

# Soluble Biodegradable Polymer-Based Cytokine Gene Delivery for Cancer Treatment

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Transgene expression and tumor regression after direct injection of plasmid DNA encoding cytokine genes, such as mL-12 and mIFN- $\gamma$ , remain very low. The objective of this study is to develop nontoxic biodegradable polymer-based cytokine gene delivery systems, which should enhance mL-12 expression, increasing the likelihood of complete tumor elimination. We synthesized poly[ $\alpha$ -(4-aminobutyl)-L-glycolic acid] (PAGA), a biodegradable nontoxic polymer, by melting condensation. Plasmids used in this study encoded luciferase (pLuc) and murine interleukin-12 (pmIL-12) genes. PAGA/plasmid complexes were prepared at different ( $\pm$ ) charge ratios and characterized in terms of particle size, zeta potential, osmolality, surface morphology, and cytotoxicity. Polyplexes prepared by complexing PAGA with pmIL-12 as well as pLuc were used for transfection into cultured CT-26 colon adenocarcinoma cells as well as into CT-26 tumor-bearing BALB/c mice. The *in vitro* and *in vivo* transfection efficiency was determined by luciferase assay (for pLuc), enzyme-linked immunosorbent assay (for mL-12, p70, and p40), and reverse transcriptase-polymerase chain reaction (RT-PCR) (for Luc and mL-12 p35). PAGA condensed and protected plasmids from nuclease degradation. The mean particle size and zeta potential of the polyplexes prepared in 5% (w/v) glucose at 3:1 ( $\pm$ ) charge ratio were approximately 100 nm and 20 mV, respectively. The surface characterization of polyplexes as determined by atomic force microscopy showed complete condensation of DNA with an ellipsoidal structure in Z direction. The levels of mL-12 p40, mL-12 p70, and mIFN- $\gamma$  were significantly higher for PAGA/pmIL-12 complexes compared to that of naked pmIL-12. This is in good agreement with RT-PCR data, which showed significant levels of mL-12 p35 expression. The PAGA/pmIL-12 complexes did not induce any cytotoxicity in CT-26 cells as evidenced by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide assay and showed enhanced antitumor activity *in vivo* compared to naked pmIL-12. PAGA/pmIL-12 complexes are nontoxic and significantly enhance mL-12 expression at mRNA and protein levels both *in vitro* and *in vivo*.

**Key Words:** biodegradable cationic polymer; interleukin-12; intratumoral delivery; gene expression; RT-PCR; ELISA.

## INTRODUCTION

The objective of cytokine gene-mediated immunotherapy of cancer lies in effective retardation of established tumor metastasis, confinement of tumor and its elimination, and prevention of reoccurrence of tumor. Cancer gene therapy has been attempted with virtually every cytokine belonging to the family of interleukins, inter-

ferons, tumor necrosis factors, and colony stimulating factors (1–6). Among them IL-12 has proven to be one of the most effective in the induction of potent antitumor immunity (7, 8). IL-12 helps in activation, maturation, and differentiation of natural killer (NK) and T cells as well as augmenting their cytolytic activity. IL-12 also induces the production of IFN- $\gamma$  and to a lesser extent TNF- $\alpha$ , which further mediate the antitumor effects of immune cells (9, 10). IL-12 is a heterodimeric cytokine consisting of p35 and p40 subunits, which form a 75-kDa protein. The p35 subunit is ubiquitous and is found in almost all the cells, whereas p40 is produced mostly by macrophages and B cells. The relative production of p35

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and p40 governs the formation of p70 and is greatly influenced by the inhibitory action of the (p40)<sub>2</sub> homodimer (11, 12).

Recombinant IL-12 (rIL-12) has been shown to mediate profound T cell-mediated antitumor effects *in vivo*, causing regression of established subcutaneous tumors and tumor metastasis (13, 14). However, systemic administration of rIL-12 protein has caused severe toxicity in rodents as well as in human clinical trials (15, 16). This limitation has created the need for alternative approaches. Localized delivery of IL-12 genes may circumvent the toxicity of systemically administered rIL-12 and provide adequate local cytokine levels for immune cell activation. Retrovirus-based *ex vivo* mIL-12 gene therapy has shown promise in inducing antitumor immune responses leading to tumor regression (17, 18). However, retroviral vectors are not safe for repeated use due to the adverse effects, such as immunogenicity, toxicity, and mutagenesis. Recently, gene gun technology has been used to deliver plasmid encoding mIL-12 to murine tumors, which resulted in tumor regression (19). However, gene gun-mediated immunotherapy of cancer is not patient friendly. Intratumoral injection of DC-chol cationic liposomes/pmIFN- $\gamma$  complexes into CT-26 subcutaneous tumor-bearing BALB/c mice has been shown to produce lower levels of transgene expression than injection with naked pDNA. Contrary to the levels of gene expression, the extent of tumor regression was more pronounced when the mice were treated with DC-chol cationic liposomes/pCMV-mIFN- $\gamma$  complexes than those treated with naked pCMV-mIFN- $\gamma$  (2). In contrast to intratumoral injection into the subcutaneous tumor-bearing mice, intravenous injection of cationic liposome/pmIL-12 complexes to the mice bearing pulmonary metastasis produced significant levels of IL-12 expression and regression of tumor growth (20, 21). However, there are several limitations in the use of cationic liposomes, some of which are similar but to a lesser degree compared to those exhibited by retroviral vectors. Cationic lipids, for example, downregulate the synthesis of protein kinase C, nitric oxide, and tumor necrosis factors and are highly toxic especially toward macrophages (22). One of the focal points of cancer gene therapy is to eradicate the tumor cells, while minimizing the damage to the surrounding normal tissues. Therefore, polyvinylpyrrolidone (PVP) was used for intratumoral delivery of pmIL-12 to murine renal and colon carcinoma (23). Although this system produced mIL-12 and mIFN- $\gamma$ , the levels of transgene expression and tumor regression were very low, possibly due to the little protection of plasmid DNA by PVP. Moreover, no comparison was made between naked pmIL-12 in saline and PVP/pmIL-12-based formulations for gene expression and tumor regression.

Compared to naked pDNA, a lower level of luciferase expression has been reported for linear polyethyleneimine (PEI)/pCMV-Luc complexes injected into the subcutaneous tumor-bearing mice, presumably because

of poor diffusion of the complexes within the tumor mass after injection (24). There was enhancement in luciferase expression when the complexes were delivered slowly into the tumor using a micropump. However, these authors did not use cytokine genes (IL-12, IFN- $\gamma$ , or IL-2) and thus the effect of PEI/pDNA complexes on tumor regression is not known. Moreover, PEI is known to be cytotoxic and thus may not be suitable for repeated injections (25). Biodegradable polymers that can protect DNA from nuclease attack and enhance cytokine gene expression after intratumoral injection into solid tumors will be an exciting option for cancer treatment. In this study, we synthesized a biodegradable analogue of poly(L-lysine) (PLL), poly[ $\alpha$ -4 aminobutyl]-L-glycolic acid] (PAGA), as described previously (26) for delivery of mIL-12 expression plasmids into cultured CT-12 colon adenocarcinoma cells and into CT-26 subcutaneous tumor-bearing BALB/c mice for the treatment of cancer. PAGA forms complexes with plasmid DNA, protects it from degradation by nucleases, and confers enhanced *in vitro* transfection compared to poly(L-lysine). PAGA shows accelerated degradation when free in aqueous solution and slow degradation when it forms complexes with pDNA. The final degradation product of PAGA is a degraded monomer, L-oxyllysine, which will be rapidly removed from cellular compartments followed by metabolism and excretion from the body (26).

