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Basic fibroblast growth factor antisense oligonucleotides inhibit renal cell carcinoma cell growth and angiogenesis

Research Article

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Summary

Renal cell carcinoma (RCC) is the most common malignancy of the kidney. A characteristic feature of RCC is evidence of abundant angiogenesis and abnormal blood vessel development. Basic fibroblast growth factor (bFGF) is a known contributor in the regulation of RCC initiated angiogenesis. In the present studies we evaluated the effects of blocking bFGF production by antisense phosphorothioate oligodeoxynucleotides (PS-ODNs) on the growth and angiogenic activity of a pre-clinical model of RCC (Caki-1). *In vitro* studies showed that treating Caki-1 cells with antisense PS-ODNs directed against bFGF mRNA led to a reduction in the levels of bFGF expression sufficient to impair the proliferation and migration of endothelial cells. In addition, such treatments exerted a direct effect on Caki-1 cell growth. The observed effects were antisense sequence specific, dose dependent, and could be achieved at a low, non-toxic concentration of PS-ODNs. When bFGF antisense treated Caki-1 cells were injected into nude mice and evaluated for their angiogenesis potential in an intradermal angiogenesis assay, the number of vessels initiated were approximately half that initiated by untreated Caki-1 cells. To test the antitumor effect of bFGF antisense, PS-ODNs were administrated to nude mice bearing macroscopic Caki-1 xenografts. The results showed that the systemic administration of two doses of bFGF antisense PS-ODNs given 1 and 4 days after the tumors reached a size of ~200 mm^3 doubled the time required for tumors to grow to 5 times the size at the start of treatment.

I. Introduction

Renal cell carcinoma (RCC) is the most common malignancy of the kidney and accounts for about 2% of all adult malignancies (McLaughlin and Lipworth, 2000). Unless discovered at an early stage, at a time when it is a still a resectable neoplasm, RCC has a very unfavorable treatment outcome to conventional measures. Unfortunately, RCC is characterized by a lack of early warning signs resulting in a high proportion of patients with metastases at diagnosis and significant relapse rates following nephrectomy. As a consequence RCC remains fatal in nearly 80% of its patients (Tsui et al, 2000).

Histopathologic evaluations of RCC reveal it to be a highly vascularized neoplasm demonstrating clear evidence of abundant angiogenesis and abnormal blood vessel development (Yoshimura et al, 1996). Not surprisingly, several studies have pointed to an important role for pro-angiogenic growth factors in RCC. Basic fibroblast growth factor (bFGF) has often been implicated. This factor has been shown to be expressed in renal cell carcinoma tissues and renal cell carcinoma cell lines (Mydlo et al, 1988; Gospodarowicz et al, 1986; Mydlo et al, 1993). Serum levels of bFGF often are elevated in RCC patients (Fujimoto et al, 1991) and renal cell carcinoma bFGF mRNA levels have been reported to be 2 - 3 fold higher than those found in surrounding normal tissues (Eguchi et al, 1992). In addition, elevated serum/urine bFGF levels have been shown to be associated with malignant progression and poor treatment outcome (Nanus et al, 1993; Nguyen et al, 1994; Duensing et al, 1995; Miyake et al, 1996; Yoshimura et al, 1996). Taken together, these findings strongly suggest an important role for bFGF in renal cell carcinoma associated angiogenesis.

Currently, there is considerable interest in developing angio-suppressive therapies for RCC. For example, interferon- , a peptide known to have anti-angiogenic effects likely due to suppression of bFGF expression (Singh et al, 1995), has been shown to prolong survival in patients with RCC(1999). Interleukin-12, a cytokine with immuno-regulatory and anti-angiogenic activity (Voest et

al, 1995), also has demonstrated antitumor activity in RCC (Motzer et al, 1998). Other drugs developed principally as angiogenesis inhibitors and studied in RCC include the fumigillin analog TNP-470, thalidomide, and a monoclonal antibody to VEGF (Gordon et al, 1998; Stadler et al, 1999).

In the present investigations, antisense phosphorothioate oligodeoxynucleotides (PS-ODNs) complementary to bFGF mRNA were designed and tested for their efficacy to block RCC angiogenesis and growth in vitro and in vivo. The therapeutic potential of bFGF antisense treatments in RCC xenografts also was evaluated.

II. Materials and methods

A. Cell culture

The clear cell RCC cell line Caki-1 was a gift from Dr. Susan Knox (Stanford University). Caki-1 cells were grown in Dulbecco's modified minimum essential medium (DMEM, Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY), 1% penicillin-streptomycin (Invitrogen, Grand Island, NY) and 1% 200 mmol/L L-glutamine (Invitrogen, Grand Island, NY). The mouse heart endothelial cell line (MHE) was a gift from Dr. Robert Auerbach (University of Wisconsin). MHE cells were grown in Dulbecco's modified minimum essential medium supplemented with 10% heat inactivated fetal bovine serum, 1% penicillin-streptomycin and 1% 200 mmol/L L-glutamine. Human microvascular endothelial cells of the lung (HMVEC-L) were obtained from Clonetics (San Diego, CA). HMVEC-L cells were grown in EBM-2-MV (Clonetics, San Diego, CA) supplemented with 5% FBS.

Phosphorothioate Oligodeoxynucleotides (PS-ODNs): Antisense and control PS-ODNs (20-mers) were custom synthesized by Gemini Biotech (Alachua, FL). PS-ODNs B460 was complementary to the translation start site (AUG codon) of bFGF mRNA: 5´ TCC CGG CTG CCA TGG TCC CT 3´; PS-ODNs B471 was complimentary to the coding region of bFGF mRNA: 5´ CGT GGT GAT GCT CCC GGC TG 3´; PS-ODNs B931 was complimentary to the 3´ UTR: 5´ GAT GTG GCC ATT AAA ATC AG 3´ A random nonsense sequence: 5´ GCC TGG ACC CTG GCT CTC TC 3'; sense sequence: 5´ AGG GAT GGC TGC CGG GA 3´ and an inverted sequence: 5´ TCC CTG GTA CCG TCG GCC CT 3´, were used as PS-ODNs controls. In the tumor distribution studies, the PS-ODNs were labeled at the 5´ end with FITC. All PS-ODNs were suspended in sterile and endotoxin free water at a concentration of 1 mM, aliquoted and stored at -20ÆC.

