Tumor Irradiation Enhances the Tumor-specific Distribution of Poly(l-glutamic acid)-conjugated Paclitaxel and Its Antitumor Efficacy

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ABSTRACT

The poly(l-glutamic acid)-paclitaxel (PG-TXL) conjugate has been shown to exhibit significantly greater antitumor activity than conventionally formulated paclitaxel (TXL) against solid tumors (Li et al., Cancer Res., 58: 2404–2409, 1998). Here we report that local tumor irradiation enhanced the distribution of PG-TXL given 24 h later to ovarian OCa-1 carcinoma implanted i.m. in C3Hf/Kam mice. Radiation significantly increased tumor uptake of PG-TXL and tumor vascular permeability, caused elevation of the serum concentration of vascular endothelial growth factor, and arrested OCa-1 cells in the G1 phase of cell cycle. The enhancement factors, as measured by incremental tumor growth delay compared with PG-TXL alone, ranged from 1.36–4.44. Complete tumor regression was also observed at a higher radiation dose (>10 Gy) and a higher PG-TXL dose (>80 mg equivalent TXL/kg). Furthermore, combined radiation and PG-TXL produced a significantly greater tumor growth delay than treatment with radiation and TXL when both drugs were given at the same equivalent TXL dose of 60 mg/kg 24 h after tumor irradiation (enhancement factors, 4.44 versus 1.50). These data suggest that conjugation of TXL to poly(l-glutamic acid) is necessary for improved response and that the supra-additive effect of combined radiation and PG-TXL therapy is due in part to modulation of the enhanced permeability and retention effect of macromolecules by radiation. We propose a treatment strategy combining radiation and macromolecular chemotherapy that may have important clinical implications in terms of scheduling and optimization of the therapeutic ratio.

INTRODUCTION

Many solid tumors have disordered capillary endothelium and are thus more permeable to macromolecules than is normal tissue (1, 2). In addition, because of the paucity of lymphatics in solid tumors, macromolecules tend to persist longer in tumors than in normal tissue. This phenomenon, called the EPR3 effect, is thought to be responsible for the enhanced uptake of macromolecular drugs seen in solid tumors (2). Therefore, the use of water-soluble macromolecular chemotherapeutic agents is an attractive approach to minimizing the systemic toxicity and improving the therapeutic efficacy of anticancer drugs (2–4).

We recently reported a new polymer-drug conjugate, PG-TXL, that is prepared by conjugating TXL with poly(l-glutamic acid) via ester bonds (5). Compared with TXL, PG-TXL exhibits greater antitumor activity against both murine tumors and human tumor xenografts (5, 6). This finding is largely attributable to a significantly higher uptake of PG-TXL (molecular weight, 40,000) than TXL (molecular weight, 854) in tumors.

Because cancer chemotherapy based on the EPR effect is becoming prominent in anticancer drug development, it is important to identify treatment modalities that may facilitate the delivery of macromolecular drugs to tumor tissue with greater selectivity. It has been shown that radiation increases the vascular permeability of solid tumors (7, 8). Moreover, tumor-secreted VEGF, also known as vascular permeability factor, has been identified as a potent vascular permeability enhancer and an angiogenesis promoter (9). These findings prompted us to hypothesize that enhancement of vascular permeability by radiation may cause enhanced tumor uptake of macromolecular drugs, leading to improved antitumor efficacy. The purpose of this study is to determine whether radiation followed by PG-TXL will produce a supra-additive antitumor effect in the treatment of murine ovarian OCa-1 tumor.

MATERIALS AND METHODS

Drugs. TXL (Hande Tech, Houston, TX) was dissolved in cremophor/alcohol (1:1, v/v) at a concentration of 30 mg/ml. The stock solution was diluted with saline (1:4, v/v) immediately before injection. PG-TXL (molecular weight, 36,000–

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3 The abbreviations used are: EPR, enhanced permeability and retention; PG-TXL, poly(l-glutamic acid)-paclitaxel; TXL, paclitaxel; VEGF, vascular endothelial growth factor.