## MATERIALS AND METHODS

**Materials.** 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), glycerol, terrific broth, ampicillin, and 3-[N-morpholino]propane sulfonic acid (Mops) were purchased from Sigma Chemical Co. (St. Louis, MO). Dimethyl sulfoxide (DMSO) and glucose were purchased from Aldrich (Milwaukee, WI). Luciferase plasmid with simian virus 40 (SV40) promoter (pLuc), DNA size marker, luciferase assay system, and bacterial strain DH5 $\alpha$  were purchased from Promega (Madison, WI). Fetal bovine serum (FBS), phosphate-buffered saline (PBS), 0.25% (w/v) trypsin-EDTA, Rosewell Park Memorial Institute Medium (RPMI 1640), Dulbecco's modified essential medium (DMEM), penicillin, streptomycin, and gentamycin were purchased from Gibco-BRL (Gaithersburg, MD). Qiagen Maxi plasmid purification kit, RNeasy Mini kits, Omniscript RT kit, and Taq PCR core kit were all purchased from Qiagen (Boulder, CO). BDOptEIA enzyme-linked immunosorbent assay (ELISA) sets for mIL-12 p70 and p40, 3,3',5,5'-tetramethylbenzidine (TMB), and hydrogen peroxide substrate reagents were obtained from Pharmingen (San Diego, CA). Bicinchoninic acid (BCA) protein assay reagent kit was purchased from Pierce Chemical Co. (Rockford, IL).

**Mice.** Five-week-old female BALB/c mice were purchased from Harlan Laboratories (Houston, TX) and housed in the Animal Care Facilities of Huntsman Cancer Institute and 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 an animal protocol approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

**Construction and purification of plasmids.** Plasmid DNA encoding murine interleukin 12 (mIL-12) was used as a model cytokine gene and was obtained as a kind gift from Dr. Jun-ichi Miyajaki of Osaka University Medical School, Japan. The p35 and p40 subunits of mIL-12 are expressed from two independent transcription units and are inserted into a single plasmid, pCAGGS (27, 28). The expression unit for mIL-12 p35, including CMV immediate early enhancer-chicken  $\beta$ -actin hybrid

promoter and rabbit  $\beta$ -globin poly(A) signal, is excised from pCAGGS-p35 and is inserted downstream of the mL-12 p40 expression unit of pCAGGS-p40 (27, 28). Another plasmid for mL-12, pCMV-mL-12, was compared with pCAGGS-mL-12 and was obtained as a kind gift from Dr. Steven Dow of the University of Colorado (20). Plasmid encoding luciferase (pLuc) was used as a reporter gene and was purchased from Promega. These plasmids were amplified in *Escherichia coli* DH5 $\alpha$  strain and then isolated and purified using Qiagen Plasmid Maxi purification kit (Qiagen, Valencia, CA). The plasmid purity and integrity were confirmed by 1% agarose gel electrophoresis followed by ethidium bromide staining, and DNA concentration was measured by ultraviolet (UV) absorbance at 260 nm. The optical density ratios at 260 to 280 nm of these plasmid preparations were in the range of 1.7–1.8.

**Restriction enzyme analysis.** To confirm that pmIL-12 contains the whole p40 and p35 genes and there is no rearrangement of the gene during cloning and propagation, restriction enzyme digestion assay was done as described by Yoshimoto *et al.* (29) with *EcoRI* and *BamHI* for p40 and *EcoRI* and *HindIII* for p35, followed by 1% agarose gel electrophoresis.

**Synthesis of poly[ $\alpha$ -(4-aminobutyl)-L-glycolic acid].** Poly[ $\alpha$ -(4-aminobutyl)-L-glycolic acid] (PAGA) was synthesized and characterized as described previously by Lim *et al.* (26). Briefly, 10 g of sodium nitrite solution was added dropwise to 20 g of CBZ-L-lysine in a mixture of 200 ml 2 N H<sub>2</sub>SO<sub>4</sub> and 200 ml acetonitrile with constant stirring on ice. Stirring was continued for an additional 7 h on ice and 12 h at room temperature. The resulting solution was then extracted with ether and precipitated with petroleum ether. CBZ-L-oxyllysine was polymerized by melting condensation at 150°C under vacuum for 5 days. The polymer was cooled and dissolved in chloroform followed by precipitation with methanol. Two grams of the dried polymer was dissolved in 28 ml DMF containing 2 g of activated palladium on carbon (10% Pd-C). Ninety milliliters of 85% formic acid was added slowly to the polymer solution, and 15 h after stirring at room temperature the solution was filtered to remove Pd-C. Forty milliliters of 2 N HCl was added and the volume was reduced under vacuum. PAGA was finally precipitated with a large excess of acetone, dried under vacuum, and stored at -70°C until further use. PAGA was characterized in terms of molecular weight and biodegradability using MALDI-TOF-mass spectrometry and in terms of purity using NMR spectroscopy.

**Gel retardation assay.** Various formulations of PAGA/pmIL-12 or PAGA/pLuc complexes, ranging from charge ratio 0.5/1 ( $\pm$ ) to 7/1 ( $\pm$ ), were prepared in the presence of 5% (w/v) glucose to adjust the osmolality to 285–295 mOsm. The polyplexes were incubated for 15–20 min at room temperature. The samples were electrophoresed on 1% agarose gel in 1 $\times$  Tris-boric acid-EDTA (TBE) buffer at 80 V until the 1 $\times$  orange-blue loading dye ran through 80% of the gel. A 200-bp marker (Promega) was used as a DNA size marker. The gel was stained with 0.5  $\mu$ g/ml ethidium bromide for 45 min and analyzed on a UV illuminator to show the location of DNA.

**DNase protection assay.** PAGA/pmIL-12 (3/1,  $\pm$ ) complexes were incubated at 37°C in the presence of DNase I (45  $\mu$ l). At 0, 5, 10, 15, 30, and 60 min postincubation, 50  $\mu$ l of the samples was taken into Eppendorf tubes and mixed with 100  $\mu$ l of stop solution (400 mM NaCl, 100 mM EDTA) under mild vortexing. The samples were then mixed with 12  $\mu$ l of 10% sodium dodecyl sulfate (SDS) and incubated at 65°C overnight. The DNA was then extracted with a mixture of phenol:chloroform:isoamyl alcohol (25:24:1, v/v). The extracted DNA was precipitated with 700  $\mu$ l of absolute ethanol. The DNA precipitate was dried by air and dissolved in 10  $\mu$ l Tris-EDTA buffer. DNA integrity was assessed by 0.8% agarose gel electrophoresis, as mentioned above.

**Atomic force microscopy.** Morphology of polymer/plasmid complexes was analyzed using atomic force microscopy (AFM) as described by Kim *et al.* (30). Briefly, red mica was freshly cleaved as a thin wafer and then soaked in 33 mM MgAc<sub>2</sub> overnight to favor the replacement of potassium ions by divalent magnesium ions for stronger DNA binding. Mica was then sonicated for 30 min in distilled water and its surface was subjected to glow discharge for 15 s in a vacuum between 100 and 200 mTorr. As soon as the mica surface was exposed to air, 20  $\mu$ l of 0.1 mg/ml of

PAGA/plasmid complexes was placed on the mica surface and allowed to stick for 2 min. The mica surface was then rinsed gently with deionized water and blown dry with nitrogen gas. A Nanoscope II SFM (Digital Instruments, Santa Barbara, CA) was used for imaging at room temperature in the attractive force regime and under 30–60% relative humidity. The microscope was operated using cantilever oscillation frequencies between 12 and 24 kHz. Force minimization was maintained by reducing the set point voltage to minimize sample damage. To remove the high-frequency noise in the slow scan direction, only minimal filtering was applied to the image.

