Antitumor Activity of Basic Fibroblast Growth Factor-Saporin Mitotoxin in Vitro and in Vivo

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Abstract

Many cancer cell lines express basic fibroblast growth factor (FGF) receptors, making them potential targets for the delivery of FGF-based cytotoxic compounds. To this end, we have investigated the antitumor activity of a novel mitotoxin, Fibroblast Growth Factor-saporin (FGF-SAP), a conjugate of FGF and the ribosome-inactivating protein, saporin. In vitro, FGF-SAP is cytotoxic for human melanoma, teratocarcinoma, and neuroblastoma cells expressing FGF-receptors. Mice treated with FGF-SAP i.v., on a variety of schedules, showed dramatic tumor growth inhibition with minimal toxicity. Thus, FGF-SAP appears to be a well-tolerated and potent antitumor agent. The potential of FGF-targeted cytotoxicity is discussed.

Introduction

The advantage of conjugating toxins from bacteria and plants to cell-targeting antibodies and ligands is well recognized (1). The conjugation of a toxin to a cytokine such as FGF to achieve local antitumor effects has been utilized less frequently, although this approach is effective with transforming growth factor α (2), interleukin 4 (3), and interleukin 6 (4), and acidic fibroblast growth factor (5). There is evidence implicating the involvement of FGF in the growth of a variety of neoplastic cell lines, including melanoma (6), astrocytoma (7), and ovarian carcinoma (8). For example, the growth of certain melanoma cell lines is stimulated by FGF (6) and can be inhibited by antagonistic FGF peptides (6). Thus, the development of therapeutic strategies utilizing FGF to deliver toxin into FGF-responsive cancer cells might prove effective against a number of malignancies.

SAP, derived from the seeds of the plant Saponaria officinalis, irreversibly inhibits the protein synthesis of eukaryotic cells by rendering the 60S subunits of ribosomes unable to bind elongation factor 2 (9). Accordingly, the conjugate of FGF to saporin (FGF-SAP) is a ligand toxin which has cellular targeting specificity and potent cytotoxicity in vitro for cells expressing FGF receptors, including baby hamster kidney cells, corneal and aortic arch endothelial cells, and AIDS-KS-3 cells (10-12). Because of the reported successful in vitro targeting efficacy of other saporin conjugates (13), we investigated the cytotoxic effects in vitro and in vivo of a saporin-based conjugate directed against the FGF-receptor found on FGF-receptor-bearing cancer cell lines.

Materials and Methods

Mitotoxin. Conjugation of saporin to basic FGF was accomplished after derivatization of saporin with N-succinimidyl-3(2-pyridyldithio)-propionate as described (10, 13). The conjugate was purified by dialysis against water and chromatography on a Mono S 5/5 NaCl cation exchange column (Pharmacia). Fractions containing the conjugate were detected by silver staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cell Lines. SK-Mel-1, a human melanoma, SK-N-MC, a human neuroblastoma, and PA-1, a human ovary teratocarcinoma cell line, were obtained from the American Type Culture Collection (Rockville, MD) and were grown in modified Eagle’s medium supplemented with 10% fetal calf serum. A431, a human epidermoid carcinoma, was also obtained from the American Type Culture Collection and was grown in RPMI 1640 supplemented with 10% fetal calf serum. FSaIIC, a murine fibrosarcoma, was a gift of Dr. Beverly Teicher (Dana Farber Cancer Institute, Boston, MA) and was grown in α modified Eagle’s medium supplemented with 5% fetal calf serum.

125I-FGF Receptor Binding Assay. Cells were seeded in 12-well tissue culture plates (Costar) at 10^4 cells/well and grown until confluent in their respective medium. 125I-FGF binding was performed using a radioreceptor assay as described (14). Briefly, cell monolayers were incubated with fresh, un-supplemented medium containing 0.2% gelatin and 3 μg/ml heparin (Sigma) for 1 h at 37°C and 5% CO2 and then washed with ice-cold medium and allowed to cool for 1 min. Cells were incubated with various concentrations of 125I-FGF in 250 μl of the same medium for 2 h on ice. The cells were then gently washed twice with ice-cold 0.9% saline buffered saline to remove unbound 125I-FGF, and remaining cell-associated radioactivity was extracted with 1% Triton X-100 and quantitated using a Beckman gamma counter. Nonspecific binding was determined by inhibiting specific binding using a 200-fold excess of nonradioabeled FGF.