B. DOTAP: DOPE liposomes

Cationic liposomes were prepared using the method described by Tang (Tang and Hughes, 1999). Briefly, cationic lipid 1,2-dioleoyloxy-3-(trimethylammonium) propane (DOTAP) was dissolved in chloroform and mixed with a helper lipid 1,2-dioleoyl-3-sn-phosphatidylethanolamine (DOPE) at a molar ratio of 1:1 (Avanti Polar-Lipids, Alabaster, Al). The mixture was evaporated to dryness in a round-bottomed flask using a rotary evaporator at room temperature. The resulting lipid film was dried by nitrogen for an additional 10 min to evaporate any residual chloroform. The lipid film was re-suspended in sterile water to a final concentration of 1 mg/ml based on the weight of cationic lipid. The resultant mixtures were shaken in a water bath at 35°C for 30 min. The suspensions then were sonicated using a Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) for 1 min at room temperature to form homogenized liposomes. The particle-size distribution of liposomes was measured using a NICOMP 380 ZLS instrument (Santa Barbara, CA). The average diameter was 144.0 ± 77.0 nm. Liposomes were stored at 4°C and used within 3 months.

C. Enzyme immunoassay of bFGF

Caki-1 cells ($1x10^5$) were set in 60 mm dishes and allowed to attach overnight. The medium then was removed and replaced with PS-ODNs in serum free medium with liposome (DOTAP:DOPE) and incubate for 5 hr. Fresh medium containing 10% FBS then was added. Caki-1 cells were collected on day 2, washed and suspended $1x10^6$ in PBS containing protease inhibitors (100 µg/ml Phenylmethanesulphonyl fluoride, 20 µg/ml leupeptin, 3 µg/ml aprotinin). The suspension was subjected to 3 freeze-thaw cycles, ultrasonication for 5 s (100 W) on ice, and centrifugation at 14,000 g for 10 min. The supernatant containing the intracellular bFGF was used for the bFGF concentration determination (human bFGF immunoassay kit, R & D Systems, Minneapolis, MN).

C. bFGF Relative quantitative RT-PCR

Caki-1 cells were set at $3x10^5$ in 100 mm dishes and allowed to attach overnight. The cells were then treated with bFGF antisense or control PS-ODNs as described. 24 hr later the cells were collected and the total RNA was isolated using a RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA concentrations were determined by UV spectrophotometry. A 2 µg total RNA sample was used to reverse synthesize cDNA using Superscript II reverse transcriptase (Invtrogen, Grand Island, NY). A 2.5 µl aliquot of the reverse transcriptase reaction product then was used for the PCR reaction. BFGF PCR reactions were carried out using a forward primer and a reverse primer with a relative RT-PCR Kit (Ambion, Austin, TX). The PCR reactions were run for 22 cycles (denature 94°C 30s, anneal 60°C 60s, extension 72°C 60s) in a DNA Engine 200 (MJ research, Waltham, MA). PCR products then were run on 2% agarose gels and stained by ethidium bromide. The gels were visualized and analyzed using a Gel Doc 2000 gel documentation system (Bio-Rad, Hercules, CA).

D. FGFR1-4 RT-PCR

Caki-1 cell total RNA was isolated using an RNeasy Mini Kit (Qiagen, Valencia, CA) and RNA concentrations were determined by UV spectrophotometry. A 2.5 µl aliquot of the reverse transcriptase reaction product then was used for the PCR reaction. Primers for human FGFR 1-4 were used (Tartaglia et al, 2001). The PCR reactions were run for 30 cycles (denature 94°C 30 s, anneal 60°C 60 s, extension 72°C 60s) in a DNA Engine 200 (MJ research, Waltham, MA). The specificities of the cDNA amplifications were then verified by endonuclease restriction analyses. All PCR preparations were carried out in a laminar flow hood using aerosol resistant plugged pipette tips. Negative controls without template DNA were included in each assay. An 18S primer set (Ambion, Austin, TX) was used as a positive control.

E. Cell cycle analysis

Caki-1 cells were plated in 60 mm dishes ($2x10^5$ cells/dish) and allowed to attach overnight. The cells were then treated with 1 µM B460 or control PS-ODNs complexed with DOTAP:DOPE as described above. 48 hr later the cells were trypsinized,

counted, and fixed in 50% ethanol overnight. Prior to FACS analysis the cells were treated with 1 mg/ml RNase (in PBS) for 30 min. The samples were then washed with PBS twice and resuspended in 25 mg/ml propidium iodine (PI) in PBS at a volume of $1x10^6$ cells/ml. The cells were stained in the dark with PI (15 min) and their cell cycle distributions were analyzed using a Beckman Dickinson flow cytometer (University of Florida Flow Cytometry Core Facility).

F. Apoptosis measurement

Caki-1 cells were set in 2-well chamber slides and treated with 1 µM B460 or control PS-ODNs as described earlier. 48 hr later, the cells were fixed in 4% para-formaldehyde solution for TdT-mediated dUTP Nick-End labeling (TUNEL) assay. Briefly, the cells were permeabilized in 0.2% Triton X-100 solution, (5 min), DNA strand breaks were labeled with Fluorescein-12-dUTP in TdT incubation buffer (37°C for 1 hr), and counterstained with 1 mg/ml PI. Localized green fluorescence of apoptotic cells (Fluorescein-12-dUTP) in a red background (PI) was detected by fluorescence microscopy. The percentage of apoptotic cells was determined by dividing the number of green fluorescent cells by the total number of cells examined. A minimum of 300 cells was counted for each condition.

