

Use of Interleukin-12 Nanocomplexes as a General Treatment Strategy against Disseminated Peritoneal Malignancies

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ABSTRACT

The strong anti-cancer properties of Interleukin-12 (IL-12) make it a sought-after target for therapeutic development. The effects of IL-12 are mediated through induction of local and systemic immune responses and strong anti-angiogenic characteristics. Specifically, IL-12 administration is associated with T-lymphocyte and natural killer (NK) cell proliferation, activation of cytotoxic T-lymphocytes and secretion of interferon-gamma (IFN- γ). The anti-angiogenic effects result from IFN- γ induced stimulation of monokine induced by IFN- γ (MIG) and IFN- γ inducible protein (IP-10) and direct inhibition of VEGF mRNA. We have developed a non-viral polymeric delivery system (PPC) formulated with a plasmid encoding for IL-12 that when delivered intraperitoneally is an effective inhibitor of experimentally induced ovarian cancer in mice. Efficacy is enhanced by combining treatment with standard chemotherapeutic agents such as Taxol[®] and Paraplatin[®]. Currently this product is being evaluated clinically in patients with recurrent ovarian cancer. We were interested in examining use of this therapy against other forms of cancer known to disseminate into the peritoneal cavity. For these studies a murine IL-12 plasmid was formulated with the polymeric delivery system PPC which is composed of a low molecular weight branched polyethyleneimine covalently linked with functional groups of polyethylene glycol and cholesterol. When formulated at 1:1 nitrogen to phosphate ratio the DNA is fully condensed into nanoparticles of ~100 nm diameter. Peritoneally disseminated tumor models of pancreatic cancer (PAN-02) and colorectal cancer (CT-26) were established in syngeneic mice. At various times after tumor implant, mice were treated with mL-12/PPC or a combination of mL-12/PPC and standard chemotherapy. In mice with pancreatic tumors, mL-12/PPC treatment resulted in dose dependent increases in median survival times of up to 127% over untreated controls. In the highest dose treatment group 42.9% achieved long-term survival of over 100 days compared to 0% in the untreated control groups. When mL-12/PPC treatment was combined with Gemzar[®] treatment survival was significantly improved over either monotherapy. In mice with colorectal tumors a 35% increase in median survival was seen in response to mL-12/PPC therapy and improved long-term survival to 40% compared to 0% for the untreated controls. Long-term survival was increased to 67% by combining mL-12/PPC administration with 5-FU/fluorouracil (5-FU). The results of these studies indicate that IL-12 plasmid formulated with a polymeric delivery system is useful in treating both pancreatic and colorectal disseminated cancer. Further, mL-12/PPC administration is compatible with standard chemotherapeutics producing added efficacy benefits and suggests broad applicability for treating a wide variety of malignancies.

INTRODUCTION

Interleukin-12 (IL-12) has proven to be one of the most active cytokines due to potent immunomodulatory properties leading to robust local and systemic immune response to cancer and strong anti-angiogenic properties (Fig 1). We have developed a non-viral gene delivery approach composed of a IL-12 expressing plasmid formulated with the polymeric delivery system PPC. Earlier results from in-vivo studies have indicated that this system is useful for treating disseminated ovarian cancer alone and in combination with standard chemotherapies. Clinical evaluation is ongoing in patients with recurrent ovarian cancer with results from a Phase I clinical trial indicating that use is safe and well tolerated. Here we show that intratumoral (IT) and intraperitoneal (IP) administration of IL-12/PPC in combination with chemotherapy inhibits the progression of solid tumors and disseminated pancreatic and colorectal cancer in animal models in a dose dependent manner. Added or synergistic responses were observed when IL-12/PPC treatment was combined with standard chemotherapies and produced acute alterations in several transcripts known to be involved with tumor metastasis suggesting additional molecular mechanisms that result in improved animal survival.

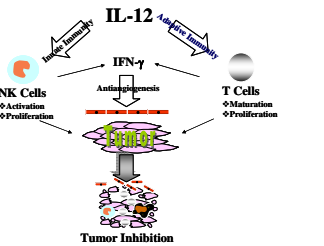


Fig. 1. Multiple mechanisms of IL-12 action on tumor growth.

MATERIALS/METHODS

Plasmid/Polymer Formulation
 The murine IL-12 plasmid (mIL-12) consists of the p35 and p40 genes each under control of a separate CMV promoter/terminator and each containing the SV40 late polyadenylation signal sequences. PPC is a cationic polymer that is composed of a low molecular weight branched polyethyleneimine (PEI) that has functional groups of methoxy polyethylene glycol (PEG) and cholesterol (CHOL) attached at an approximately 2.5:1:1 ratio (PEG-PEI-CHOL). Plasmid and PPC were formulated at a 1:1:1 nitrogen to phosphate molar ratio. When high DNA concentrations were used (>0.5 mg/ml) a lyophilization process was incorporated. Prior to use the final solutions were reconstituted at various DNA concentrations with water for injection.

