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Intratumoral Delivery of p2CMVmIL-12 Using Water-Soluble Lipopolymers

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Our objective was to design a water-soluble lipopolymer (WSLP) and an interleukin-12 (IL-12) expression plasmid for enhanced delivery of the IL-12 gene. We synthesized WSLP using branched polyethylenimine (PEI) of 1800 Da and cholesteryl chloroformate, and constructed p2CMVmIL-12, encoding the IL-12 subunits p35 and p40, each under the transcriptional control of a separate cytomegalovirus (CMV) promoter. The percentage of cholesterol conjugated to PEI was about 47% and the average molecular weight of WSLP was approximately 2000 Da. The mean particle size of WSLP/p2CMVmIL-12 complexes formulated in 5% glucose was 26 to 62 nm and ζ potential was 8 to 60 mV. The WSLP/p2CMVmIL-12 complexes were nontoxic to CT-26 colon carcinoma cells at the N/P ratio (nitrogen atoms of WSLP/phosphate of plasmid DNA) of 20 and below; PEI25000/pDNA complexes were highly toxic. WSLP/p2CMVmIL-12 complexes demonstrated higher transfection in CT-26 cells compared with the DNA formulations prepared using PEI of molecular weights 1800, 10,000 and 25,000 Da. Transfection efficiency increased with an increase in N/P ratios from 5 to 15, then there was no significant increase in transfection up to the N/P ratio of 30/1. There was an increase in the level of IL-12 when free or complexed p2CMVmIL-12 was compared with free or complexed pIRESmIL-12 in which the p35 and p40 subunits were linked to the internal ribosome entry sites (IRES). At 48 hours post-injection of WSLP/p2CMVmIL-12 complexes into BALB/c mice bearing CT-26 subcutaneous tumors, the levels of IL-12, IFN-γ, and nitric oxide (NO) in the supernatant of the cultured tumors were higher for the WSLP/p2CMVmIL-12 complexes than for the naked p2CMVmIL-12, WSLP, and 5% glucose injected groups. There was a significant improvement in the survival rate and the inhibition of tumor growth after a single injection of WSLP/p2CMVmIL-12 complexes. We have designed an effective, nontoxic WSLP and an IL-12 expression plasmid with two CMV promoters.

Key Words: lipopolymer, polyethylenimine, cholesterol, water-soluble, gene expression, plasmid, cytotoxicity, interleukin-12, tumor regression

INTRODUCTION

Gene therapy can be used to deliver cytokine genes to the site of a tumor, altering the local tumor environment to induce an anti-tumor immune response and subsequent tumor eradication [1]. Among various cytokine genes, *IL12* (encoding interleukin-12, IL-12) has the most potent antitumor activity [2]. Although *ex vivo* retrovirus-based mouse *Il12* gene therapy has shown promise in inducing antitumor immune responses, this strategy is laborious and requires that cancer cells are cultured from each patient [3]. A more attractive strategy is to deliver *IL12* to primary and metastatic tumors using an adenoviral vector [4]; however, adenoviral vectors elicit an immune response, which reduces their gene transfer efficiency. The recent death of a young patient with liver ornithine transcarbamylase deficiency following intrahepatic adenoviral injection raises many questions regarding the safety of viral vectors [5].

Over the past decade there have been many attempts to design a nonviral vector that could achieve the level of gene expression and specificity offered by viral vectors, while still maintaining the flexible characteristics of cDNA size, bypassing the immune response, and safety [6,7]. Several nonviral approaches are being investigated for *IL12* delivery to treat a variety of cancers. For example, gene gun technology has been used to deliver plasmid encoding *Il12* to mouse tumors, which resulted in tumor regression [8]. However, this method is not patient-friendly and requires gold particles to be coated with plasmid DNA. Intravenous injection of cationic liposome/pmIL-12 complexes to mice bearing pulmonary metastasis has also been shown to inhibit tumor growth [9,10]. However, plasmid DNA and cationic liposomes produce large complexes, which show

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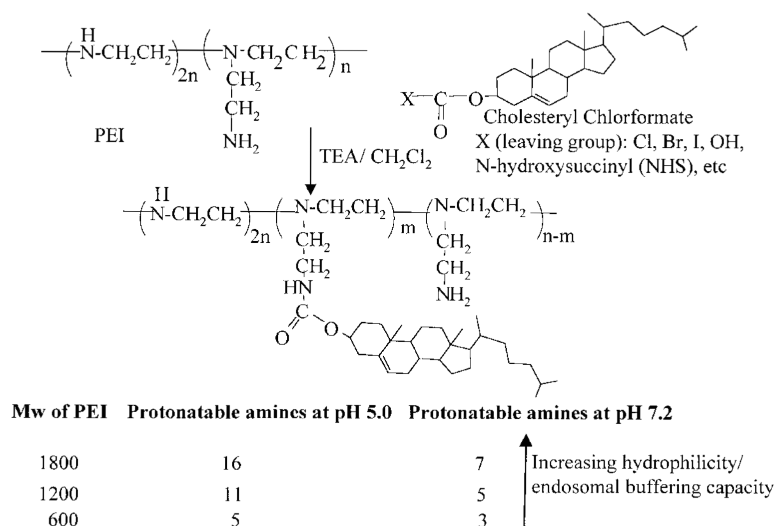
poor *in vivo* gene expression, high toxicity, and poor storage stability. These liposome/pDNA complexes are efficient at transfecting cells in culture, yet are poor at diffusing within tumor tissues [11]. Among various polycations used for gene delivery, polyethylenimine (PEI) is one of the most effective transfection reagents, in that plasmid DNA is delivered to the cytoplasm by means of endosomes due to the proton-sponge effect of PEI [12,13].

Transgene expression depends not only on the type of gene carrier, but also on the components of gene expression systems. There are two methods to coexpress heterologous gene products in a single vector: independent promoters or internal ribosome entry site (IRES) sequences [14]. In many gene transfer experiments, cDNA is simply inserted after the promoter or IRES sequence. In such cases, the translation efficiency is not optimized. Therefore, the way in which the plasmid is constructed can modulate the expression of the IRES-dependent second gene relative to that of the first gene. Both the capacity of IRES and the translation efficiency should be taken into consideration in relation to the relative expression of the first and second genes. IL-12 is a disulfide-linked heterodimer composed of 35 kDa (p35; encoded by the gene *Il12a*) and 40 kDa (p40; encoded by *Il12b*) subunits. Coexpression of p35 and p40 is required for efficient production of bioactive IL-12. The expression vector used for IL-12 employs IRES to express both p35 and p40. Excess expression of p40 can form a homodimer, however, which then prevents the efficient expression of IL-12 [15].

There is a growing need to develop nontoxic gene carriers that can avoid the preparation of cationic liposomes and the variability associated with it. We have designed an effective water-soluble lipopolymer (WSLP) by combining the advantages of both cationic liposomes and polycations, which condense DNA, while the lipid coating on the DNA increases its permeability through cell membranes. In addition, we also constructed p2CMVmIL-12, with *Il12a* and *Il12b* each under the transcriptional control of a separate cytomegalovirus (CMV) promoter.

FIG. 1. Synthesis scheme and properties of WSLP.



