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Nanoparticle-Mediated Wild-Type p53 Gene Delivery Results in Sustained Antiproliferative Activity in Breast Cancer Cells

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Abstract: Gene expression with nonviral vectors is usually transient and lasts for only a few days. Therefore, repeated injection of the expression vector is required to maintain a therapeutic protein concentration in the target tissue. Biodegradable nanoparticles (~200 nm diameter) formulated using a biocompatible polymer, poly(D,L-lactide-*co*-glycolide) (PLGA), have the potential for sustained gene delivery. Our hypothesis is that nanoparticle-mediated gene delivery would result in sustained gene expression, and hence better efficacy with a therapeutic gene. In this study, we have determined the antiproliferative activity of wild-type (*wt*) p53 gene-loaded nanoparticles in a breast cancer cell line. Nanoparticles containing plasmid DNA were formulated using a multiple-emulsion−solvent evaporation technique. To understand the mechanism of sustained gene expression with nanoparticles, we monitored the intracellular trafficking of both the nanoparticles and the nanoparticle-entrapped DNA, and also determined p53 mRNA levels over a period of time. Cells transfected with *wt*-p53 DNA-loaded nanoparticles demonstrated a sustained and significantly greater antiproliferative effect than those with naked *wt*-p53 DNA or *wt*-p53 DNA complexed with a commercially available transfecting agent (Lipofectamine). Cells transfected with *wt*-p53 DNA-loaded nanoparticles demonstrated sustained p53 mRNA levels compared to cells which were transfected with naked *wt*-p53 DNA or the *wt*-p53 DNA−Lipofectamine complex, thus explaining the sustained antiproliferative activity of nanoparticles. Studies with fluorescently labeled DNA using confocal microscopy and quantitative analyses using a microplate reader demonstrated sustained intracellular localization of DNA with nanoparticles, suggesting the slow release of DNA from nanoparticles localized inside the cells. Cells which were transfected with naked DNA demonstrated transient intracellular DNA retention. In conclusion, nanoparticle-mediated *wt*-p53 gene delivery results in sustained antiproliferative activity, which could be therapeutically beneficial in cancer treatment.

Keywords: Sustained release; biodegradable polymers; cellular gene delivery; gene transfection; cancer

Introduction

Gene delivery using nonviral systems such as liposomes and cationic lipid− or polymer−DNA complexes is usually transient and requires repeated delivery of the expression vector for the maintenance of a therapeutic level of the expressed protein in the target tissue.[1,2] The frequency of dosing of the expression vector, depending on the need of a

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particular disease condition, would depend on the efficiency of gene expression and the stability of the expressed protein in the tissue.[3] Repeated delivery of the vector may cause toxicity, and the therapy may not be effective.[4,5] To avoid these problems, various sustained release gene delivery systems such as polymeric implants, gels, etc., are being investigated.[3−7] We have been investigating sustained release nanoparticles formulated using biodegradable polymers, poly(D,L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA), as a gene delivery system. Although nanoparticles have been extensively investigated as a carrier for various therapeutic agents, including macromolecules such as proteins and peptides, their application as a gene expression vector is recent.[8−13]

Recently, we have demonstrated rapid escape (within 10 min) of nanoparticles from the endolysosomal compartment to the cytoplasmic compartment following their intracellular uptake via an endocytic process.[11] The escape of nanoparticles was attributed to the reversal of their surface charge from anionic to cationic in the acidic pH of the endolysosomal compartment, causing nanoparticles to interact with the endolysosomal membrane and then escape into the cytoplasmic compartment.[11] The rapid escape of nanoparticles from the endolysosomal compartment could protect nanoparticles as well as the encapsulated DNA from the degradative environment of the endolysosomes.[12] Our hypothesis is that the nanoparticles localized in the cytoplasmic compartment would release the encapsulated DNA slowly, thus resulting in sustained gene expression. Sustained gene expression could be advantageous, especially if the half-life of the expressed protein is very low and/or a chronic gene delivery is required for therapeutic efficacy.[3]