**Particle size and zeta potential measurement.** Zeta potential and particle size of PAGA/plasmid complexes were measured as described by Mahato *et al.* (31). Briefly, polymer/plasmid complexes were prepared as discussed above and diluted four times in the cuvette. The electrophoretic mobility was determined with ZetaPALS (Brookhaven Instruments Corp., Holtsville, NY). All experiments were performed at 25°C, pH 7.0, and 677-nm wavelength at a constant angle of 15°. The zeta potential was automatically calculated from the electrophoretic mobility based on Smoluchowski's formula. Following the determination of electrophoretic mobility, the samples were subjected to mean particle size measurement by the same equipment using the same light source and wavelength. The particle size was reported as effective mean diameter.

**Tumor cell lines.** CT-26 colon adenocarcinoma cell lines was a kind gift from Dr. Charles Tannenbaum of the Cleveland Clinic Foundation (Cleveland, OH) (32). Tumor cells were grown and maintained in RPMI 1640 medium which was supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin, and 50  $\mu$ g/ml gentamycin (all from Gibco-BRL) at 37°C and humidified 5% CO<sub>2</sub>.

**Cytotoxicity assay.** Cytotoxicity of PAGA/pmIL-12 complexes prepared at different ( $\pm$ ) charge ratios were assessed using MTT assay as described by Fisher *et al.* (33). Briefly, CT-26 cell lines were seeded in 96-well plates at 4000 cells/well and incubated for 24 h. After checking the cell confluency, which was over 80%, PAGA/pmIL-12 complexes prepared at different ( $\pm$ ) charge ratios were added to the cells at a dose of 0.15  $\mu$ g pmIL-12/well. Following 48 h of incubation, 25  $\mu$ l of MTT stock solution in phosphate-buffered saline (5 mg/ml) was poured into each well reaching a final concentration of 0.5 mg/ml MTT. The plate was then incubated at 37°C in 5% CO<sub>2</sub> for 4 h. The medium was removed and 150  $\mu$ l of DMSO was added to dissolve the formazan crystals. The plate was read spectrophotometrically at 570 nm in an ELISA plate reader. The relative cell viability was calculated as  $[\text{Abs}]_{\text{sample}}/[\text{Abs}]_{\text{control}} \times 100$ .

**In vitro transfection.** In the case of the luciferase gene, CT-26 cells were seeded in six-well tissue culture plates at  $3 \times 10^5$  cells per/well in 10% FBS containing RPMI 1640 medium. Cells achieved 80% confluency within 24 h after which they were transfected with PAGA/DNA complexes prepared at different charge ratios ranging from 0.5/1 ( $\pm$ ) to 5/1 ( $\pm$ ). The total amount of DNA loaded was maintained constant at 2.5  $\mu$ g/well and transfection was carried out in the absence of serum. The cells were allowed to incubate in the presence of complexes for 5 h in a CO<sub>2</sub> incubator followed by replacement of 2 ml of RPMI 1640 containing 10% FBS. Thereafter the cells were incubated for an additional 36 h. Cells were lysed using 1 $\times$  lysis buffer (Promega) after washing with cold PBS. Total protein assays were carried out using a BCA protein assay kit (Pierce Chemical Co.). Luciferase activity was measured in terms of relative light units (RLU) using a 96-well plate luminometer (Dynex Technologies Inc., Chantilly, VA). Luciferase activity was monitored and integrated over a period of 30 s. The final values of luciferase were reported in terms of RLU/mg total protein. In all the above experiments, both naked DNA and untreated cultures were used as positive and negative controls, respectively.

In the case of the mL-12 gene, CT-26 cells were seeded in 75-cm<sup>2</sup> flasks at  $2 \times 10^6$  cells/flask in 10% FBS containing RPMI 1640. Cells achieved 80% confluency in 24 h after which they were transfected with PAGA/pmIL-12 complexes prepared at different charge ratios ranging from 2/1 ( $\pm$ ) to 5/1 ( $\pm$ ). The total amount of DNA loaded was maintained at 15  $\mu$ g/flask and transfection was carried out in the absence of serum. The cells were allowed to incubate in the presence of complexes for 5 h in a CO<sub>2</sub> incubator followed by replacement of 10 ml of RPMI 1640 con-

taining 10% FBS. Thereafter the cells were incubated for an additional 36 h. Culture supernatants were assayed for mL-12 p70 and p40 using ELISA kits as suggested by the manufacturer.

**Tumor implantation and treatment.** Subcutaneous tumor-bearing BALB/c mice were used as an animal model for evaluation of PAGA/pLuc and PAGA/pmIL-12 complexes for transgene expression. To generate tumors, 5-week-old BALB/c mice were subcutaneously injected in the middle of the left flank with 100  $\mu$ l of a single-cell suspension containing  $4 \times 10^5$  CT-26 cells. Tumor volume was calculated by using its mean diameter measured with calipers across its two perpendicular diameters and its depth using the formula  $v = \frac{4}{3} \pi abc$ . Treatment of the tumors was started after about 2 weeks when they reached a size of about 100–120 mm<sup>3</sup>. PAGA/pmIL-12 complexes were prepared at a 3/1 ( $\pm$ ) charge ratio and 50  $\mu$ l of the complexes was injected directly into the tumors of BALB/c mice at a dose of 25  $\mu$ g DNA/mouse. The treated mice were sacrificed on days 1, 3, 5, and 7 postinjection, whereas the control mice were sacrificed on day 7. Tumors were isolated and cut into two pieces, one for ELISA and the other for RT-PCR. The tumor for RT-PCR was frozen immediately in liquid nitrogen and stored at  $-70^\circ\text{C}$  until further use. For ELISA, the tumor was chopped into small pieces, recultured into six-well plates, and incubated at  $37^\circ\text{C}$  for 24 h. Supernatants were separated from the cells by centrifugation and measured by ELISA for mL-12 p70, mL-12 p40, and mIFN- $\gamma$ . In the case of the luciferase gene, isolated tumors were cut into two pieces, one for luciferase assay and the other for RT-PCR. Luciferase activity was assessed following homogenization and digestion of the tumor with  $1 \times$  passive lysis buffer.

**ELISA for mL-12 and induced mIFN- $\gamma$ .** Measurement of mL-12 p70 and p40 as well as induced mIFN- $\gamma$  was done using ELISA set BDOptEIA for mL-12 p70, mL-12 p40, and mIFN- $\gamma$  (Pharmingen, San Diego, CA) and used according to the manufacturer's instructions. Briefly, ELISA plates (Nunc, Maxisorp, Denmark) were coated with capture antibody, sealed, and kept overnight for antibody binding. The plate was washed several times followed by incubation with assay diluent to block any non-specific binding for 1 h. After washing several times, 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 the enzymatic reaction. The reaction was stopped with 2 N H<sub>2</sub>SO<sub>4</sub> and the plate was read at 450 nm using a Bio-Rad (Hercules, CA) Model 3550 ELISA reader. The mL-12 p70 and p40 as well as mIFN- $\gamma$  concentrations were reported in terms of pg/ml.