G. Co-culture conditions

Transwell 6-well dishes (Corning, Corning, NY) with a membrane pore size of 0.4 µM were used. Caki-1 cells were seeded at $5x10^4$ in the transwell inserts. After allowing the cells to attach overnight, the Caki-1 cell medium was replaced with serum free medium containing 1 µM B460 PS-ODNs or control PS-ODNs complexed with liposome (DOTAP:DOPE). 5 hr later, medium containing 10% heat inactivated FBS was added to yield a final FBS concentration of 2.5%. The transwells containing treated Caki-1 cells were inserted into the 6-well dishes containing MHE or HMVEC-L cells ($5x10^4$) and incubated at 37°C for 48 hr. The number of endothelial cells then was determined by hemocytometer count.

H. Endothelial cell migration

Caki-1 cells were set at $1x10^5$ per well in 24-well dishes and allowed to attach overnight. The Caki-1 cells then were treated with 1 µM control or B460 PS-ODNs for 24 hr. HTS FluoroBlok inserts (Becton Dickinson, Franklin Lakes, NJ) with a pore size of 8.0 µm were assembled into the 24-well dish with the Caki-1 cells. MHE or HMVEC-L cells ($5x10^4$) were plated into the FluoroBlok inserts. These endothelial cells had been previously stained in medium containing 10 µg/ml Di-I (Molecular Probes, Eugene, OR) for 24 hr and washed 4 times with PBS. After a 24 hr incubation period, the number of migrated endothelial cells was determined by direct measurement of the fluorescence in the bottom well using a CytoFluor 4000 plate reader (Perceptive BioSystems, St. Paul, MN).

I. Tumor cell-induced angiogenesis

Caki-1 cells ($5x10^4$) were inoculated (10 µl) intradermally at 4 sites in the ventral surface of mice. Three days later, the mice were killed, the skin carefully separated from the underlying muscle and the number of vessels entering the scoring area was counted under a dissecting microscope (Sidky and Auerbach, 1976).

J. Caki-1 xenografts

Female nude mice (NCR, nu/nu), age 8 - 10 weeks were maintained under specific-pathogen-free conditions (University of Florida Health Science Center) with food and water supplied *ad libitum*. Animals were inoculated subcutaneously in a single flank with $5x10^6$ tumor cells. When the tumors reached a size of ~200 mm^3, animals were randomly assigned to the different treatment groups.

K. bFGF western blot preparation and analysis

bFGF antisense PS-ODNs B460 were injected via tail vein at a dose of 10 mg/kg. At various times after injection (24, 48 and 72 hr), the mice were killed, the tumors excised and frozen in liquid nitrogen. The tumors were then homogenized (Dounce tissue grinder, Wheaton, Millville, NJ) and the homogenates were lysed on ice for 30 min with 1 ml of hypotonic buffer (20 mm Tris-HCl, pH 6.8, 1 mm $MgCl_2$, 2 mm EGTA, 0.5% Nonidet P-40, 2 µM Phenylmethanesulphonyl fluoride (PMSF), 200 U/ml Approtinin, 2 µg/ml leupetin) (Giannakakou et al, 1998) per 0.1 g tissue. Following a brief but vigorous vortex the samples were centrifuged at 14,000 rpm for 10 min at 4_C. A 30 µl aliquot of each sample was mixed with 10 µl 4x SDS-PAGE sample buffer (0.3 M Tris-HCl, pH 6.8, 45% glycerol, 20% -mercaptoethanol, 9.2% SDS and 0.04 g/100ml bromophenol blue) and heated at 100_C for 10 min. 30 µl of each sample was then analyzed by SDS-PAGE on a 12% separating gel and 3% stacking gel. Following transfer, the membrane was immunoblotted using a bFGF primary antibody (Upstate Biotechnology, Lake Placid, NY) 1:1000 diluted in antibody solution (3% dry milk, 25 mm Tris, pH 7.5, 0.5 M NaCl, 0.05% Tween 20) overnight at 4°C. After washing, a secondary antibody labeled with horseradish peroxidase was applied and incubated at room temperature for 1 hr. Protein bands were visualized and densitometry was performed.

L. Tumor response assessments

Once the Caki-1 xenografts reached a size of ~200 mm^3, animals were assigned randomly to various treatment groups. B460 or control PS-ODNs were administrated via the tail vein with DOTAP:DOPE liposomes at a dose of 5 mg/kg or 10 mg/kg 1 and 4 days later. Tumors were measured using calipers and volumes were approximated by the formula, volume=$1/6(ab^2)$, with a and b represent two perpendicular tumor diameters. The times for the tumors in the various treatment groups to grow from 200 to 1000 mm^3 were recorded and compared.

III. Results

Caki-1 cell bFGF levels were significantly reduced from a normal of 720 pg/10^6 cells after treatment with 1 µM antisense PS-ODNs (**Figure 1**). This effect was sequence and target region specific. The antisense PS-ODNs complimentary to the start codon (AUG) region (B460) was found to be the most effective. For example, the cellular bFGF levels of B460 treated Caki-1 cells were found to be about 41% of those found in control or untreated cells ($p<0.05$). In comparison, the antisense PS-ODNs complimentary to the 3´ UTR (B931) or coding region (B471) were less effective at down regulating bFGF expression (57% and 65% of control respectively, $p<0.05$).

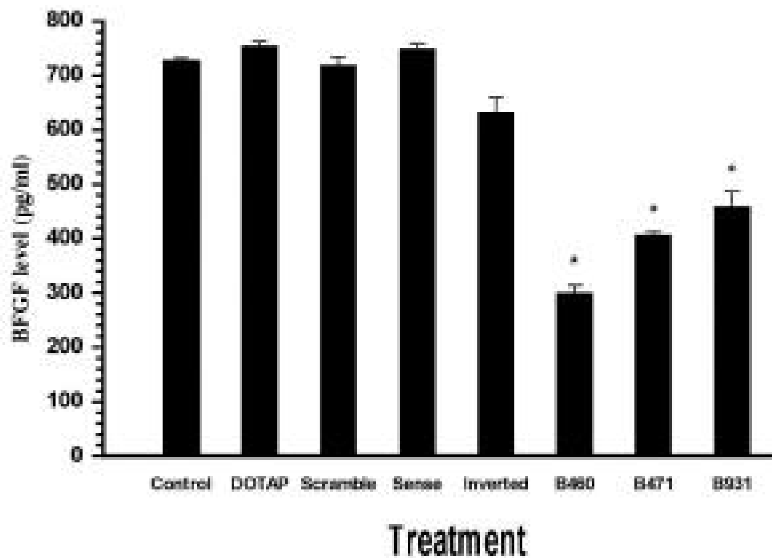


Figure 1. Cellular bFGF levels in Caki-1 tumor cells treated with different antisense PS-ODNs. The cells were either untreated (Control), liposome vehicle treated (DOTAP), or treated with a 1 μM dose of control PS-ODNs antisense sequences (Scramble, Sense, Inverted) or a 1 μM dose of PS-ODNs antisense sequences targeted to different regions of bFGF mRNA. Each bar represents the mean ± S.E. of at least 3 different experiments. Stars indicate significant differences ($p<0.05$) from the untreated control group.