The aim of the proposed studies was to determine the efficacy of p53 gene-loaded nanoparticles in inducing antiproliferative activity in a breast cancer cell line. The p53 tumor suppressor gene is the most frequently mutated gene identified in many tumors, including in breast cancer.[14] Mutations in the p53 gene lead to the loss of vital cellular functions that govern cell division and apoptosis, leading to tumor growth.[15] Several studies have indicated that the restoration of wild-type (*wt*) p53 function could result in tumor inhibition,[16−18] and has been suggested as a potential therapeutic strategy for the treatment of cancers.[19−21] In this study, we have determined the antiproliferative activity of *wt*-p53 DNA-loaded nanoparticles in MDAMB-435S cells. This cell line is derived from a human ductal carcinoma from patients with metastatic disease and had not been subjected to chemotherapy.[22] This cell line has a p53 gene rearrangement that causes a reduced level of expression of p53 and, hence, is a preferred cell line for studying the effect of p53 gene therapy.[23]

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Experimental Section

Plasmid DNA Isolation and Purification. The *Escherichia coli* (DH5α) pCEP4 vector either alone [p53(−ve)] or containing CMV-driven wild-type human p53 cDNA was provided by P.-W. Cheng (University of Nebraska Medical Center). The luciferase protein-encoding gene (pGL3) was purchased from Promega (Madison, WI). Plasmid DNA was extracted and purified using a Qiagen mega/giga DNA extraction and purification kit. The concentration and purity of the DNA preparation were determined by measuring the absorbance at 260 and 280 nm using a UV spectrophotometer (Shimadzu, Columbia, MD).

Nanoparticle Formulation. Nanoparticles containing plasmid DNA were formulated using a multiple-emulsion solvent evaporation technique as per our previously published protocol.[13] In brief, 1 mg of DNA and 2 mg of nuclease free bovine serum albumin (BSA, Sigma, St. Louis, MO) were dissolved in 200 μL of Tris-EDTA buffer (pH 8). The primary emulsion was formulated by sonicating 30 mg of PLGA 50:50 (intrinsic viscosity of 1.32 g/dL, Birmingham Polymers) dissolved in 1 mL of chloroform with the above DNA solution for 2 min over an ice bath using a probe sonicator set at 55 W of energy output (Sonicator XL, Misonix). The resulting emulsion was further emulsified into a 2% w/v polyvinyl alcohol (PVA, 30−70 kDa, Sigma) solution using sonication as described above for 5 min to form a multiple (water-in-oil-in-water) emulsion. The emulsion was stirred overnight on a magnetic stir plate and kept in a vacuum desiccator for 1 h to evaporate chloroform. Nanoparticles were separated using ultracentrifugation (35 000 rpm for 20 min at 4 °C, Optima LE-80K, Beckman, Palo Alto, CA) and washed twice to remove PVA and unencapsulated DNA. The nanoparticle pallet was then resuspended in 5 mL of sterile water by sonication as described above for 30 s, and the suspension was lyophilized (−80 °C and <10 μmHg, LYPH-LOCK 12, Labconco, Kansas City, MO) for 48 h. The DNA loading in nanoparticles was determined using the protocol described in our earlier studies.[12] In brief, the supernatant following recovery of nanoparticles and the washings were carefully collected to determine the amount of DNA that was not entrapped in nanoparticles. The supernatant and washings were combined and analyzed for the DNA levels by measuring the UV absorbance at 260 nm with washings from the nanoparticles formulated under identical conditions but without DNA as a blank. The DNA loading in nanoparticles was determined from the amount of DNA that is not entrapped and subtracting this from the total amount of DNA added in the formulation.

Nanoparticles containing YOYO- and TOTO-labeled DNA (luciferase) were prepared in a similar manner as described above except that the DNA was prelabeled by incubation with either YOYO or TOTO (Molecular Probes, Eugene, OR). In a typical labeling procedure, 1 mg of DNA in 200 μL of TE buffer was incubated in the dark with a 0.1 μM stock solution of the dye (TOTO or YOYO) for 1 h. In addition, nanoparticles containing DNA (TOTO-labeled) and a fluorescent dye (6-coumarin, Polysciences Inc., Warrington, PA) were formulated using an identical procedure. The dye was dissolved in the PLGA polymer solution prior to emulsification. The incorporated dye acts as a probe for nanoparticles and hence can be used to quantitatively determine the cellular uptake of nanoparticles as well as to study their intracellular distribution using confocal microscopy.[24] The green fluorescence of nanoparticles (due to the dye incorporated in nanoparticles) and the red fluorescence of the encapsulated DNA (TOTO-labeled) can be used to study the intracellular distribution of nanoparticles as well as that of the DNA released from nanoparticles. In some of the studies, YOYO-labeled (green fluorescence) DNA was used for encapsulation in nanoparticles to study the intracellular distribution of the released DNA along with a marker for endolysosomes (LysoTraker Red, Molecular Probes).