In Vitro Survival Studies. Cells were plated in 96-well tissue culture plates (Costar) at 10^4 cells/well in their respective medium. One day later, the medium was removed, and medium containing 1 μM to 1 μM of the conjugate FGF-SAP or free SAP was added. Cells were treated in triplicate and maintained at 37°C and 5% CO2. Seventy-two h after the treatment was initiated, the medium was removed and the cells were trypsinized and counted using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL). Results are expressed as the mean cell number from treated wells, normalized to media controls, as a function of the FGF-SAP or SAP which resulted in a 50% reduction in cell number.

In Vivo Antitumor Studies. Experiments with SK-Mel-1, SK-N-MC, and A431 cell lines were performed in 8-10-week-old male nu/nu mice, while those with PA-1 cells used 8-10-week-old female nu/nu mice. Nude mice were bred and maintained by the Roger Williams Hospital Animal Care Facility. FSaIIC cells were carried in 8-10-week-old male C3H/HeN mice (Taconic Laboratories, Germantown, NY). The 50% lethal dose of FGF-SAP in BALB/c mice was found to be 500 μg/kg, with toxicity manifested as extensive hemorrhage, often in the intestinal tract (11). For in vivo studies, groups of five mice were inoculated with...
concentrations of saporin in each treatment group) were dissolved in
vals, for a total of four doses. The progress of each tumor was measured
of i.v. injections of 0.5 μg/kg FGF-SAP administered at weekly inter
after tumor implantation. In subsequent studies, mice received a course
of i.v. injections of 0.5 μg/kg FGF-SAP administered at weekly inter-
vals, for a total of four doses. The progress of each tumor was measured
at least twice weekly, beginning 5 days after tumor implantation, and
tumor volumes were calculated as (15)

(Minimum measurement)2 (maximum measurement)

Results

Inhibition of Tumor Cell Proliferation. The FGF-SAP conjugate
has been shown to interact with the high-affinity FGF
receptor and to be cytotoxic to cells expressing this receptor
(10–12). Furthermore, FGF-SAP cytotoxic activity is inhibited
in a dose-dependent fashion by exogenous FGF (10, 12). To
assess the potential cytotoxicity of FGF-SAP against different
tumor cell lines, we performed 125I-FGF binding studies to
demonstrate the presence of high-affinity binding sites for FGF
on these cells. Scatchard analysis showed that SK-Mel-1, PA-
1, SK-N-MC, and FSA1IC cells expressed high-affinity FGF
receptors, whereas A431 cells were devoid of FGF receptors (Table 1).

Next, we determined cell survival after treatment with various
concentrations of FGF-SAP or SAP for 72 h in vitro. FGF-
SAP is a potent inhibitor of cell growth for each of the cell
lines expressing FGF receptors (Table 1 and Fig. 1A). FGF-
SAP demonstrated minimal cytotoxic effects in A431 cells. SAP-associated growth inhibition was observed for PA-1 and
SK-N-MC cells, but only after exposure to SAP concentrations
that were 2–6 orders of magnitude greater than that of the
conjugate. The addition of FGF and SAP in a noncovalent
mixture had no cytotoxic effects.

