Inhibition of Angiogenic Activity of Renal Carcinoma by an Antisense Oligonucleotide Targeting Fibroblast Growth Factor-2

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Abstract. Background: Fibroblast growth factor-2 (FGF-2) induces angiogenesis, critical for the growth and metastatic spread of tumors. Materials and Methods: The effect of blocking FGF-2 synthesis by an antisense phosphorothioate oligodeoxynucleotide (PS-ODN2) was evaluated on the angiogenic activity of Caki-1 and of a cell line isolated from a renal carcinoma bone metastasis (CRBM-1990). After the transfection with PS-ODN2, FGF-2 mRNA, protein expression and angiogenic activity were evaluated. Results: In Caki-1, a not significant decrease in the released FGF-2 was observed after 72 hours. In CRBM-1990, a not significant decrease in intracellular FGF-2 protein was observed after 72 hours. Endothelial cell migration induced by the conditioned media from Caki-1 treated with PS-ODN2 for 72 hours was significantly reduced. Conclusion: PS-ODN2 treatment of the established line Caki-1 induced minimal variations in FGF-2 expression, but inhibited endothelial cell migration. In CRBM-1990 cells, PS-ODN2 determined a decrease in intracellular protein without reducing the ability to induce endothelial cell migration and proliferation.

Renal cell carcinoma (RCC) is one of the most highly vascularized solid tumors, which suggests that the neovascularization contributes greatly to the disease. Anti-angiogenic therapy might, therefore, represent a potentially useful approach for the treatment of this tumor, which is refractory to radiotherapy, chemotherapy and hormonal therapy.

Several authors agree that fibroblast growth factor-2 (FGF-2) plays an important role in the angiogenesis of RCC. The serum and tissue expressions of this protein seem to correlate with the metastatic potential of RCC (1), and it has been reported that this pro-angiogenic factor is positively correlated with the stage of the tumor and nuclear grade (2).

In the human genome, there is only one copy of the FGF-2 gene that codifies for four isoforms with different molecular weight, each one representing a primary product of the translation: 18-, 22-, 23- and 25 kDa (3). The 18 kDa isof orm is localized mainly in the cytoplasm and extracellular environment, and stimulates cell migration and proliferation, whereas high molecular weight isoforms are found mainly in the nucleus (4).

In the present study, the anti-angiogenic effect of a FGF-2 antisense phosphorothioate oligodeoxynucleotide was evaluated on an established RCC line and on a cell line isolated from a bone metastasis of a renal carcinoma.

Materials and Methods

Cell culture. Two cell lines of human renal carcinoma were tested. Caki-1, isolated from a skin metastasis of renal clear cell carcinoma, were purchased from ATCC (Manassas, VA, USA). CRBM-1990 cells were isolated and characterized in our laboratory from a bone metastasis of a RCC, and were used after 10 to 25 passages (5). The angiogenesis inhibition was tested on a bovine bone endothelial cell line (BBE), originally cloned from fetal bovine sternum (6). The cell lines were maintained in Ham’s F12 medium modified by Coon (Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Mascia Brunelli, Milan, Italy).

Antisense oligonucleotide. A 21-mer antisense oligodeoxynucleotide (5’-GTGATGCTCCCGGCTGCCATG-3’), phosphorothioate in the first and in the last base (PS-ODN2), was synthesized to complement and block the region of the FGF-2 mRNA from 467 to 487 (GenBank™ NM_002006.3) (TIB MolBiol, Genova, Italy).
A 22-mer degenerate PS-ODN (PS-ODNdeg 5’-NNNNNNNNNNNNNNNNNNNNNNN-3’, where N was G or C, or A, or T) was used as control for non-antisense effects. Artificial cationic lipids (Lipofectamine™ 2000, Invitrogen, Carlsbad, CA, USA) were used at the concentration DNA (µg): Lipofectamine™ (µl) of 1:2 to improve PS-ODNs cellular uptake.

