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RESEARCH ARTICLE

Treatment of disseminated ovarian cancer using nonviral interleukin-12 gene therapy delivered intraperitoneally

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Abstract

Background The poor prognosis associated with ovarian cancer is primarily the result of delayed diagnosis and the lack of an effective treatment for advanced disease. Use of novel immunotherapy strategies are being evaluated that work to enhance local and systemic immune responses against cancer cells and can possibly work together with traditional cytotoxic chemotherapy regimens to produce more effective treatment options.

Methods In the present study, we describe a gene-based therapy whereby the anticancer cytokine interleukin-12 gene (pmIL-12) is formulated with a synthetic polymeric delivery vehicle (PPC) and administered intraperitoneally into a mouse model of disseminated ovarian cancer.

Results The administration of pmIL-12/PPC in tumor-bearing mice was associated with a shift towards a Th1 immune state, including significant increases in murine IL-12 (mIL-12) and interferon (IFN)-γ (mIFN-γ) in ascites fluid, with little change in systemic levels of these proteins. The mIL-12 protein was detectable for several days and could be reintroduced with subsequent injections. We show that treatment delayed the onset of ascites formation and improved survival in a dose-dependent manner. A significant decrease in vascular endothelial growth factor was associated with pmIL-12/PPC delivery and believed to play a predominant role in inhibiting ascites accumulation. Administration of pmIL-12/PPC was associated with minimal toxicity and, when combined with standard chemotherapies, resulted in additive improvement in survival.

Conclusions Taken together, these results suggest that pmIL-12/PPC may be an effective strategy for inhibiting progression of disseminated ovarian cancer and may offer a new option for treatment of advanced disease that can be used to complement standard therapies. Copyright © 2009 John Wiley & Sons, Ltd.

Keywords gene therapy; interleukin-12; nonviral; ovarian cancer; PEI; polymer

Introduction

In North America, ovarian cancer is the fifth most common form of cancer in women, the second most common gynecologic malignant disease and is the leading cause of death among women with gynecologic cancer [1]. The high case-fatality rate is partly the result of delayed diagnosis and the lack of an

effective treatment for women who have advanced disease. Ovarian cancer of the epithelial cells accounts for more than 85–90% of all ovarian cancers. Approximately 75% of women are not initially diagnosed until Stages III or IV, when the disease has spread outside the pelvis and has become symptomatic resulting in 5-year survival rates of 25–41% and 11%, respectively [2,3]. The ultimate goal of treating ovarian cancer is to achieve a complete remission without recurrence. However, median survival rates of ovarian cancer fall rapidly at later disease stages. Therefore, the immediate goal of treatment is to prolong survival in this difficult-to-treat group.

Immunotherapy-based strategies for treating cancer have continued to gain acceptance during the last decade [4,5]. Although traditional chemotherapy regimens kill the fast growing ovarian cancer cells, immunotherapy enhances the local and systemic immune response against the cancer cells and may offer a more targeted and possibly less toxic approach to cancer treatment. A number of immunostimulatory cytokines have been invoked for the treatment of cancer [6], with interleukin (IL)-12 proving to be one of the most active cytokines for the induction of potent anti-cancer immunity [7]. Immunomodulatory properties associated with IL-12 include T-lymphocyte and natural killer (NK) cell proliferation and cytotoxic activation and secretion of interferon (IFN)-γ subsequently leading to tumor inhibition. In addition, the action of IL-12 mediated through IFN-γ has been reported to be strongly antiangiogenic [8,9], offering the possibility of anti-tumor activity through multiple pathways. Use of recombinant human (rh) IL-12 has been shown to induce tumor regression in a number of experimental cancer models [7,10] and clinical responses to rhIL-12 administered by intravenous (i.v.), subcutaneous or intraperitoneal (i.p.) injection have been observed in patients with renal cell carcinoma, melanoma, cutaneous T-cell lymphoma, and peritoneal carcinoma that metastasize from ovarian and other peritoneal malignancies [11–17]. However, despite initial aggressive testing in the clinic, rhIL-12 therapy has not advanced to an approved therapy primarily because of toxicity concerns, which have led researchers to explore alternative means of IL-12 delivery [18,19].

Gene-based cancer therapies that are designed to be delivered locally offer the potential for therapeutic proteins to be expressed specifically in the tumor environment with the goal of achieving increased efficacy at the same time minimizing potential systemic toxicity. Cytokine gene therapies such as IL-12 seek to enhance both local and systemic immune response against tumor cells. It has been demonstrated that, for an optimal effect, cytokines must be present over an extended period of time, which is not achievable with recombinant IL-12 protein because of its short half-life [20,21]. Thus, gene therapy may offer a significant advantage over conventional recombinant protein therapy because of the ability to provide sustained concentrations of IL-12 within the tumor environment at levels sufficient to be therapeutic without causing dose-limiting systemic toxicities. Moreover, sustained local concentrations of IL-12 and IFN-γ after the administration of an IL-12 plasmid may lead to prolonged infiltration of macrophages and other immune cells in the tumor environment, which is not achievable with a bolus injection of the recombinant protein.

Significant progress in the development of nonviral gene carriers has been made in the last decade, producing novel synthetic gene delivery systems that appear to improve upon safety and manufacturing issues associated with viral vectors [22–25]. Using a polymer functionalization approach, we have shown that a small molecular weight polyethylenimine (PEI) of low transfection activity and toxicity can be covalently modified with biocompatible functional groups of polyethyleneglycol (PEG) and cholesterol to significantly increase its *in vivo* transfection activity without significantly increasing toxicity [26]. This functionalized polymer, PPC, is less than 5 kDa and contains a relatively small 1.8 kDa PEI backbone. PPC has the ability to condense DNA through electrostatic interaction with negatively-charged DNA which helps to protect the DNA from degradation by nucleases and can help to prolong DNA integrity in biological systems. Previously, we have shown that PPC used to deliver plasmid encoding for mouse IL-12 (pmIL-12) intratumorally into a mouse model of head and neck cancer can inhibit tumor growth [26]. Using this murine ovarian cancer cell line, another group has also evaluated intratumoral administration of pmIL-12/PPC into subcutaneous solid tumors in combination with an experimental paclitaxel-based chemotherapy. The results from this study also demonstrated tumor inhibition and suggested that some benefit could be obtained by combining the treatments [27]. We have significantly extended those findings in this study by using a regional delivery modality (i.p. injection) for the treatment of a therapeutically relevant model of end-stage disseminated ovarian cancer. We show that pmIL-12/PPC can be repeatedly administered producing high levels of IL-12 protein within the peritoneal cavity and significantly extend survival via inhibition of ascites accumulation in tumor-bearing animals. Survival was further improved by combining pmIL-12/PPC administration with a chemotherapy regimen consisting of Taxol (Bristol-Myers Squibb, New York, NY, USA) and Paraplatin (Bristol-Myers Squibb, New York, NY) without inducing a concomitant increase in systemic toxicity.