RESULTS

Synthesis and Characterization of WSLP

We synthesized WSLP using branched PEI of 1800 Da as a cationic headgroup and cholesteryl chloroformate as a hydrophobic lipid anchor (Fig. 1). Following synthesis and purification, we determined the structure and molecular weight of WSLP using ^{1}H NMR and MALDI-TOF mass spectrometry. The amount of cholesterol conjugated to PEI was 47% and the molecular weight of WSLP was approximately 2000 Da. This indicates that most WSLP synthesized was of 1/1 molar ratio of cholesterol and PEI of 1800 Da.

The mean particle size of WSLP/pDNA complexes was in the range of 26 to 148.5 nm and was dependent on N/P ratios. At the N/P ratio of 1.25, plasmid DNA was not completely neutralized, resulting in the formation of heterogeneous complexes with a mean diameter of 148.5 nm. However, the mean particle size of WSLP/pDNA complexes was 40 nm or below when formulated at the N/P ratios of 2.5 and 10. At 20/1 (N/P) ratio, these complexes had a narrow size distribution with a mean diameter of 61.8 nm. The ζ potential was greatly dependent on the charge ratios and linearly increased with the increase in N/P ratios. It was –41.35 at the N/P ratio of 2.5/1, but increased to 61.67 mV when formulated at the N/P ratio of 20/1. No aggregation occurred when WSLP/pDNA complexes were prepared at 0.2 mg/ml DNA concentration, but the degree of aggregation increased as the DNA concentration increased beyond 0.3 mg/ml. Aggregates formed at high DNA concentrations were insoluble and thus we did not test them for transfection efficiency. For *in vivo* applications, WSLP/pDNA complexes were formulated at 0.2 mg/ml DNA and then concentrated to 0.5 mg/ml by centifugation. Aggregation of WSLP/pDNA complexes was not observed following storage at 4°C for up to 24 hours. WSLP condensed pDNA into small spherical particles with a mean diameter of 25 to 62 nm and protected pDNA from digestion by DNase at least for 2 hours at 37°C. WSLP/pDNA complexes were nontoxic to CT-26 colon carcinoma cells when formulated at N/P ratio of 20 and below, whereas PEI25000/pDNA complexes were highly toxic to the cells.

Construction of IL-12 Expression Plasmid

Transgene expression depends on the types of gene delivery and expression systems. IL-12 is

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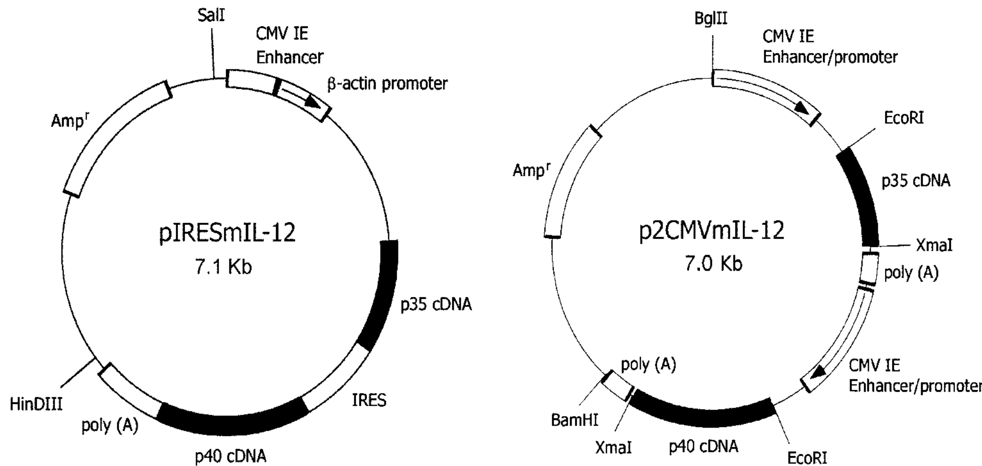

FIG. 2. Construction of IL-12 expression plasmids.

a disulfide-linked heterodimer comprising 35-kDa light chain (p35) and 40-kDa heavy chain (p40) subunits. To express p35 and p40 using a single vector, we used an IRES in pIRESmIL-12. The coding sequence located downstream of the IRES is expressed at a much lower level than the coding sequence that is located upstream. Therefore, for more efficient expression of the second gene, we constructed the plasmid p2CMVmIL-12, in which the expression of p35 and p40 was driven by two independent CMV promoters (Fig. 2). To construct p2CMVmIL-12, we amplified the p35 and p40 cDNAs by PCR using pIRESmIL-12 as a template. The amplified p35 and p40 cDNAs were inserted into pCI plasmid carrying a CMV promoter, resulting in the construction of pCMV-p35 and pCMV-p40, respectively. We isolated the p40 expression unit in pCMV-p40 and inserted it into pCMV-p35 at a *Bam*HI site. The construction of p2CMVmIL-12 was confirmed by a restriction enzyme assay.

In Vitro Transfection

We next determined the effect of N/P ratio on transfection efficiency of WSLP/p2CMVmIL-12 complexes in CT-26 cells (Fig. 3). The levels of IL-12 increased with an increase in the N/P ratio from 5 to 15. There was no significant increase in transfection up to the N/P ratio of 30/1. No significant difference in the levels of IL-12 was found when WSLP/p2CMVmIL-12 complexes were prepared at the N/P ratios of 15/1, 20/1, 25/1, and 30/1 (Fig. 3). Therefore, we decided to formulate the complexes at the N/P ratio of 20/1 to avoid any toxic effects, which may occur *in vivo*.

To determine the effect of PEI molecular weights on *Il12* expression, branched PEIs of molecular weights 1800, 10,000, and 25,000 Da were formulated with p2CMVmIL-12 and compared for *in vitro* transfection efficiency. The levels of *Il12* expression were very low for formulations based on a PEI of 1800, but increased with increasing molecular weight of PEI (Fig. 4). The transfection efficiency of PEI of 25,000 Da, which is very toxic to the cells, was the highest among all the PEI-based formulations, but lower than those of WSLP/p2CMVmIL-12 complexes (Fig. 4). The potencies of pIERSmIL-12 and p2CMVmIL-12 were determined after complex formation with WSLP followed by transfection into cultured CT-26 cells. There was increase in the levels of IL-12 when free or complexed p2CMVmIL-12 was compared with free or complexed pIRESmIL-12 (Fig. 5). The IL-12 concentration (pg/ml) for WSLP/p2CMVmIL-12 complexes was several orders of magnitude higher than that for naked p2CMVmIL-12 (Fig. 5).