**PS-ODNs uptake.** Caki-1 cells were seeded in Ham’s F12 medium modified by Coon (Sigma) with 0.1% fetal bovine serum (FBS) (Mascia Brunelli), without antibiotics, in 6-well plates (500,000 cells/w) and in 12-well plates (200,000 cells/w); CRBM-1990 cells were seeded in 6-well plates (600,000 cells/w) and in 12-well plates (300,000 cells/w). All the samples were in duplicate. After 24 hours at 37°C, PS-ODNs 200nM (PS-ODN2 or PS-ODNdeg) were added. Only medium was added to the negative controls. Forty-eight hours after the treatment, the cultures were treated again with PS-ODNs at a concentration of 100 nM. Twenty-four hours after the first and the second treatment, total RNA and proteins were extracted and the supernatants were collected.

**Semiquantitative RT-PCR.** The RNA was isolated from cells at confluence using the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Total RNA was reverse transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech Laboratories Inc., Palo Alto, CA, USA). Semiquantitative RT-PCR amplification for human FGF-2 was determined using primers from the GenBank™ accession number NM_002006.3 (forward: CTCTTTCAACGATTCAACAC; reverse: TCCCCCTAACAAACATCAC). The RT-PCR consisted of one denaturation at 94°C for 5 minutes, and then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 30 seconds and extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. Parallel reactions were performed for every assay using primers designed to amplify human β-actin, from the GenBank™ accession number NM_001101 (forward: ATCTCGG CACCAACCTTCTCAATGAGCTGGC; reverse: TG TCATA CTCTCTGCCTGATCCACATCTGC). Specific cDNA for β-actin mRNA levels was assayed by denaturation at 94°C for 10 minutes, and then 30 cycles of denaturation at 94°C for 30 seconds, annealing at 65°C for 45 seconds and extension at 72°C for 30 seconds, and final extension at 72°C for 10 minutes. The products were separated by electrophoresis using 2% agarose gel stained with ethidium bromide (0.5 µg/ml). DNA Ladder 100 bp was run in parallel as a molecular weight marker (New England Biolabs, Beverly, MA, USA). The pictures of the gel were transferred to the computer by camcorder and quantified by dedicated software for densitometric evaluation of the bands (Quantity One, Biorad Laboratories Headquarters, Hercules, CA, USA). FGF-2 signals were normalized to β-actin signals determined in parallel for each sample.

**FGF-2 Western blot.** Western blot for FGF-2 was performed both on cells and conditioned media. The cells were lysed with a boiling solution made of one part of 10% SDS, one part of 0.5M Tris-HCl pH 6.8, and two parts of water. The lysates were boiled for 3 minutes and sonicated 3 times for 15 seconds. The conditioned media were concentrated by Centricon® Plus-20 filter (Amicon Bioseparations). The protein concentration of the samples was determined using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Cell lysates (30 µg /lane) and conditioned medium concentrates (60 µg/lane) were subsequently electrophoresed on a 15% polyacrylamide gel and transferred to nitrocellulose sheets. The membrane was incubated with a polyclonal anti-FGF2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) 1:500 for 2 hours and then with anti-rabbit Ig horseradish peroxidase linked whole antibody 1:1000 (Amersham, Little Chalfont, Buckinghamshire, UK) for 30 minutes. The signal was visualized using an enhanced chemiluminescence assay ECL (Amersham) after 2 minutes of exposure. The protein bands were quantified by dedicated software (Quantity One, Biorad).

**Endothelial cell chemotaxis inhibition.** The chemotaxis assay on BBE was performed with the Boyden chamber technique using a 24-well plate. In the lower compartment of the wells, serum-free medium (0.8 ml), or undiluted PS-ODNs-treated or untreated Caki-1- and CRBM-1990-conditioned media (0.8 ml) were placed. BBE were resuspended in serum-free medium at a final density of 2 x 10^4 cells/ml. After placing polycarbonate filters (Transwells, Costar, Corning, Acton, MA, USA) with a pore size of 8 µm over the wells, 200 µl of the cell suspension was seeded in the upper compartment. The cells were allowed to migrate for 5 hours at 37°C in a humidified atmosphere with 5% CO2. Then, the filter was removed and migrated cells on the lower side were fixed in 11% glutheraldheyde, stained with crystal violet solution, and counted from 13 random fields (magnification x20) in each well.