Inhibition of Tumor Growth in Vivo. In subsequent studies we
explored the FGF-SAP mitotxin's efficacy in vivo for each cell
line grown s.c. as solid tumor xenografts in mice. Preliminary
toxicological evaluation of FGF-SAP showed a dose of 500 μg/
kg to be lethal in BALB/c mice (11) and 250 μg/kg to be
nonlethal. Accordingly, we chose 125 μg/kg as an initial dose
of FGF-SAP. We performed pilot studies in nude mice bearing
human tumor xenografts in which a single i.v. dose of FGF-
SAP was administered 1, 5, 10, or 15 days after tumor implan-

Table 1. High-affinity FGF receptor number, FGF dissociation constant,
and growth inhibition of tumor cell lines in the presence of FGF-SAP or SAP

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>K_a (nM)</th>
<th>FGF-R Number</th>
<th>bFGF-SAP I_C50</th>
<th>SAP I_C50</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-Mel-1</td>
<td>167</td>
<td>19,000</td>
<td>0.1</td>
<td>No effect</td>
</tr>
<tr>
<td>PA-1</td>
<td>33,000</td>
<td>10</td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>45,000</td>
<td>0.01</td>
<td>1000</td>
<td>500</td>
</tr>
<tr>
<td>FSA1IC</td>
<td>41</td>
<td>7,000</td>
<td>2.5</td>
<td>No effect</td>
</tr>
<tr>
<td>A431</td>
<td>NA</td>
<td>0</td>
<td>No effect</td>
<td>No effect</td>
</tr>
</tbody>
</table>

K_a in pm.

b Concentration (in nM) calculated from dose-response curves which resulted
in a 50% reduction in cell number. Each value is the mean of at least three
determinations.

Fig. 1. A, inhibition of PA-1 cell proliferation after treatment with various
concentrations of FGF-SAP (●), unconjugated FGF (●), unconjugated SAP (●),
or a noncovalent mixture of FGF plus SAP (●) in vitro. Cells were seeded into
96-well plates, treated in triplicate for 72 h with concentrations of FGF-SAP or
controls as indicated, trypsinized, and counted. Results are expressed as the mean
cell number from treated wells, normalized to media controls, as a function of
dose responses. Mean tumor volumes on day 30 for FGF-SAP 125 μg/kg (●), FGF-
SAP 0.5 μg/kg weekly for four doses (●), SAP 85 μg/kg (●), or no treatment (●).

B, Tumor growth inhibition in nude mice bearing PA-1 xenografts treated
with FGF-SAP or free SAP administered 5 days after tumor implantation. Mean
tumor volumes are expressed as a function of time for mice receiving a single
dose of FGF-SAP 125 μg/kg (●), FGF-SAP 0.5 μg/kg weekly for four doses (●),
SAP 85 μg/kg (●), or no treatment (●). n = 10 mice/treatment group. Bars, SE.

tation. FGF-SAP caused marked inhibition of tumor growth,
and in some animals, complete tumor regression was observed
(Fig. 1B, data not shown). Tumor inhibition was observed even
when treatment was delayed until day 15, when tumor volumes
are approximately 50–100 mm3. By day 30, mean volumes of
tumors in treated mice measured only 5–33% of those of control
tumors. Since FGF-SAP at this dose level appeared equally
efficacious when administered on day 1, 5, or 10 (data not
shown), we chose day 5 as the treatment day for further inves-
tigations. At this time, tumors are approximately 40–50 mm3
in volume.

The next series of studies compared a broad range of FGF-
SAP doses with equivalent doses of SAP to determine in vivo
dose responses. Mean tumor volumes on day 30 for FGF-SAP-
or SAP-treated xenografts compared to those of untreated
controls are displayed in Table 2. Studies performed in nude
mice bearing SK-Mel-1, PA-1, or SK-N-MC xenografts dem-
ronstrated growth inhibition with FGF-SAP and lack of efficacy
with free SAP (Table 2 and Fig. 1B). Complete tumor regres-
sion was observed on day 30 in 16% of SK-N-MC xenografts
treated with a single dose of FGF-SAP (125 μg/kg) on day 5.
Antitumor responses to FGF-SAP were also observed in immunocompetent host mice bearing FSaIIC tumors (Table 2), although the effects of FGF-SAP were characteristically short-lived in this single-dose regimen (Table 3). No growth inhibition was observed in mice bearing A43l xenografts following treatment with FGF-SAP or SAP.