In Vivo Gene Expression

We studied the gene expression levels of *Il12* and induced IFN-γ after intratumoral injection of WSLP/p2CMVmIL-12 complexes into BALB/c mice bearing CT-26 subcutaneous tumors. Mice injected with naked p2CMVmIL-12, WSLP alone, and 5% (w/v) glucose were used to compare the efficiency of WSLP/p2CMVmIL-12 complexes. At 48 hours postinjection, tumors were harvested, chopped into small pieces, and recultured for 24 hours. Culture supernatants were analyzed by ELISA. IL-12 expression levels were

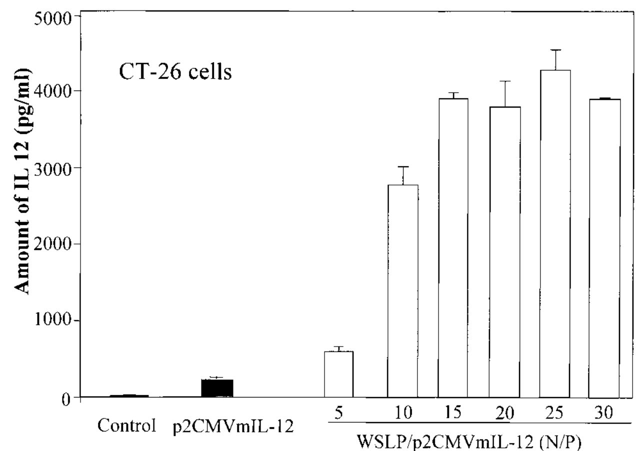

FIG. 3. Effect of N/P ratio on *in vitro* transfection of WSLP/p2CMVmIL-12 complexes. The results are expressed as the mean ± S.D. of three samples. $P < 0.05$ compared with N/P ratio of 5/1, but no statistical significance among the N/P ratios of 15/1, 20/1, 25/1, and 30/1.

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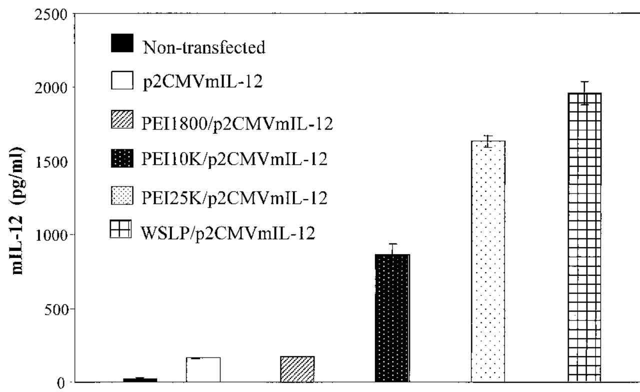

FIG. 4. Effect of PEI molecular weights on Il12 expression in CT-26 cells. The results are expressed as the mean (S.D. of three samples. *P, 0.05. **P, 0.01 (compared with PEI 1800/p2CMVmIL-12 complexes).

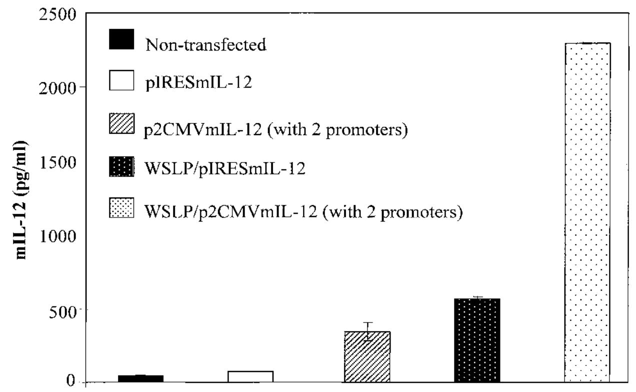

FIG. 5. Effect of plasmid construct design on IL-12 expression in CT-26 cells. The results are expressed as the mean ± S.D. of three samples. *P, 0.05 (compared with pIRESmIL-12 group). **P, 0.01 (compared with WSLP/pIRESmIL-12 complex group).

higher than those of naked p2CMVmIL-12 (Fig. 6). To determine whether complex formation with WSLP can enhance the level and duration of *Il12* expression, tumors were isolated, chopped, and cultured for IL-12 at days 1, 3, and 5 postinjection of WSLP/p2CMVmIL-12 complexes. IL-12 levels were highest at day 1 and then decreased slowly with time. At day 3 postinjection, IL-12 levels were higher compared with the naked DNA group (Fig. 6).

IL-12 primarily exerts its antitumor effect by inducing the production of IFN-γ. We, therefore, determined the production of IFN-γ, at 48 hours post-injection, induced by WSLP/p2CMVmIL-12 complexes in BALB/c mice bearing CT-26 subcutaneous tumors to see whether IL-12 was able to upregulate these two cytokines. In case of IFN-γ, the levels were higher for WSLP/p2CMVmIL-12 complexes compared with naked p2CMVmIL-12 (Fig. 7). Upon injection of WSLP alone and 5% glucose, there was little induction of these cytokines, which confirms that the production was solely due to p2CMVmIL-12 and not due to any charge or antigenicity associated with WSLP.

Induction of Nitric Oxide

IL-12 regulates the production of nitric oxide (NO), which is an important signaling and cytotoxic molecule that acts against tumor growth and metastasis. Therefore, we measured NO levels in terms of nitrite (NO_2^-) using the Griess reagent system. At 48 hours postinjection of WSLP/p2CMVmIL-12 complexes into BALB/c mice bearing CT-26 subcutaneous tumors, tumors were chopped into small pieces, recultured for 24 hours, and culture supernatants analyzed for NO_2^-. The nitrite levels were higher in tumors of mice injected with WSLP/p2CMVmIL-12 complexes compared with those injected with naked p2CMVmIL-12 or 5% glucose (Fig. 8).

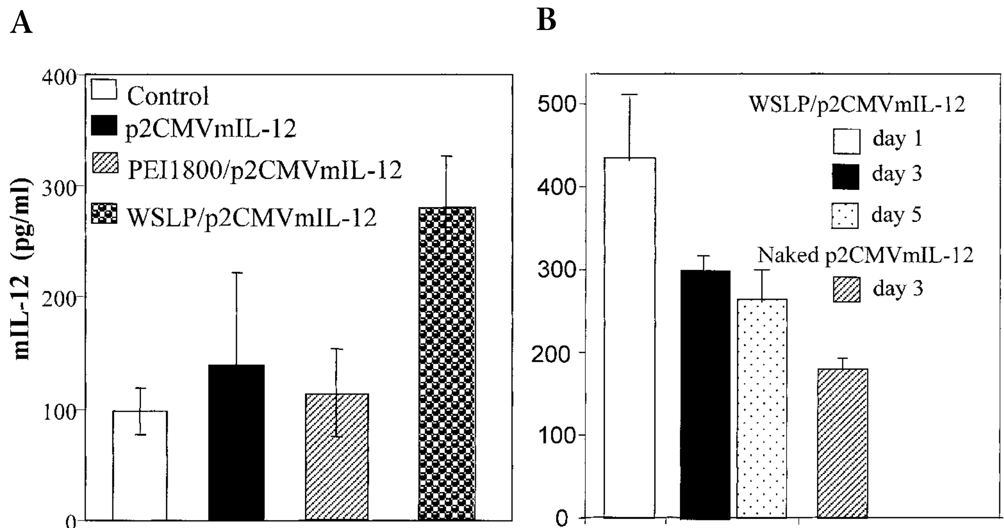

FIG. 6. *Il12* expression after intratumoral injection of WSLP/p2CMVmIL-12 complexes into subcutaneous CT-26 tumor bearing BALB/c mice. (A) Effect of plasmid construct on gene expression. (B) Persistence of gene expression. At 48 h postinjection, tumors were isolated, chopped into small pieces, and cultured. Supernatants were analyzed by ELISA for IL-12. Mice injected with 5% (w/v) glucose were used as negative controls. WSLP was also tested for its IL-12 inducing capability. IL-12 expression levels for WSLP/p2CMVmIL-12 complexes were higher than naked p2CMVmIL-12. The results are expressed as the mean ± S.D. of three samples. *$P < 0.05$ compared with PEI 1800/p2CMVmIL-12, but no statistical significance between p2CMVmIL-12 and PEI 1800/p2CMVmIL-12 groups.