4 C. Li et al., manuscript submitted.
after i.v. injection and is used as a marker for the EPR effect of macromolecules in solid tumors. Evans Blue solution in saline (1 mg/ml) was injected i.v. at a dose of 10 mg/kg into groups of OCa-1 tumor-bearing mice (4–8 mice/group) that were either unirradiated or had been exposed to 15 Gy of local radiation. Sixteen h later, the mice were sacrificed, and the tumors were perfused with saline solution to remove the dye-albumin complex in the vascular lumen. Tumors were removed, weighed, and immersed in 3 ml of formamide (Sigma) and then incubated at 60°C for 48 h to extract the dye. The concentration of the dye was determined spectrophotometrically at 620 nm. Skin specimens were removed and quantified for dye extravasation in the same experiment, and the data were used to normalize tumor accumulation of the dye.

Analysis of Serum Levels of VEGF. Concentrations of VEGF in mouse serum were determined using ELISAs according to the instructions of the manufacturer (Oncogene Research Products, Cambridge, MA). Briefly, blood samples (~100 µl) were collected immediately before and 48 h after tumors were irradiated with 10–15 Gy. The blood was centrifuged, and the serum was stored at −20°C. Standards and test samples were dispensed in duplicate into 96-well microtiter plates that had been precoated with polyclonal antibody directed against VEGF. After incubation at room temperature for 2 h and washing away of unbound proteins, a second VEGF-specific antibody conjugated to horseradish peroxidase was added, and the plates were reincubated. After another washing cycle, the wells were incubated with tetramethylbenzidine. The intensity of the color developed, which was proportional to the amount of VEGF present, was measured in a microplate reader (Molecular Devices, Sunnyvale, CA) at dual wavelengths of 450 and 540 nm. The concentration of VEGF in the serum samples was determined by extrapolation from the standard curve determined by the serially diluted VEGF standard.

Flow Cytometry Analysis of Cell Cycle. OCa-1 tumors were collected 24 h after the tumors were irradiated with 15 Gy and transferred to Petri dishes with 10 ml of basic MEM with 5% fetal bovine serum. Cells were released by cutting the tumor in half and scraping the surface. The cell suspension was filtered through a 53-µm nylon mesh (Spectrum Laboratories, Laguna Hills, CA) and washed with the culture medium. The cell concentration was then adjusted to 1–3 × 10⁶ cells/ml, and cells were stained with fluorochrome buffer (0.1% sodium citrate, 0.3% Triton X-100, 20 µg/ml RNase, and 50 µg/ml propidium iodide) in the dark at 4°C overnight. The cellular content was analyzed using Coulter SL flow cytometer (Coulter, Miami, FL).

Data Analysis. Student’s t test was used to compare differences in the tumor uptake of PG-TXL and Evans Blue dye and differences in the percentages of cells in various phases of the cell cycle between irradiated and nonirradiated tumors. A value of P < 0.05 was considered significant.

RESULTS
Tumor Irradiation Enhanced the Antitumor Activity of PG-TXL. To determine whether tumor preirradiation enhances the tumor response to PG-TXL, mice bearing 8-mm-diameter OCa-1 tumors were divided into groups of six mice each and treated with saline, radiation alone, drugs alone, or
radiation plus drugs. In the first experiment, mice received a single dose of 15 Gy, followed 24 h later by i.v. injection of either PG-TXL or TXL at an equivalent TXL dose of 60 mg/kg. Tumor growth delay, i.e., the time in days needed for tumors to grow from 8 to 12 mm, was used as the treatment end point. Both PG-TXL and TXL at the equivalent TXL dose of 60 mg/kg were effective as single treatments. When tumors were irradiated 24 h before PG-TXL or TXL injection, the tumor growth was delayed more than the additive effect of individual treatments. However, combined radiation and PG-TXL therapy produced remarkably greater tumor growth delay than treatment with radiation and TXL (Fig. 1). The enhancement factors over the respective individual drug effect were 4.44 and 1.50 for PG-TXL and TXL, respectively (Table 1, Exp. 1).

In the second experiment, mice received a single dose of 15 Gy, followed 24 h later by i.v. injection of either PG-TXL or TXL at an equivalent TXL dose of 60 mg/kg. Tumor growth delay, i.e., the time in days needed for tumors to grow from 8 to 12 mm, was used as the treatment end point. Both PG-TXL and TXL at the equivalent TXL dose of 60 mg/kg were effective as single treatments. When tumors were irradiated 24 h before PG-TXL or TXL injection, the tumor growth was delayed more than the additive effect of individual treatments. However, combined radiation and PG-TXL therapy produced remarkably greater tumor growth delay than treatment with radiation and TXL (Fig. 1). The enhancement factors over the respective individual drug effect were 4.44 and 1.50 for PG-TXL and TXL, respectively (Table 1, Exp. 1).

