Antitumor Activity of Poly(L-glutamic acid)-Paclitaxel\(^1\) on Syngeneic and Xenografted Tumors\(^2\)

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ABSTRACT

Poly(L-glutamic acid)-paclitaxel (PG-TXL) is a new water-soluble paclitaxel derivative that has shown remarkable antitumor activity against both ovarian and breast tumors. The purpose of this study was to test whether the antitumor efficacy of PG-TXL depends on tumor type, as is the case for paclitaxel, and to test whether paclitaxel-resistant tumors could be responsive to PG-TXL. We evaluated the therapeutic activity of PG-TXL against four syngeneic murine tumors (MCA-4, MCA-35, HCa-1, and FSA-II) inoculated i.m. into C3Hf/Kam mice, a human SKOV3ip1 ovarian tumor injected i.p. into nude mice, and a human MDA-MB-435Lung2 breast tumor grown in the mammary fat pad of nude mice. Two paclitaxel-responsive murine tumors, MCA-4 and MCA-35, showed significant growth delay with a single i.v. injection at its maximum tolerated dose of 160 mg of equivalent paclitaxel/kg or even at a lower dose of 120 mg of equivalent paclitaxel/kg. The other two murine tumors, HCa-1 and FSA-II, did not respond particularly well to either of the two agents, although significant growth delay was observed for both tumors with PG-TXL. In mice with SKOV3ip1 tumors, the median survival times for mice treated with PG alone and PG-TXL at doses of 60 or 120 mg of equivalent paclitaxel/kg were 43, 61, and 75 days, respectively; no survival difference was found between paclitaxel-treated and Cremophor vehicle-treated mice. In mice with MDA-MB-435Lung2 tumor, PG-TXL at a dose of 120 mg of equivalent paclitaxel/kg produced regression of the tumor in 50% of the animals, and in the remaining mice, micrometastases in the lung were found only in 25% of the animals. In comparison, treatment with paclitaxel at 60 mg/kg did not result in tumor regression, and the rate of lung metastases was 42%. These results clearly demonstrate that PG-TXL has significant therapeutic activity against breast and ovarian tumors tested in this study. Future studies to elucidate the mechanism of action of PG-TXL and to assess its clinical applications are warranted.

INTRODUCTION

Taxanes represent a new class of antitumor agents that exert their action by promoting tubulin polymerization and microtubule assembly. Paclitaxel, the first taxane used in clinical practice, has shown significant antitumor activity in patients with ovarian cancer, breast cancer, head and neck cancer, non-small cell lung cancer, and sarcoma (1, 2). Because paclitaxel has limited solubility in water, it is presently formulated as Taxol\(^3\), a concentrated solution containing 6 mg of paclitaxel/ml of Cremophor EL (polyoxyethylated castor oil) and ethanol (50% v/v) that must be further diluted before administration (3). Several toxic effects have been attributed to Cremophor, including serious hypersensitivity reactions (4, 5). Present clinical protocols call for infusion of paclitaxel over 3–24 h and premedication with corticosteroids and antihistamines (1).

Conjugation of chemotherapeutic agents to water-soluble macromolecular carriers is an alternative approach to improving the solubility of the agents. In addition, polymer-drug conjugates may provide desirable pharmacokinetics and an improved therapeutic index (6). We have recently reported a highly water-soluble polymer-paclitaxel conjugate that uses PG\(^4\) as the polymeric carrier (7). PG-TXL has water solubility of 20 mg of equivalent paclitaxel/ml of Cremophor EL (polyoxyethylated castor oil) and ethanol (50% v/v) that must be further diluted before administration. PG-TXL has demonstrated significant antitumor activity against both breast and ovarian tumors tested in this study. Future studies to elucidate the mechanism of action of PG-TXL and to assess its clinical applications are warranted.