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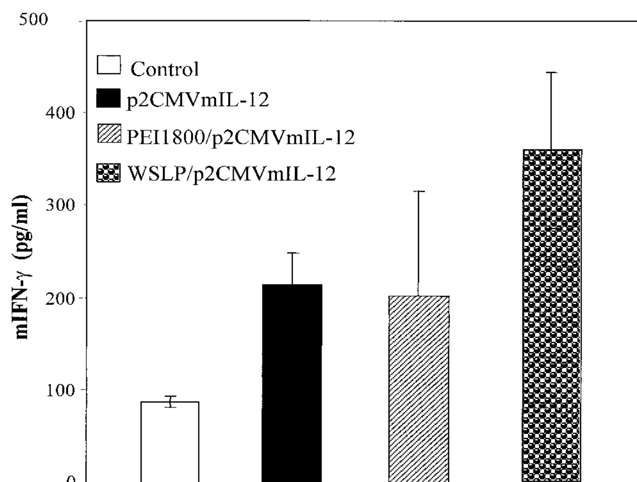

FIG. 7. Levels of induced IFN-γ after intratumoral injection of WSLP/p2CMVmIL-12 complexes. At 48 h postinjection, tumors were isolated, chopped into small pieces, and cultured. Supernatants were analyzed by ELISA for mIFN-γ. Mice injected with 5% (w/v) glucose were used as negative controls. WSLP was also tested for its IFN-γ inducing capability. IFN-γ expression levels for WSLP/p2CMVmIL-12 complexes were higher than naked p2CMVmIL-12. The results are expressed as the mean ± S.D. of three samples. *$P < 0.05$ compared with PEI 1800/p2CMVmIL-12, but no statistical significance between p2CMVmIL-12 and PEI 1800/p2CMVmIL-12 groups.

Anti-tumor Response

To determine the therapeutic outcome of WSLP/p2CMVmIL-12 complexes after intratumoral injection, we measured the rate of tumor progression with time. There was a substantial improvement in the inhibition of tumor growth after a single injection of WSLP/p2CMVmIL-12 complexes (Fig. 9). The mice treated with WSLP/p2CMVmIL-12 complexes had greater physical activity and a better response to change in environment compared with the control mice treated with naked p2CMVmIL-12 and 5% glucose. Mice injected with 5% glucose faced early death compared with the mice injected with WSLP/p2CMVmIL-12 complexes. The deaths of p2CMVmIL-12 injected mice were quicker compared with those of WSLP/p2CMVmIL-12 injected mice.

DISCUSSION

IL-12 is unique among cytokines due to its high potency and ability to induce other antitumor cytokines. Recombinant IL-12 (rIL-12) has been demonstrated to induce profound T-cell-mediated antitumor effects *in vivo* causing regression of established primary and metastatic tumors. The serum half-life of IL-12 is 3.5 hours in rodents and 16 hours in monkey. The relatively long serum half-life of IL-12 compared with other cytokines, such as IL-2 and IFN-γ, should permit more flexibility in dose scheduling. However, systemic administration of rIL-12 has been shown to cause severe dose- and schedule-dependent toxicity in rodents, nonhuman primates, and humans. In monkeys the maximum tolerated dose was 10 μg/kg/d and the minimally toxic dose was 1 μg/kg/d [16,17]. This limitation has created the need for localized *IL12* delivery to circumvent the toxicity of systemically administered rIL-12 and provide adequate local cytokine levels for immune cell activation.

The general structure of a cationic lipid has three parts: (1) a hydrophobic lipid anchor group (cholesterol or fatty acid chains), which helps in forming micellar structure and can interact with cell membranes; (2) a potentially biodegradable linker group, such as ester, amides, or carbamate; and (3) a positively charged headgroup, which interacts with plasmid DNA, leading to its condensation [18]. The linker groups should be biodegradable yet strong enough to survive in a biological environment. The active complex consists of a mixture of cationic lipid, neutral lipid, and pDNA and is strongly influenced by the relative proportions of each. Cholesterol is a naturally occurring lipid and is metabolized in the body. The early success of 3β[*N*,(*N′*,*N′*-dimethylaminoethane)-carbamoyl]cholesterol (DC-Chol) lipid-based gene delivery systems spurred interest in the development of novel cholesterol-based cationic lipids [19–21]. The levels of gene expression obtained with spermine cholesteryl carbamate and spermidine cholesteryl carbamate was 50–100-fold higher both *in vitro* and *in vivo* than that observed with DC-Chol, which has only a single protonatable amine [20].

To combine the advantages of lipids and polycations, Zhou *et al.* [22] synthesized lipopolylysine by mixing poly-L-lysine of 3300 Da with two molar equivalents of *N*-hydroxysuccinimide ester of dipalmitoylsuccinylglycerol in dimethyl sulfoxide. Similarly, Choi *et al.* [23] recently synthesized 3β(L-lysineamide carbamoyl)cholesterol (K-Chol) and 3β(L-ornithinamide carbamoyl)cholesterol (O-

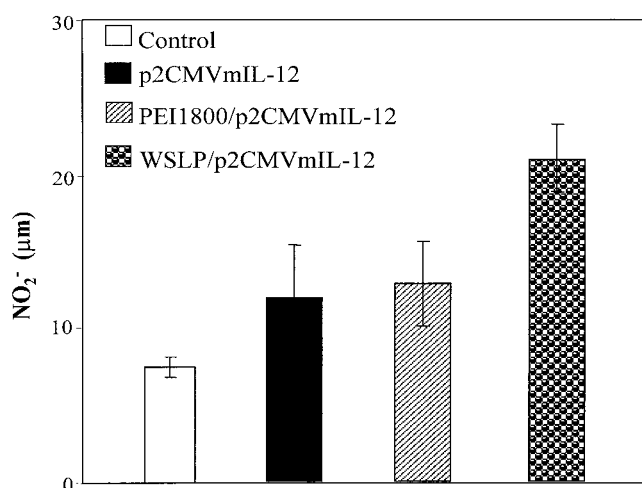

FIG. 8. Production of nitric oxide (NO) after intratumoral injection of WSLP/p2CMVmIL-12 complexes into BALB/c mice. At 48 h postinjection, tumors were isolated, chopped into small pieces, and cultured. Supernatants were analyzed by Griess reagent assay for assessing the levels of NO in terms of nitrite (NO_2^-). Mice injected with 5% (w/v) glucose were used as negative controls.