**Reverse transcriptase-polymerase chain reaction.** RT-PCR was performed to detect mRNA transcripts for mL-12 and luciferase in transfected tumor

cells as well as in tumor tissues of mice. In the case of the mL-12 gene, tumors were isolated on days 1, 3, 5, and 7 after intratumoral injection of PAGA/pmIL-12 complexes (3/1,  $\pm$ ) and total RNA was isolated using an RNeasy Qiagen kit (Qiagen Inc., Valencia, CA). Samples were lysed and homogenized in the presence of guanidine isothiocyanate and then reverse transcribed using an Omniscript reverse transcriptase kit (Qiagen, Valencia, CA). The reverse-transcribed samples were amplified by PCR technique using a *Taq* polymerase core kit (Qiagen, Valencia, CA). RT-PCR was used to detect the p35 subunit as well as  $\beta$ -actin promoter and pCAGGS. The primers synthesized from 5' to 3' were as follows: for pmIL-12 (p35), 5'-GTC TCC CAA GGT CAG CGT TCC-3' upstream and 5'-CTG GTT TGG TCC CGT GTG ATG-3' downstream; for  $\beta$ -actin, 5'-ATG GTG GGA ATG GGT CAG AAG-3' upstream and 5'-CAC GCA GCT CAT TGT AGA AGG-3' downstream; and for pCAGGS, 5'-GCC AAT AGG GAC TTT CCA T-3' upstream and 5'-GGT CAT GTA CTG GGC ATA ATG-3' downstream, respectively. The PCR cycling conditions were as follows: Denaturing at  $95^\circ\text{C}$  for 15 s, annealing at  $56^\circ\text{C}$  for 15 s, and extension at  $72^\circ\text{C}$  for 30 s. A total of 35 cycles were run for product amplification. The PCR product was separated by electrophoresis using a 1% agarose gel. The expected sizes of the PCR products from mL-12 p35 mRNA and  $\beta$ -actin were 297 and 150 bp, respectively. For detection of luciferase transcripts, the following primers from 5' to 3' were used: 5'-ATG AAG AGA TAC GCC CTG GTT-3' upstream and 5'-CGG GAG GTA GAT GAG ATG TGA-3' downstream. The primers for  $\beta$ -actin were the same as mentioned above. The PCR cycling conditions for luciferase were as follows: denaturing at  $95^\circ\text{C}$  for 1 min, annealing at  $54.4^\circ\text{C}$  for 1 min, and extension at  $72^\circ\text{C}$  for 2 min. A total of 35 cycles were run for product amplification.

**Antitumor effect after single injection.** PAGA/pmIL-12 (3/1,  $\pm$ ) complexes were injected directly into CT-26 subcutaneous tumor-bearing BALB/c mice at a dose of 25  $\mu$ g pmIL-12/mouse. Naked pmIL-12 (25  $\mu$ g/mouse) as well as 5% glucose was also injected into separate CT-26 tumor-bearing mice and these were used as controls. After a single intratumoral injection, mice were closely monitored twice a week for tumor growth.. Tumor progression was reported in terms of tumor volume (mm<sup>3</sup>) over a period of 56 days.

## RESULTS

### Physicochemical Properties of PAGA/Plasmid Complexes

MALDI-TOF-mass spectrometric analysis of PAGA showed its molecular weight to be approximately 3200.

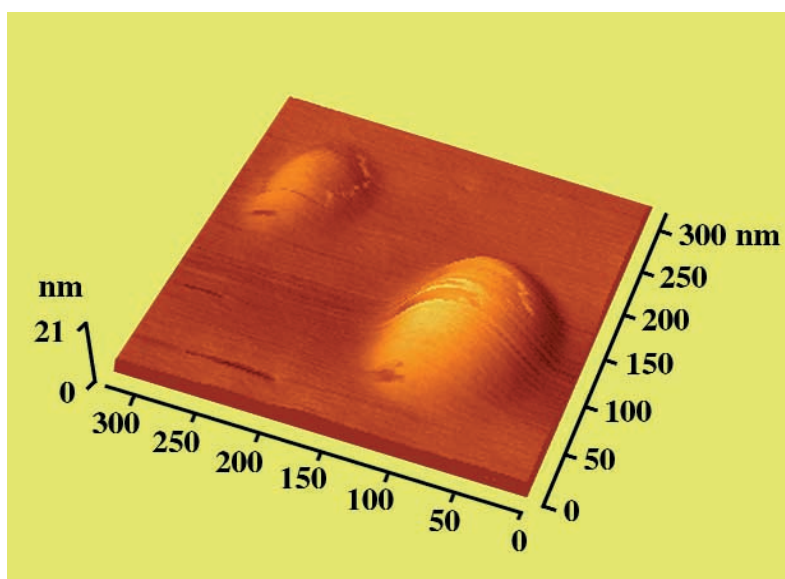


FIG. 1. Atomic force microscopy (AFM) image of a PAGA/pDNA complex (3/1,  $\pm$ ). Condensed plasmid DNA forms an ellipsoidal structure as seen in Z direction.

PAGA was characterized in terms of purity using NMR [ $^1\text{H}$  NMR( $\text{D}_2\text{O}$ ); 1.4(2H), 1.6(2H), 1.8(2H), 2.9(2H), 5.2(1H)] and the disappearance of the CBZ peak at 7.5 ppm indicated that the deprotection was complete (26). Fast degradation of PAGA could be attributed mainly to the autohydrolysis of the ester bond, which was replaced by the  $\epsilon$ -amine bond.

DNA condensing ability of PAGA was determined by gel retardation assay. The positively charged PAGA makes strong complexes with the negatively charged phosphate ions on sugar backbone of DNA. When the charge ratio ( $\pm$ ) reached 1.5:1, no free DNA was seen (data not shown). PAGA could protect plasmids from degradation by nucleases up to 60 min postincubation in the presence of DNase at charge ratios 2/1 ( $\pm$ ) (data not shown). DNA condensation, its reduction in overall size, and the resulting surface morphology were captured by AFM (Fig. 1). pmIL-12 when complexed with PAGA at charge ratio 3/1 ( $\pm$ ) was completely condensed and formed an ellipsoidal structure in  $Z$  dimensions. The ellipsoidal particle morphology was supplemented with its particle size of 100 nm along the longest axis of the complexes. The AFM imaging revealed that the naked DNA was condensed from its natural extended state in water to about one-sixth of its size in the complex. This ellipsoid morphology might be of special significance as it could facilitate enhanced mobility of the complex through the plasma membrane as well as cytosol.

The mean particle size of PAGA/pmIL-12 complexes when measured using laser light scattering was within a range of 57–130 nm for charge ratios 1.5/1 ( $\pm$ ). The narrow particle size distribution, for example at 3/1 ( $\pm$ ) charge ratio, revealed that the equal volume mixing of DNA and PAGA during formulation resulted in well-dispersed, uniform-sized particles with little aggregation. The decreasing size of the particles with the increase in charge ratio reaffirmed the calculations based on molecular weight that beyond 1/1 ( $\pm$ ) charge ratio and especially at 3/1 ( $\pm$ ) ratio the size of the polyplexes was an order of magnitude lower than that of naked DNA due to charge-charge interactions.

Zeta potential was in the range of 12–20 mV for the polyplexes prepared at different charge ratios ranging from 1.5/1 ( $\pm$ ) to 3/1 ( $\pm$ ). The zeta potential proportionally increased with the increase in charge ratios and at the ratio 3/1 ( $\pm$ ) was close to 20 mV. A zeta potential close to zero would be the most ideal for gene delivery as it would not induce electric polarizability inside the cell due to low positive charge. The gel retardation data (not shown) confirm the above finding as there was no DNA movement seen at the 1.5/1 ( $\pm$ ) charge ratio or above.