Treating Caki-1 cells with control scramble PS-ODNs or liposome vehicles did not affect bFGF levels in Caki-1 cells. Similarly, treatment with sense or inverted sequence PS-ODNs failed to reduce bFGF expression. Because B460 treatment led to the greatest inhibition of bFGF expression, this PS-ODN was used in all subsequent investigations.

The results of **Figure 2** illustrate that the inhibitory effect of B460 was clearly dose dependent with doses as low as 0.5 μM leading to significant reductions in the cellular bFGF levels. When higher doses of B460 were applied, bFGF levels could be suppressed to 20% of control values. Levels of bFGF mRNA in Caki-1 cells treated with PS-ODNs also were determined (**Figure 3**). The results indicated a marked inhibition of bFGF mRNA after treatment with B460 that was absent in cells treated with scramble PS-ODNs. Because bFGF can have mitogenic effects on renal cells (Gospodarowicz et al, 1986; Issandou and Darbon, 1991), the influence of antisense and control PS-ODNs treatment on Caki-1 cell growth was investigated. Control PS-ODNs or liposome vehicles showed no effect on Caki-1 cell growth (**Figure 4**). However, Caki-1 cell growth was inhibited by PS-ODNs targeted against different regions of bFGF mRNA. B460 was found to be the most effective while antisense PS-ODNs targeting the 3´UTR (B931) or coding region (B471) showed less cell growth inhibition. When comparing these data to those illustrated in **Figure 1,** it is readily apparent that the extent of Caki-1 cell growth inhibition by different antisense PS-ODNs is closely related to their potency in down regulating bFGF expression.

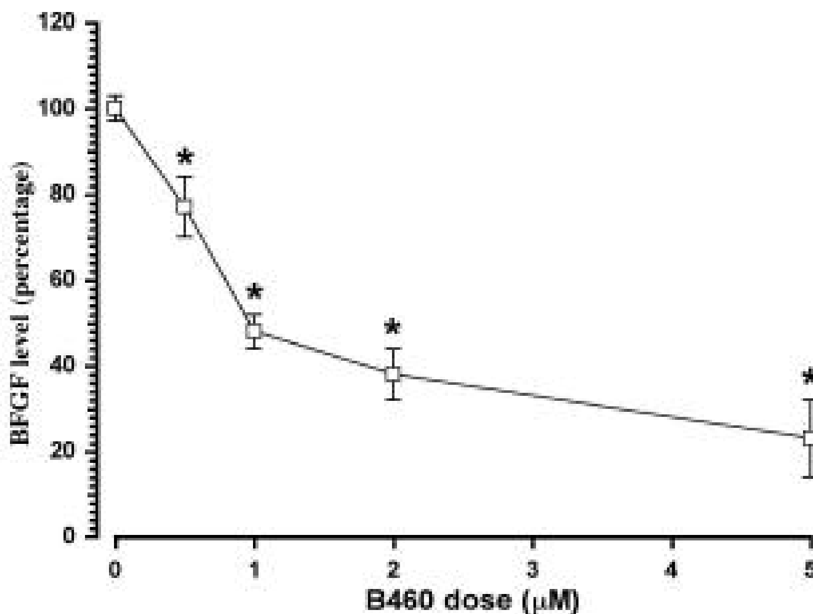
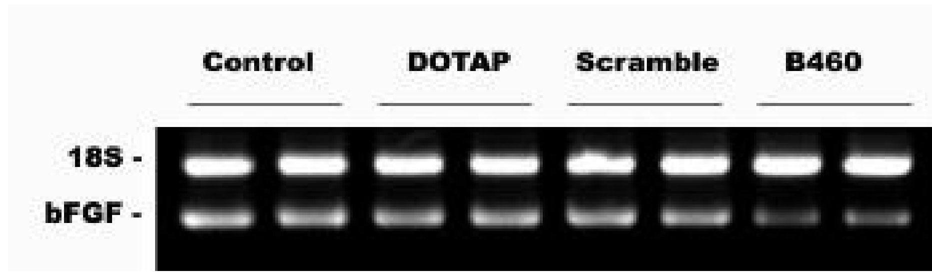


Figure 2. Effect of different doses of bFGF antisense PS-ODNs (B460) on Caki-1 cell bFGF expression level. The 0 μM dose corresponds to cells treated with scramble control oligomers. The bFGF levels were determined after 3-day treatment; each datum point represents the mean ± S.E. of 3 independent experiments. Stars indicate significant differences ($p<0.05$) from untreated cells.



