Radiation is frequently used in treating the ∼20% to 40% of newly diagnosed pancreatic cancer patients who present with locally advanced unresectable disease (1). In this group of patients, radiotherapy in combination with chemotherapy is superior to chemotherapy alone. Based on phase III clinical data, 5-fluorouracil in combination with radiation has been the standard of care in clinical practice (2). However, recent laboratory studies as well as various phase I/II trials have shown that the combination of gemcitabine plus radiation may also be effective (3, 4). It is felt that both 5-fluorouracil and gemcitabine sensitize the tumor cells to ionizing radiation resulting in increased cell death (5, 6). However, the prognosis of patients with locally advanced unresectable disease treated with chemoradiotherapy is poor with a median survival of <1 year (1).

Given the disappointing results of chemoradiotherapy, there is a need to evaluate other agents that may enhance the response to radiation in pancreatic cancer. There are now experimental and preliminary clinical data to suggest that combining radiation with drugs that target specific molecular pathways can improve tumor control (7). An example of this approach includes enhancing radiosensitivity by combining radiation with epidermal growth factor receptor or farnesyltransferase inhibitors (8, 9). Enhanced radiosensitivity has also been noted against breast cancer cell line by using the monoclonal antibody to HER-2/neu (10).

Because sensitivity of tumor cells to radiation can be enhanced by agents targeting growth factor receptors, we wanted to determine if targeting tumor differentiation antigens in combination with radiation could lead to improved antitumor activity. SS1(dsFv)PE38 (SS1P) is a recombinant immunotoxin consisting of an antimesothelin Fv linked to a truncated Pseudomonas exotoxin, which is currently undergoing clinical testing in patients (11). Mesothelin is a glycosylphosphatidylinositol-linked cell surface protein present on normal mesothelial cells that is overexpressed in several cancers, including mesotheliomas and ovarian and pancreatic cancers (12–14). Whereas mesothelin expression is absent in normal pancreas, almost all pancreatic cancers express mesothelin, making it a good target for SS1P therapy (15, 16). The antitumor activity of the combination of SS1P plus radiation was evaluated in a human xenograft model in nude mice. Our

**Abstract**

**Purpose:** Mesothelin is a cell surface protein overexpressed in mesotheliomas and pancreatic and ovarian cancers. The goal of this study was to determine if radiation therapy in combination with the antimesothelin immunotoxin SS1 (dsFv)PE38 (SS1P) would result in enhanced antitumor activity against mesothelin-expressing xenografts in nude mice.

**Experimental Design:** Female athymic nude mice bearing s.c. mesothelin-expressing xenografts were treated with SS1P alone, tumor-focused radiation alone, or the combination of the two. Two different regimens of the combination therapy were tested. In the low-dose combination schedule, mice were treated with either 5 Gy radiation alone, 0.2 mg/kg SS1P alone, or the same doses of radiation and SS1P in combination. In the high-dose combination experiments, mice were treated with either 15 Gy radiation alone, 0.3 mg/kg SS1P alone, or the combination of radiation and SS1P.

**Results:** In the low-dose radiation and SS1P combination studies, mice treated with the combination of radiation and SS1P had a statistically significant prolongation in time to tumor doubling or tripling compared with control, SS1P, or radiation alone. A similar increase in time to tumor doubling or tripling was seen in mice treated with high-dose radiation and SS1P combination.

**Conclusions:** Combination of SS1P with tumor-directed radiation results in enhanced antitumor activity against mesothelin-expressing tumor xenografts. This effect was seen when either low or high doses of radiation were used.

**Radiation Oncology Branch, Center for Cancer Research, National Cancer Institute, NIH, 37 Convent Drive, Room 5116, Bethesda, Maryland**

**Requests for reprints:** Rafit Hassan, Laboratory of Molecular Biology, Center for Cancer Research, National Cancer Institute, NIH, 37 Convent Drive, Room 5116, Bethesda, MD 20892-4264, Phone: 301-451-8742; Fax: 301-402-1344; E-mail: hasann@mail.nih.gov.

**Grant support:** Intramural Research Program of the Center for Cancer Research, National Cancer Institute, NIH.

**Acknowledgments:** This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**doi:** 10.1158/1078-0432.CCR-06-0441

**Keywords:** Radiation; Mesothelin; Immunotoxin; Pancreatic cancer; Xenografts.
results show that SS1P given in combination with either low or high doses of tumor-focused radiation results in superior antitumor activity. These results suggest that a combination of radiation with SS1P could have a potential benefit for the treatment of pancreatic cancer.