Particle Size Analysis and ζ Potential. Particle size was determined using a quasi-elastic light scattering technique. A dilute suspension of nanoparticles (0.1 mg/mL) was prepared in double-distilled water and sonicated on an ice bath for 30 s as described above. The sample was subjected to a particle size analysis in the ZetaPlus particle size analyzer (Brookhaven Instrument Corp., Holtsville, NY) and ζ potential measurement using the ZetaPlus ζ potential analyzer.

Cell Culture. The human breast carcinoma cell line (MDA-MB-435S) was obtained as a kind gift from Dr. Singh (Department of Pathology and Microbiology, University of Nebraska Medical Center). Cells were grown in DMEM supplemented with 10% FBS and incubated at 5% CO_2 and 37 °C. The medium was changed on every alternate day.

Antiproliferative studies. Cells were seeded in 96-well plates at a cell density of 2500 cells per well per 100 μL 1 day prior to the experiment. Suspensions of nanoparticles at three different doses (4, 6, and 8 mg, DNA loading in nanoparticles = 2.1 mg of DNA/100 mg of nanoparticles) were prepared in 0.5 mL of serum free medium using a water bath sonicator (FS140, Fisher Scientific, Pittsburgh, PA) for 10 min. Each sample was then diluted to 8 mL with serum containing medium to give nanoparticle concentrations of 500, 750, and 1000 μg/mL, respectively. DNA in serum-containing medium, corresponding to the DNA dose in nanoparticles (10.5, 15.75, and 21 μg/mL), was used for the transfection as respective controls. The DNA−Lipofectamine complex was prepared as per the protocol described by the supplier of the reagent (Invitrogen, Carlsbad, CA). In a typical procedure, a DNA solution (1 μg diluted in 62.5 μL of serum free medium) and Lipofectamine (6 μL stock diluted in 31.25 μL of serum free medium) were mixed

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together. The mixture described above was incubated at room temperature for 45 min to allow the complexation to take place and then diluted to 0.2 mL with serum free medium. Cells were incubated with 20 μL of serum free medium prior to transfection. The DNA−Lipofectamine complex (30 μL) was added to each well for transfection. The final DNA concentration used for transfection with the DNA−Lipofectamine complex was 1.5 μg/mL. A higher concentration of the DNA−Lipofectamine complex could not be used because of cell toxicity. Thus, the DNA dose used with the DNA−Lipofectamine complex was ~7-fold lower than the lowest DNA dose used with nanoparticles (1.5 μg/mL vs 10.5 μg/mL). Cells were incubated for 2 h and then supplemented with 50 μL of 2× serum-containing medium as per the protocol described in the manufacturer's instructions. Untreated cells, p53(-ve) DNA nanoparticles, control (without DNA) nanoparticles, and Lipofectamine (without DNA) were used as respective controls. Cell proliferation was followed as a function of time using a standard MTS assay (CellTiter 96 AQ_{ueous}, Promega). The medium was changed on day 2 following transfection and on every other day thereafter, and no further dose of DNA was added. For the MTS assay, cells were washed twice with PBS and incubated with fresh medium for 2 h. The MTS reagent (20 μL) was added to each well and the mixture incubated for 150 min, and the absorbance was measured at 490 nm using a microplate reader (BT 2000 Microkinetics Reader, BioTek Instruments, Inc., Winooski, VT).

Determination of p53 mRNA Levels by RT-PCR. Cells (9×10^5) were seeded in T-75 flasks 1 day prior to the experiment. The medium was aspirated, and the nanoparticle suspension (4 mg/10 mL, DNA dose of 8.4 μg/mL) in serum-containing medium was added to each flask. Cells were incubated, and the medium was changed 1 day after the transfection and on every alternate day thereafter. DNA in solution (8.4 μg/mL) and the DNA−Lipofectamine complex (DNA dose of 1.5 μg/mL) were added to a separate set of cells. Nanoparticles loaded with p53(-ve) DNA were used as a control. The cells were trypsinized at different time points, washed twice with 1× PBS, and processed for isolation of RNA as follows.