Materials and methods

Formation of polymer/DNA complexes

PPC was synthesized as previously described [26] by covalently conjugating a low molecular weight branched polyethylenimine (BPEI; MW = 1800) purchased from Polysciences, Inc. (Warrington, PA, USA), with cholesteryl chloroformate (Sigma-Aldrich, Inc., St Louis, MO,

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USA) and activated methoxypolyethyleneglycol (*N*-hydroxysuccinimidyl ester of methoxypolyethyleneglycol-propionic acid, MPEG-SPA; MW = 550, ester MW 719, obtained from Nektar Therapeutics, Huntsville, AL, USA). For synthesis, BPEI and dry chloroform (Acros Organics, Thermo Fisher Scientific Inc., Waltham, MA, USA) were mixed together to dissolve the BPEI. A solution containing cholesteryl chloroformate and MPEG550-SPA in dry chloroform was then added to the reaction mixture. After extensive stirring, the reaction mixture was concentrated and residual chloroform removed under vacuum. The resulting residue was dissolved with aqueous HCl and again concentrated in vacuum to thick syrup. To isolate PPC hydrochloride and remove the reaction byproducts and unreacted starting materials, the concentrated syrup was mixed with acetone (<0.4% water) and stirred, leading to PPC hydrochloride precipitation as a free-flowing material. After precipitation, the supernatant liquid was discarded. The washing step was repeated to ensure complete removal of residual reaction byproducts and unreacted starting materials. The hygroscopic PPC hydrochloride was dried under vacuum and stored under argon at −20 °C until use. Nuclear magnetic resonance (NMR) was used to evaluate the relative ratios of the functional groups of PPC with a targeted ratio of 2.5 : 1 : 0.6 (PEG : PEI : CHOL). For analysis, the dried polymer was dissolved in D_2O and NMR spectra were recorded at 500 MHz on Varian-500 spectrometer (Varian Inc., Palo Alto, CA, USA). A fluorescently-labeled PPC was synthesized using a similar scheme by incorporating lissamine sulfonylchloride (Sigma-Aldrich), during the conjugation step.

The plasmid encoding pmIL-12 contains both the p35 and p40 genes each under control of a separate cytomegalovirus (CMV) promoter/enhancer. Each gene is followed by the SV40 late polyadenylation signal sequence. The plasmid expresses ampicillin resistance to select for positive bacteria in ampicillin containing medium. The plasmid encoding for human IL-12 (phIL-12) contains both the p35 and p40 genes each under control of a separate CMV promoter/enhancer, with each gene followed by the human growth hormone polyadenylation signal sequence and expresses ampicillin resistance. All plasmid constructs were purified using the Qiagen EndoFree plasmid giga kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's instructions. Polymer DNA complexes were generated by first preparing PPC and plasmid stock solutions in 10% lactose. Plasmid was then complexed with PPC under controlled mixing conditions and incubated for 15 min at room temperature to ensure stabilization of the plasmid/polymer complex. Determinations of the particle size and ζ-potential of the plasmid/polymer complexes were performed by dynamic light scattering using a 90Plus/BI-MAS Brookhaven particle size analyser (Brookhaven Instruments Ltd, Redditch, UK).

Cell culture

The ID8 cell line (gift from Dr Sung Wan Kim, University of Utah) used in our studies is derived from spontaneous *in vitro* malignant transformation of ovarian surface epithelial cells of the C57BL/6 mouse. This cell line has been modified by the stable transformation of vascular endothelial growth factor (VEGF)164 and enhanced green fluorescent protein (GFP) [28]. ID8 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 4% fetal bovine serum, 1× insulin-transferrin-sodium selenite supplement, and 1% penicillin and streptomycin.

In vivo experiments

All procedures involving animals were undertaken in compliance with the federal and local principles and procedures and overseen by the Institutional Animal Care and Use Committee. Disseminated ovarian cancer was created in 8-week-old C57BL/6 mice (Harlan Laboratories, Indianapolis, IN, USA) by injecting i.p. 2.5×10^6 ID8 cells in 500 µl of phosphate-buffered saline. After injection, the animal weights were monitored twice weekly to assess tumor burden and ascites accumulation resulting from progressive peritoneal carcinomatosis. Survival was the endpoint of efficacy studies and moribund animals were humanely euthanized. Administration of pmIL-12/PPC (prepared at nitrogen to phosphate ratio of 11 : 1 and at final DNA concentration of 0.5 mg/ml) was via i.p. injection. In dose–response studies, the plasmid dose was altered by adjusting the injection volumes. Quantification of cytokine protein levels was performed by enzyme-linked immunosorbent assay using commercially available kits for mIL-12, mIFN-γ, hIL-12 and mVEGF (R&S Systems, Minneapolis, MN, USA). Serum samples used for analysis were collected via retro-orbital bleeds. Complete blood count (CBC) and serum chemistry analysis was performed by IDEXX laboratories (West Sacramento, CA, USA) using whole blood and serum samples that had been shipped via overnight courier.

For combination therapy studies involving Taxol and Paraplatin, chemotherapy reagents were diluted in saline to the appropriate concentrations and administered i.v. or i.p. in a 250 µl volume for either two or four cycles (depending on the experiment. Taxol was routinely administered first, followed by Paraplatin with an intervening 2-h delay between reagents.

Reverse transcriptase-polymerase chain reaction (PCR) array

Transcript analysis was performed using a Mouse Th1-Th2-Th3 RT^2 Profiler PCR Array (SuperArray, Frederick, MD, USA). Samples (ascites fluid and ID8 tumors) were collected from the peritoneal cavity of mice 1 day after i.p. administration of pmIL-12/PPC. RNA

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was isolated using TRI Reagent (Molecular Research Centre, Cincinnati, OH, USA) in accordance with the manufacturer's instructions. For ascites, prior to RNA isolation, samples were submitted to a red blood cell lysis procedure using an eBioscience 1× RBC lysis buffer (eBioscience, San Diego, CA, USA) in accordance with the manufacturer's instructions for mouse blood. After RNA isolation, optical density (OD) readings at 260/280 nm were obtained and 25 μg of each sample of purified RNA was subjected to an additional purification using RNeasy Plus Mini Kit (Qiagen) including the optional on-column DNase digestion using the RNase-Free DNase Set. After purification, OD readings at 260/280 nm were obtained and 1 μg of each sample of RNA was used in a first strand synthesis reaction using an RT^2 First Strand Kit (SuperArray). A 102-μl volume of each sample of the first strand synthesis reaction was combined with an RT^2 SYBR Green/ROX qPCR Master Mix then used in the RT^2 Profiler PCR Array.

Statistical analysis

Statistical analysis was performed using SigmaStat 3.0 (SPSS Inc., Chicago, IL, USA). Survival was plotted using a Kaplan–Meier survival curve and statistical significance was determined by the Gehan–Breslow nonparametric analysis of variance (ANOVA). Comparison between two groups was performed by Student's *t*-test between multiple groups one-way ANOVA with Holm–Sidak pos-hoc comparisons. $p < 0.05$ was considered statistically significant.

Results

Initial studies were focused on establishing the expression kinetics of mIL-12 protein and downstream cytokines after mIL-12 gene transfer in animals with advanced disseminated ovarian cancer. The modified ID8 cell line used for these studies has proven to be useful for establishing a syngeneic murine model of disseminated ovarian cancer that results in the accumulation of large amounts of hemorrhagic ascites by approximately 4 weeks after injection. The mIL-12 plasmid when formulated with PPC produced positively-charged nanoparticles having a zeta-potential of approximately 22 mV and a diameter in the range 70–150 nm. These pmIL-12/PPC complexes were then injected i.p. into mice 32 days after tumor implant, a point at which the animals generally have advanced disease with high tumor burden and significant ascites. At various times after pmIL-12/PPC injections, ascites fluid was collected and analysed for mIL-12, mIFN-γ and mVEGF protein expression levels (Figure 1). The results obtained indicated that peak expression levels of mIL-12 are detected 1 day after injection and declined to near baseline by 7 days. Expression of mIFN-γ is delayed with respect to mIL-12, with peak levels occurring 3 days after treatment. The mIFN-γ levels had decreased to 25% of peak by 7 days but were still significantly above background (Figure 1A). The ID8 cell line has been engineered to constitutively express mVEGF to promote aggressive tumor growth, consequently resulting in high mVEGF levels in the ascites. A significant reduction in ascites mVEGF protein levels was noted after treatment with pmIL-12/PPC (Figure 1B) that lasted for approximately 1 week, indicating an inverse relationship to the mIL-12 expression kinetics. Light microscopy analysis of cells harvested from ascites of animals that were administered fluorescently-labeled PPC complexed with mIL-12 indicated that both the GFP expressing ID8 cells and other cells found within the hemorrhagic ascites fluid readily take up the fluorescent PPC and presumably contribute to mIL-12 protein expression (Figure 1C).