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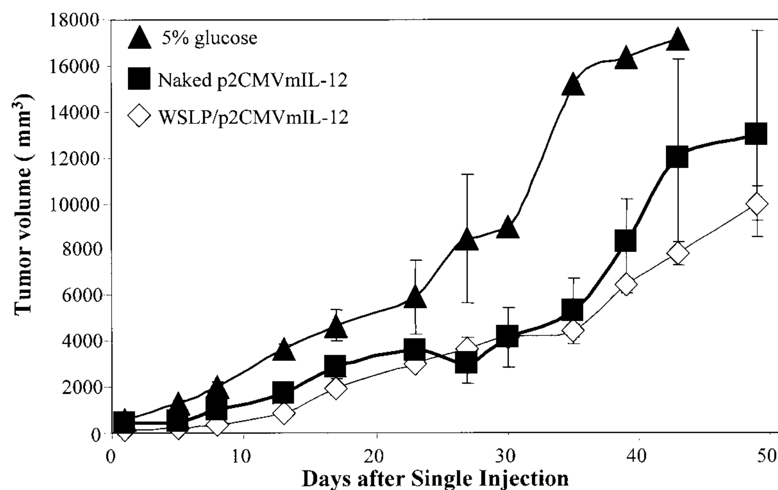

FIG. 9. Profiles of tumor progression after single intratumoral injection of WSLP/p2CMVmIl-12 complexes into CT-26 subcutaneous tumor-bearing BALB/c mice. Mean tumor volumes ± S.D. were recorded every three days until the animals died naturally or were killed due to humane reasons.

Chol) by the solid phase synthesis method. However, liposome preparation with colipid DOPE was essential for enhanced gene transfer by these cationic amphiphiles. Yamazaki *et al.* [24] grafted cetyl groups as hydrophobic lipid anchors on to PEI of 1800 and 25,000 Da, and prepared polycation liposomes for gene transfer. There is a growing need, however, to avoid the formation of cationic liposomes and the large size complexes (> 200 nm), as these DNA particles do not disperse well inside the tumor tissues.

Behr [25] synthesized lipopolyamines such as dioctadecylamidoglycylspermine (DOGS), which mediates transfection by itself and does not require any phospholipid to form liposomes. The tertiary amine of bis(guanidinium)-tren-cholesterol (BGTC) has also been suggested to buffer the acidic environment of endosomes; thus BGTC can be used as cationic micelles for gene delivery [26]. Here we conjugated cholesterol as a hydrophobic lipid anchor to PEI, a cationic polymer known to have high transfection activity. The hydrophilicity and endosomolytic capacity of WSLP depend on the molecular weight of PEI and the number of cholesterols conjugated per molecule of PEI. Micellization should concentrate positive charge on the surface and thus PEI of high molecular weights may not be required. Among 600, 1200, and 1800 Da PEI, it is likely that PEI of 1800 Da will offer the highest endosomal buffering capacity and water solubility. PEI has many protonation sites and thus should confer endosomolytic properties to WSLP. Thus, WSLP should deliver plasmid DNA without the requirement of any colipid as a liposomal compartment. The percentage of cholesterol conjugated to PEI was about 47% and the average molecular weight of WSLP was about 2000 Da. Because WSLP forms micellar solutions, we investigated its gene transfer efficiency by direct mixing of its solution with pDNA. The mean particle size of WSLP/pDNA complexes was in the range of 26 to 62 nm. WSLP and WSLP/pDNA complexes were not toxic to CT-26 colon carcinoma cells even at N/P ratio of 20 or below, whereas PEI25000-based formulations were toxic to these cells.

Transfection efficiency and cytotoxicity increase with an increase in the molecular weight of PEI. We, and others, have shown previously that DNA formulations based on a PEI of 25,000 Da or above show efficient gene transfer, as these polymers offer more protection to the plasmid DNA they carry. However, high molecular weight PEIs are highly toxic to cells. In contrast, low molecular weight PEIs (600, 1200, and 1800 Da) are not toxic, but do not transfect cultured cells [27,28]. This is because interpolymer systems with relatively few salt bonds dissociate during dilution, which implies that PEI/pDNA complexes prepared using small molecular weight PEI molecules will dissociate more easily and provide lower transfection yields [29]. Unlike PEI, WSLP is likely to interact with pDNA through both ionic and hydrophobic interactions. Moreover, micellization may concentrate positive charge on the surface and thus PEI of high molecular weight may not be required. In our study, the levels of secreted IL-12 for samples treated with WSLP/p2CMVmIL-12 were higher than those for PEI1800 and PEI25000-based formulations, and were dependent on the N/P ratios (Figs. 3 and 4).

The IRES segment between *Il12a* and *Il12b* of pIRESmIL-12 may not allow the regulated production of p35 and p40 to confer increased production of bioactive IL-12 p70. We have previously shown at least 10–15-fold higher production of IL-12 p40 compared with IL-12 p70 for tumor-bearing BALB/c mice treated with PAGA/pIRESmIL-12 complexes [30]. IL-12 p40 can act as an antagonist towards IL-12 p70 by forming a $(p40)_2$ homodimer. The IL-12 expression plasmid with two separate CMV promoters driving *Il12a* and *Il12b* cDNAs produced at least twofold IL-12 p70 compared with the bicistronic IRES vector of IL-12 both *in vivo* and *in vitro* [8]. Therefore, for more efficient expression of the second gene, p2CMVmIL-12 was constructed, in which the expression of *Il12a* and *Il12b* was driven by two independent CMV promoters. The levels of secreted IL-12 were much higher when CT-26 cells were transfected with WSLP/p2CMVmIL-12 complexes compared with WSLP/pIRESmIL-12 complexes (Fig. 5).

Following *in vitro* characterization, we injected WSLP/p2CMVmIL-12 complexes into subcutaneous CT-26 tumor bearing BALB/c mice and determined the levels of IL-12 by ELISA of the supernatant of the cultured tumors

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at 48 hours postinjection. There is evidence that IL-12 inhibits angiogenesis and induces apoptosis as well as extensive necrosis in mouse tumor. Our studies show that use of WSLP/p2CMVmIL-12 complexes substantially enhances the levels of IL-12 p70 production compared with naked p2CMVmIL-12 and control groups (Fig. 6), which would reduce the potential of tumor growth and metastasis. Complex formation between WSLP and p2CMVmIL-12 enhanced the level and duration of *IL12* expression, which were highest at day 1 and then decreased slowly with time.

At day three postinjection, IL-12 levels of samples treated with the WSLP/p2CMVmIL-12 complex were higher than those treated with naked p2CMVmIL-12 (Fig. 6). Compared with naked DNA, there was only a modest (1.5- to 2-fold) increase in IL-12 expression after intratumoral injection of WSLP/pDNA complexes into CT-26 subcutaneous tumor bearing BALB/c mice. We believe this is an important finding, especially because WSLP is nontoxic and may be used for repeated intratumoral injection to provide sustained transgene expression. To our knowledge, this is the first report on the use of WSLP for intratumoral gene delivery, with some enhancement in *IL12* expression. Although polyvinylpyrrolidone (PVP) has been used for intratumoral delivery of pmIL-12 into CT-26 tumor bearing BALB/c mice, no comparison was made between naked pDNA in saline and PVP-based formulations [31]. Intratumoral injection of DC-chol cationic liposome/pmIFN-γ complexes into CT-26 tumor bearing mice have been shown to produce a lower level of transgene expression than those injected with naked pDNA. Contrary to the levels of gene expression, the extent of tumor regression was more pronounced in the CT-26 subcutaneous tumor bearing mice treated with DC-chol liposome/pCMV-mIFN-γ complexes than those treated with naked pCMV-mIFN-γ [32]. Compared with naked pDNA, a lower level of luciferase expression has also been reported for PEI/pCMV-Luc complexes when injected into mice bearing subcutaneous tumors, presumably because of poor diffusion of the complexes within the tumor mass after injection [33]. An enhancement in luciferase expression was seen when the complexes were delivered slowly into the tumor using a micropump, but the use of PEI 25,000 for cytokine gene delivery was not investigated. Moreover, PEI 25,000 is cytotoxic and thus may not be suitable for repeated injections [34].