Materials and Methods

Materials. Purified SS1P, a recombinant antimtesothelin immunotoxin, was obtained from the Laboratory of Molecular Biology, National Cancer Institute. SS1P at 0.250 mg/mL was kept frozen at −70°C until use.

Cell line and tissue culture. A431-K5 is a cell line derived from A431, an epidermoid carcinoma cell line that has been stably transfected with a plasmid encoding mesothelin (17). A431-K5 cells were grown in DMEM with 10% fetal bovine serum, 2 mmol/L l-glutamine, 1 mmol/L sodium pyruvate, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 7.5 μg/mL G418. For antimetastasis experiments, cells were plated at a density of 1.5 × 10^4 in 35 mL medium in a T75 flask 3 days before animal injection. On the day of injection, cells were harvested by trypsinizing flask for 3 minutes with 0.05% trypsin/0.02% versene and rinsing flask with complete medium. Once all flask were trypsinized, cells were spun down, washed thrice with culture medium, and then resuspended in the medium at a density of 1.5 × 10^6/mL.

Irradiation of A431-K5 tumor cells. The cell line A431-K5 was irradiated as described previously (18, 19). Tumor cells were harvested in log growth phase and were placed on ice and irradiated at 10 Gy by a cesium 137 source (Gammacell-1000, AECL/Nordion, Kanata, Ontario, Canada) at a dose of 0.74 Gy/min. Control samples were also placed on ice but not irradiated. Irradiated and nonirradiated cells were then washed in fresh medium and seeded in 75 cm^2 cell culture flasks. Cells were harvested after 72 hours in culture to determine cell surface mesothelin expression by flow cytometry using K1 antibody.

Animals. Four- to 6-week-old female athymic nude mice were purchased from the National Cancer Institute-Frederick Animal Production Area (Frederick, MD) and housed in microisolator cages for the course of the experiment. The research protocol was approved and the mice were maintained as per the institutional guidelines of the NIH.

A431-K5 xenografts in nude mice. Tumors were implanted by injecting mice in the right flank s.c. with 3 × 10^6 A431-K5 cells in 0.2 mL DMEM with 2 mol/L l-glutamine 13 to 15 days before receiving treatment. Tumors were measured every 2 to 4 days and tumor volumes were calculated using the formula: tumor volume = length × width^2 × 0.4.

Tumor irradiation. Once the tumors reached the appropriate size, the mice were randomized to radiation alone or to the combination of radiation plus SS1P. The mice received a single dose of focused radiation to the tumor. Mice were briefly restrained in a Plexiglas jig with an opening that exposed only the tumor.

Treatment of A431-K5 xenografts with low-dose radiation and SS1P combination. In these experiments, A431-K5 tumor-bearing mice were randomized into four treatment groups: (a) the control group was treated with PBS i.v. q.o.d. × 3 doses, (b) the group treated with SS1P alone received 0.2 mg/kg SS1P i.v. q.o.d. × 3 doses, (c) the group treated with radiation alone received a single dose of 5 Gy focused radiation to the tumor, and (d) the combination treatment group received SS1P 0.2 mg/kg i.v. q.o.d. × 3 doses with the first dose given 6 to 8 hours after administration of a single dose of 5 Gy focused radiation to the tumor. This experiment was repeated four times with 4 mice per treatment group in two of the experiments and 10 mice per group in one of the experiments. The activity of these different treatment regimens was tested against both small and large volume tumors.

Statistical analysis. The primary objective of the study was to determine if the use of SS1P plus radiation would be associated with a lengthening of the time to tumor volume doubling or tripling compared with controls, or each agent alone, and whether SS1P alone or radiation alone was associated with lengthening of the doubling or tripling time compared with a control group. Because both high-dose and low-dose SS1P and radiation combinations were used, analyses were done according to the dose level used.

A series of actuarial analyses was done using the Kaplan-Meier method to test whether the double or tripling time of the tumor volume was the same among the treatments. Because the exact doubling or tripling time was not measured (only the tumor volume at various time points was measured), the doubling or tripling time of the tumor volume was calculated for each mouse using linear interpolation. If the doubling or tripling time was never reached for a particular mouse, then that observation was censored at the last time period. We tested for homogeneity of doubling or tripling time curves with a log-rank test.

To ensure that doubling or tripling times of each experiment were not dependent on any differences in baseline volume among the arms for each of the two baseline measurements, we first tested for a difference among the baseline treatment means by performing a repeated-measures one-way ANOVA. By this analysis, we determined that baseline tumor volumes never differed significantly among treatments by experiment.