In the second experiment, the dose response of the chemopotentiating effect of radiation was studied. Mice received single doses of radiation ranging from 5–15 Gy, followed 24 h later by i.v. injection of PG-TXL at an equivalent TXL dose of 80 mg/kg. Radiation alone produced an absolute tumor growth delay of 5.7–15.7 days when the doses were increased from 5 to 15 Gy. At a constant PG-TXL dose of 80 mg equivalent TXL/kg, the combined therapy produced an absolute tumor growth delay of 28.3–63.4 days, yielding enhancement factors of 1.37–2.87 as the radiation dose increased from 5 to 15 Gy. A total of three mice in combination groups (one at a dose of 10 Gy and two at a dose of 15 Gy) had achieved complete pathologically confirmed tumor regression (Table 1, Exp. 2).

**Tumor Irradiation Increased Uptake of PG-TXL.** Because radiation has been reported to increase tumor vascular permeability (8), we hypothesized that the observed increase in chemoresponse in previously irradiated tumors could be due to elevated concentrations of PG-TXL in these tumors. To determine whether prior irradiation affects the tumor uptake of PG-TXL, $[^3]H$PG-TXL was injected into mice with OCa-1 tumors 24 h after irradiation with 15 Gy. Fig. 2 shows the uptake of $[^3]H$PG-TXL in irradiated and nonirradiated tumors determined at 5, 24, and 144 h after injection of $[^3]H$PG-TXL. Irradiated tumors showed a significantly higher uptake of $[^3]H$PG-TXL than did nonirradiated tumors at all three time points. The uptake of $[^3]H$PG-TXL in irradiated tumors was 35%, 28%, and 38% higher than that in nonirradiated tumors at 5, 24, and 144 h after $[^3]H$PG-TXL injection, respectively. In contrast, no difference was found in the uptake of $[^3]H$PG-TXL in muscle tissues taken from the contralateral, unexposed thigh in irradiated and nonirradiated mice (Fig. 2). In addition, no differences were found in the concentrations of $[^3]H$PG-TXL in blood, liver, kidney, and spleen between irradiated and nonirradiated mice (data not shown). Thus, tumor irradiation increased the accumulation of PG-TXL in tumors for at least 6 days.

To determine whether increased uptake of PG-TXL was associated with increased vascular permeability, Evans Blue dye was injected into mice bearing irradiated and nonirradiated tumors, and its uptake in tumors 24 h after irradiation was quantified. The mean uptake of the dye increased from 3.32 ng/g in nonirradiated tumors to 4.18 ng/g in irradiated tumors ($P = 0.025$). Thus, irradiation increased tumor vascular permeability by 26%.

**Serum VEGF Levels Were Elevated in Irradiated Mice.** To examine whether irradiation induced a release of VEGF, we measured the concentrations of VEGF in the sera of mice before
and 48 h after irradiation with 10 and 15 Gy using ELISA. The mean serum VEGF concentrations increased from a pretreatment level of 60.4 ± 13.8 pg/ml to a posttreatment level of 119.5 ± 37.9 pg/ml at 48 h after irradiation (P = 0.005).

Irradiation Arrests Tumor Cells in G1 Phase of the Cell Cycle. To determine whether irradiation increased cellular sensitivity to PG-TXL, we analyzed the cell cycle distribution of OCa-1 tumor cells 24 h after irradiation with 15 Gy. Flow cytometry revealed that the percentage of cells in the G1 phase was 64% in control samples and 77% 24 h after irradiation (P = 0.014). This resulted in a decrease in the percentage of cells in S phase from 26% in control samples to 13% in irradiated samples (P = 0.0105). Irradiation did not cause measurable changes in the population of G2-M-phase cells (9.4% and 9.5% for control and irradiated samples, respectively).