**Endothelial cell growth inhibition.** For the endothelial cell growth inhibition assay, BBE (2 x 10^4) were plated in 12-well plates (Costar) in complete medium. After 24 hours, the cells of two wells were detached and counted in duplicate (time 0). In the remaining wells, the medium was removed and replaced respectively with serum-free medium previously placed at 37°C for 24 hours and 72 hours, or PS-ODNs-treated or untreated Caki-1- and CRBM-1990-conditioned media. The serum-free media and the conditioned media were tested diluted 1:2 with fresh medium. Each sample was tested in duplicate. After 96 hours, the BBE cells were determined in duplicate.

**Statistics.** Statistical analysis was performed with the StatView™ 5.0.1 software for Windows (SAS Institute Inc., Cary, NC, USA) and with the SPSS software (SPSS Inc., Chicago, IL, USA). The results were reported as means and standard error. The effect of the PS-ODN2 on cell number, FGF-2-specific mRNA and FGF-2 isoforms at Western blotting was analyzed by the Mann-Whitney U-test, calculated according to the Monte Carlo method. The statistical analysis of the effect of the PS-ODN2 on the inhibition of chemotaxis and growth of endothelial cells was performed by variance analysis (ANOVA), and Bonferroni’s multiple comparison

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation</th>
<th>Caki-1</th>
<th>CRBM-1990</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>24h</td>
<td>1.16±0.21</td>
<td>0.51±0.14</td>
</tr>
<tr>
<td>PS-ODN2</td>
<td>24h</td>
<td>1.08±0.13</td>
<td>0.34±0.20</td>
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<tr>
<td>PS-ODNdeg</td>
<td>24h</td>
<td>0.89±0.07</td>
<td>0.45±0.16</td>
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<tr>
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<td>0.30±0.04</td>
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<td>0.48±0.23</td>
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</tr>
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</table>

Table I. Mean and standard error of FGF-2/β-actin cDNA ratio of treated cells.
test was applied to detect specific differences between groups. The level of significance was set at \( p < 0.05 \).

**Results**

Three independent experiments were performed.

**FGF-2 semi-quantitative RT-PCR.** In Caki-1 cells, the expression of FGF-2 mRNA was inhibited by PS-ODN2 only after 72 hours' incubation (24 hours from the second treatment) in comparison both with the untreated cells and PS-ODNdeg. The reduction was equal to 51% of the untreated cells and 25% compared to PS-ODNdeg. However, this variation is not significant. In CRBM-1990 cells, PS-ODN2 inhibited the FGF-2 cDNA expression not significantly after 24 hours, but with a large variability (33% of the untreated cells, 24% of the PS-ODNdeg) (Table I). No inhibition was demonstrated after 72 hours. No products were obtained in controls lacking cDNA.

**FGF-2 Western blot.** In Caki-1, no changes were demonstrated in intracellular FGF-2 protein (18 kDa) after treatment with PS-ODN2; after 72 hours, there was a non-significant reduction in the 22 kDa band, equal to 26% of the untreated cells and 24% of PS-ODNdeg, whereas the 25 kDa band had a similar trend to the 18 kDa band. In CRBM-1990, a non-significant decrease of the 18 kDa band after 72 hours was observed (66% of the untreated cells, 52% of PS-ODNdeg). The trend of the 22 kDa band and 25 kDa band in CRBM-1990 reflected that of the 18 kDa band (Table II).