We also did actuarial analyses to test whether the initial tumor volume within an arm would have affected the doubling or tripling time. For experiments using low-dose radiation plus SS1P (combination of four separate experiments) and experiments using high-dose radiation plus SS1P (combination of three separate experiments), the baseline data for each analysis were divided into four groups using the quartiles as dividing points. We did a separate analysis for each of the 2 baseline days as well as an analysis of the average of the 2 baseline days. Because we found no strong evidence that the initial tumor volume affected either the doubling or tripling time, all remaining analyses of the doubling or tripling times were done without regard to the baseline data.

Finally, if the log-rank test *P* < 0.01, we also did all pair-wise comparisons between treatments curves and report unadjusted log-rank test *Ps*.

Results

Effect of radiation on mesothelin expression by A431-K5 cells. We have shown previously that sublethal doses of radiation can increase cell surface mesothelin expression in some cancer cell lines (20). To determine whether radiation altered mesothelin expression of A431-K5 cells, these cells were irradiated and mesothelin expression was determined by flow cytometry. As shown in Fig. 1, there was no difference in the percentage of cells expressing mesothelin after radiation. However, there was significant increase in the levels of mesothelin expression per cell as determined by the increase in mean fluorescence intensity from 99 without radiation up to 261 after 10 Gy radiation.
Effect of single-dose focused radiation on growth of mesothelin-expressing tumor xenografts. To determine the appropriate radiation dosage, we completed a pilot study testing the activity of a single dose of tumor-directed radiation against A431-K5 tumor xenografts. Athymic nude mice (four mice per group) bearing \( \sim 200 \text{ mm}^3 \) tumors were treated with 5, 10, or 15 Gy radiation. A dose-dependent antitumor effect was observed (although no complete remissions were seen and the tumors recurred in all the groups; data not shown). For the combination studies, we initially started with the lowest dose of radiation (5 Gy) that had some antitumor activity. Subsequently, we also tested the hypothesis whether a higher dose of radiation (15 Gy) that is well tolerated by mice can result in enhanced antitumor activity when combined with SS1P. Regarding SS1P, we chose two doses (0.2 and 0.3 mg/kg) that are both below the dose (0.4 mg/kg) that can be safely given to athymic nude mice.

Effect of combined administration of low-dose radiation and SS1P on tumor growth. The activity of combined treatment with low-dose radiation and SS1P on tumor growth of mesothelin-expressing A431-K5 tumors was tested in four separate experiments and the results of a typical experiment are shown in Fig. 2. In this experiment, the median size of the tumors in the different groups at the time of treatment initiation was 459 mm\(^3\) (25%, 358; 75%, 563). As can be seen in Fig. 2, mice that received the combination of 5 Gy radiation to the tumor followed by 0.2 mg/kg SS1P i.v. q.o.d. \( \times \) 3 doses had much smaller tumors compared with mice that received no treatment or were treated with SS1P or radiation alone. In addition, the time for tumors receiving radiation plus SS1P to reach 2\( \times \) (doubling time) or 3\( \times \) (tripling time) the median baseline tumor size was significantly prolonged compared with control, SS1P, or radiation alone treated mice.

The effect of the different treatment arms on the growth of tumors was determined, using an actuarial analysis, by measuring the time until a tumor doubles or triples in volume from baseline (day 0). Data from four separate experiments using the same dose of SS1P and radiation either alone or in combination were combined for one actuarial analysis (Fig. 3). Actuarial analyses were done using the Kaplan-Meier method to test the hypothesis that either the doubling or tripling time of the tumor volume was the same among the treatments. Figure 3 shows the Kaplan-Meier curve for tumor doubling time from baseline. In case of either tumor doubling or tripling curves, the overall log-rank test \( P < 0.0001 \), indicating that the curves differ significantly from one another. For both tumor doubling and tripling, all the curves were significantly different from each other \( (P < 0.01) \), except for the SS1P and radiation curves. Actuarial analysis of the baseline data showed no evidence that initial tumor volume affected either the doubling or tripling time.

Table 1 summarizes the median tumor doubling and tripling times for the different treatment groups in experiments using low-dose radiation plus SS1P. As shown, the median tumor doubling and tripling time of 17.4 and 24.8 days, respectively, is significantly longer than that in the control, SS1P, or radiation alone treated mice.