### Cytotoxicity

PAGA-based polyplexes were tested for cytotoxicity using MTT assay in CT-26 cells over a wide range of charge ratios. Commercially available cationic liposomes (LipofectAMINE)/pmIL-12 (5/1, w/w) and dendrimer

(SuperFect)/pmIL-12 (6/1, w/w) complexes were used for comparison. LipofectAMINE reagent is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleoyloxy- $N$ -[2(spermincarboxamido)ethyl]- $N,N$ -dimethyl-1-propanaminium trifluoroacetate (DOSPA) (MW 867) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) (MW 744) in membrane-filtered water. Based on its chemical structure, LipofectAMINE has two primary amines, two secondary amines, and one quaternary amine, totaling five positive charges per molecule (34). Subsequent calculations show that 5/1 (w/w) corresponds to 6.823/1 ( $\pm$ ) for LipofectAMINE/pDNA complexes. Similarly, SuperFect, a G6 dendrimer, has a 30,000 MW, with approximately 140 total protonatable amines, so that 6/1 (w/w) corresponds to 8.8/1 ( $\pm$ ) for SuperFect/pDNA complexes. Following normalization by ( $\pm$ ) charge ratios, we thus confirmed that PAGA/pmIL-12 complexes were indeed nontoxic to the cells when formulated at a charge ratio of 7/1 ( $\pm$ ) and below (Fig. 2). In contrast, SuperFect/pmIL-12 and LipofectAMINE/pmIL-12 were toxic to the cells. Superfect- and Lipofectamine-treated cells granulated and the cell population decreased under the same or lower concentration as PAGA. PAGA alone was also put to test for its cytotoxicity at amounts equivalent to those used in charge ratios 7/1 ( $\pm$ ) and 9/1 ( $\pm$ ). Even at such high charge ratios the cell viability attained a decent range of 72–79% (data not shown). Previously, we compared PAGA/p $\beta$ -Gal and PLL/p $\beta$ -Gal complexes for cytotoxicity in 293 cells and found PAGA-based formulations to be nontoxic (26). By taking care of high cytotoxicity and a reported low level of transgene expression in subcutaneous tumor models (2, 24), we did not test cationic liposome-

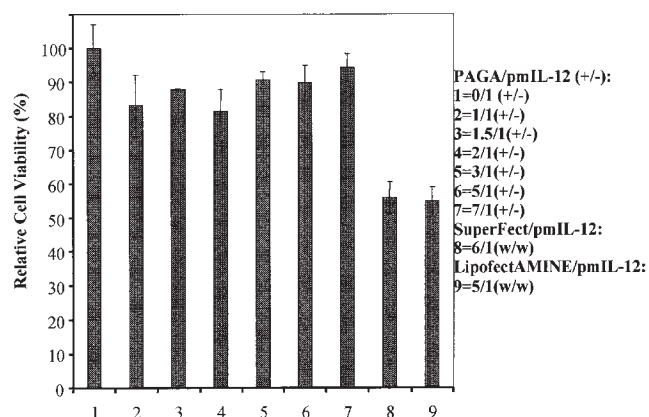


FIG. 2. Viability of CT-26 colon adenocarcinoma cells after transfection with PAGA/pmIL-12 ( $\pm$ ) complexes prepared at different ( $\pm$ ) charge ratios in 5% (w/v) glucose. Naked pmIL-12, SuperFect/pmIL-12 (6/1, w/w), and LipofectAMINE/pmIL-12 (5/1, w/w) were used for comparison. Relative cell viability was at least 80% for all PAGA/pmIL-12 complexes. In contrast, SuperFect/pmIL-12 and LipofectAMINE/pmIL-12 complex-based transfection resulted in less than 60% cell viability.

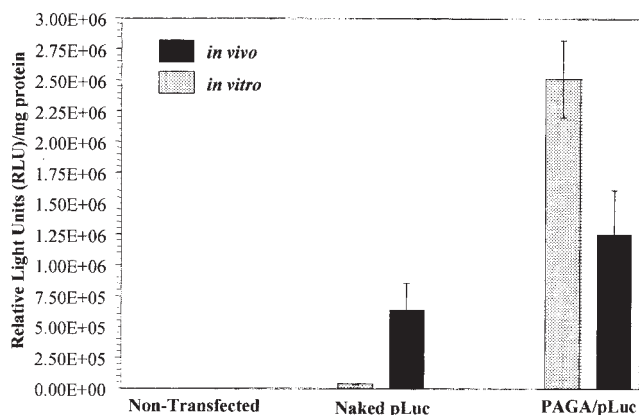


FIG. 3. Luciferase activity in cultured cells as well as CT-26 subcutaneous tumors 48 h after transfection. PAGA/pLuc (3/1,  $\pm$ ) complexes and naked pLuc were used for *in vitro* transfection as well as intratumoral injections in subcutaneous tumor-bearing BALB/c mice. Nontransfected cells (*in vitro*) and 5% (w/v) glucose-injected mice (*in vivo*) were used as negative controls. Luciferase activity is expressed as RLU/mg of total protein.

(LipofectAMINE) and cationic polymer (SuperFect)-based formulations for direct intratumoral injections.

#### *In Vitro* Transfection

PAGA/pLuc and PAGA/pmIL-12 complexes formulated at different charge ratios in 5% (w/v) glucose were evaluated for their transfection efficiency in CT-26 colon carcinoma cell lines. In the case of the luciferase gene, cells were lysed after transfection and RLU and total protein concentration were determined. PAGA/pLuc complexes were used over a wide range of charge ratios 1/1 ( $\pm$ ) but <9/1 ( $\pm$ ). The transfection efficiency increased with the increase in charge ratios but diminished slightly with ratios 7/1 ( $\pm$ ) (data not shown). We found similar levels of luciferase expression at charge ratios 3/1 ( $\pm$ ) and 5/1 ( $\pm$ ). Thus, we chose polyplexes at charge ratio 3/1 ( $\pm$ ) for further evaluation. As shown in Fig. 3, the RLU values for PAGA/pLuc complexes gave several orders of magnitude higher luciferase levels compared to naked pLuc. We have previously compared PAGA with PLL for *in vitro* transfection efficiency and cytotoxicity in 293 cells. *In vitro* transfection efficiency of PAGA/pDNA complexes was higher than that of the PLL/pDNA complex (26).

In the case of cytokine genes, the culture medium was collected after transfection and the levels of mIL-12 p70, mIL-12 p40, and mIFN- $\gamma$  were determined using ELISA. Trends similar to luciferase transfection followed when a gradient of various charge ratios 1/1 ( $\pm$ ) was used. However, in the case of mIL-12, the transfection efficiency upward of 7/1 ( $\pm$ ) regressed more sharply than that exhibited for condensed luciferase at various ratios (data not shown). As shown in Fig. 4, the mIL-12 levels for PAGA/pmIL-12 complexes (3/1,  $\pm$ ) were substantially higher than those for naked pmIL-12. Plasmid DNA with CMV promoter produced only slightly higher levels of

mIL-12 compared to the plasmid with  $\beta$ -actin promoter (576 pg/ml vs 690 pg/ml).

To complement with the ELISA results of *in vitro* transfected samples, RT-PCR was performed to assess the transfection efficiency of PAGA/pmIL-12 at mRNA levels and the results are shown in Fig. 5. RT-PCR results show that the mIL-12 p35 production at the mRNA level is sufficient enough to induce the formation of mIL-12 p70 by forming disulfide linkages with mIL-12 p40. The  $\beta$ -actin control confirmed that mIL-12 gene expression was indeed from the plasmid encoding mIL-12 and not from endogenous production of mIL-12 by the cells. The bands obtained from RT-PCR suggest that the mIL-12 expression at protein levels should be considerably high and mIL-12 p70 secreted from the transfected CT-26 cells should also be very high if the relative production levels of mIL-12 p35 and IL-12 p40 are close to each other. This was not the case when the levels of mIL-12 p70 and p40 were compared by ELISA (Fig. 7).