MATERIALS AND METHODS

Materials

Paclitaxel was obtained from Hande Tech. (Houston, TX). It was dissolved in a Cremophor EL vehicle (Cremophor:alco-
Hol = 50:50, v/v) at a concentration of 30 mg/ml. This stock solution was further diluted with saline (1:4, v/v) immediately before injection. PG-TXL was synthesized in our laboratory, as described previously (7). PG-TXL was dissolved in saline at an equivalent paclitaxel concentration of 8 mg/ml and filtered through a 0.22-mm sterile Millex-GV filter before injection. Cremophor EL vehicle [Cremophor:alcohol (1:1) diluted with saline (1:4)] and PG solution in saline (600–800 mg/kg) were used as controls.

**Animals**

Female C3Hf/Kam mice (25–30 g) were bred and maintained in a specific pathogen-free mouse colony in the Department of Experimental Radiation Oncology. Female nude mice (nu/nu; 18–22 g; 6–8 weeks of age) were purchased from Harlan (Indianapolis, IN). All experiments involving animals were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

**Syngeneic Murine Tumors**

Solid tumors were generated in the muscle of the right legs of C3Hf/Kam mice by inoculation of $5 \times 10^5$ viable tumor cells in suspension. The following tumors were studied: mammary carcinomas (MCa-4 and MCa-35); a hepatocellular carcinoma (HCa-1); and a soft-tissue sarcoma (FSa-II). All tumors, originally developed spontaneously, were syngeneic to this strain of mouse. MCa-4 and MCa-35 are responsive to paclitaxel treatment, and HCa-1 and FSa-II are resistant to paclitaxel treatment (8).

The antitumor effects of PG-TXL and paclitaxel were determined by their ability to delay tumor growth. When the tumors had grown to 400–600 mm$^3$, mice were randomly allocated into groups, with each group typically consisting of five mice. A single dose of paclitaxel ranging from 40–80 mg/kg or PG-TXL at an equivalent paclitaxel dose of 40–160 mg/kg (total dose of PG-TXL of 200–800 mg/kg) was given i.v. The MTDoS of paclitaxel and PG-TXL in C3Hf/Kam mice have been determined previously to be 80 mg/kg and 160 mg of equivalent paclitaxel/kg, respectively (7). Tumor growth was determined daily by measuring three orthogonal tumor diameters. Tumor volume was calculated according to the formula $(A \times B \times C)/2$. The effect of treatment on tumor growth was expressed as the AGD, defined as the time in days for tumors in the treated groups to grow from 500 mm$^3$ to 2000 mm$^3$ minus the time in days for tumors in the control saline-treated group to grow from 500 mm$^3$ to 2000 mm$^3$. Mice with HCa-1 and FSa-II tumors were also weighed daily to estimate the effect of treatment on tumor burden.

**Human Tumor Xenografts**

**SKOV3ip1 Ovarian Tumor.** Nude mice received i.p. injections of $1 \times 10^6$ SKOV3ip1 human ovarian cancer cells. On day 8 after tumor injection, mice were treated i.v. with PG alone (control), paclitaxel, or PG-TXL. PG was given at a dose of 600 mg/kg. Paclitaxel was given at a dose of 60 mg/kg. PG-TXL was given as a saline solution at equivalent paclitaxel doses of 60 and 120 mg/kg. Mice were sacrificed when they became moribund because of tumor burden or when they had weight loss of $\geq 25\%$. The experiment was terminated on day 100. Mice sacrificed on day 100 were censored from the survival analysis and median survival calculations. In a separate experiment, PG-TXL at a dose of 120 mg of equivalent paclitaxel/kg was given at 7-day intervals for a total of three injections starting on day 5 after tumor inoculation.