To further characterize immunomodulatory changes associated with pmIL-12/PPC administration, a PCR array targeted towards genes involved in immune activation was perfomed on ascites and tumor nodules that were collected from mice 1 day after treatment. A total of 84 genes were examined and transcripts that were shown to be significantly up- or down-regulated compared to samples from untreated animals are presented (Figure 2). As expected, there was a general shift towards Th1 type immune response, with IL-12 being the most significantly upregulated transcript. In ascites (Figure 2A), 54% of the upregulated transcripts were Th1 cytokines or related genes, whereas only 19% were Th2 genes. Only 11% of the down-regulated transcripts were Th1 related and 44% were Th2 related. Two cytokines that are associated with Th17 cells (IL23a and IL17a) were also shown to be down-regulated. A similar response was noted in the tumors (Figure 2B) and, although the extent of the immunomodulatory response is attenuated compared to ascites, the results do indicate that tumors nodules found within the peritoneal cavity also are likely transfected with mIL-12/PPC.

An effective therapy for ovarian cancer is envisioned to involve multiple treatments given over extended periods of time. Thus, we evaluated the ability to achieve high mIL-12 levels after multiple i.p. injections of pmIL-12/PPC. The expression kinetics of mIL-12 and mIFN-γ after a second injection (1-week interval) follow a pattern similar to the initial injection (Figure 3). Both mIL-12 and mIFN-γ were detected systemically in the serum but at levels approximately ten-fold lower than those in the ascites fluid (on a pg/ml basis), suggesting that the mIL-12 expression is primarily restricted to the peritoneal cavity. In a parallel study, PPC formulated phIL-12 was injected i.p. into healthy mice, resulting in expression of hIL-12 in serum (Figure 3B) that followed a similar expression time course to mIl–12. Use of this construct confirms that high plasmid specific IL-12 expression can be produced from delivery of PPC formulated plasmid. The higher systemic hIL-12 expression levels that were observed (relative to mIL-12) probably reflects, in part, the species specificity of IL-12 and that hIL-12 is not readily consumed in the mouse via binding to mIL-12 receptors.

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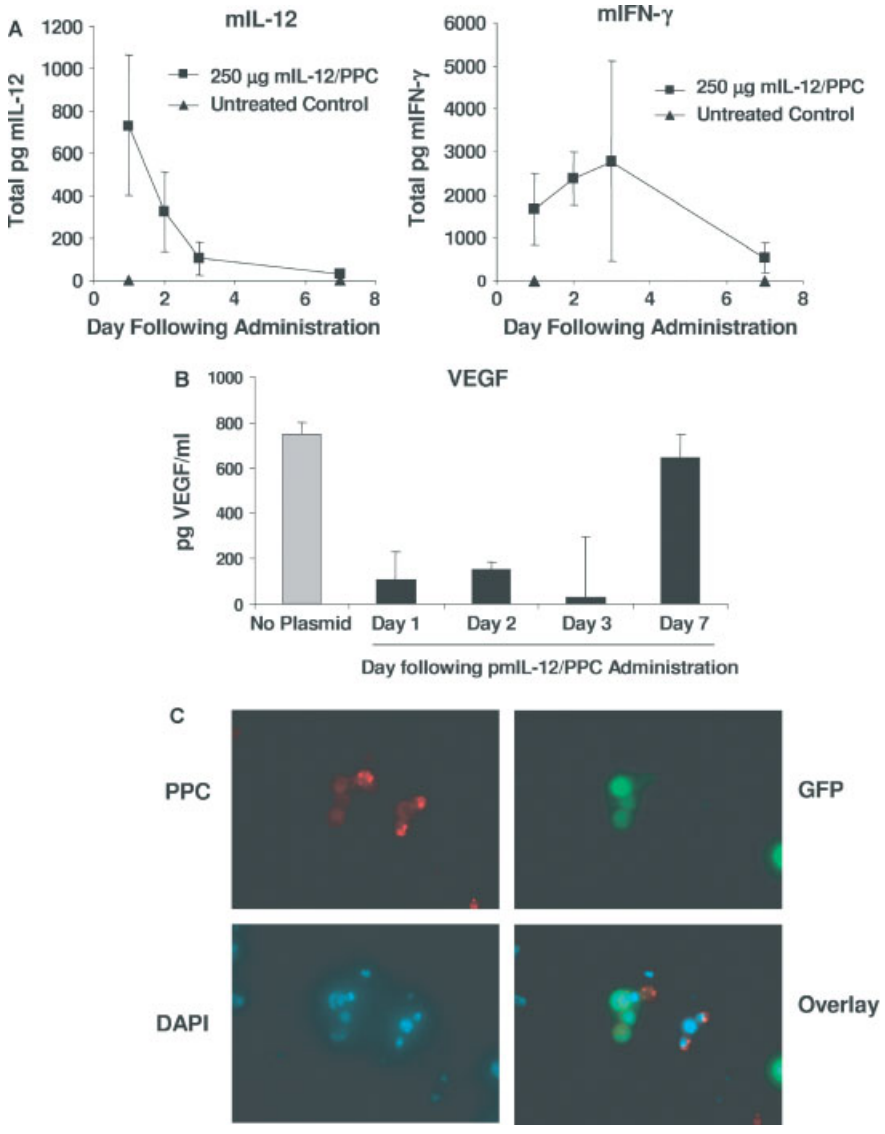

Figure 1. Protein expression levels following a single administration (i.p.) of pmIL-12/PPC into ID8 tumor-bearing mice. (A) Expression time-course of mIL-12 and mIFN-γ in ascites fluid. Values are normalized to total ascites volume. (B) Total protein mVEGF expression levels in ascties over time are shown in comparison to untreated animals. In all cases, values are the mean ± SD, with $n = 5$ for each point. (C) Fluorescent images (×400 magnification) of cells obtained from ascites fluid of animals 24 h after the administration of fluorescently-labeled PPC complexed with mIL-12 plasmid. The panels indicate the localization of PPC (red) in GFP expressing ID8 cells (green) and nuclear staining with DAPI (blue)

To determine whether pmIL-12/PPC could inhibit tumor growth and enhance animal survival in mice with disseminated ovarian cancer, we first characterized the relationship between tumor burden and ascites accumulation. Tumor-bearing animals were injected with pmIL-12/PPC starting 18 days after tumor implant with repeated weekly injections for a total of four pmIL-12/PPC administrations. At various times after treatment, all visible tumors were collected from the peritoneal cavity and weighed and the total ascites volume determined. In untreated animals, rapid tumor growth occurred between 5 and 7 weeks after tumor implant, with progression being delayed in the pmIL-12/PPC treated animals (Figure 4A). A strong correlation existed between tumor burden and accumulated ascites for both treated and untreated animals; however, pmIL-12/PPC treatment resulted in a significant decrease in the slope of the ascites volume/tumor volume relationship curve, indicating a pronounced inhibition of ascites accumulation in these animals at any given tumor burden (Figure 4B). For subsequent studies, we used the accumulation of ascites and resulting weight gain as a surrogate marker of disease progression. Total injected DNA amounts in the range 10–250 µg were evaluated to establish optimum dose levels. The pmIL-12/PPC was administered to mice starting 18 days after tumor implant using a 4-week treatment regimen. As indicated in Figure 4A, pmIL-12/PPC- treated mice have significantly less ascites accumulation 2 days after the final treatment (i.e. 42 days after tumor implant) than untreated animals and a clear dose response was established that appeared to plateau at plasmid dose levels of ≥50 µg. Survival plots