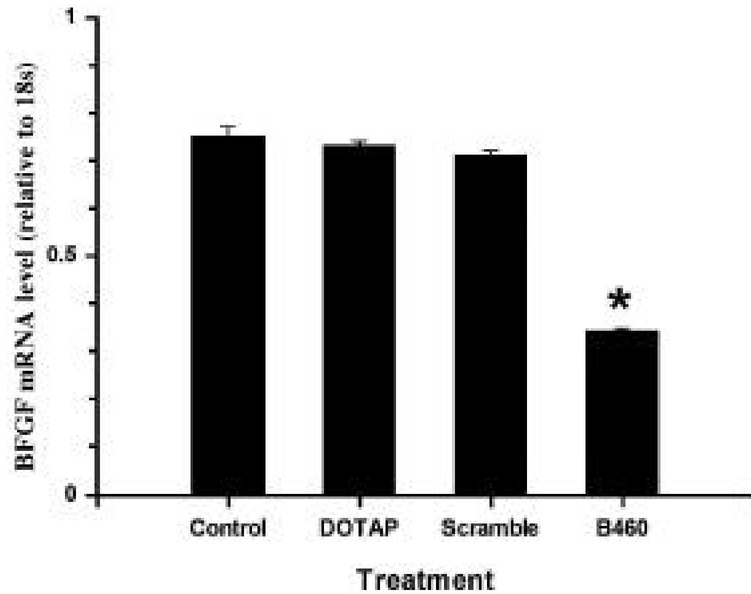


Figure 3. Message RNA levels in Caki-1 cells which were either untreated (Control), liposome vehicle treated (DOTAP) or treated with a 1 μM dose of control PS-ODNs antisense sequence (Scramble) or bFGF antisense PS-ODNs (B460). **A**. Representative relative RT-PCR results, each group was performed in duplicate; **B** Relative bFGF mRNA levels of Caki-1 cells treated with different PS-ODNs. Each bar shows the mean ± S.E. of 3 independent experiments. The star indicates a significant difference (p<0.05) from the untreated control group.

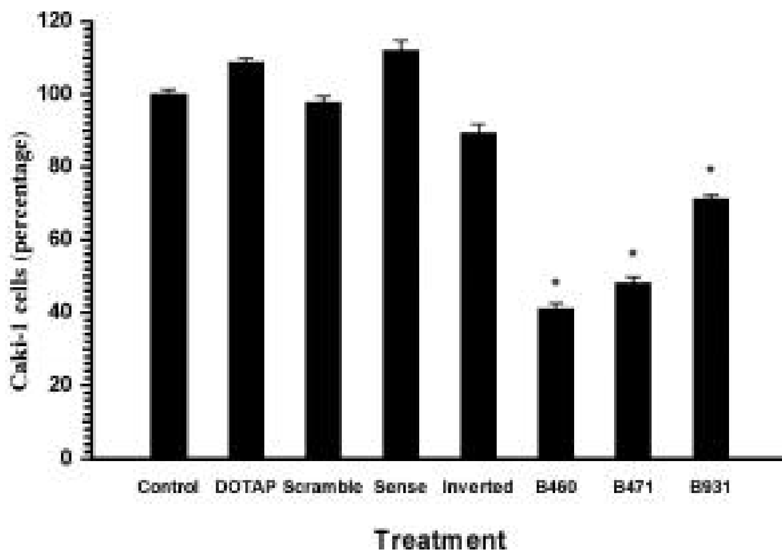


Figure 4. Percentage of Caki-1 cells 3 days after treatment with liposome vehicle (DOTAP), 1 μM control PS-ODNs sequences (Scramble, Sense, Inverted), or different PS-ODNs sequences targeted to bFGF mRNA (B460, B471, B931). Control cells were untreated. Data are the mean ± S.E. of 3 different experiments. Stars indicate significant differences (p<0.05) from the untreated control group.

To gain a better understanding of the underlying mechanisms involved in the observed growth inhibitory effects, the expression of FGF receptors on Caki-1 cells was determined. The results (**Figure 5a**) showed that Caki-1 cells expressed 3 of 4 FGF receptors involved in the bFGF signal transduction pathway. B460 treatment also led to small but significant changes in cell cycle (**Figure 5b**) and induction of apoptosis (**Figure 5c**). Clonogenicity of Caki-1 cells was not however affected by B460 treatment (data not shown).

Since the ultimate goal of bFGF antisense therapy is to inhibit cancer cell induced angiogenic signaling, experiments designed to mimic the in vivo paracrine interaction between tumor and endothelial cells were conducted using a Transwell co-culture system to evaluate the effect of bFGF expression in Caki-1 cells on endothelial cell growth and migration. Caki-1 tumor cells, which had been pretreated with bFGF antisense, were grown in transwells inserts while endothelial cells (MHE and HMVEC-L) were set on the bottom of the wells.

Figure 5a. FGF receptors expression by Caki-1 cells. Caki-1 cells express FGFR 1-3 but not FGFR4.

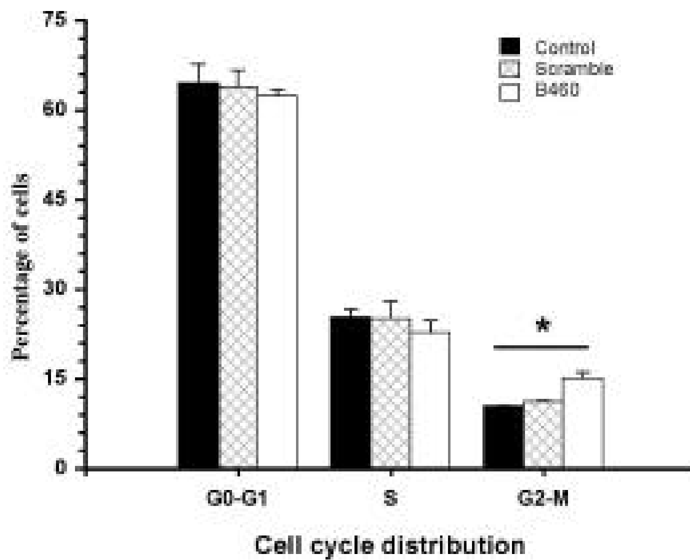


Figure 5b. Caki-1 cell cycle analysis performed 72 hr after treatment with a 1 μM dose of B460 or scramble PS-ODNs. The control group was untreated. Each bar represents the mean ± S.E. of 3 experiments. The star indicates a significant difference ($p<0.05$) compared to the untreated control group.