**MDA-MB-435 Breast Tumor.** Nude mice were anesthetized with methoxyflurane inhalation, and a small incision was made in the skin over the lateral thorax. The fat pad was exposed, and MDA-MB-435Lung2 cells (a variant of the MDA-MB-435 human breast cancer cell line, $2 \times 10^6$ cells in 0.1 ml PBS) were injected. The incision was closed with surgical clips, and mice were allowed to recover. When the tumors reached 5 mm in mean diameter (about 27 days after tumor injection), mice received i.v. injections of each drug. Tumors were measured weekly using calipers. Tumors that reached 1.5 cm in diameter were removed surgically. On day 120, all mice were sacrificed, and remaining tumors were removed and weighed. The mice were examined for metastases, and the lungs were processed for histological examination, with single sections of the organs scored for the presence of micrometastases. In another experiment, animals received three injections of PG-TXL, the first given when the tumors reached 5 mm in diameter and the others given at 14-day intervals after that.

**Data Analysis**

Mean differences in the tumor growth delay (number of days required to grow from 500 mm$^3$ to 2000 mm$^3$) were analyzed by Student’s $t$ test. Survival was analyzed using the SAS software package (SAS Institute, Cary, NC), and differences in median survival between groups were analyzed using the Wilcoxon test.

**RESULTS**

**Murine Tumors**

The antitumor effect of PG-TXL against murine tumors was considerably better than that of paclitaxel (Figs. 1–4). However, the magnitude of antitumor activity of PG-TXL varied considerably among tumor types.

**Breast Tumor.** PG-TXL was very efficacious against MCa-4 tumor. At an equivalent paclitaxel dose of 120 mg/kg, total tumor regression was observed for 11 days (from days 8–19 after drug injection) in all animals. At day 21, tumors reappeared; however, their growth rate was slower than that of tumors in the control group (Fig. 1). PG-TXL at 120 mg of equivalent paclitaxel/kg caused an AGD of 39.6 days, whereas paclitaxel at 60 mg/kg caused an AGD of only 4.5 days. It is interesting that MCa-4 has previously shown an AGD of about 14 days when local tumor irradiation (15 Gy) is combined with paclitaxel treatment (60 mg/kg; Ref. 9).

MCa-35 tumor has been shown previously to be nonresponsive to i.v. injection of paclitaxel at 40 mg/kg (8). However, in MCa-35 tumor, paclitaxel at a dose of 80 mg/kg induced a small yet significant growth delay compared with growth in Cremophor-treated controls ($P = 0.0005$; Fig. 2A). PG-TXL at the MTD of 160 mg of equivalent paclitaxel/kg induced a highly significant growth delay compared with growth in saline-treated controls ($P < 0.0001$). The AGD induced by PG-TXL at 160
mg of equivalent paclitaxel/kg was also significantly higher than the AGD induced by paclitaxel at its MTD of 80 mg/kg (16 days versus 7 days; \(P, 0.05\)).

**Hepatocarcinoma and Sarcoma.** For HCa-1 tumor, there was no apparent tumor growth delay in the paclitaxel-treated groups. However, statistically significant growth delay was observed with PG-TXL treatment (Fig. 3B). A physical combination of PG and paclitaxel (80 mg/kg) did not inhibit tumor growth (Fig. 3A). Evidence of treatment effect was also observed in the form of weight loss. Mice in the control group and the paclitaxel-treated group quickly lost weight due to the increased tumor burden. PG-TXL-treated animals maintained their body weight in a dose-dependent manner (Fig. 3C), indicating that the treatment was effective in relieving the tumor burden.

FSa-II tumor showed similar patterns of sensitivity to PG-TXL and paclitaxel (Fig. 4). Both PG-TXL and paclitaxel reduced body weight loss in comparison with weight loss in saline-treated control mice (Fig. 4C). Statistical analysis revealed that all treatments except the Cremophor vehicle caused slight yet significant growth delay as compared with growth in the saline-treated group.