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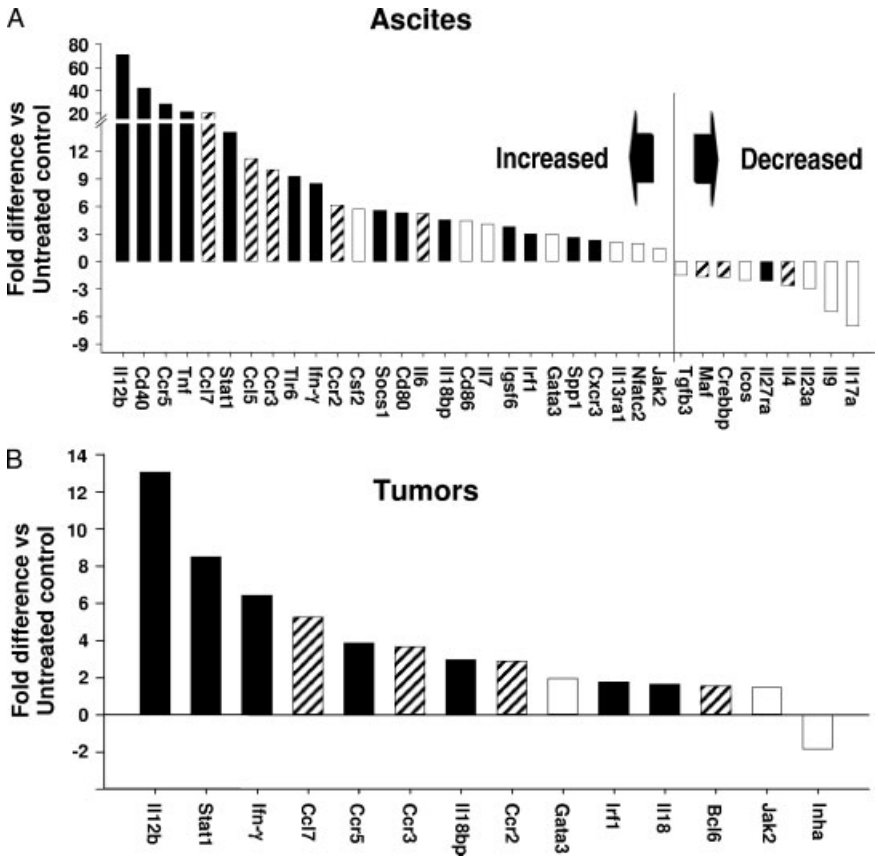

Figure 2. Cytokine transcript array analysis. Ascites (A) and tumors (B) were harvested from ID8 tumor-bearing animals 1 day after i.p. administration of pmIL-12/PPC. Transcript levels are expressed relative to untreated controls. Only transcripts that were determined to be significantly changed ($p < 0.05$) are shown. Dark bars indicate Th-1 associated transcripts. Cross-hatched bars indicate Th-2 associated transcripts. A total of six treated and six untreated control animals were used for analysis

also indicate a dose response where the higher doses prolonged survival compared to untreated and 10 μg treated animals (Figure 5A). In the most effective dosing groups compared to untreated, a significant 53% increase in median survival time was noted.

The initial chemotherapy treatment strategy for patients with ovarian cancer is a combination of platinum- and paclitaxel-based chemotherapeutics. We were interested in evaluating use of pmIL-12/PPC treatment in combination with standard chemotherapies in this murine model of disseminated ovarian cancer. A Taxol/Paraplatin chemotherapy treatment regimen was established in ID8 tumor-bearing mice that was shown to produce therapeutic efficacy without overt toxicity and consisted of Paraplatin 30 mg/kg (body weight) and Taxol (6 mg/kg) given i.v. In combination studies, chemotherapy was initiated 14 days after tumors were implanted and pmIL-12/PPC treatment was started 18 days after tumor implant. A total of nine pmIL-12/PPC and four chemotherapy administrations were given. Relative to untreated controls, both the pmIL-12/PPC and chemotherapy produce similar significant delays in weight gain and increased survival (Figure 6). Relative to either monotherapy, a significant prolongation in animal survival was observed when pmIL-12/PPC and chemotherapy were combined. (Figure 6B).

The potential for systemic toxicity associated with i.p. delivery of pmIL-12/PPC in combination with chemotherapy was evaluated. Serum chemistry and CBC were performed on healthy animals 24 h after a treatment regimen consisting of multiple weekly administrations of pmIL-12/PPC, chemotherapy, or both. Two chemotherapy doses were used, a low dose consisting of 15 mg/kg Paraplatin and 3 mg/kg Taxol and an additional higher dose (40 mg/kg Paraplatin with 8 mg/kg Taxol) that was shown to produce some gross toxicity (data not shown). Notable findings are presented in Figure 7. A dose-dependent decrease in white blood cells and platelets was observed with chemotherapy administration that did not have a significant dependence on pmIL-12/PPC treatment. Small but statistically significant changes in hematocrit levels were also associated with the high-dose chemotherapy groups. In general, all of these changes are consistent with the dose-dependent myelosuppressive effects associated chemotherapy. In addition, there was a small increase in AST levels in groups of animals given chemotherapy, which is a well characterized side-effect of platinum-based therapies [29] with no additional increases associated with pmIL-12/PPC administration. A comprehensive safety/toxicity analysis of four weekly i.p. injections of pmIL-12/PPC (10–250 μg pmIL-12) in healthy animals performed under Good

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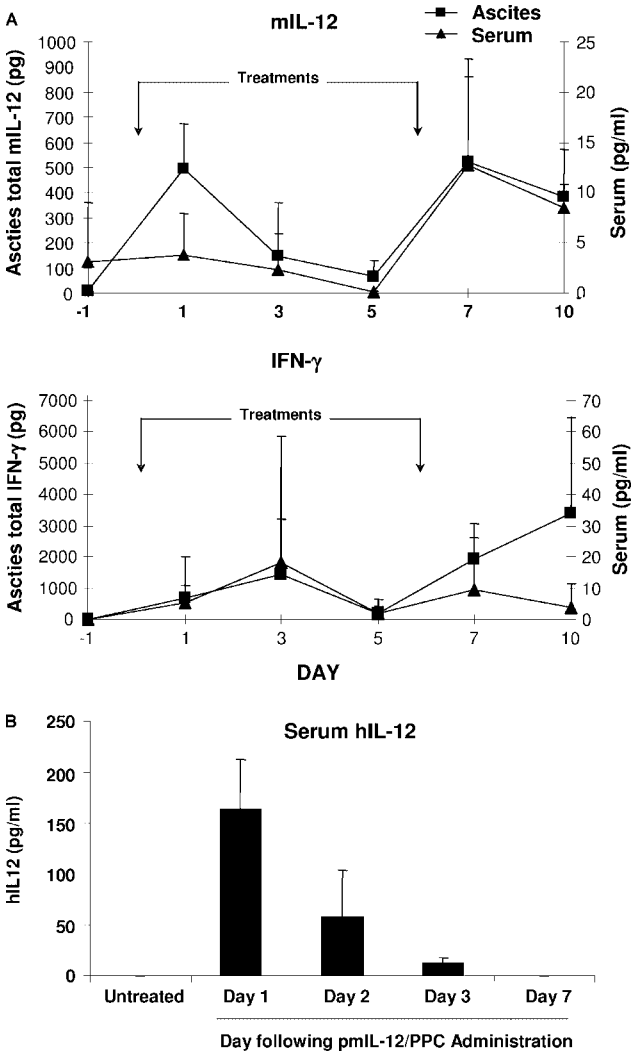