Effect of combined administration of higher-dose radiation and SS1P on tumor growth. The antitumor effect of combined therapy with radiation and SS1P was also evaluated using a treatment regimen of higher-dose radiation and SS1P. The experimental design was similar to that described above, except that the dose of radiation was increased to 15 Gy and SS1P was given at a dose of 0.3 mg/kg q.o.d. \( \times \) 3 doses. This experiment was repeated three separate times and the activity of this regimen using mice bearing either small or large tumors at the time of treatment initiation is shown in Fig. 4. As shown in Fig. 4A, the median tumor volume in mice with small tumors at the start of treatment was 192 mm\(^3\) (25%, 148; 75%, 274). In mice that received the combination of radiation and SS1P, there was significant tumor shrinkage compared with tumors in the control group or in mice treated with SS1P or radiation alone. In the combination treatment group 3, mice had complete tumor regression. In one mouse, the tumor regression was noted on day 8 of treatment and persisted until follow-up on day 68. Two other mice had complete tumor regression lasting 6 and 4 days.

**Fig. 1.** Flow cytometry analysis of mesothelin surface expression on A431-K5 cells without radiation (0 Gy) and following radiation (10 Gy). Results are expressed as percent positive cells (mean fluorescence intensity).

**Fig. 2.** Antitumor activity of low-dose radiation and SS1P against established mesothelin-expressing A431-K5 tumor xenografts in nude mice. Horizontal lines, median baseline (BL) tumor size as well as 2\( \times \) and 3\( \times \) of the median baseline value. The median tumor size in the different treatment arms at the time of treatment initiation was 459 mm\(^3\). A single dose of 5 Gy radiation was given on day 0 in the radiation alone (RT) and radiation plus SS1P groups. SS1P (0.2 mg/kg) was given i.v. on days 0, 2, and 4 to mice in the SS1P alone and SS1P plus radiation group.
The antitumor activity of higher-dose radiation and SS1P was also evaluated using mice with very bulky tumors at time of treatment initiation. As shown in Fig. 4B, the median tumor volume of mice in the different treatment arms was 440 mm$^3$ (25%, 315; 75%, 563). Mice that received the combination of tumor-directed radiation followed by SS1P had much greater shrinkage of the tumor compared with tumors in mice treated with SS1P or radiation alone. However, unlike the similar experiment in mice with smaller tumors at start of treatment (Fig. 4A), no complete tumor regressions were noted in the combined SS1P and radiation group. This is most likely because the tumors were much bigger at the start of treatment. As shown in Fig. 4A and B, the time to median tumor size $2^{\times}$ and $3^{\times}$ the baseline median tumor volume was prolonged in mice that received the combination treatment with radiation plus SS1P compared with other treatment groups.

An actuarial analysis was done using the Kaplan-Meier method to test the hypotheses that either the doubling or tripling of the tumor volume was the same among the four treatment groups (control, SS1P or radiation alone, and SS1P in combination with radiation) in experiments ($n = 3$) where high-dose radiation and SS1P was used. The homogeneity of doubling or tripling time curves was tested using a log-rank test. Figure 5 represents the Kaplan-Meier curve for tumor doubling from baseline. Both tumor doubling and tripling time were significantly prolonged in mice treated with SS1P in combination with radiation compared with control or mice treated with SS1P or radiation alone.

Table 2 summarizes the median doubling and tripling times among the different treatment groups in experiments in which high-dose radiation and SS1P was used. The median doubling time of 40.1 days and median tripling time of 47 days in mice treated with radiation plus SS1P is greater than that observed in mice in the other treatment groups.

**Discussion**

The combined antitumor activity of radiation and the antimesothelin immunotoxin SS1P was tested in vivo using a human tumor xenograft expressing mesothelin. Our results show that low doses of tumor-directed radiation (5 Gy) combined with systemically given SS1P result in increased antitumor activity compared with radiation or SS1P alone. This activity was seen against well-established s.c. tumors with a median tumor size of 459 mm$^3$ at time of treatment initiation. The combined treatment of SS1P and radiation significantly prolonged both the tumor doubling and tripling times compared with the control, SS1P alone, or radiation alone groups.

Our experiments combining higher doses of radiation (15 Gy) with SS1P showed similar results. This effect was seen regardless of whether the median tumor volume at treatment initiation was relatively small (192 mm$^3$) or large (440 mm$^3$). As with the low-dose radiation group, the tumor doubling and tripling times for mice receiving 15 Gy radiation in combination with SS1P were significantly prolonged compared with the control, SS1P alone, or radiation alone groups. All mice tolerated the treatment well with no deaths observed in mice that received the combination treatment.