The determination by Western blot of FGF-2 released by the cells demonstrated a non-significant decrease in the 18 kDa fraction in the samples of Caki-1 treated with PS-ODN2 for 72 hours, but not in the samples treated for 24 hours. No decrease in the released FGF-2 was observed in CRBM-1990 cells after 24 hours' treatment compared to controls, but only compared to the degenerate (Table III). The 25 kDa fraction, mostly bound to the nucleus, was not detectable in the supernatants, with the exception of Caki-1 after 72 hours' treatment.
Endothelial cell chemotaxis inhibition. The number of spontaneously migrated BBE cells was 31.4±2.62. A significant increase was induced by incubation with the supernatants of untreated Caki-1 (p<0.0001) and untreated CRBM-1990 (p=0.0133). Treatment of Caki-1 with PS-ODN2 for 24 hours induced a significant reduction in migration compared to treatment with PS-ODNdeg (untreated: 123.0±8.93 migrated BBE vs. PS-ODN2: 111.0±6.88 migrated BBE; p=0.3168; PS-ODN2 vs. PS-ODNdeg: 150.0±9.40 migrated BBE; p=0.0015). After 72 hours' treatment with PS-ODN2, inhibition of migration was significant also with respect to the untreated control (untreated: 155.5±7.06 migrated BBE vs. PS-ODN2: 117.0±6.99 migrated BBE; p=0.0007; PS-ODN2 vs. PS-ODNdeg: 145.9±9.11 migrated BBE; p=0.01). There were no significant differences between the control and PS-ODNdeg at 24 hours or 72 hours. Treatment of CRBM-1990 with PS-ODN2 for 24 hours prompted a non-significant reduction in endothelial cell migration (untreated: 49.0±3.81 migrated BBE vs. PS-ODN2: 39.9±4.61 migrated BBE; p=0.1673; PS-ODN2 vs. PS-ODNdeg: 47.56±5.25 migrated BBE; p=0.2434; untreated vs. PS-ODNdeg: p=0.8285). Also after treatment with PS-ODN2 for 72 hours, there were no significant variations in the number of migrated endothelial cells (untreated: 34.2±4.5 migrated BBE; PS-ODN2: 48.1±5.66 migrated BBE; PS-ODNdeg: 44.2±4.33 migrated BBE).

Endothelial cell growth inhibition. Twenty-four hours after seeding in the wells and before treating BBE, the number of cells was 26.1±1.2 x 10³/well. Ninety-six hours after seeding in the wells and before treating BBE, the number of migrated endothelial cells (untreated: 26.1±1.2 x 10³/well). Ninety-six hours after seeding in the wells and before treating BBE, the number of migrated endothelial cells (untreated: 26.1±1.2 x 10³/well) migrated BBE).

Discussion

The modulation of FGF-2 expression by antisense PS-ODN enables its role to be studied in the pathogenesis of RCC and is the starting point for developing therapies aimed at inhibiting this factor.

Cells were treated with PS-ODNs by using medium with 0.1% FBS, instead of 10%. This choice was made because FGF-2 and other angiogenic factors are present in serum and, therefore, might have interfered with the functional tests of angiogenesis inhibition in vitro.

The PS-ODN2 sequence was chosen so as to include the AUG codon where the translation of the 18 kDa protein isoform of FGF-2 initiates, localised among the 468-470 bases. Lipofectamine was used to carry the PS-ODN into the cell, which permits lower concentrations to be used compared to DOTAP, and produces transfection more efficiently. A dose of 200 nM was chosen because, in preliminary experiments, lower doses had no effect, whereas at higher doses detectable amounts of RNA or protein could not be extracted because of the cytotoxic effect. In preliminary experiments, PS-ODN marked with fluorescein isothiocyanate in the extremity 5' was used in order to estimate the efficiency of the uptake of the PS-ODN. After 6 hours, by confocal microscopy it was demonstrated that PS-ODN was localized into the cytoplasm of the cells.

The control groups consisted of untreated cells, to observe FGF-2 expression in normal conditions, and of cells treated with PS-ODNdeg. Other types of PS-ODN used as controls, such as sense, scramble, or inverted, in our opinion are not optimal, because they are composed of a single molecular species, the effects of which are unpredictable. The PS-ODNdeg we used as a control was a collection of random sequences in the same molar concentration, which was so low that the possibility of a sequence-specific effect can be ruled out a priori.