### DISCUSSION

In the treatment of many solid tumors, combining chemotherapy and radiotherapy has produced significantly improved response and survival rates compared to treatment with either modality alone (12, 13). However, local recurrence and systemic relapse remain the major obstacles to cure. There is still a compelling need to establish the most effective sequence of therapies, the best chemotherapeutic agents, and the most appropriate way to deliver these agents. Both in vitro and in vivo studies have demonstrated that TXL given before radiation can strongly enhance tumor radioresponse (14–16). In animal studies, the enhancement factors range from 1.2 to more than 2.0, depending on the tumor type, drug concentration, and dose scheduling (10, 16). However, to our knowledge, no previous in vivo study has examined the ability of radiation to increase the therapeutic effectiveness of TXL. In fact, in vitro experiments in which TXL was added postirradiation demonstrated a subadditive effect of the two modalities (17–19).

The present study attempts to examine whether radiation can be used to enhance tumor response to macromolecular chemotherapy in vivo. We found that treatment with radiation followed 24 h later by PG-TXL caused a significantly longer tumor growth delay than did treatment with PG-TXL alone, implying that radiation increased tumor responsiveness to PG-TXL (Fig. 1). Furthermore, the enhanced tumor growth delay was much greater when radiation was combined with PG-TXL than when it was combined with TXL, suggesting that conjugation to poly(L-glutamic acid) is necessary for improved antitumor efficacy (Table 1). The enhanced chemoresponsiveness was evident at a radiation dose as low as 5 Gy, and the enhancement increased as the radiation dose increased (10, 16).

### Table 1: Effect of radiation on response of OCa-1 tumor to PG-TXL treatment as measured by tumor growth delay

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Treatment</th>
<th>Time in days for tumors to grow from 8–12 mm (mean ± SE)</th>
<th>Absolute growth delay</th>
<th>Normalized growth delay</th>
<th>Enhancement factor (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<tr>
<td>Exp. 1</td>
<td>Saline</td>
<td>14.0 ± 0.4</td>
<td>9.0 ± 1.2</td>
<td></td>
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<tr>
<td></td>
<td>PG-TXL</td>
<td>23.0 ± 1.2</td>
<td>8.0 ± 3.8</td>
<td></td>
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<tr>
<td></td>
<td>TXL</td>
<td>22.0 ± 3.8</td>
<td>15.0 ± 2.0</td>
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<tr>
<td></td>
<td>15 Gy</td>
<td>69.0 ± 6.5</td>
<td>55.0 ± 6.5</td>
<td>40.0 ± 6.5</td>
<td>4.44 (1.42–6.52)</td>
</tr>
<tr>
<td></td>
<td>15 Gy + PG-TXL</td>
<td>41.0 ± 2.9</td>
<td>27.0 ± 2.9</td>
<td>12.0 ± 2.9</td>
<td>1.50 (0.74–2.28)</td>
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<tr>
<td>Exp. 2</td>
<td>Saline</td>
<td>12.8 ± 0.7</td>
<td>16.6 ± 4.4</td>
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<td></td>
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<tr>
<td></td>
<td>PG-TXL</td>
<td>29.4 ± 4.4</td>
<td>5.7 ± 1.3</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>5 Gy</td>
<td>18.5 ± 1.3</td>
<td>11.2 ± 1.6</td>
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<tr>
<td></td>
<td>10 Gy</td>
<td>24.0 ± 1.6</td>
<td>15.7 ± 2.1</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>15 Gy</td>
<td>41.2 ± 3.4</td>
<td>28.3 ± 3.4</td>
<td>22.6 ± 3.4</td>
<td>1.36 (0.85–1.89)</td>
</tr>
<tr>
<td></td>
<td>5 Gy + PG-TXL</td>
<td>63.0 ± 7.8</td>
<td>50.2 ± 7.8</td>
<td>39.0 ± 7.8</td>
<td>2.35 (0.70–3.73)</td>
</tr>
<tr>
<td></td>
<td>15 Gy + PG-TXL</td>
<td>76.3 ± 11.0</td>
<td>63.4 ± 11.0</td>
<td>47.7 ± 11.0</td>
<td>2.87 (0.77–4.99)</td>
</tr>
</tbody>
</table>

* Exp. experiment.

* In Exp. 1, mice were given PG-TXL or TXL at an equivalent TXL dose of 60 mg/kg as a single i.v. injection. In Exp. 2, the dose of PG-TXL was 80 mg equivalent TXL/kg. When taxane and irradiation were combined, local irradiation was delivered 24 h before PG-TXL treatment. The groups consisted of six mice each.