#### *In Vivo* Gene Expression

We implanted CT-26 colon adenocarcinoma cells into BALB/c mice to assess the *in vivo* gene transfer efficiency of PAGA, PAGA/pLuc (3/1,  $\pm$ ), PAGA/pmIL-12 (3/1,  $\pm$ ), and PAGA/pCMV-IL-12 (3/1,  $\pm$ ) complexes injected intratumorally. For the luciferase gene, transgene expression was assessed at mRNA and protein levels using RT-PCR and luciferase assay, respectively (Fig. 3 and Fig. 6). RLU/mg of protein was significantly higher for PAGA/pLuc complexes, compared to naked pLuc in cultured cells. RLU/mg protein values for intratumorally injected naked pLuc were only twofold lower than that

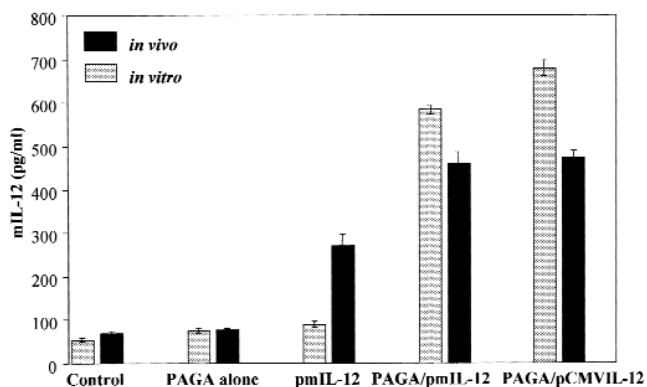


FIG. 4. ELISA for mIL-12 levels in cultured CT-26 colon adenocarcinoma cells as well as CT-26 subcutaneous tumor-bearing BALB/c mice 48 h after transfection. PAGA/pmIL-12 and PAGA/pCMV-mIL-12 (3/1,  $\pm$ ) complexes as well as naked pmIL-12 were used for *in vitro* and *in vivo* transfection. In the case of *in vivo* gene expression, tumors were isolated, chopped into small pieces, and cultured in RPMI 1640 supplement with 10% FBS for 48 h at 37°C. Supernatants from both *in vitro* and *in vivo* samples were assessed by ELISA for mIL-12. Nontransfected cells (*in vitro*) and 5% (w/v) glucose-injected mice (*in vivo*) were used as negative controls. PAGA alone was also tested for its mIL-12-inducing capability. mIL-12 protein levels for PAGA/pmIL-12 complexes were several fold higher than naked pmIL-12.

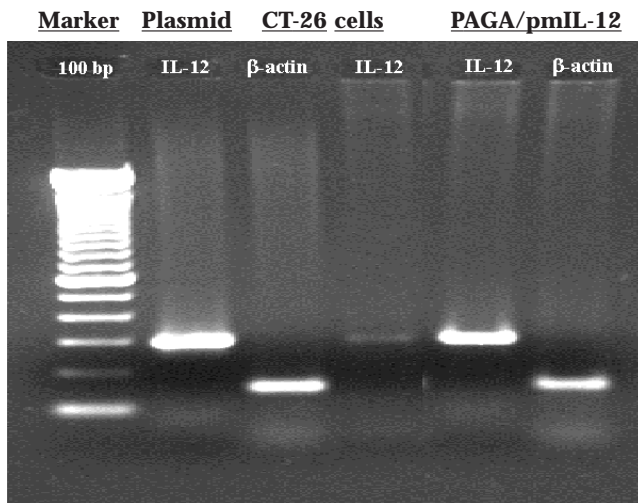


FIG. 5. RT-PCR after *in vitro* transfection of PAGA/pmIL-12 complexes into cultured CT-26 colon adenocarcinoma cells. DNA size marker, 100 bp (lane 1), control plasmid pmIL-12 (lane 2), and nontransfected CT-26 cells (lane 3) for β-actin, (lane 4) for mIL-12 p35, PAGA/pmIL-12 transfected cells, (lane 5) for mIL-12 p35, and (lane 6) for β-actin.

of PAGA/pLuc complexes. The RT-PCR bands were much thicker for PAGA/pLuc complexes compared to those of naked pLuc (Fig. 6).

In the case of mIL-12 plasmid, at 48 h after intratumoral injection of PAGA/pmIL-12 complexes, tumors were harvested, chopped into small pieces, and recultured for 24 h and the culture supernatants were analyzed by ELISA. As shown in Fig. 4, PAGA/pmIL-12 (3/1, ±) complexes produced higher levels of mIL-12 compared to naked pmIL-12 (473 pg/ml vs 250 pg/ml). mIL-12 production was insignificant in mice injected with 5% (w/v) glucose or the polymer (PAGA) alone. The use of CMV promoter for mIL-12 expression was found not to be as efficient as reported for plasmids encoding genes other than cytokines, as both PAGA/pmIL-12 complexes (with β-actin promoter) and PAGA/pCMV-mIL-12 complexes (with CMV promoter) produced similar amounts of mIL-12 (Fig. 4). mIL-12 p40 homodimer may function as a natural mIL-12 p70 antagonist that binds to the mIL-12 receptor but does not mediate a biologic response (35). Therefore, we determined the relative production of mIL-12 p70 and p40 and found that mIL-12 p40 was 10- to 15-fold higher than p70 heterodimer production (Fig. 7). The level of mIL-12 p40 was very high not only for the PAGA/pmIL-12 and PAGA/pCMV-mIL-12 complexes, but also for the mice injected with naked pmIL-12 (Fig. 7). One of the most important properties of mIL-12 is its ability to induce production of large amounts of mIFN-γ (9). Therefore, we also measured the levels of mIFN-γ induced by mIL-12. As shown in Fig. 8, both PAGA/pmIL-12 and PAGA/pCMV-mIL-12 complexes produced much higher levels of mIFN-γ compared to naked pmIL-12 or PAGA alone (530 pg/ml vs 78 pg/ml).

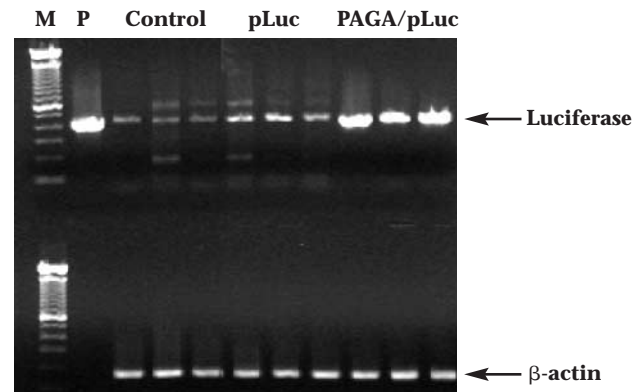


FIG. 6. RT-PCR after intratumoral injection of PAGA/pLuc (3/1, ±) complexes and naked pLuc in CT-26 subcutaneous tumor-bearing BALB/c mice. Lanes in the upper half are for luciferase while those in the lower half are for β-actin controls. DNA size marker, 100 bp (lane 1), luciferase plasmid (lane 2), 5% (w/v) glucose-injected negative controls (lanes 3 to 5), naked pLuc (lanes 6 to 8), and PAGA/pLuc complexes (lanes 9 to 11). Band intensity for PAGA/pLuc complexes is greater than that of naked DNA.