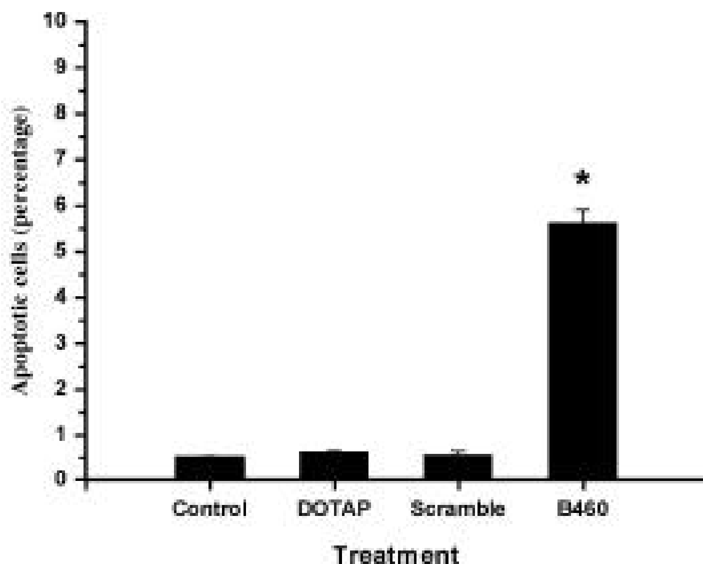


Figure 5c. Apoptosis rate of Caki-1 cells after B460 treatment. Caki-1 cells were untreated (Control), treated with liposome vehicle (DOTAP), or treated with a 1 μM dose of either scramble PS-ODNs (Scramble) or B460 for a period of 72 hr. Each bar shows the results of 3 experiments ± S.E. The star indicates a significant difference ($p<0.05$) from the untreated control group.

The two cell types were separated by a membrane with 0.4 μm pores, chosen to allow the exchange of growth factors while preventing any direct cell-cell interactions. The results (**Figure 6**) showed that Caki-1 cells pre-treated with B460 significantly inhibited endothelial cell proliferation whereas treating the tumor cells with scramble antisense PS-ODNs had no effect on MHE or HMVEC-L cell growth. Media derived from B460 treated tumor cells also impaired the migration rate of both MHE and HMVEC-L cells whereas media from

control or scramble treated tumor cells did not (**Figure 7**).

To demonstrate that bFGF antisense treatments could affect the induction of angiogenesis by Caki-1 cells in vivo, tumor cells pretreated with B460 were injected into mice and the number of blood vessels induced 3 days later was determined. The results (**Figure 8**) showed that untreated or scramble sequence PS-ODNs treated Caki-1 cells had very similar angiogenic potency, inducing ~45 new vessels in the assay period. In contrast, the angiogenic potency of Caki-1 cells pre-treated with B460 was found to be severely impaired; only ~26 new blood vessels were observed.

In order to investigate whether the in vivo administration of bFGF antisense could lead to reductions in tumor bFGF expression levels, B460 PS-ODNs were mixed with cationic liposome DOTAP:DOPE in 5% dextrose and injected (10 mg/kg) via tail vein into Caki-1 xenograft-bearing mice. Western blot analysis of tumor samples collected at various times after B460 injection showed significant reductions in bFGF levels 24, 48 and 72 hr after treatment, with the maximum suppression occurring between 48 hr to 72 hr post B460 administration (**Figure 9**).

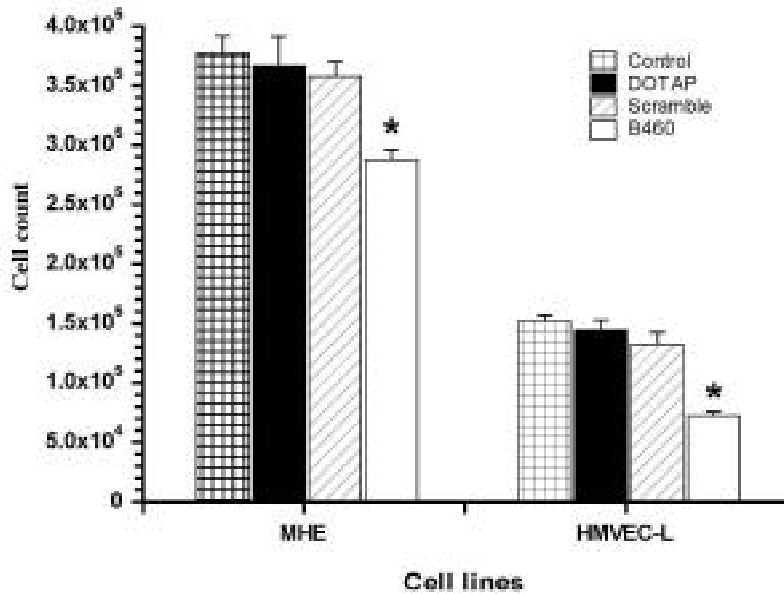


Figure 6. Effect of Caki-1 cell co-culture on the growth of MHE and HMVEC-L cells. Caki-1 cells were untreated (Control) or pre-treated with either liposome vehicle (DOTAP), 1 μM control antisense PS-ODNs (Scramble) or 1 μM bFGF antisense PS-ODNs (B460). Cells were counted at the end of a 4-day treatment period. Each bar represents the mean ± S.E. of 3 experiments. Stars indicate significant differences ($p<0.05$) from the untreated control group.

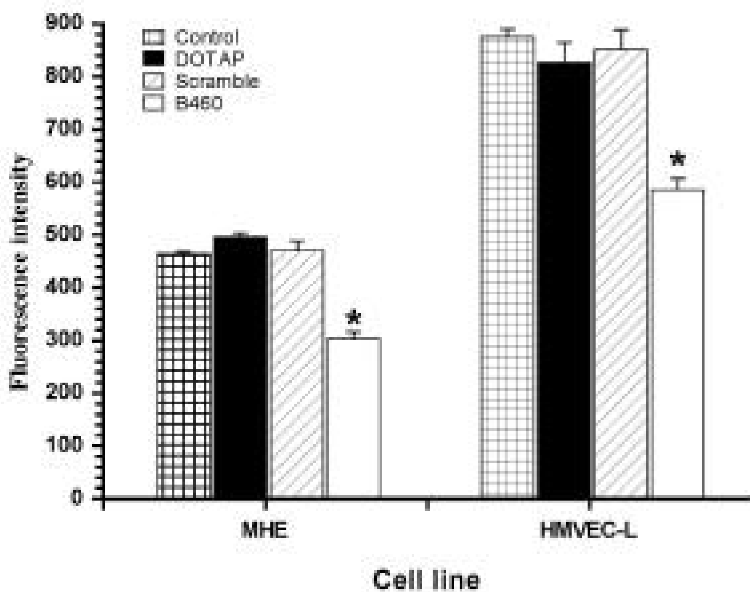


Figure 7. Effect of conditioned media derived from Caki-1 cells on MHE and HMVEC-L cell migration. Media were obtained from Caki-1 cells which were not treated (Control), liposome vehicle treated (DOTAP), control PS-ODNs treated (Scramble) or bFGF antisense PS-ODNs (B460) treated. Each treatment was carried out in quadruplicate and the data shown are the mean ± S.E. Stars indicate significant differences ($p<0.05$) from the untreated control group.