Tumor Cell Lines
 CT-26 colon carcinoma cell line was obtained from American Type Culture Collection (ATCC) (Manassas, VA). PAN-02 pancreatic ductal adenocarcinoma was purchased from the DCTO, NCI, (Frederick, MD).

ELISAs
 mL-12, mIFN- γ , mVEGF and mTNF- α ELISAs were obtained from R&D systems (Minneapolis, MN).

qRT-PCR ARRAY
 The murine metastasis PCR array was obtained from SuperArray Bioscience Corporation (Frederick, MD) and analysis was performed using an Applied Biosystems Real Time PCR system.

Animals/Treatment
 Syngeneic mouse strains were used for these studies. Both C57BL/6 (PAN-02) and BALB/c (CT-26) strains of mice were obtained from Harlan Laboratories (Indianapolis, IN). All animal experiments were performed under approved IACUC protocols and adhered to accepted animal use guidelines. Chemotherapy reagents (Gemzar, 5-FU) were kindly provided by Clint Gregory and Dr. Marshall Schreeder at the Clearview Cancer Institute (Huntsville, AL).

RESULTS

> Treatment of subcutaneous (SC) CT-26 tumors with mL-12/PPC resulted in dose-dependent inhibition of tumor growth and tumor rejection. Animals that rejected tumors exhibited long-term immunity to CT-26 re-challenge (Fig 2). Modest changes in tumor cytokine levels were associated with a single mL-12/PPC treatment (Fig 3).

> In a disseminated (IP) CT-26 tumor model, combined treatment of 5-FU + mL-12/PPC significantly improved long-term survival over either monotherapy (Fig 4).

> Most potent inhibition of SC PAN-02 tumors are noted following intra-tumoral (IT) treatment with mL-12/PPC (Fig 5). However, significant changes in tumor cytokine levels are detected 24 hours after mL-12/PPC (Fig 6). Cytokine changes included an unexpected increase in VEGF. Earlier studies have shown that peritoneal exudates of animals with disseminated ovarian cancer treated intra-peritoneally (IP) with mL-12/PPC there is a rapid and significant decrease in VEGF (data not shown).

> mL-12/PPC treatment led to significant changes in several transcripts linked to tumor metastasis including those corresponding to extracellular matrix proteins (Mmp10, Mmp11), Cell cycle genes (Cdkn2a, Vegfa), Cell growth and proliferation (Ccr7) and apoptosis (Trnf10). Transcripts for tumor necrosis factor and VEGF both increased consistent with the protein data (Table 1).

> A weekly treatment regimen of mL-12/PPC (4 weeks) resulted in dose-dependent increases in survival of animals with a disseminated pancreatic tumors (Fig 7). Combining mL-12/PPC treatment with Gemzar and increasing treatment duration improved survival compared to shorter treatment regimens and monotherapies (Fig 8).

Dose dependent CT-26 tumor inhibition and re-challenge following IT treatment with mL-12/PPC

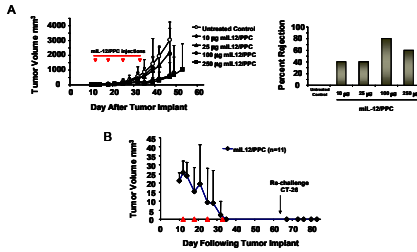


Figure 2. Subcutaneous tumors were grown on the flanks of mice. A. Tumors (~40mm³) were treated (IT) with various doses of mL-12/PPC (adjusted by varying DNA concentration) n=6 for each group. B. Animals from all groups that had a complete tumor rejection response were pooled and re-challenged with CT-26 tumor cells 64 days after the original tumor implant. 100% of the animals did not grow tumors following re-challenge.

Cytokine expression levels in CT-26 tumors following intra-tumoral mL-12/PPC administration (30 µg)

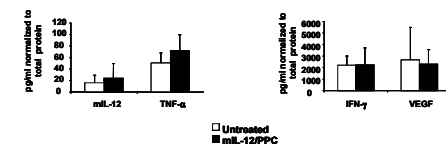


Figure 3. Tumor protein expression levels in CT-26 tumors following intra-tumoral mL-12/PPC administration (IT). Subcutaneous tumors were grown on the flanks of mice. Tumors were treated when they reached a volume of ~25mm³. Tumors were harvested 24 hours after treatment, homogenized and analyzed for cytokine expression levels by ELISA. Values are means \pm SD, n=3 for each group.

Synergistic improvement in survival in animals with disseminated (IP) colorectal cancer treated with a combination of mL-12/PPC and 5-FU

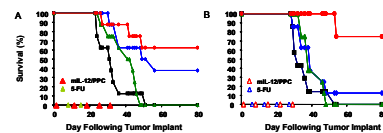


Figure 4. BALB/c mice were inoculated with 1x10⁶ CT-26 cells (IP). One day after tumor implant the mL-12/PPC treatments (IP) were initiated and given weekly for a total of 5 treatments. Weekly chemotherapy administrations (IP) were started 6 days after tumor implant. A. Animals were given 2 5-FU treatments (n=7 for each group). B. Animals were given 4 5-FU treatments (n=6 for each group).