To study the persistence of mIL-12 expression, we injected PAGA/pmIL-12 (3/1, ±) complexes intratumorally into subcutaneous CT-26 tumor-bearing BALB/c mice. At 1, 3, and 7 days after intratumoral injection, tumors were harvested and analyzed for mIL-12 p35 mRNA using RT-PCR. Figure 9 shows the levels of mIL-12 expression at different time points after injection of PAGA/pmIL-12 complexes. The samples obtained on day 1 showed significantly higher levels of mIL-12 compared to days 3, 5, and 7 as evidenced by much thicker RT-PCR bands for mIL-12 on day 1. The level of transcription for mIL-12 p35 on day 3 does show substantial intensity followed by sharp regression on day 5 and day 7.

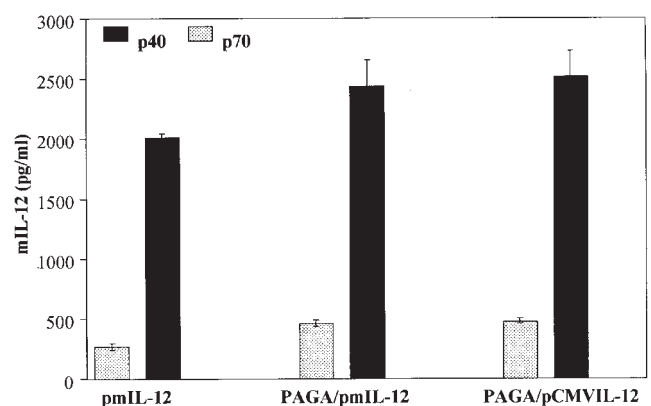


FIG. 7. Relative production of mIL-12 p40 and p70 48 h after intratumoral injection of PAGA/pmIL-12 and PAGA/pCMV-mIL-12 (3/1, ±) complexes or naked pmIL-12 into CT-26 subcutaneous tumor-bearing mice. Tumors were isolated, chopped into small pieces, and cultured in RPMI 1640 supplemented with 10% FBS for 48 h at 37°C. Supernatants were analyzed by ELISA for assessing the levels of mIL-12 p40 and p70. mIL-12 p40 levels are more than 10-fold higher than that of pmIL-12 p70.

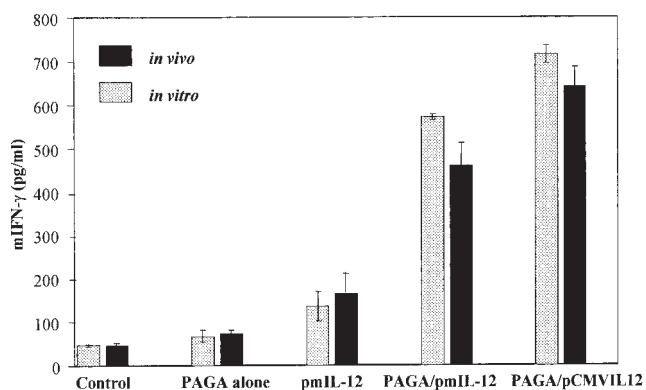


FIG. 8. ELISA for mIFN- $\gamma$  levels in cultured CT-26 colon adenocarcinoma cells as well as CT-26 subcutaneous tumor-bearing BALB/c mice. PAGA/pmIL-12 and PAGA/pCMV-mIL-12 (3/1,  $\pm$ ) complexes as well as naked pmIL-12 were used for transfection. In the case of *in vivo* gene expression, tumors were isolated, chopped into small pieces, and cultured in RPMI 1640 supplemented with 10% FBS for 48 h at 37°C. Supernatants were analyzed by ELISA for assessing the levels of mIFN- $\gamma$ . Nontransfected cells (*in vitro*) and 5% (w/v) glucose-injected mice (*in vivo*) were used as negative controls. PAGA alone was also tested for its induced mIFN- $\gamma$ -producing capability. mIFN- $\gamma$  levels for both condensed plasmids are several fold higher than for noncondensed pmIL-12.

#### *In Vivo* Tumor Growth after pmIL-12 Administration

We studied the effect of PAGA/pmIL-12 (3/1,  $\pm$ ) complexes on tumor regression after single-dose intratumoral administration and compared it with naked pmIL-12 and the control mice injected with 5% glucose. We found over a period of 56 days that retardation of tumor progression as well as life span was greater for mice injected with PAGA/pmIL-12 complexes compared to mice injected with 5% glucose and pmIL-12 alone. Figure 10 shows the trends for tumor growth in terms of mean tumor volume versus time (days). In addition, 33% of the mice injected with naked pmIL-12 and 5% glucose (w/v) showed metastasis compared to none in PAGA/pmIL-12-injected mice. Mouse deaths were recorded as follows: mice injected with 5% glucose on days 42, 46, and 48; mice injected with naked pmIL-12 on days 43, 45, and 52; and mice injected with PAGA/pmIL-12 complexes on days 52, 54, and 56. Since PAGA is a nontoxic biodegradable polymer, we envision that it can be used for repeated injections to further regress tumor growth compared to single-dose effects shown in Fig. 10.

#### DISCUSSION

Often times stress has been laid on high transgene expression; however, the issues of toxicity and the role of gene delivery vehicle posttransfection are relatively sidelined. An effective gene carrier should be nontoxic and possibly biodegradable to achieve a safe and meaningful gene therapy during clinical trials. Most synthetic gene carriers currently under investigation can be subdivided into cationic lipids, polymers, and peptides. Most of the

cationic polymers have amide (e.g., PLL) or vinyl bonds (e.g., polyethyleneimine). These bonds are very stable in aqueous solution and there is no direct evidence of their degradation in body fluids. Both cationic lipids and polymers are known to be cytotoxic and do not boost transgene expression after repeated injection of cationic carriers/pDNA complexes (25, 36). To make nontoxic gene carriers, we designed PAGA as a polymer with a hydrolytically labile ester bond. Degraded monomers or small oligomers after polymer dissociation from plasmid DNA will not elicit immune responses. The amide backbone linkage of PLL was converted into ester linkage to confer biodegradability upon PAGA (26). The small size of degraded oligomers and monomers will not elicit complement activation which can be a problem for repeated administration of therapeutic genes into patients (37).

In this study, we prepared PAGA/pDNA complexes using plasmid encoding luciferase as a reporter gene and mIL-12 as a therapeutic gene and assessed their gene transfer efficiency. PAGA successfully condensed and protected these plasmids from degradation by nucleases. PAGA/pDNA complexes were characterized in terms of particle size, zeta potential, surface morphology, and osmolality. The particle size of PAGA/pmIL-12 complexes was in the range of 58–130 nm and had an ellipsoidal structure in Z direction. PAGA/pDNA complexes display minimal aggregation and a high level of homogeneity in aqueous solution. Low aggregate formation and small size would enhance the diffusivity and rapid cellular uptake of pDNA. Adjusting the osmolality using glucose instead of saline would augment the diffusion of PAGA/pDNA complexes and will help in maintaining the