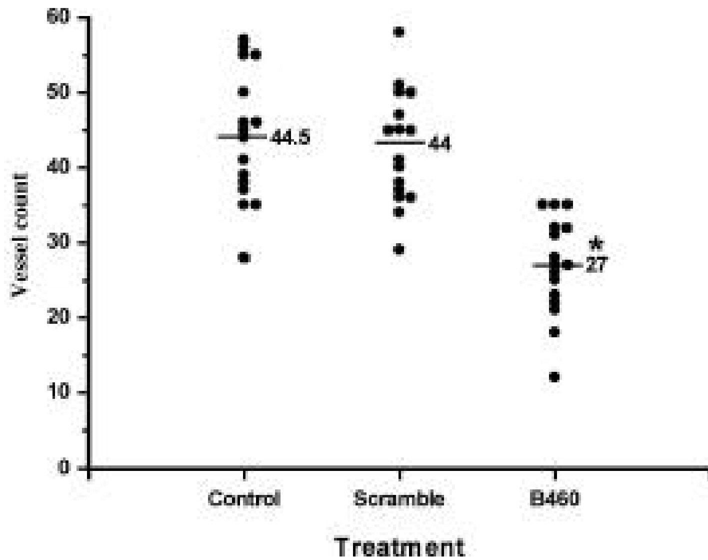
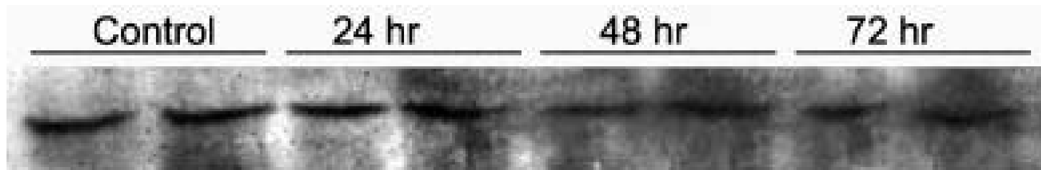


Figure 8. Number of blood vessels induced 3 days after injecting 5 x 10^4 Caki-1 cells intradermally at 3-4 sites per mouse. Caki-1 cells were either untreated (Control) or pretreated with a 1 μM dose of PS-ODNs for 2 hr prior to injection. The Scramble group refers to cells pretreated with scramble sequence PS-ODNs whereas the B460 group represents Caki-1 cells pretreated with bFGF antisense PS-ODNs. Each circle represents one injection site; the bar shows the median of 16 sites. Results for B460 treated cells are significantly different ($p<0.05$, Wilcoxon rank test) from untreated or scramble PS-ODNs treated cells.

A



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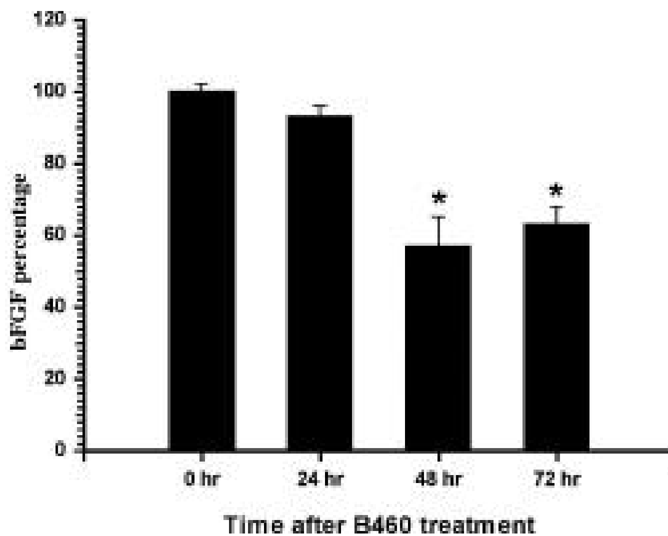

Figure 9. bFGF protein levels in Caki-1 tumors at different times after treatment with 10 mg/kg bFGF antisense PD-ODNs (B460). **A** Representative bFGF western blot results, showing two tumor samples per group; **B** Relative bFGF protein levels of Caki-1 tumors in mice treated bFGF antisense PD-ODNs (B460). Each bar represents the mean ± S.E. of 6 tumors. The stars indicate significant differences from time zero ($p<0.05$).

Subsequent experiments were designed to determine the antitumor efficacy of the systemic delivery of bFGF antisense PS-ODNs by examining the effect of such treatments on Caki-1 tumor growth. Caki-1 xenograft-bearing mice were treated with two doses of bFGF antisense PS-ODNs B460 (5 or 10 mg/kg) 1 and 4 days after the tumors reached a size of ~200 mm^3. The time for the tumors to grow from 200 to 1000 mm^3 then was recorded (**Figure 10**). The data show that the median time for the tumors to grow to 5 times the original starting size was significantly prolonged in the bFGF antisense PS-ODNs (B460) treated groups, and that this increase in growth delay was treatment dose dependent.

IV. Discussion

Evidence exists to strongly implicate bFGF as an important growth-promoting and angiogenic factor in RCC. First described in this disease 10 to 15 years ago (Mydlo et al, 1988; Mydlo et al, 1993) higher bFGF mRNA levels now have been noted in RCC than adjacent normal kidney (Eguchi et al, 1992). Associations between serum and urine bFGF levels and malignant progression as well as treatment outcome also have been made (Nanus et al, 1993; Nguyen et al, 1994; Duensing et al, 1995; Miyake et al, 1996; Yoshimura et al, 1996).