(1) Isolation of RNA. Cells were lysed by incubating them with 1.2 mL of TRIzol reagent (Invitrogen) per flask for 5 min at room temperature, and the contents were transferred to Eppendorf tubes. Chloroform (240 μL) was added to each sample, and the tubes were shaken vigorously for 15 s and incubated at room temperature for 3 min. The samples were then centrifuged at 12000*g* (Eppendorf 5417R microcentrifuge, Brinkmann Instruments, Westbury, NY) for 15 min at 4 °C. The upper aqueous phase containing RNA was collected in separate Eppendorf tubes. For separation of RNA, the RNA was precipitated using 0.6 mL of isopropyl alcohol, incubated at room temperature for 10 min, and the RNA pallet was recovered by centrifugation at 12000*g* for 10 min at 4 °C. The pellet was washed once with 1.2 mL of 75% ethanol, and the RNA was recovered by centrifugation at 7500*g* for 5 min at room temperature.

(2) Reverse Transcription of RNA and PCR Amplification of cDNA. Approximately 1 μg of RNA was used for the RT-PCR using a GeneAmp PCR system (Applied Biosystems, Foster City, CA). The primers used for the gene amplification were synthesized at the molecular biology core facility of the University of Nebraska Medical Center. Sequences of the primers that were used were GAGCGCTGCTCAGATAGCGA (forward) and CTGTTCCGTCCCAGTAGATT (reverse).

(3) Determination of mRNA Levels. PCR products were resolved alongside a DNA marker on a 1.7% agarose gel and stained with an ethidium bromide solution. The band intensity were quantitated using a densitometer (GelExpert software, Nucleotech, San Mateo, CA). β-Actin was used as an internal standard. The data were expressed as the ratio of p53 to β-actin band intensities.

Intracellular DNA Distribution Using Confocal Microscopy. For an intracellular distribution study, luciferase DNA-loaded nanoparticles were used. Nanoparticles containing YOYO-DNA were used to study endolysosomal colocalization of DNA, and nanoparticles containing TOTO-DNA and 6-coumarin dye were used to demonstrate sustained intracellular retention of DNA and nanoparticles following transfection. Cells were seeded at a cell density of 35 000 cells/mL in Bioptec culture plates (Bioptechs, Butler, PA) 1 day prior to the experiment. The medium in the Bioptec culture plates was replaced with a nanoparticle suspension (450 μg/mL), and the plates were incubated. DNA in solution (8 μg/plate) equivalent to the dose used in nanoparticles was also added to a separate group of cells. Untreated cells were used as a control to account for autofluorescence. The medium was changed on the second day following transfection and on every alternate day thereafter. The images were captured by using a 488 nm filter (6-coumarin), a 633 nm filter (TOTO), and differential interference contrast using a Zeiss confocal microscope equipped with an argon−krypton laser (model LSM410, Carl Zeiss Microimaging, Thornwood, NY). Nanoparticles are green in color, and the released DNA is red in color. The images obtained using a 588 nm filter (rhodamine) and a 488 nm filter (YOYO-labeled DNA) were overlaid to determine the colocalization of the released DNA with an endolysosomal marker, LysoTracker Red. LysoTracker Red emits red fluorescence in the acidic pH of the endosomes but is colorless at physiologic pH. Therefore, colocalization of nanoparticles or DNA (green fluorescence) in the presence of LysoTracker Red (red fluorescence) is expected to give a yellow fluorescence.

Quantitative Analysis of Intracellular DNA Using Fluorimetry. Cells were seeded at a cell density of 35 000 cells/mL in 24-well plates. The following day, cells were transfected with 450 μg per well per milliliter of YOYO-labeled DNA-loaded nanoparticles. YOYO-labeled naked DNA and the DNA−Lipofectamine complex were also added to a separate set of cells. After 1, 3, 5, and 7 days, cells were washed twice with PBS, and the fluorescence associated with the cells was measured using a Dynex fluorimeter plate

reader and quantitated using Dynex software (Dynex Revelation 4.21, Dynex Technologies, Ashford, U.K.). These measurements provided overall relative DNA levels in the transfected cells.

Statistical Analysis. Statistical analysis was performed using a Student's *t* test. Differences were considered significant for p values of <0.05.