The mechanism employed by the combinatorial treatment of SS1P and radiation remains unknown; the two agents achieve cytotoxicity through different means. SS1P, an immunotoxin, acts by inhibiting ADP-ribosylation of elongation factor 2, resulting in the inhibition of protein synthesis and cell death (21, 22). Unlike SS1P, radiation mediates cell death by damaging a cell's DNA (23). In addition, damage to the tumor microvasculature also seems to play an important role in tumor response to radiation (24).

Prior studies of radiation and monoclonal antibodies in mouse models have shown increased antibody uptake in tumors following radiation (25). It is possible that the effect
of radiation on the vascular endothelial cells may improve the delivery of SS1P to the tumor, thereby enhancing its antitumor activity. Another proposed synergistic mechanism is the up-regulation of the tumor antigen mesothelin. Previously, it has been shown that sublethal doses of radiation increase mesothelin gene expression in the human colon carcinoma cell line (WiDr; ref. 19). In addition, in vitro studies have also shown that radiation results in increased expression of mesothelin on the cell surface (20). Treatment of the ovarian cancer cell line OVCAR-3 and the pancreatic cancer cell line CFPAC-1 showed increased mesothelin expression after treatment with 10 Gy radiation. This increased mesothelin

![Fig. 4. Antitumor activity of high-dose radiation and SS1P against established small (A) or large (B) mesothelin-expressing A431-K5 tumor xenografts in nude mice. Horizontal lines, median baseline tumor size as well as 2× and 3× of the median baseline value. The median tumor volume in the different treatment arms at the time of treatment initiation was 192 mm³ in experiment A and 440 mm³ in experiment B. A single dose of 15 Gy radiation was given on day 0 in the radiation alone and radiation plus SS1P groups. SS1P (0.3 mg/kg) was given i.v. on days 0, 2, and 4 to mice in the SS1P alone and SS1P plus radiation group.](image)

![Fig. 5. Kaplan-Meier curve for tumor doubling time from baseline of high-dose radiation and SS1P against established mesothelin-expressing A431-K5 tumor xenografts in nude mice. Median doubling time for control group, radiation alone (15 Gy), SS1P alone (0.3 mg/kg), and radiation in combination with SS1P (15 Gy radiation plus 0.3 mg/kg SS1P). Top right, pair-wise log-rank test two-tailed P's for tumor doubling.](image)
expression was associated with increased susceptibility to lysis by mesothelin-specific T cells (20). Our results also show that radiation increases the cell surface expression of mesothelin in A431-K5 cells that were used in our animal studies. It is possible that the increased antitumor activity of SS1P in combination with radiation is partly due to enhanced tumor mesothelin expression, making the cells more sensitive to SS1P treatment.

SS1P is currently undergoing clinical evaluation in patients with mesothelin-expressing cancers. Two phase I studies of SS1P, one using a 10-day continuous infusion schedule and the other using a bolus i.v. injection q.o.d. × 3 doses, have been recently completed (26, 27). These studies show that SS1P is well tolerated with antitumor activity observed in several heavily pretreated patients. Clinical studies of SS1P in pancreatic cancer are especially appealing because healthy pancreatic tissue does not express mesothelin, whereas all pancreatic adenocarcinomas highly express mesothelin (15, 16). Given the efficacy of SS1P plus radiation in animal models of mesothelin-expressing cancers, this combination could be potentially useful for treating patients and needs to be studied in well-designed clinical trials.

References

Table 2. Median tumor doubling and tripling times in mice treated in the high-dose radiation and SS1P combination experiments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median doubling time (d)</th>
<th>Median tripling time (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.0</td>
<td>8.0</td>
</tr>
<tr>
<td>SS1P</td>
<td>10.1</td>
<td>15.3</td>
</tr>
<tr>
<td>Radiation</td>
<td>24.1</td>
<td>32.8</td>
</tr>
<tr>
<td>SS1P + radiation</td>
<td>40.1</td>
<td>47.0</td>
</tr>
</tbody>
</table>

Cancer Therapy: Preclinical
Tumor-Directed Radiation and the Immunotoxin SS1P in the Treatment of Mesothelin-Expressing Tumor Xenografts

Raffit Hassan, Juanita Williams-Gould, Seth M. Steinberg, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/16/4983

Cited articles
This article cites 26 articles, 13 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/16/4983.full.html#ref-list-1

Citing articles
This article has been cited by 3 HighWire-hosted articles. Access the articles at:
/content/12/16/4983.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.