The RCC model used in the present investigations (Caki-1) expresses 3 of 4 FGF receptors involved in bFGF signal transduction (**Figure 5a**). Blocking the production of bFGF by antisense PS-ODNs treatment causes a

moderate inhibition of RCC growth in vitro (**Figure 4**). This result was sequence specific, dose dependent and achieved at low concentrations (**Figures 1-3**). In general, the effects of different antisense PS-ODNs appeared to be directly related to their ability to suppress bFGF expression (**Figure 4 vs. 1**).

The most probable explanation for the observed growth inhibition associated with the bFGF treatment is the small but significant modulation of the cell cycle (increase in G2-M, decrease in S (**Figure 5b**) coupled with the induction of apoptosis (**Figure 5c**)).

To evaluate whether bFGF mRNA targeted PS-ODNs could inhibit tumor cell induced angiogenesis, both in vitro and in vivo assessments of this process were made. Since endothelial cell proliferation and migration are key elements in angiogenesis, the ability of Caki-1 cells to induce these components after bFGF antisense PS-ODNs treatment was investigated under conditions that allowed growth factor exchange between tumor and endothelial cells or by exposing endothelial cells to media collected from antisense treated tumor cells. These in vitro experiments were conducted under reduced serum conditions to minimize interference of other growth factors. The results showed that the inhibition of bFGF production in tumor cells by antisense PS-ODNs treatment significantly reduced endothelial cell proliferation (**Figure 6**) and migration (**Figure 7**).

Subsequent studies demonstrated that inhibiting the production of bFGF by pre-treating Caki-1 cells with bFGF antisense PS-ODNs could significantly impair their ability to induce the angiogenic process in vivo (**Figure 8**). While these results support the role of bFGF as an important pro-angiogenic growth factor in Caki-1 cell-induced angiogenesis, the direct effect of B460 treatment on Caki-1 cell proliferation (**Figure 4**) may also be contributing to the reduced vessel counts observed in vivo (**Figure 8**).

When administered in vivo, B460 not only significantly reduced bFGF expression levels in established Caki-1 xenografts (**Figure 9**) but also resulted in a dose-dependent tumor growth delay (**Figure 10**). Previous studies had already shown that down regulating bFGF expression by antisense treatment could inhibit endothelial and tumor cell proliferation (Masood et al, 1997). For example, transfection of bFGF antisense cDNA or treatment with bFGF antisense PS-ODNs led to growth inhibition in several malignant cell types in vitro (Becker et al, 1989; Murphy et al, 1992; Ensoli et al, 1994; Redekop and Naus, 1995). Also, pretreating Kaposi's sarcoma cells with bFGF antisense oligomers prior to injecting them into nude mice led not only to a reduction in the number of KS-like lesions present 4 days later but also to a reduced histopathology and lower levels of bFGF in those lesions that did occur (Ensoli et al, 1994). However, the present investigations provide the first experimental evidence that the systemic administration of bFGF antisense PS-ODNs to mice bearing macroscopic tumors can have significant antitumor efficacy. Indeed, the tumor growth delays observed (**Figure 10**) were achieved without overt toxicity and with doses well below the LD_{10} dose.

In summary, the results of this study indicate that bFGF is an important factor for the growth and angiogenic potential of Caki-1 cells. Treatment with the novel bFGF antisense PS-ODNs (B460) proved to be an effective means of down-regulating bFGF production and impairing both Caki-1 growth and angiogenic signaling in vitro and in vivo. Moreover, the systemic administration of bFGF antisense PS-ODNs resulted in a significant inhibition of tumor growth when mice bearing established Caki-1 xenografts were treated. Taken together, these findings suggest that the application of an antisense treatment strategy based on targeting the angiogenic growth factor bFGF may have utility in the management of renal cell carcinoma.

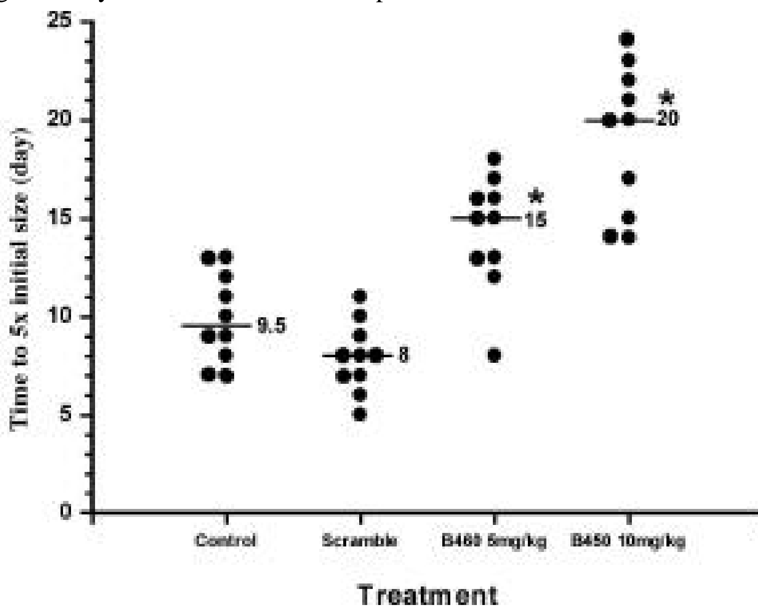

Figure 10. The effect of antisense PS-ODNs targeted to bFGF mRNA treatment on the growth of Caki-1 xenografts. Anti-bFGF (B460) or control PS-ODNs (Scramble) were administered with cationic liposomes (DOTAP:DOPE) *via* the tail vein 1 and 4 days after the tumors reached a size of ~200 mm^3. Control mice were untreated. Liposome vehicle administration on its own had no effect on Caki-1 tumor growth (data not shown). Each circle represents a single tumor; the bar shows the response of the median tumor in each group of 10 mice. The stars show significant differences ($p<0.05$, Wilcoxon rank test) from control or scramble PS-ODNs treated mice.

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