Figure 3. Protein expression levels in serum and ascites after repeated i.p. administration of formulated IL-12 plasmid. (A) Initial injection of pmIL-12/PPC occurred 32 days after tumors were implanted. Protein expression levels of mIL-12 and mIFN-γ in ascites (left axis) and serum (right axis) are shown in each graph. Arrows indicate pmIL-12/PPC administration (day 0 and day 6). (B) Serum expression levels of hIL-12 following i.p. administration of phIL-12/PPC into healthy mice. Values are the mean ± SD, with $n = 6$ for each point

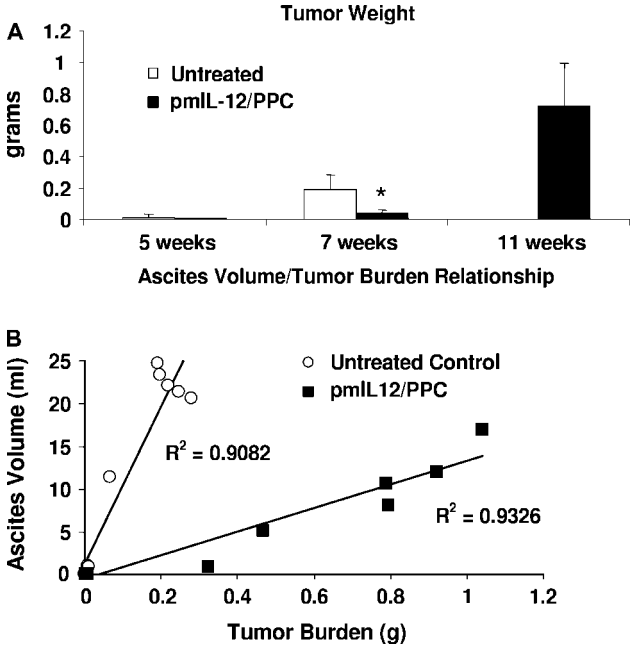

Figure 4. Relationship between tumor burden and ascites accumulation. (A) At selected times, mice were euthanized and all visible peritoneal tumor nodules were removed and weighed. No animals from the untreated group survived to 11 weeks post-tumor implant. Values are the mean ± SD, with $n = 6$ for each group. *Significant difference from untreated animals ($p < 0.01$). (B) Correlation between tumor weight and ascites accumulation in ID8 tumor-bearing mice. Tumors were harvested from animals and weighed at 5 weeks and 7 weeks after being implanted

Laboratory Practices similarly did not show significant evidence of systemic toxicity. The findings from that study were small (but statistically significant) dose-dependent increases in spleen weight after administration. No other organs were affected. There was some indication (not statistically significant) of a dose-dependent decrease in white blood cells, with the loss of absolute lymphocytes being most pronounced. This result may be consistent with myelosuppression associated with recombinant IL-12 protein administration. There were no changes noted in any serum chemistry parameters. Microscopic findings after i.p. dosing consisted of a granulamatous to pyogranulamatos inflammatory reaction occurring in a dose-dependent manner in multiple organs and tissues of the peritoneal cavity. The most severe reaction (still considered mild) was noted in the serosal surface lining in five of eight female mice at the highest dose level. Histopathological observations were diminished in a recovery group of animals (30 days post-injection), suggesting resolution of the lesions.

Discussion

It is widely recognized that a single treatment strategy against cancer is generally ineffective because of the multi-factorial nature of this disease and the use of combinations of more than one drug to maximize anticancer response is increasingly becoming an accepted therapeutic approach [30,31]. Combination therapies employing two cytokines or a cytokine with chemotherapy have also been evaluated and the results obtained often indicate that synergistic effects can be achieved by pursuing this approach [32–34]. Specifically, enhanced efficacy has been demonstrated after a combination treatment of IL-12 gene therapy and IFN-γ gene therapy [32]. Our interests were to evaluate the effectiveness of a combination approach using pmIL-12/PPC gene therapy with chemotherapy in an effort to increase local efficacy of the combined treatments without inducing systemic toxicities that have been associated with recombinant IL-12 protein therapy [35]. We believe this approach could act to maximize the activity of traditional chemotherapies by boosting the immune response at the time of tumor

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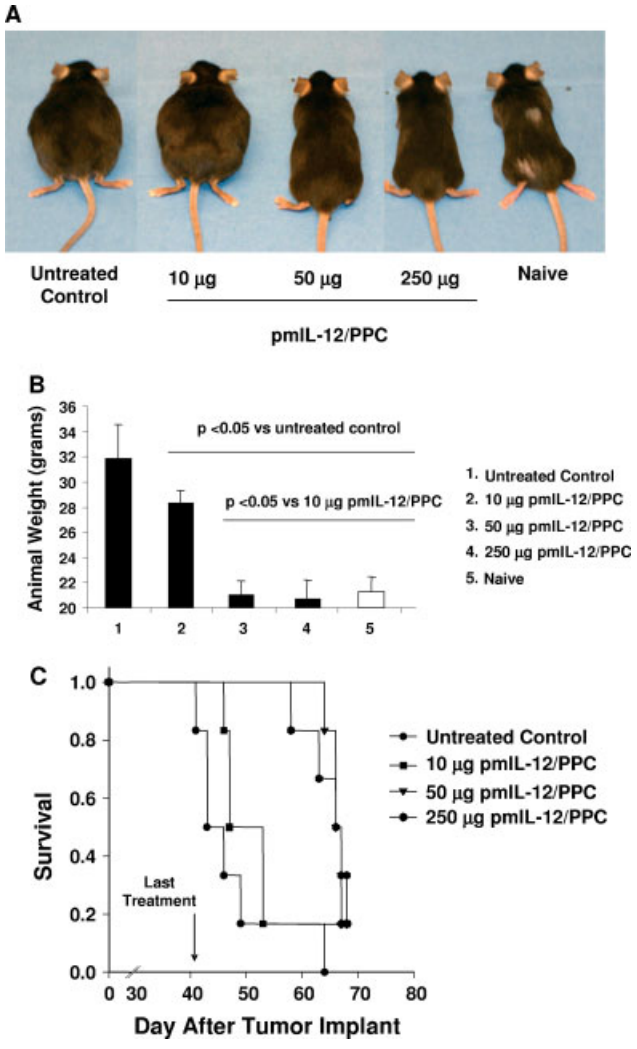

Figure 5. Animal survival following increasing doses of pmIL-12/PPC. Animals were given 4-weekly pmIL-12/PPC administrations (i.p.) starting 20 days after tumor implant. A constant volume was used for all injections in all groups. Alterations in DNA concentration were used to adjust dosages. (A) Photographic montage of animals taken 42 days after tumor implant (1 day after final pmIL-12/PPC treatment) and (B) comparison of animal weights at 42 days after tumor implant. Values are the mean ± SD, with $n = 5$ for each point. (C) Kaplan–Meier survival curves for various treatment groups ($n = 5$ for each group)

cell death antigen release, resulting in a more complete anti-tumor activity.