In conjunction with our evaluation of lower FGF-SAP doses in vivo, we performed studies in which multiple doses of FGF-SAP were administered i.v. Because we observed only modest antitumor effects with FGF-SAP at a single dose of 0.5 μg/kg (Table 2; data not shown), we treated groups of mice bearing SK-Mel-1, PA-1, SK-N-MC, or FSaIIC tumors with FGF-SAP 0.5 μg/kg beginning on day 5, and then once a week for a total of four doses. Table 3 compares mean tumor volumes for the multiple low-dose FGF-SAP regimen to volumes for the single high-dose regimen. Significant tumor inhibition was seen on day 35 for each of the tumor types examined under the multiple low-dose regimen of FGF-SAP. Thus, chronic treatment of tumors in vivo with low doses of FGF-SAP appeared to be both efficacious and nontoxic. No toxic side effects or premature deaths were observed either in mice receiving free SAP in the doses used or in untreated control mice.

### Discussion

The ribosome-inactivating protein, saporin, covalently linked to FGF exerts potent cytotoxic effects in vitro against a variety of tumor cell types expressing cell surface receptors for FGF (Table 1). The in vitro data accurately predict the superior cytotoxicity of FGF-SAP as compared with free SAP for FGF-receptor-bearing SK-Mel-1, PA-1, and SK-N-MC xenografts (Table 2) and the absence of significant antitumor effects on A43l xenografts. The extent to which FGF-SAP cytotoxicity directly resulted in tumor growth inhibition was expected, since A43l cells did not express cell surface receptors for FGF.

The efficacy of FGF-SAP administered as multiple low doses in vivo is particularly impressive. This regimen affords the advantage of delivering repeated, relatively nontoxic FGF-SAP doses to tumor cells that survive the initial treatment. Certainly, from the studies presented here, some tumor cells survive FGF-SAP treatment, be it after a 72-h exposure in vitro, or after a single, i.v. high dose in vivo. Resistant tumor subpopulations may thus be capable of continued proliferation in vivo. Poor vascular access of the conjugate; alterations in FGF-receptor number, occupancy, or internalization in resistant cells; or, perhaps, the stage in cell cycle of tumor cells at the time of treatment must all be considered. Thus, from a therapeutic standpoint, it is of great practical importance that more than one dose of conjugate be administered with safety.

Formal pharmacokinetic studies of FGF-SAP, including blood half-life, stability, and elimination of the conjugate, have not yet been performed. In the absence of these studies, however, we speculate that FGF-SAP persists in vivo long enough to exert both its antitumor effects and its toxic effects. Since saporin is not glycosylated, saporin conjugates may have an enhanced ability to escape hepatic clearance from the circulation (16). Basic FGF has a high affinity for natural glycosaminoglycans in vitro and in vivo, which is also conferred to SAP when conjugated. Studies in which FGF was injected locally in the rat brain have shown that FGF binds to the extracellular matrix initially and is subsequently transferred and internalized within target tissues, in this case, the vasculature, 2 days later.

### Table 3 Comparison of FGF-SAP treatment regimens in tumor-bearing mice

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Day</th>
<th>Single high dose</th>
<th>Multiple low doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>SK-Mel-1</td>
<td>12</td>
<td>38 ± 12&quot;</td>
<td>68 ± 12</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>26 ± 7&quot;</td>
<td>11 ± 2&quot;</td>
</tr>
<tr>
<td>PA-1</td>
<td>12</td>
<td>54 ± 10&quot;</td>
<td>72 ± 13</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>56 ± 11</td>
<td>19 ± 6&quot;</td>
</tr>
<tr>
<td>SK-N-MC</td>
<td>12</td>
<td>58 ± 11</td>
<td>84 ± 5</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>30 ± 11&quot;</td>
<td>18 ± 7&quot;</td>
</tr>
<tr>
<td>FSaIIC</td>
<td>12</td>
<td>34 ± 12&quot;</td>
<td>51 ± 3&quot;</td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>92 ± 18</td>
<td>42 ± 4&quot;</td>
</tr>
</tbody>
</table>

* Tumor cells (2 × 10⁶) were inoculated s.c. into the right rear flank of host mice.