**Human Tumor Xenografts**

Nude mice were less tolerant of PG-TXL and paclitaxel than were C3Hf/Kam mice. At 160 mg of equivalent paclitaxel/kg, 5 of 10 mice died within a week after PG-TXL injection. Therefore, only the dose of \(\leq 120\) mg of equivalent paclitaxel/kg was used in subsequent experiments. One mouse in the PG-TXL group treated with 120 mg of equivalent paclitaxel/kg died with extreme weight loss; no other obvious therapy-related deaths were observed in the PG-TXL-treated mice throughout these experiments. A total of seven mice died immediately after the injection of the Cremophor vehicle (three mice) or paclitaxel (four mice), possibly because of hypersensitivity reactions.

**SKOV3ip1 Ovarian Cancer.** In the first experiment, all drugs were given by a single bolus injection. Compared with PG alone, PG-TXL significantly extended the survival of the mice with i.p. SKOV3ip1 (\(P = 0.0058\) and \(P = 0.0001\) at 60 and 120 mg of equivalent paclitaxel/kg, respectively; Fig. 5B). The median survival times for mice treated with PG alone or PG-TXL at doses of 60 and 120 mg of equivalent paclitaxel/kg were 43, 61, and 75 days, respectively.

In comparison, no survival difference was found between paclitaxel-treated mice and Cremophor vehicle-treated mice (\(P = 0.872\)). The median survival times for the Cremophor and the paclitaxel control groups were 59 and 56 days, respectively (Fig. 5A). Cremophor alone seems to have some impact on the survival time.

The second experiment with SKOV3ip1 used three injections of PG-TXL at 7-day intervals. Repeated injections of PG-TXL extended the survival time of tumor-bearing mice. The
median survival time after three injections of PG-TXL at 120 mg of equivalent paclitaxel/kg was 82 days (Fig. 5C). However, no significant difference was noted between survival after a single injection and multiple injections ($P = 0.837$), although these were not given in the same experiment.

**MDA-MB-435 Breast Tumor.** In mice with MDA-MB-435Lung2 breast tumors, the 60 and 120 mg of equivalent paclitaxel/kg dosage groups of PG-TXL produced regression of the tumor in 20% and 50% of the cases, respectively. At the dose of 120 mg of equivalent paclitaxel/kg, PG-TXL also caused reduction of lung metastases. Of the four animals with progressively growing tumors, only one had micrometastasis in the lung. The size of the metastases varied from microscopic to 1–2 mm in diameter. Fig. 6 shows the histology of lungs from nude mice bearing MDA-MB-435Lung2 tumor. The control groups treated with either PBS, PG, or Cremophor vehicle, or with paclitaxel at 60 mg/kg (MTD: 80 mg/kg) showed extensive metastatic disease in the lungs. In contrast, the lungs of mice treated with PG-TXL showed few metastases or no metastases at equivalent paclitaxel doses of 60 and 120 mg/kg, respectively (Fig. 6).

PG-TXL administered at 60 or 120 mg of equivalent paclitaxel on a 14-day schedule inhibited tumor growth during the experimental period (100 days). Multiple injections seemed to have antitumor activity similar to that of a single injection in terms of reducing the primary tumor mass and the incidence of lung metastases.

**DISCUSSION**

PG-TXL is a water-soluble conjugate of paclitaxel and PG that exhibits excellent antitumor activity, including induction of complete regression, in rodent breast and ovarian tumor models (7). To confirm and extend previously reported activity, a range of tumor models, including paclitaxel-refractory tumor models were tested for their responses to PG-TXL. The objectives of this study were 2-fold: (a) to test whether antitumor efficacy of PG-TXL depends on tumor type, as was the case for paclitaxel; and (b) to test whether paclitaxel-resistant tumors could be responsive to PG-TXL. The antitumor activity of PG-TXL demonstrated in this study is particularly significant because pacli-
Drug as a result of reduced toxicity, increased injection dose, and enhanced permeability and retention effect of the polymer-drug conjugate. However, other mechanisms of action in addition to paclitaxel-induced cell death may also exist. The antitumor effects of paclitaxel against syngeneic murine tumors are due, in large part, to apoptosis (8). Previously, we observed that PG-TXL induced less apoptosis than paclitaxel in a paclitaxel-responsive tumor, OCa-1 (7). Because paclitaxel induces only low levels of apoptosis in MCA-35 and HCA-1 tumors (8), it is plausible that the observed activity for PG-TXL may not be related to a direct cell killing effect of paclitaxel, which is released only very slowly from PG-TXL in the tumor (7). The in vivo activity of PG-TXL may be the result of its complex interactions with various genetic and molecular factors of the tumor (including p53, bcl-2, epidermal growth factor, and HER-2/neu) and with various host factors including angiogenic response, hormones, cytokines, and immune response effector cells. We have begun to study the functionality and temporal expression of some of these factors in response to PG-TXL.