We have developed a polymeric delivery system that can be safely used for repeated delivery of plasmids, which is composed of a small branched PEI backbone that has been modified by the addition of functional groups (PEG and cholesterol). The addition of the PEG moiety helps to promote the stability of the particle by shielding the surface charge and helping to prevent association with serum proteins, thus resulting in reduced clearance rates. The cholesterol, a naturally occurring lipid, increases complex permeability across cellular membranes and is readily metabolized in the body, making it highly compatible for *in vivo* application. PPC also offers high endosomolytic activity, allowing for DNA escape from the endosome and theoretically leading to higher transfection efficiencies. This effect is believed to be mediated through the many available amines within the PEI polymer backbone that are protonated at the low pH associated with the endosome. The extensive protonation causes a high influx chloride ions across the endosomal membranes followed by osmotic swelling and, ultimately, leads to endosomal release of the DNA polymer complex [36].

In the present study, we show that PPC allowed for efficient i.p. delivery and cellular uptake of plasmid, leading to the production of locally high levels of mIL-12 protein. Elevated protein levels of both mIL-12 and mIFN-γ were detectable for several days after treatment and could be re-introduced by weekly injections, thus providing a pharmacokinetic profile not achievable with recombinant protein administration. Repeated delivery of pmIL-12/PPC resulted in significantly elevated IL-12 and IFN-γ levels in the peritoneal cavity with a much attenuated response observed systemically. This ability to restrict elevated cytokine expression levels to sites where malignancies are present may not only enhance the efficacy of treatment, but also also help to reduce systemic toxicities.

Cytokine array analysis indicated a preferential upregulation of Th1 cytokines and related genes in ascites and tumors after pmIL-12/PPC administration. Immunomodulation strategies that can shift towards a Th1 dominant state overexpressing IL-12 and driving IFN-γ expression may play an important role in promoting a better clinical outcome. Specifically, for ovarian cancer, it has been shown that a significant correlation exists between a low Th1/Th2 ratio defined as IFN-γ*IL-12/IL-6 and poor survival for advanced stage ovarian cancer patients [37] and that higher levels of IL-12 in serum and ascites were correlated with patients having no evidence of disease during follow-up laparotomy [38]. In other malignancies, such as renal cell carcinoma, melanoma and B-cell lymphoma, a more positive clinical outcome is associated with circulating levels of Th1 cytokines or, conversely, a less favorable outcome has been correlated with the expression of Th2 cytokines [39].

Growth factors, and in particular VEGF, are believed to have a key role in the development of ascites formation, a well characterized complication and negative prognostic indicator of ovarian cancer [40,41]. Expression of IL-12 after pmIL-12/PPC administration in our tumor model resulted in a strong inhibition of VEGF protein, which is a result that has also been noted in other studies after the administration of IL-12 into solid tumors [42,43]. This observation would suggest that pmIL-12/PPC administration could have a dramatic impact on ascites formation. Indeed, in our animal model of disseminated ovarian cancer, the high levels of VEGF expressed from the engineered ID8 cells likely contributes to the promotion of leaky vasculature in the peritoneal cavity, resulting in the massive accumulation of hemorrhagic ascites, which is the predominant determinant of animal survival. The clinical importance of ameliorating VEGF was highlighted recently in a study demonstrating that the secretion of

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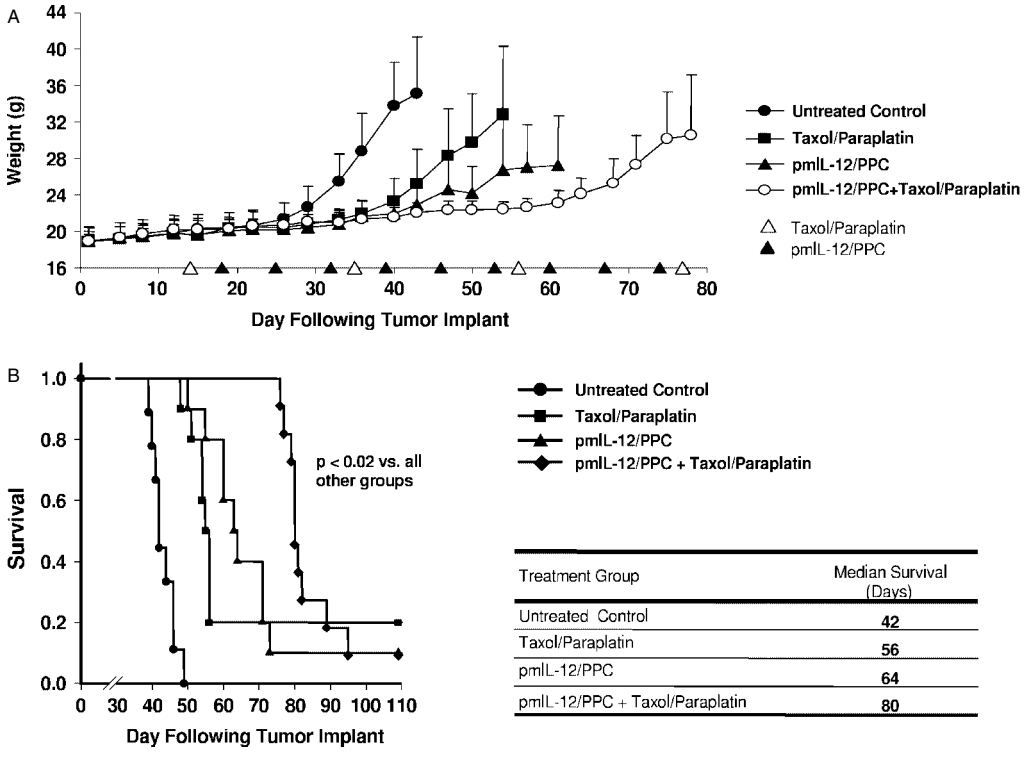

Treatment Group	Median Survival (Days)
Untreated Control	42
Taxol/Paraplatin	56
pmIL-12/PPC	64
pmIL-12/PPC + Taxol/Paraplatin	80

Figure 6. Animal weights and survival analysis of animals given combination treatment of pmIL-12/PPC with a Taxol/Paraplatin treatment regimen. Taxol/Paraplatin, treatments started 14 days after tumor implant. The pmIL-12/PPC was administered at a total dose of 100 μg per treatment starting 18 days after tumor implant. (A) Animal weight over time. Mean values are plotted until the maximum group weight is achieved. (B) Kaplan–Meier curves of treatment groups compared to untreated controls. Median survival times are presented in the table next to the graph, with $n = 10$ for each group

angiogenic growth factors is an important step in the tumorgenicity of epithelial ovarian cancer and can be modulated by blocking VEGF mediated signaling [44].

The administration of pmIL-12/PPC was well tolerated up to doses of 250 μg with no signs of overt toxicity in mice with disseminated ovarian cancer as well as in healthy animals. The safety profile offered by using the i.p. route of administration is an improvement over some earlier IL-12 gene therapy approaches using i.v. administered nonviral (i.e. cationic liposome) delivery systems, which showed not only anti-tumor efficacy, but also significant toxicities, as indicated by sustained elevation in liver transaminases [45]. A similar tolerance to a linear PEI-based system delivered i.p. was reported by Louis *et al*. [46] in female Swiss/nude mice bearing human ovarian adenocarcinomas (SKOV3) tumors. Importantly, the results from our studies show that the addition of pmIL-12/PPC administration to Paraplatin and Taxol treatment did not augment the side-effects associated with the chemotherapy treatments.

