogenin may indeed contribute to the phenotype of clinical prostate cancer. If so, antagonists of angiogenin, including antisense [S]ODNs as described here, should be useful for preventing the establishment and progression of prostate cancer in patients.

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