There is a strong induction of IFN-γ as a result of rIL-12 administration, which acts synergistically along with IL-12 to generate a potent anti-tumor response. Low systemic levels of IL-12 have been shown to induce IFN-γ production and, through a feedback control mechanism, IL-12 activates its own upregulation through macrophages, dendritic cells, and other stimulatory factors induced by IFN-γ as part of adaptive immune response [35]. We determined the induced levels of IFN-γ after intratumoral injection of WSLP/p2CMVmIL-12 complexes. The production of induced IFN-γ was significantly higher compared with control mice injected with naked p2CMVmIL-12 and 5% glucose (Fig. 7).

Paracrine stimulation with IFN-γ can lead to endogenous synthesis of nitric oxide synthase via macrophages as well as type I interferons [36]. However, it has not been found to be as effective as IL-12 for its own production *in vivo* as well as for its antitumor and antimetastatic activity. Because antitumor activity of IL-12 depends strongly on IFN-γ induction, it is imperative that use of a plasmid encoding IFN-γ should result in similar trends in terms of anti tumor activity as found via *IL12* delivery. We have previously shown that intratumoral injection of PAGA/pmIL-12 and PAGA/pmIFN-γ complexes produced similar levels of IFN-γ [37], but IL-12 is superior to IFN-γ in terms of tumor regression even at lower pDNA dose [2]. This may be due to the fact that IL-12 has greater potential to induce a host of cytokines compared with IFN-γ, thereby resulting in a stronger Th1 type immune development.

NO is an important regulator of the immune response and is a highly cytotoxic molecule. NO is endogenously produced in activated macrophages and natural killer cells by inducible nitric oxide synthase (iNOS) after stimulation by induced IFN-γ and IL-12 [38]. We measured the levels of NO in terms of moles of nitrite (NO_2^-) in the supernatant of the cultured tumors of CT-26 subcutaneous tumor bearing BALB/c mice. NO levels were significantly upregulated within a period of 48 hours, when the animals were injected with WSLP/p2CMVmIL-12 complexes compared with the control animals (Fig. 8).

To assess the IL-12 treatment outcome, subcutaneous CT-26 tumor bearing BALB/c mice treated with WSLP/p2CMVmIL-12 complexes were studied for their long-term survival and tumor progression. The tumor size of the animals receiving a single injection of WSLP/p2CMVmIL-12 complexes progressed slower compared with animals receiving single injections comprising WSLP/p2CMVmIL-12 and naked p2CMVmIL-12 groups (Fig. 9). As WSLP is nontoxic, we envision that it can be used for repeated injections to further regress tumor growth compared with single-dose effects (Fig. 9).

Our results indicate that WSLP will be a suitable carrier for intratumoral cytokine gene delivery. Our findings on the usefulness of WSLP are in good agreement with Vigneron *et al.* [21], who reported the synthesis of guanidinium cholesterol lipids and their use for the efficient transfection of various mammalian cell lines. We have designed a nontoxic WSLP that can efficiently condense and protect plasmid DNA from degradation by nucleases, and shows enhanced transgene expression both *in vitro* and *in vivo*. We also constructed an effective IL-12 expression plasmid driven by two promoters.

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MATERIALS AND METHODS

Materials. Branched PEI (mw 1800) was purchased from Polysciences, Inc. (Warrington, PA). Cholesteryl chloroformate, branched PEI (mw 25,000), triethyleneamine, glucose, and deuterium oxide (D_2O) were purchased from Aldrich Chemical Co. (Milwaukee, WI). Terrific broth, ampicillin, Rosewell Park Memorial Institute (RPMI-1640) medium, Dulbecco's modified essential medium (DMEM), and 1,4-dioxane-d_8 were purchased from Sigma Chemical Co. (St. Louis, MO). Methylene chloride and dimethyl sulfoxide (DMSO) were purchased from Fisher Scientific (Fair Lawn, NJ). pCI plasmid, RQ1 RNase-free DNase I, *Hin*dIII, *Sca*I, *Xma*I, *Bam*HI, *Eco*RI, T4 lygase, calf intestinal phosphatase, 100-bp DNA stepladder, 1-kb DNA stepladder, reporter lysis buffer, and bacterial strain DH5α were purchased from Promega (Madison, WI). Glucose, sodium dodecyl sulfate (SDS), molecular biology grade isopropyl alcohol, ethyl alcohol, and methanol were all purchased from Aldrich (Milwaukee, WI). Library efficiency DH5α competent cells, DNA grade high melting agarose, bovine serum albumin (BSA), fetal bovine serum (FBS), phosphate buffered saline (PBS), ethidium bromide, 0.25% (w/v) trypsin-EDTA, and Qiagen EndoFree Plasmid Maxi Kit was purchased from Qiagen (Valencia, CA). BDOptEIA ELISA sets for IL-12 p70 and IFN-γ were obtained from Pharmingen (San Diego, CA). Bicinchoninic acid (BCA) protein assay reagent kit was purchased from Pierce Chemical Co. (Rockford, IL).

Mice. We purchased five-week-old female BALB/c mice from Simonsen Laboratories (Gilroy, CA) and housed tham in the Animal Care Facility, Biomedical Polymers Research Building, University of Utah. Mice were maintained on *ad libitum* rodent feed and water at room temperature, 40% humidity. All mice were acclimated for at least 1 week before tumor implantation. All studies were performed in accordance with the approved animal protocol.

Synthesis of WSLP. We synthesized WSLP as shown in Fig. 1 [39]. Briefly, we stirred 3 g branched PEI (mw 1800) on ice in a mixture of 10 ml anhydrous methylene chloride and 100 μl triethylamine for 30 min. We dissolved 1 g cholesteryl chloroformate in 5 ml anhydrous ice-cold methylene chloride and then slowly added it to the PEI solution for 30 min. We stirred the mixture for 12 h on ice and dried the resulting product on a rotary evaporator. We dissolved the powder in 50 ml of 0.1 N HCl and then filtered through the glass microfiber filter. Cholesteryl chloroformate is soluble in methylene chloride, diethyl ether, and acetone. Therefore, nonreacted cholesteryl chloroformate was removed from the product by washing three times in these different organic solvents as follows: the aqueous solution was extracted 3 times with 100 ml methylene chloride to remove non-reacted cholesterol chloroformate, then extracted aqueous solution was filtered through a glass microfiber filter. We concentrated the product by solvent evaporation, precipitated with large excess acetone, and dried under vacuum. The powder was dissolved in water and precipitated three times with acetone. After washing with methanol, the product was washed three times with diethyl ether. The white powder product was analyzed using matrix-assisted laser desorption-time of flight (MALDI-TOF) mass spectrophotometry (Perspective Voyager-DE STR, PE Applied Biosystem Co., Framingham, MA) with *trans*-4-hydroxy-3-methoxy-cinnamic acid as matrix and 400-MHz ^{1}H nuclear magnetic resonance (^{1}H NMR, Varian, Inc., Palo Alto, CA) and then stored at –20°C until used.