Results

Nanoparticle Formulation. The formulation protocol resulted in 60−63% entrapment of p53 plasmid DNA in nanoparticles with a DNA loading of ∼1.99−2.10% (w/w). Nanoparticles had a mean hydrodynamic diameter of 280 nm (polydispersity index of 0.143) and mean ζ potential of −18.9 mV. Nanoparticle formulations prepared using YOYO- and TOTO-labeled DNA exhibited similar physical characteristics.

***In Vitro* Antiproliferative Studies.** The antiproliferative effect was more sustained and greater in cells which were transfected with *wt*-p53 DNA-loaded nanoparticles than that with plasmid DNA or the DNA−Lipofectamine complex. The antiproliferative effect became stronger with incubation time in the case of nanoparticles, whereas the effect was transient and lasted for 1 day when the cells were transfected with naked *wt*-p53 DNA (Figure 1). There was no significant difference in the antiproliferative effect of *wt*-p53 DNA nanoparticles with an increase in the dose (Figure 1). Transfection of cells with the *wt*-p53 DNA−Lipofectamine complex resulted in relatively stronger inhibition of cell proliferation 1 day following transfection than that with naked *wt*-p53 DNA or *wt*-p53 DNA-loaded nanoparticles, but the inhibitory effect with the complex did not extend beyond 3 days post-transfection (Figure 2).

p53 mRNA Levels. Since the p53 protein has a shorter half-life (∼6 min), mRNA levels were used as an indicator of gene expression. Cells demonstrated constitutive levels of p53 mRNA. Hence, mRNA levels in the transfected cells were expressed as the percentage above the mRNA levels in nontransfected cells (Figure 3D). Cells transfected with *wt*-p53 DNA-loaded nanoparticles and the *wt*-p53 DNA−Lipofectamine complex demonstrated relatively higher levels of p53 mRNA than cells which were transfected with naked *wt*-p53 DNA. The mRNA levels in the cells transfected with naked *wt*-p53 DNA were close to that in the nontransfected cells. Cells transfected with *wt*-p53 DNA-loaded nanoparticles demonstrated an increase in mRNA levels with time, whereas these levels dropped gradually in the cells which were transfected with the *wt*-p53 DNA−Lipofectamine complex.

Intracellular DNA Distribution. Cells transfected with DNA-loaded nanoparticles at 2 h demonstrated mainly the green fluorescence of nanoparticles (due to 6-coumarin incorporated in nanoparticles) but no red fluorescence of the DNA (TOTO-labeled) that is incorporated in nanoparticles. However, with incubation time, the red color of the DNA became more prominent gradually and was more prominent at 7 days than the green color of the nanoparticle-