* Absolute growth delay was defined as the time in days for tumors in treated groups to grow from 8 to 12 mm minus the time in days for tumors in the saline-treated control group to grow to the same size.

* Normalized growth delay was defined as the time in days for tumors to grow from 8 to 12 mm in mice treated with the combination of radiation and drug minus the time in days for tumors to grow to the same size in mice treated with the same dose of radiation alone.

* Enhancement factors were obtained by dividing normalized tumor growth delay by the absolute growth delay in mice treated with drug only. CI, confidence interval.

* Analysis was based on five mice; one of six mice had total tumor control at 180 days after the treatment.

* Analysis was based on four mice; two of six mice had total tumor control at 180 days after the treatment.
could be maximized using fractionated clinically relevant radiation doses (i.e., 5 Gy/dose for five doses) and multiple injections of PG-TXL.

Enhancement of chemoresponse to PG-TXL by radiation can be explained in part on the basis of increased tumor vascular permeability induced by local tumor irradiation. As measured by the Evans Blue dye extravasation assay, the vascular permeability increased 28–38% at 24 h after irradiation. Consistent with the role of VEGF as a potent mediator of tumor angiogenesis and vascular permeability, we observed elevated serum VEGF concentrations after irradiation at doses ranging from 10–15 Gy, possibly resulting from tumor response to radiation stress. Interestingly, Gorski et al. (20) reported recently that exposure to ionization radiation induces VEGF expression in Lewis lung carcinomas both in vitro and in vivo and in human tumor cell lines and that blocking the effect of VEGF enhances the antitumor effects of irradiation.

Our results support the view that radiation enhances the EPR effect of macromolecules, causing an increase in tumor vascular permeability and allowing more PG-TXL to enter the tumor. This results in a high and prolonged concentration of PG-TXL in surviving tumor cells. The amount of TXL per viable tumor cell was higher as the radiation dose was increased because fewer viable cells remained in the tumor after higher radiation doses. This could explain the observed increase in enhancement factor values as the radiation dose was increased (Table 1). In a previous study, we demonstrated that increased tumor uptake of PG-TXL and sustained release of TXL from PG-TXL in the tumor were responsible for the enhanced antitumor activity of PG-TXL compared with TXL formulated in a cremophor/alcohol vehicle.4 Both this previous study and the current study reached the same conclusion: that the EPR effect is an important mechanism through which use of macromolecular chemotherapeutic agents enhances antitumor efficacy and improves the therapeutic index.

Radiation treatment of OCa-1 tumor in the present study also caused cell cycle redistribution and resulted in a significant increase in the percentage of G1 cells at the time of PG-TXL administration (24 h after irradiation). Talwar and Redpath (18) examined the interaction of radiation and TXL in HeLa cells using a protocol in which cells were exposed to 7 Gy and then treated with TXL after a variable interval (0–24 h). The authors analyzed their data in terms of redistribution of cells in the phases of the cell cycle and concluded that subadditive, additive, and supra-additive interactions can be observed, depending on the treatment schedule and the corresponding cell cycle distribution. Maximum cell killing occurred when the percentage of cells in G1 phase was at a minimum at the time of TXL treatment (18). According to the findings of Talwar and Redpath (18), the schedule used in the present study should have resulted in reduced efficacy of PG-TXL and TXL. However, our data showed potentiation of the efficacy of TXL (enhancement factor, 1.50) and a remarkably strong potentiation of the efficacy of PG-TXL (enhancement factor, 4.44). Therefore, the radiation-induced enhancement of OCa-1 response to PG-TXL and TXL observed in the in vivo setting cannot be explained on the basis of radiation-induced cell cycle redistribution. Obviously, additional detailed studies are needed to clarify the contradictory results between in vitro and in vivo findings.

In summary, our data suggest that radiation may be used to modulate tumor vascular permeability and the EPR effect of macromolecular chemotherapeutic agents. The treatment strategy that combines radiotherapy and macromolecular chemotherapy as demonstrated in the present study may have important clinical implications in terms of treatment scheduling and optimization of the therapeutic ratio. Additional studies are needed to determine whether enhancement of the EPR effect by radiation also occurs in other tumors and with other macromolecular chemotherapeutic agents.

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