Combination approaches using pmIL-12/PPC with a Taxol/Paraplatin chemotherapy treatment regimen significantly improved survival times in treated mice. Another study revealed some anti-tumor activity when using a subcutaneous tumor model and administering (intratumorally) IL-12 plasmid formulated with PPC in combination with biweekly administrations (i.v.) of paclitaxel [27]. Our results significantly advance these earlier findings by evaluating the i.p. delivery of pmIL-12/PPC in a

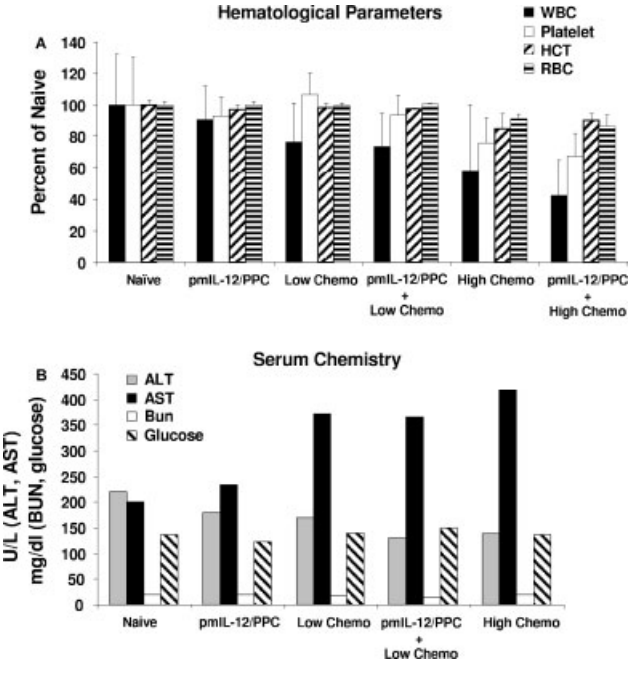

Figure 7. Serum chemistry and CBC analysis. Animals underwent a treatment regimen that consisted of 3-weekly chemotherapy administrations (administered i.p.) and 2-weekly pmIL-12/PPC administrations. Serum and whole blood were collected via retro-orbiral plexus from animals 1 day after the final chemotherapy treatment. Individual samples were used for (A) CBC analysis ($n = 4$ for each group) and pooled samples were used for (B) serum chemistries

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therapeutically relevant animal model of ovarian cancer that closely emulates the human disease and by characterizing the local and systemic biological effects after administration. Of special consideration when combining an immunomodulatory therapy with standard cytotoxic chemotherapies is the primary anti-cancer function of Taxol and other taxanes, which aims to disrupt microtubule function, resulting in cell cycle disruption. Immune cells are particularly sensitive to this action, which leads to significant immune suppression in treated patients. Somewhat paradoxically, it has also been suggested that Taxol can work to reverse T-induced macrophage immune suppression through stimulation of IL-12 production [47] and the use of IL-12 recombinant protein can restore Taxol-induced deficits in T-cell proliferation *in vitro* [48,49]. Results obtained *in vivo* have shown that optimum tumor inhibitory response occurs when there is a temporal spacing between Taxol and IL-12 administration to allow for recovery of lymphocytes from the Taxol treatment and maximum responsiveness to IL-12 [49]. In the present study, Taxol/Paraplatin administration and pmIL-12/PPC administration were separated by 3–4 days, which would allow for mIL-12 expression levels to be at peak levels 1–2 days prior to the next chemotherapy administration. In experiments where pmIL-12/PPC was administered 1 day prior to Taxol/Paraplatin, which would result in peak mIL-12 levels being coincident with chemotherapy administration, there was a moderate loss in efficacy of combined treatment (data not shown). These data suggest that the temporal spacing of treatment regimens will be an important consideration in the further development of combination studies.

In summary, we have used a gene-based approach to inhibit the progression of advanced disseminated ovarian cancer and improved survival in a murine model that closely resembles the human condition. A dose-dependent inhibition in ascites accumulation was noted and relatively high doses were administered i.p. with little increase in toxicity. An additive efficacy effect was achieved when pmIL-12/PPC was used in combination with platinum/paclitaxel-based chemotherapy regimens with no significant increase in systemic toxicities over that observed with chemotherapy alone. These pre-clinical results suggest that i.p. delivery of pmIL-12/PPC may be a useful therapeutic for the treatment of ovarian cancer alone and in combination with standard chemotherapy treatment regimens. Because of these encouraging results, the use of phIL-12/PPC delivered i.p. has advanced into clinical testing in women with advanced recurrent ovarian cancer. Favorable treatment safety, biological activity and encouraging efficacy results have been observed from initial human trials [50] and suggest that this IL-12 gene therapeutic has the potential to safely offer benefits to ovarian cancer patients.

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References

1. American Cancer Society. *Cancer Facts and Figures 2008*. American Cancer Society: Atlanta, GA, 2008.
2. Hasenburg A, Tong XW, Rojas-Martinez A, *et al*. Thymidine kinase gene therapy with concomitant topotecan chemotherapy for recurrent ovarian cancer. *Cancer Gene Ther* 2000; **7**: 839–844.
3. Bast RC, Yu Y, Xu FJ, Le XF, Mills GB. Molecular approaches to management of epithelial ovarian cancer. *Int J Gynecol Cancer* 2000; **10**: 2–7.
4. Weiss JM, Subleski JJ, Wigginton JM, Wiltrout RH. Immunotherapy of cancer by IL-12-based cytokine combinations. *Expert Opin Biol Ther* 2007; **7**: 1705–1721.
5. Schuster M, Nechansky A, Kircheis R. Cancer immunotherapy. *Biotechnol J* 2006; **1**: 138–147.
6. Li CY, Huang Q, Kung HF. Cytokine and immuno-gene therapy for solid tumors. *Cell Mol Immunol* 2005; **2**: 81–91.
7. Robertson MJ, Ritz J. Interleukin 12: basic biology and potential applications in cancer treatment. *Oncologist* 1996; **1**: 88–97.
8. Oshikawa K, Rakhmilevich AL, Shi F, Sondel PM, Yang N, Mahvi DM, 2001. Interleukin 12 gene transfer into skin distant from the tumor site elicits antimetastatic effects equivalent to local gene transfer. *Hum Gene Ther* 2001; **12**: 149–160.
9. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. *J Natl Cancer Inst* 1995; **87**: 581–586.
10. Freedman RS, Lenzi R, Kudelka AP, Lawrence DD, Rosenblum M, Platsoucas CD. Intraperitoneal immunotherapy of peritoneal carcinomatosis. *Cytokines Cell Mol Ther* 1998; **4**: 121–140.
11. Lenzi R, Edwards R, June C, *et al*. Phase II study of intraperitoneal recombinant interleukin-12 (rhIL-12) in patients with peritoneal carcinomatosis (residual disease <1 cm) associated with ovarian cancer or primary peritoneal carcinoma. *J Transl Med* 2007; **5**: 66.
12. Sangro B, Mazzolini G, Ruiz J, *et al*. Phase I trial of intratumoral injection of an adenovirus encoding interleukin-12 for advanced digestive tumors. *J Clin Oncol* 2004; **22**: 1389–1397.
13. van Herpen CM, Looman M, Zonneveld M, *et al*. Intratumoral administration of recombinant human interleukin 12 in head and neck squamous cell carcinoma patients elicits a T-helper 1 profile in the locoregional lymph nodes. *Clin Cancer Res* 2004; **10**: 2626–2635.
14. Gollob JA, Veenstra KG, Parker RA, *et al*. Phase I trial of concurrent twice-weekly recombinant human interleukin-12 plus low-dose IL-2 in patients with melanoma or renal cell carcinoma. *J Clin Oncol* 2003; **21**: 2564–2573.
15. Lenzi R, Rosenblum M, Verschraegen C, *et al*. Phase I study of intraperitoneal recombinant human interleukin 12 in patients with Müllerian carcinoma, gastrointestinal primary malignancies, and mesothelioma. *Clin Cancer Res* 2002; **8**: 3686–3695.
16. Motzer RJ, Rakhit A, Thompson JA, *et al*. Randomized multicenter phase II trial of subcutaneous recombinant human interleukin-12 versus interferon-alpha 2a for patients with advanced renal cell carcinoma. *J Interferon Cytokine Res* 2001; **21**: 257–263.
17. Rook AH, Wood GS, Yoo EK, *et al*. Interleukin-12 therapy of cutaneous T-cell lymphoma induces lesion regression and cytotoxic T-cell responses. *Blood* 1999; **94**: 902–908.
18. Salem ML, Gillanders WE, Kadima AN, *et al*. Review: novel nonviral delivery approaches for interleukin-12 protein and gene systems: curbing toxicity and enhancing adjuvant activity. *J Interferon Cytokine Res* 2006; **26**: 593–608.
19. Imboden M, Shi F, Pugh TD, *et al*. Safety of interleukin-12 gene therapy against cancer: a murine biodistribution and toxicity study. *Hum Gene Ther* 2003; **14**: 1037–1048.
20. Nastala CL, Edington HD, McKinney TG, *et al*. Recombinant IL-12 administration induces tumor regression in association with IFN-gamma production. *J Immunol* 1994; **153**: 1697–1706.