For in vitro assessment of angiogenesis, we used a continuous line of bovine bone endothelial cells, which expressed the type-1 receptor for FGF-2. A line from the microcirculation of the bone was chosen, because this study is part of a wider study about osteolytic metastases, and differences in patterns between endothelial cells of different segments were highlighted. We did not use cells of the microcirculation of human bone, due to the extreme difficulty in isolating and maintaining them in culture.

In Caki-1, PS-ODN2 induced a non-significant reduction in RNA after 72 hours, intracellular protein did not vary, whereas secreted protein was reduced after 72 hours. Correspondingly, the samples of Caki-1 treated for 72 hours with PS-ODN2 had a chemotactic effect on the endothelial cells that was significantly less than that of both controls.

In CRBM-1990, the administration of PS-ODN2 200 nM brought about a slight, non-significant, reduction in mRNA...
for FGF-2, 24 hours after transfection, without a reduction in the protein. The chemotaxis assay also revealed at that time a non-significant reduction in the migration of endothelial cells. The amount of FGF-2 secreted was lower in the supernatants of the cells treated with PS-ODN2 for 24 hours, compared to those with PS-ODNdeg. The isoform mostly secreted by CRBM-1990 is 18 kDa, in agreement with what is reported in the literature on the main role of this isoform in mediating the paracrine effects of FGF-2. Twenty-four hours after the second transfection, a non-significant reduction in intracellular protein, but not in mRNA, was found in the CRBM-1990. This is compatible with the trend observed in the preliminary experiments on untreated cells; in CRBM-1990, the presence of transcripts peaked 72 hours after seeding, thus evidently the dose used in the second administration was not sufficient to obtain appreciable inhibition. Protein synthesis, which was constant after 48 hours in the untreated cells, was inhibited by the action of PS-ODN2. The minimal inhibiting effect on endothelial cell proliferation might be due to the fact that other pro-angiogenic factors promote such growth.

Other authors have tested antisense oligonucleotides targeting FGF-2 for the inhibition of tumor growth and angiogenesis. Morrison (7) observed a 70% reduction in the growth of the human glioma cell line SNB-19 after treatment with FGF-2 antisense oligonucleotides aimed at two different sites of mRNA, namely, the translation initiation site (AUG codon), similar to our PS-ODN2, or the first splice donor-acceptor site (codon 60). Oligonucleotides complementary to the codon 60 also significantly inhibited the growth of HT-29 human colon cancer cells (8). We too observed a reduction in the number of tumor cells after incubation with oligonucleotides, but it was probably due to an aspecific effect, because it also occurred with the degenerate oligonucleotide to a greater extent than with the antisense. Antisense oligonucleotides, anti-FGF-2, or anti-FGF-receptor-1, inhibited growth and angiogenesis of melanoma (9). It would seem that the inhibition of FGF-2 and its receptor affects the growth of the tumor, by acting directly on the proliferation of tumor cells and reducing angiogenesis (10). Our results, although in vitro, also support this hypothesis.

The results we obtained were not compatible with previous studies, probably due to the fact that this tumor produces large amounts of FGF-2 in basal conditions, as well as other pro-angiogenic factors. The antichemotactic effect observed supports the role of FGF-2 secreted by the tumor cell in recalling endothelial cells, whereas the slight inhibiting effect of the proliferation of endothelial cells might be connected to the fact that other pro-angiogenic factors promote this growth. The different response by the two lines might depend on differences in the expression mechanism of FGF-2 in tumor lines of different origins.

In conclusion, the PS-ODN2 treatment of the established line Caki-1 induced slight variations in FGF-2 expression, but inhibited endothelial cell migration. In CRBM-1990, isolated from a bone metastasis, PS-ODN2 determined a decrease in intracellular protein without reducing the ability to induce endothelial cell migration and proliferation.

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References