Construction and purification of IL-12 expression plasmids. To express *Il12a* and *Il12b* using a single vector (obtained from Jun-ichi Miyazaki of Osaka University, Japan [40]), an IRES segment was used to construct pIERSmIL-12 plasmid. However, IRES has been reported to be inefficient in expression of the second gene, which may lead to low production of bioactive IL-12 p70. Therefore, p2CMVmIL-12 was constructed with *Il12a* and *Il12b* under the transcriptional control of a separate CMV promoter. The *Eco*RI and *Xma*I restriction enzyme sites were introduced into the *Il12a* and *Il12b* cDNAs by PCR using pCAGGS-IL12 as a template. The sequences of the primers are as follows: p35 forward, 5′-GGAATTCTATGTGTCAATCACGCTACCTCC-3′; p35 backward, 5′-CCCCCCGGGCGATCAGGCGGAGCTCAGATAGCCC-3′; p40 forward, 5′-GGAATTCTATGTGTCCTCAGAAGCTAACCA-3′; p40 backward, 5′-CCCCCCGGGCGACTAGGATCGGACCCTGCAGGGA-3′ (*Eco*RI and *Xma*I restriction enzyme sites are underlined). After PCR reactions, we purified the p35 and p40 cDNAs by 1% agarose gel electrophoresis and electroelution. The purified cDNAs were digested with *Eco*RI and *Xma*I and inserted into pCI plasmid, resulting in construction of pCMV-p35 and pCMV-p40, respectively. The expression unit of p40 in pCMV-p40 was then isolated by digestion with *Bgl*II and *Bam*HI followed by 1% agarose gel electrophoresis. The isolated p40 expression unit was inserted at *Bam*HI site of pCMV-p35. The resulting plasmid, p2CMVmIL-12, was confirmed by restriction enzyme assays. Plasmids were amplified using DH5α strain of *Escherichia coli* and purified using the EndoFree kit (Qiagen) following the manufacturer's protocols. Plasmids were characterized using the following methods: (1) UV spectrophotometic assay at 260/280 nm and 1% agarose gel electrophoresis to determine the purity, integrity, and concentration of the plasmids; and (2) restriction enzyme assay to confirm that there was no rearrangement of the genes during cloning and propagation.

Formulation and in vitro characterization. Following synthesis, purification and characterization, equal volume of 5% glucose solution containing WSLP was mixed directly with IL-12 expression plasmids containing 5% glucose and complex formation was allowed to proceed at room temperature for 15 min. WSLP/p2CMVmIL-12 complexes were characterized in terms of particle size, ζ potential, osmolality, and surface morphology as described [39].

Tumor cell lines. CT-26 colon adeno-carcinoma cell lines were a gift from Charles Tannenbaum (Cleveland Clinic Foundation, Cleveland, OH) [41]. Tumor cells were grown and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 U/ml streptomycin, and 50 μg/ml gentamycin (all from Gibco-BRL, Gaithersburg, MD) at 37°C and humidified 5% CO_2.

In vitro transfection. CT-26 cells were seeded in 6-well tissue culture plates at 3×10^5 cells per well in 10% FBS containing RPMI 1640 media. Cells achieved 70% confluency within 24 h, after which they were transfected with WSLP/p2CMVmIL-12 and pIRESmIL-12 complexes. The total amount of plasmid DNA loaded was maintained constant at 2.5 μg/well and transfection was carried out in absence of serum. The cells were incubated at 37°C in the presence of complexes for 6 h in CO_2 incubator followed by replacement with 2 ml RPMI 1640 containing 10% FBS. Thereafter the cells were incubated at 37°C for additional 36 h. Following transfection, the levels of IL-12 secreted in culture supernatants were determined by ELISA. Both naked DNA as well as untreated cells were used as positive and negative controls, respectively.

Tumor implantation and treatment. To generate tumors, we injected 5-week-old female BALB/c mice subcutaneously in the middle of the left flank with 100 ml of a single-cell suspension containing 1×10^6 CT-26 cells. Tumor size was measured using a vernier caliper across its two perpendicular diameters and its depth, and its volume was calculated using the formula $V = (4/3)\pi abc$. Treatment of the tumors was started after 10–15 d when the tumor size reached approximately 100–120 mm^3. WSLP/p2CMVmIL-12 complexes were prepared in 5% glucose and 50 ml of the complexes were injected directly into the tumors of BALB/c mice at a dose of 25 μg pDNA/mouse. The treated mice were sacrificed at 48 h postinjection. Tumors were chopped into small pieces, recultured into 6-well plates and incubated at 37°C for 24 h. Supernatants were separated from the cells by centrifugation and assayed for IL-12 p70, IFN-γ, and nitric oxide.

ELISA for IL-12 and induced cytokines. Measurement of IL-12 p70 and IFN-γ was carried out using BDOptEIA ELISA sets (Pharmingen, San Diego, CA) according to the manufacturer's instructions. ELISA plates (Nunc, Maxisorp, Denmark) were coated with capture antibody, sealed, and kept overnight for antibody binding. The plates were washed several times followed by incubation with assay diluent to block any nonspecific binding for 1 h. After several washings, the plate was then incubated with samples and standards for 2 h. After incubating with detection antibody solution containing avidin-HRP reagent for 1 h the substrate solution was added to carry out enzymatic reaction. The reaction was stopped by 2N H_2SO_4 and the plate was read at 450 nm using Bio-Rad (model 3550) ELISA reader (Hercules, CA). The IL-12 p70 and IFN-γ concentrations were reported in terms of pg/ml.

Tumor regression studies. WSLP/p2CMVmIL-12 complexes were injected directly into CT-26 subcutaneous tumor bearing BALB/c mice at a dose of 25 μg pDNA/mouse. Naked p2CMVmIL-12 (25 μg/mouse) as well as 5% glucose were also injected into CT-26 tumor bearing mice to use as the pos-

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itive and negative controls, respectively. After a single intratumoral injection, mice were closely monitored at an interval of every 3 d for tumor growth. Tumor progression was reported in terms of tumor volume over a period of 48 d.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) and Student's *t* test. Data were considered statistically significant if *P* values were < 0.05.