21. Tan J, Newton CA, Djeu JY, *et al*. Injection of complementary DNA encoding interleukin-12 inhibits tumor establishment at a distant site in a murine renal carcinoma model. *Cancer Res* 1996; **56**: 3399–3403.
22. Quezada A, Larson J, French M, *et al*. Biodistribution and safety studies of hDEL-1 plasmid based gene therapy in mice and rabbit models. *J Pharm Pharmacol* 2004; **56**: 177–185.
23. Han S, Mahato RI, Sung YK, Kim SW. Development of biomaterials for gene therapy. *Mol Ther* 2000; **2**: 302–317.
24. Mahato RI, Lee M, Han S, Maheshwari A, Kim SW. Intratumoral delivery of p2CMVmIL-12 using water soluble lipopolymers. *Mol Ther* 2000; **4**: 130–138.
25. Anwer K, Rhee BG, Mendiratta SK. Recent progress in polymeric gene delivery systems. *Crit Rev Ther Drug Carrier Syst* 2003; **20**: 249–293.
26. Fewell JG, Matar M, Slobodkin G, *et al*. Synthesis and application of a non-viral gene delivery system for immunogene therapy of cancer. *J Control Release* 2005; **109**: 288–298.
27. Janát-Amsbury MM, Yockman JW, Anderson ML, Kieback DG, Kim SW. Combination of local, non-viral IL12 gene therapy and systemic paclitaxel chemotherapy in a syngeneic ID8 mouse model for human ovarian cancer. *Anticancer Res* 2006; **26**: 3223–3228.
28. Roby KF, Taylor CC, Sweetwood JP, *et al*. Development of a syngeneic mouse model for events related to ovarian cancer. *Carcinogenesis* 2000; **4**: 585–591.
29. King PD, Perry MC. Hepatotoxicity of chemotherapy. *Oncologist* 2001; **6**: 162–176.
30. Bracci L, Proietti E, Belardelli F. IFN-alpha and novel strategies of combination therapy for cancer. *Ann NY Acad Sci* 2007; **1112**: 256–268.
31. Hurwitz H, Kabbinavar F. Bevacizumab combined with standard fluoropyrimidine-based chemotherapy regimens to treat colorectal cancer. *Oncology* 2005; **69**(Suppl 3): 17–24.
32. Mendiratta SK, Quezada A, Matar M, *et al*. Combination of interleukin 12 and interferon alpha gene therapy induces a synergistic antitumor response against colon and renal cell carcinoma. *Human Gene Ther* 2000; **11**: 1851–1862.
33. Zagozdzon R, Go3ab J, Stok3osa T, *et al*. Effective chemo-immunotherapy of L1210 leukemia in vivo using interleukin-12 combined with doxorubicin but not with cyclophosphamide, paclitaxel or cisplatin. *Int J Cancer* 1998; **77**: 720–727.
34. Teicher BA, Ara G, Buxton D, Leonard J, Schaub RG. Optimal scheduling of interleukin 12 and chemotherapy in the murine MB-49 bladder carcinoma and B16 melanoma. *Clin Cancer Res* 1997; **3**: 1661–1667.
35. Leonard JP, Sherman ML, Fisher GL, *et al*. Effects of Single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon production. *Blood* 1997; **90**: 2541–2548.
36. Sonawane ND, Szoka FC Jr, Verkman AS. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J Biol Chem* 2003; **278**: 44826–44831.
37. Kusuda T, Shigemasa K, Arihiro K, Fujii T, Nagai N, Ohama K. Relative expression levels of Th1 and Th2 cytokine mRNA are independent prognostic factors in patients with ovarian cancer. *Oncol Rep* 2005; **13**: 1153–1158.
38. Bozkurt N, Yuce K, Basaran M, Gariboglu S, Kose F, Ayhan A. Correlation of serum and ascitic IL-12 levels with second-look laparotomy results and disease progression in advanced epithelial ovarian cancer patients. *Int J Gynecol Cancer* 2006; **16**: 83–86.
39. Ichim CV, 2005. Revisiting immunosurveillance and immunostimuation: Implications for cancer immunotherapy. *J Transl Res* 2005; **3**: 8.
40. Mesiano S, Ferrara N, Jaffe RB. Role of vascular endothelial growth factor in ovarian cancer: inhibition of ascites formation by immunoneutralization. *Am J Pathol* 1998; **153**: 1249–1256.
41. Nagy JA, Masse EM, Herzberg KT, *et al*. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res* 1995; **55**: 360–368.
42. Duda DG, Sunamura M, Lozonschi L, *et al*. Direct in vitro evidence and in vivo analysis of the antiangiogenic effects of interleukin 12. *Cancer Res* 2000; **60**: 1111–1116.
43. Li S, Zhang X, Xia X. Regression of tumor growth and induction of long-term antitumor memory by interleukin 12 electro-gene therapy. *J Nat Cancer Inst* 2002; **94**: 762–768.
44. Schumacher JJ, Dings RP, Cosin J, Subramanian IV, Auersperg N, Ramakrishnan S. Modulation of angiogenic phenotype alters tumorigenicity in rat ovarian epithelial cells. *Cancer Res* 2007; **67**: 3683–3690.
45. Anwer K, Meaney C, Kao G, Hussain N, Shelvin R, Earls RM. Cationic lipid-based delivery system for systemic cancer gene therapy. *Cancer Gene Ther* 2000; **7**: 1156–1164.
46. Louis MH, Dutoit S, Denoux Y, Erbacher P, Deslandes E, Behr JP. Intraperitoneal linear polyethylenimine (L-PEI)-mediated gene delivery to ovarian carcinoma nodes in mice. *Cancer Gene Ther* 2006; **13**: 367–374.
47. Mullins DW, Burger CJ, Elgert KD. Paclitaxel enhances macrophage IL-12 production in tumor-bearing hosts through nitric oxide. *J Immunol* 1999; **162**: 6811–6818.
48. Mullins DW, Koci MD, Burger CJ, Elgert KD. Interleukin-12 overcomes paclitaxel-mediated suppression of T-cell proliferation. *Immunopharmacol Immunotoxicol* 1998; **20**: 473–492.
49. Pressley JS, Elgert KD. Post-chemotherapeutic administration of interleukin-12 retards tumor growth and enhances immune cell function: combination therapy using paclitaxel and IL-12. *Cancer Invest* 2006; **24**: 351–359.
50. Anwer K, Barnes MN, Anderson ML, *et al*. Progress in the clinical development of a novel IL-12 gene therapeutic for the treatment of recurrent ovarian cancer. *Mol Ther* 2007; **15**: S69.