Inhibition of PAN-02 tumor growth in mice following mL-12/PPC treatment

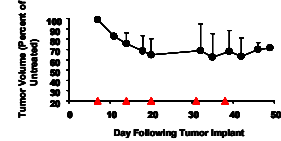


Figure 5. Summarized data of tumor inhibition of PAN-02 tumor bearing C57BL/6 mice (SC). Animals were implanted with 2x10⁶ - 5x10⁶ cells into the right flank. The mL-12/PPC treatments (IT) were initiated 7 days after tumor implant when tumors were ~30mm³ in size.

Cytokine expression levels in PAN-02 tumors following intra-tumoral mL-12/PPC administration (15 µg)

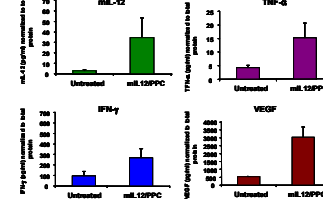


Figure 6. Tumor protein expression levels in PAN-02 tumors following mL-12/PPC administration (IT). Subcutaneous tumors were grown on the flanks of mice. Tumor were treated when they reached a volume of ~30mm³. The tumor were harvested 24 hours after treatment and homogenates were analyzed for cytokine expression levels. Values are means \pm SD.

Summary of significant changes in transcripts related to metastasis in PAN-02 tumors treated with mL-12/PPC vs. untreated tumors

GENE SYMBOL	GENE	GENE NAME	FOLD CHANGE
Ccr7	Chemokine (C-C motif) receptor 7	CCR7-304paf	+1.8
Blca	Blastic sarcoma 1, lung-specific	P089191BLCA1	+0.8
	Blca 2	AV256F	+0.28
Tnfrsf9	Tumor necrosis factor receptor superfamily member 9	A33M221BLCA4 SBT1	+0.61
Vegfa	Vascular endothelial growth factor A	VEGF-JA06P129	+1.57
Col6a3	Collagen type VI, alpha 3	AMP-06A041	-1.3
Mmp10	Matrix metalloproteinase 10	BT028p07	-1.6

Changes in 4 transcripts were consistent with metastasis inhibition and/or inflammation.
 ↓ Ccr7, ↑ Blca2, ↑ Tnfrsf9, ↓ Mmp11

Changes in 2 transcripts were consistent with pro-metastasis or pro-angiogenic.
 ↑ Vegfa, ↓ Col6a3

Changes in 1 transcript uncertain.
 ↓ Mmp10

Dose dependent improvement in survival mice with disseminated pancreatic tumors following treatment with mL-12/PPC

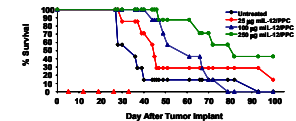


Figure 7. IP administration of mL-12/PPC in mice with disseminated pancreatic tumors. Effect of increasing mL-12/PPC administration (IP) on survival is evaluated. Animals (C57BL/6) were injected with 5x10⁶ PAN-02 cells in 500 µl. The mL-12/PPC treatments were started 5 days after tumor implant and were given weekly for 4 additional weeks.

Improved animal survival following combination treatment of mL-12/PPC with Gemzar.

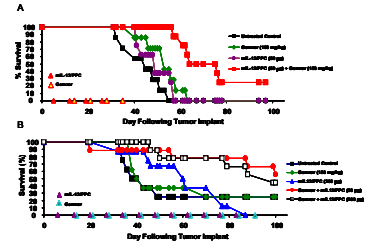


Figure 8. C57BL/6 mice were inoculated with 5x10⁶ PAN-02 cells (IP). Five days after tumor implant the mL-12/PPC treatments (IP) were initiated and given weekly. Weekly chemotherapy administrations (Gemzar, 150 mg/kg, IP) were started 16 days after tumor implant and were given weekly. A. Short treatment regimen (4 weeks). B. Long treatment regimen (12 weeks).

CONCLUSIONS

- > Use of mL-12/PPC in combination with chemotherapy improves survival in animals with disseminated colorectal and pancreatic cancers compared to either monotherapy.
- > Changes in tumor cytokine protein expression levels (mIL-12, IFN- γ , VEGF and TNF- α) 24 hours after intra-tumoral mL-12/PPC administration were tumor-type dependent. Route of delivery (IT vs. IP) is also important as earlier studies have indicated a significant decrease in VEGF levels in peritoneal exudates following IP delivery into mice with disseminated ovarian cancer.
- > Following mL-12/PPC administration, alterations in transcripts associated with metastasis, on balance, lean towards inhibition of metastasis in PAN-02 tumors.
- > The IP administration of mL-12/PPC appears to have broad applicability for treating a variety of peritoneal malignancies in combination with standard chemotherapeutics.