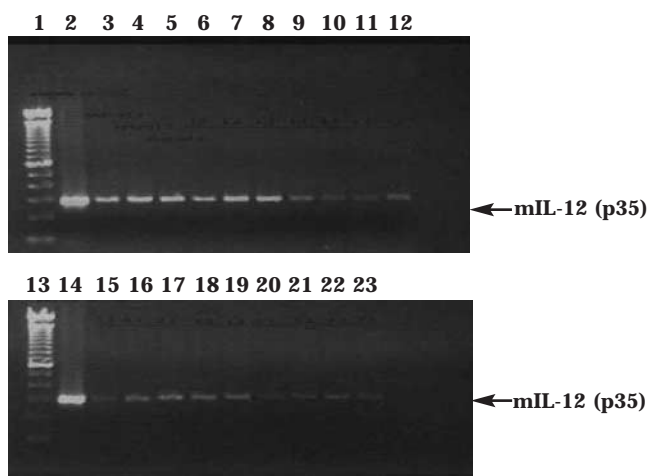


FIG. 9. RT-PCR after intratumoral injection of PAGA/pmIL-12 (3/1,  $\pm$ ) complexes in CT-26 subcutaneous tumor-bearing BALB/c mice. DNA size marker, 100 bp (lanes 1 and 13), control plasmid pmIL-12 (lanes 2 and 14), mIL-12 p35 [day 1 posttransfection (lanes 3 to 5), day 3 (lanes 6 to 8), day 5 (lanes 9 to 12), and day 7 (lanes 15 to 18)], and 5% (w/v) glucose-injected negative controls (lanes 19 to 22). Band intensity for PAGA/pmIL-12 complexes is much greater on day 1 and day 3 but starts decreasing by day 5 and is almost negligible on day 7.

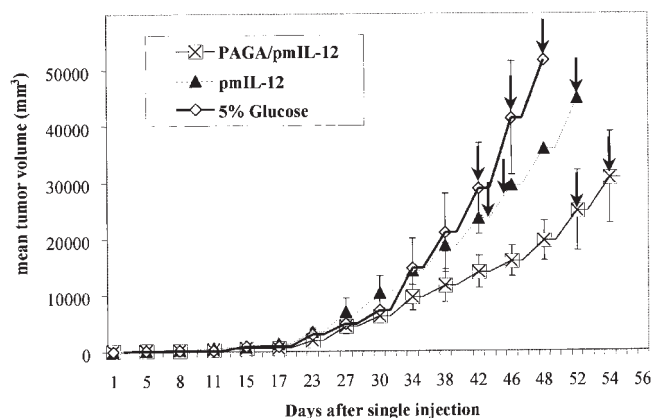


FIG. 10. Profile of tumor progression after single intratumoral injection of PAGA/pmIL-12 (3/1,  $\pm$ ) complexes, naked pmIL-12, and 5% glucose (w/v) in CT-26 subcutaneous tumor-bearing BALB/c mice. Mean tumor volumes  $\pm$  SD were recorded twice a week for a period of 56 days. Mice treated with PAGA/pmIL-12 complexes showed retarded tumor growth and lived longer compared to naked pmIL-12 and 5% glucose-injected mice. Vertical arrows indicate death of mice at various time points.

biophysical structure of these polyplexes until they are in contact with plasma membrane (36).

Gene expression levels for naked plasmid have been shown to be higher compared to condensed pDNA; however, tumor regression has been found to be higher for condensed plasmid (2). Tightly condensed plasmid using polyethyleneimine when injected intratumorally has been suggested to result in a reservoir inside the tumor. This can lead to slow dissociation of DNA, resulting in lower short-term gene expression, but higher gene expression at later time points compared to naked plasmid (24). In the present study, luciferase expression *in vitro* was several orders of magnitude higher for PAGA/pLuc complexes compared to naked pLuc. However, *in vivo* transgene expression for PAGA/pLuc complexes was only twofold higher than naked pLuc. This trend clearly suggests that although PAGA efficiently condensed and protected pLuc, owing to its biodegradable nature it might have released pLuc in large amounts in a short span of time inside the tumor resulting in transgene expression higher than naked pLuc. This also suggests that PAGA might be useful for repeated injections.

Efforts have been concentrated on increasing the level of gene expression by using strong promoters like CMV and SV40 which have been found to be highly potent in transgene expression (38). Therefore, we compared mIL-12 expression plasmids with  $\beta$ -actin, a constitutive cellular promoter and CMV viral promoter after intratumoral injection of PAGA/pmIL-12 (with  $\beta$ -actin promoter) and PAGA/pCMV-mIL-12 complexes. However, the results clearly suggest that there was little difference in the levels of mIL-12 and mIFN- $\gamma$  by these two formulations (Figs. 4 and 8). mIL-12 production would induce the activation of mIFN- $\gamma$  and mTNF- $\alpha$ ,

both acting as strong antagonists of viral and tumorigenic elements inside the body. This enhanced induction of mIFN- $\gamma$  due to mIL-12 might inhibit the viral promoter within cells, rendering the CMV promoter less effective (39). On the other hand,  $\beta$ -actin, although weaker but being a constitutive cellular promoter, would be less sensitive to immunomodulatory effects of cytokines.

In normal cells, whenever there is activation of immune response by antigenic components there is increased production of mIL-12 p70 as well as 20-fold higher production of mIL-12 p40. The same is held true for tumor cells treated with cytokine genes. mIL-12 p40 can act as an antagonist toward mIL-12 p70 by forming (p40)<sub>2</sub> homodimer (9). We therefore measured the relative production levels of both p70 and p40 subunits of mIL-12 by ELISA and found that mIL-12 p40 was at least 10- to 15-fold higher than the mIL-12 p70 segment for tumors treated with PAGA/pmIL-12 complexes (Fig. 7). The relative production of p40 and p35 subunits should be regulated for further enhancement in mIL-12 p70 gene expression. This would also result in higher induced production of mIFN- $\gamma$  and consequently greater antitumor response through the feedback control mechanism related to mIL-12 and mIFN- $\gamma$ . Replacing the internal ribosomal entry site (IRES) segment between the p35 and p40 genes of mIL-12 with a peptide linker has demonstrated regulated production of mIL-12 p40 in comparison with mIL-12 p35, which should increase the levels of mIL-12 p70 (40). Using two different promoters to regulate the relative production of p35 and p40 segments would be another approach to increase the mIL-12 p70 levels inside tumor cells (1). However, the conformational stability and antiproliferation activity of mIL-12 produced using these approaches need to be investigated in greater detail.

It has been consistently shown that immunomodulatory activities of IL-12 p70 and induced IFN- $\gamma$  regulate the function of tumor-infiltrating immune cells like T cells, NK cells, and macrophages leading to retarded tumor growth and even significant tumor regression after treatment with IL-12 (13, 14). We therefore administered single-dose injections of PAGA/pmIL-12 (3/1,  $\pm$ ) complexes intratumorally in subcutaneous tumor-bearing mice. Tumor growth and longer life span of mice injected with our formulations indeed reconfirmed that PAGA/pmIL-12 (3/1,  $\pm$ ) polyplexes induced higher production of IFN- $\gamma$  compared to naked pmIL-12, which could lead to significantly enhanced antitumor activity.

PAGA/pmIL-12 polyplexes are likely to degrade quickly after entering the cytosol due to autohydrolysis of ester linkage in PAGA and consequently DNA transfection efficiency was not as high as we would like it to be. To slow down the degradation rate of PAGA, high-molecular-weight PAGA derivatives are currently being synthesized for our future gene transfer studies. In addition, we are currently synthesizing lysine-histidine-grafted PAGA to enhance the endosomolytic properties

of PAGA which might lead to enhanced cytokine gene expression. Since PAGA is nontoxic and given its high level of biodegradability, we envisage it to be fit for repeated administration to maintain sustained gene expression. In conclusion, nontoxic biodegradable polymer-based gene delivery offers an exciting incentive for cancer treatment.

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