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REFERENCES

1. Brunda, M. J., *et al.* (1993). Antitumor and antimetastatic activity of interleukin-12 against murine tumors. *J. Exp. Med.* **178**: 1223–1230.
2. Rakhmilevich, A. L., Janssen, K., Turner, J., Culp, J., and Yang, N.-S. (1997). Cytokine gene therapy of cancer using gene gun technology: Superior antitumor activity of Interleukin-12. *Hum. Gene Ther.* **8**: 1303–1311.
3. Tahara, H., *et al.* (1995). Effective eradication of established murine tumors with IL-12 gene therapy using a polycistronic retroviral vector. *J. Immunol.* **154**: 6466–6474.
4. Caruso, M., *et al.* (1996). Adenovirus mediated interleukin-12 gene therapy for metastatic colon carcinoma. *Proc. Natl. Acad. Sci. USA* **93**: 1302–11306.
5. Zallen, D. T. (2000). US gene therapy in crisis. *Trends Genet.* **16**: 272–275.
6. Han, S.-O., Mahato, R. I., Sung, Y. K., and Kim, S. W. (2000). Development of biomaterials for gene therapy. *Mol. Ther.* **2**: 302–317.
7. Mahato, R. I., Smith, L. C., and Rolland, A. (1999). Pharmaceutical perspectives of nonviral gene therapy. *Adv. Genet.* **41**: 95–156.
8. Rakhmilevich, A. I., and Yang, N.-S. (1999). IL-12 gene therapy of tumors. *US PT* 5,922,685.
9. Dow, S.W., *et al.* (1999). Intravenous cytokine gene delivery by lipid-DNA complexes controls the growth of established lung metastases. *Hum. Gene Ther.* **10**: 2961–2972.
10. Whitmore, M., Li, S., and Huang, L. (1999). LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Ther.* **6**: 1867–1875.
11. Nomura, T., *et al.* (1997). Intratumoral pharmacokinetics and in vivo gene expression of naked plasmid and its cationic liposome complexes after direct gene transfer. *Cancer Res.* **57**: 2681–2686.
12. Boussif, O., *et al.* (1995). A versatile vector for gene and oligonucleotide transfer into cells in culture and *in vivo*: polyethylenimine. *Proc. Natl. Acad. Sci. USA* **92**: 7297–7301.
13. Abdullah, B., *et al.* (1996). A powerful nonviral vector for in vivo gene transfer into the adult mammalian brain: polyethylenimine. *Hum. Gene Ther.* **7**: 1947–1954.
14. Mountford, P. S., and Smith, A. G. (1995). Internal ribosome entry site and dicistronic RNAs in mammalian transgenesis. *Trends Genet.* **11**: 179–184.
15. Mizuguchi, H., Xu, Z., Ishii-Watanabe A., Uchida, E., and Hayakawa, T. (2000). IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Mol. Ther.* **1**: 376–382.
16. Sarmiento, U. M., *et al.* (1994). Biologic effects of recombinant human interleukin-12 in squirrel monkeys (Sciureus saimiri). *Lab. Invest.* **71**: 862–873.
17. Leonard, J. P., *et al.* (1994). Effects of single-dose inteleukin-12 exposure on interleukin-12-associated toxicity and interferon-γ production. *Blood* **90**: 2541–2548.
18. Mahato, R. I., Rolland, A., and Tomlinson, E. (1997). Cationic lipid-based gene delivery systems: pharmaceutical perspectives. *Pharm. Res.* **14**: 853–859.
19. Gao, X., and Haung, L. (1991). A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **179**: 280–285.
20. Lee, E. D., *et al.* (1996). Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum. Gene Ther.* **7**: 1701–1717.
21. Vigneron, J.-P., *et al.* (1996). Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. *Proc. Natl. Acad. Sci. USA* **93**: 9682–9686.
22. Zhou, X., and Huang, L. (1994). DNA transfection mediated by cationic liposomes containing lipopolylysine. Characterization and mechanism of action. *Biochim. Biophys. Acta* **1189**: 195–205.
23. Choi, J. S., Lee, E. J., Jang, H. S., and Park, J. S. (2001). New cationic liposomes for gene transfer into mammalian cells with high efficiency and low toxicity. *Bioconjug. Chem.* **12**: 108–113.
24. Yamazaki, Y., *et al.* (2000). Polycation liposomes, a novel nonviral gene transfer system, constructed from cetylated polyethylenimine. *Gene Ther.* **7**: 1148–1156.
25. Behr, J. P. (1986). DNA strongly binds to micelles and vesicles containing lipopolyamines or lipointercalants. *Tetrahedron Lett.* **27**: 5861–5864.
26. Pitard, B., *et al.* (1997). Virus-size self-assembling lamellar complexes between plasmid DNA and cationic micelles promote gene transfer. *Proc. Natl. Acad. Sci. USA* **94**: 14412–14417.
27. Godbey, W. T., Wu, K. K., and Mikos, A. G. (1999). Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. *J. Biomed. Mater. Res.* **45**: 268–275.
28. Fischer, D., Bieber, T., Li, Y., Elsasser, H.-P., and Kissel, T. (1999). A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm. Res.* **16**: 1273–1279.
29. Godbey, W. T., Wu, K. K., and Mikos, A. G. (1999). Poly(ethylenimine) and its role in gene delivery. *J. Control. Release* **60**: 149–160.
30. Maheshwari, A., *et al.* (2000). Soluble biodegradable polymer-based cytokine gene delivery for cancer treatment. *Mol. Ther.* **2**: 121–130.
31. Mendiratta, S. K., *et al.* (1999). Intratumoral delivery of IL-12 gene by polyvinyl polymeric vector system to murine renal and colon carcinoma results in potent antitumor immunity. *Gene Ther.* **6**: 833–839.
32. Nomura, T., *et al.* (1999). Gene expression and antitumor effects following direct interferon (IFN)-γ gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice. *Gene Ther.* **6**: 121–129.
33. Coll, J. L., *et al.* (1999). *In vivo* delivery to tumors of DNA complexed with linear polyethylenimine. *Hum. Gene Ther.* **10**: 1659–1666.
34. Kircheis, R., *et al.* (1999). Polycation-based DNA complexes for tumor-targeted gene delivery *in vivo. J. Gene Med.* **1**: 111–120.
35. Brunda, M.J., *et al.* (1995). Role of interferon-γ in mediating the anti-tumor efficacy of interleukin-12. *J. Immunother.* **17**: 71–77.
36. Huang, F.-P., *et al.* (1998). Nitric oxide regulates Th1 cell development through the inhibition of IL-12 synthesis by macrophages. *Eur. J. Immunol.* **28**: 4062–4070.
37. Mahato, R. I., Furgeson, D. Y., Maheshwari, A., Han, S.-O. and Kim, S. W. (2000). Polymeric gene delivery for cancer treatment. In *Biomaterials and Drug Delivery towards New Millennium* (K. D. Park, I. C. Kwon, N. Yui, S. Y. Jeong, and K. Park, Eds.), pp. 249–280. Han Rim Won Publishing Co., Seoul, Korea.
38. Salvucci, O., Kolb, J. P., Dugas, B., Dugas, N. and Chouaib, S. (1998). The induction of nitric oxide by interleukin-12 and tumor necrosis factor-α in human natural killer cells: relationship with the regulation of lytic activity. *Blood* **92**: 2093–2102.
39. Niwa, H., Yamamura, K., and Miyazaki, J. (1991). Efficient selection of high expression transfectants with a novel eukaryotic vector. *Gene* **108**: 193–199.
40. Han, S.-O., Mahato, R. I., and Kim, S. W. (2001). Water soluble lipopolymer-based gene delivery. *Bioconjug. Chem.* **12**: 337–345.
41. Tannenbaum, C. S., *et al.* (1996). Cytokine and chemokine expression in tumors of mice receiving systemic therapy with IL-12. *J. Immunol.* **156**: 693–699.

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