SKOV3ip1 is a human ovarian cancer cell line which overexpresses HER2/neu (10). Amplification and overexpression of the HER-2/neu proto-oncogene has been found to correlate with both poor prognosis and decreased survival in breast and ovarian cancer patients (11, 12). Using human breast tumor lines, Yu et al. (13) demonstrated that cells with HER-2/neu overexpression are highly resistant to paclitaxel treatment. The finding that PG-TXL at 60 and 120 mg of equivalent paclitaxel/kg significantly extended the median survival time of mice with i.p. SKOV3ip1 tumor could have important clinical implications.

The orthotopic human breast MDA-MB435 cancer forms metastases in the lung (14). Price et al. (14, 15) have shown that human breast carcinomas grow better when injected in the mammary fat pad of nude mice than when injected s.c.. These results indicate that the progression and metastatic pattern of tumor cells are influenced by both the biological behavior of the tumor cells and the surrounding microenvironments. Therefore, in examining the antitumor effect of any agent, it is important to consider the impact of relevant organ condition. As shown in Fig. 6, PG-TXL at 120 mg of equivalent paclitaxel/kg induced tumor regression in 50% of animals and reduced the incidence of lung metastases to only 25%. Although the number of mice in the experiment was small, the results suggest that the therapy was effective in controlling both local tumor growth and tumor metastasis. In this study design, it is not possible to distinguish whether the lower incidence of metastasis was a result of reduction in primary tumor mass or a direct effect of PG-TXL on metastases that may have already been established at the time of therapy.

In both human tumor xenograft experiments, a single bolus injection of PG-TXL was given i.v. To resolve the question whether repeated treatments were more beneficial, we also gave mice multiple injections of PG-TXL. The results, shown in Fig. 5, indicate that although repeated injections of PG-TXL at the accumulated dose of 360 mg of equivalent paclitaxel/kg were well tolerated, the activity was similar to that of a single injection of the drug at 120 mg of equivalent paclitaxel/kg. These results, combined with our previous finding that PG-TXL at its maximum dose was most active, suggest that a schedule de-
signed to intensify the initial dose of PG-TXL may be most beneficial in clinical practice.

In conclusion, PG-TXL possesses therapeutic potential against a variety of solid tumors, including paclitaxel-resistant tumors. Additional studies will be required to fully characterize the nature of the tumor uptake of PG-TXL, interaction with various tumor and host factors, and ultimate mechanism of cytotoxicity. The antitumor activity of PG-TXL demonstrated in

Fig. 6 Histological examination of the lungs of MDA-MB-435Lung2 tumor-bearing mice. The lungs were fixed in 10% formalin, and sections were stained with H&E. Representative sections from the three control groups treated with either PBS (top left), PG (top right), or Cremophor vehicle (center left), or with paclitaxel (bottom left) showed extensive metastatic disease in the lungs. In contrast, the lungs of mice treated with PG-TXL at 60 mg of equivalent paclitaxel/kg showed few metastases (center right), and no metastases were found in sections of lung from mice treated with PG-TXL at 120 mg of equivalent paclitaxel/kg (bottom right). Magnification × 70.
tumor models used in this study clearly warrants clinical investigation of this novel agent for possible use against human solid tumors.

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