Mean tumor volumes were calculated using 2—14 mice/treatment group. Errors are SEM.

* A single dose of FGF-SAP 125 μg/kg was administered on day 5.

* A dose of FGF-SAP 0.5 μg/kg was administered on day 5, followed by weekly injections for a total of four doses.

* Statistical difference between treatment and control tumor volumes, P < 0.01.

### Table 2 Dose efficacy of FGF-SAP versus SAP administered as a single i.v. injection 5 days after tumor implantation in mice

<table>
<thead>
<tr>
<th>Dose (μg/kg)</th>
<th>Mean tumor volume on day 30 (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SK-Mel-1</td>
</tr>
<tr>
<td>bFGF-SAP 125</td>
<td>39 ± 14&quot;</td>
</tr>
<tr>
<td>SAP 85.0</td>
<td>118 ± 22</td>
</tr>
<tr>
<td>bFGF-SAP 0.5</td>
<td>61 ± 19</td>
</tr>
<tr>
<td>SAP 0.025</td>
<td>92 ± 18</td>
</tr>
<tr>
<td>SAP 0.017</td>
<td>92 ± 18</td>
</tr>
</tbody>
</table>

* Tumor cells (2 × 10⁶) were inoculated s.c. into the right rear flank of host mice.

* Mean tumor volumes for treated xenografts were calculated using 2—11 mice/treatment group. There were 2—24 mice in the control groups. Errors are SEM.

* Significant difference between treatment and control tumor volumes, P < 0.01.
Thus, it is plausible that the single FGF-SAP dose exerts its effects on target tissues over a relatively long time, perhaps days.

In-depth toxicological studies need to be performed, including evaluations of bone marrow, hepatic, and renal function, in mice receiving each of the various schedules of FGF-SAP. With the exception of the highest FGF-SAP dose, the conjugate was well tolerated in vivo, even when multiple low doses were administered. In addition, autoradiographic or immunohistochemical studies designed to localize FGF-SAP binding within tumor xenografts and their supporting vasculature are being planned since they will be crucial for a complete understanding of the mechanism of action of FGF-SAP. Particularly critical to the delineation of FGF-SAP-induced toxicities is the binding of FGF-SAP within normal tissues expressing FGF receptors.

Along these lines, a number of equally important references can be extrapolated from the data presented here. Like Lindner et al. (18), we find that there is minimal toxicity of FGF-SAP for normal tissues. We thus conclude that, under normal conditions, the basic FGF receptor is not expressed at high enough levels to mediate the internalization effects of the conjugate. This proposal is compatible with our unpublished finding and the results of Whalen et al. (19) that the systemic administration of basic FGF has little or no effect. Accordingly and surprisingly, the selective expression of the FGF receptor in tumors makes them exquisitely susceptible to FGF-SAP action, almost as if they were exposing a unique antigen to immunotoxins.

No detectable toxicity was observed in vivo with free SAP. Others have shown that SAP is rapidly cleared from the circulation, with only 4% of free SAP detectable in the blood 10 min after i.v. injection (16). In addition, the doses of SAP used in our studies were several orders of magnitude lower than the reported 50% lethal dose for SAP of 6.8 mg/kg mouse (16). Not surprisingly, we failed to observe significant antitumor effects with SAP administration in vivo.

In conclusion, we believe that therapies utilizing mitotoxins designed to target cells expressing cell surface receptors for a particular growth factor or cytokine will provide viable alternative approaches to conventional cancer treatments, including immunotherapy. Toxins coupled to epidermal growth factor and to interleukin 2 are already the focus of clinical cancer trials, and some excellent clinical responses have been reported with the latter (20). In light of these recent successes using other targeted agents, the antitumor activity of FGF-SAP against FGF-receptor-bearing tumors is immensely exciting and is potentially immediately applicable to cancer therapy.

References


Unpublished observations.
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