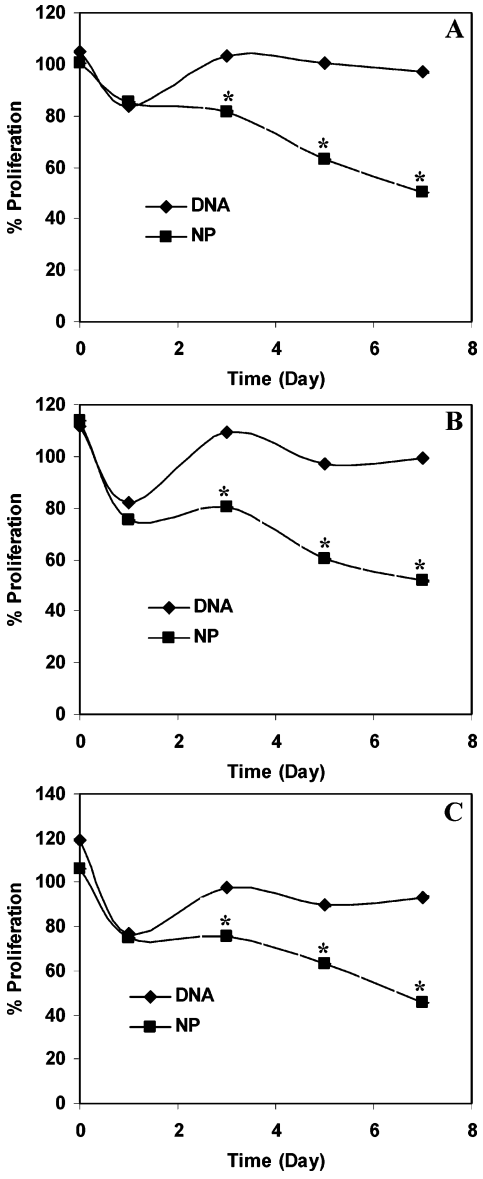

Figure 1. Antiproliferative activity of *wt*-p53 DNA-loaded nanoparticles (NP) and naked *wt*-p53 DNA (DNA) in MDAMB-435S cells. Cells (2500 cells/well) grown in 96-well plates were incubated with (A) 500, (B) 750, and (C) 1000 μg/mL nanoparticles and an equivalent amount of naked DNA [(A) 10.5, (B) 15.75, and (C) 21 μg/mL, respectively]. Medium control or nanoparticles without DNA were used as controls. Cell growth was followed using a standard MTS assay where the absorbance is directly proportional to the number of viable cells. Nanoparticles demonstrated an increase in antiproliferative activity with incubation time. Data are represented as the mean ± the standard error of the mean ($n = 6$; $p < 0.01$ for points marked with asterisks).

incorporated dye. The dye remains with nanoparticles because it is incorporated (dissolved state) in the polymer phase, whereas DNA is dispersed in the polymer phase and is released slowly from the nanoparticles. The green color of nanoparticles became less prominent gradually because either the particles undergo degradation or the red color of the released DNA is masking the green color of nano-

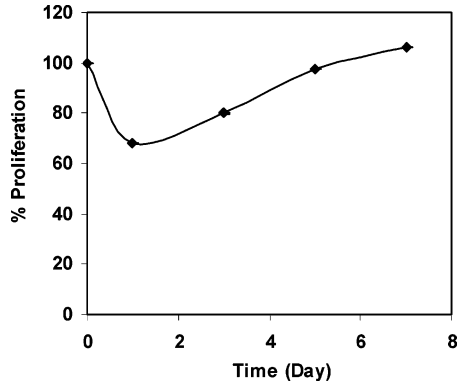

Figure 2. Antiproliferative activity of the *wt*-p53 DNA−Lipofectamine complex. MDAMB-435S cells (2500 cells/well) grown in 96-well plates were incubated with the DNA−Lipofectamine complex (DNA dose of 1.5 μg/mL), and cell proliferation was followed with a standard MTS assay. The DNA−Lipofectamine complex resulted in the transient inhibition of cell proliferation. Data are represented as the mean ± the standard error of the mean ($n = 6$).

particles. The increase in red fluorescence indicates the sustained release of DNA from the nanoparticles which are localized inside the cells. The cells transfected with naked DNA exhibited the red color of DNA 2 h post-transfection, but its intensity was reduced significantly at 3 days, suggesting a transient intracellular retention of DNA (Figure 4). The studies with YOYO-labeled DNA (green fluorescence)-loaded nanoparticles along with LysoTracker Red (red fluorescence) demonstrated yellow fluorescence inside the cells, suggesting that there is localization of a fraction of the released DNA into the endolysosomal compartment. However, the predominant green fluorescence seen inside the cells suggests that most of the released DNA is probably in the cytoplasmic compartment (Figure 5). However, since nanoparticles and the released DNA have green fluorescence, it is difficult to distinguish between them in the confocal microscopic picture. Like the results with TOTO-labeled DNA (Figure 4), these studies also demonstrated sustained intracellular retention of DNA in the cells which were transfected with nanoparticles as compared those which were transfected with naked DNA. In all the studies described above, the released DNA was seen in the perinuclear area.

Quantitative Analysis of Intracellular DNA. The above confocal microscopic observation of sustained intracellular DNA delivery with nanoparticles was further confirmed using a quantitative method. For this purpose, YOYO-labeled DNA-loaded nanoparticles were used. Cells transfected with nanoparticles demonstrated an increase in intracellular fluorescence with incubation time, while the cells transfected with plasmid DNA demonstrated transient intracellular DNA localization (Figure 6). Cells transfected with the DNA−Lipofectamine complex demonstrated almost constant intracellular DNA levels that were below the levels observed in the cells which were transfected with either naked DNA or nanoparticles (data not shown). The lower levels with the complex could probably be due to the smaller dose of DNA

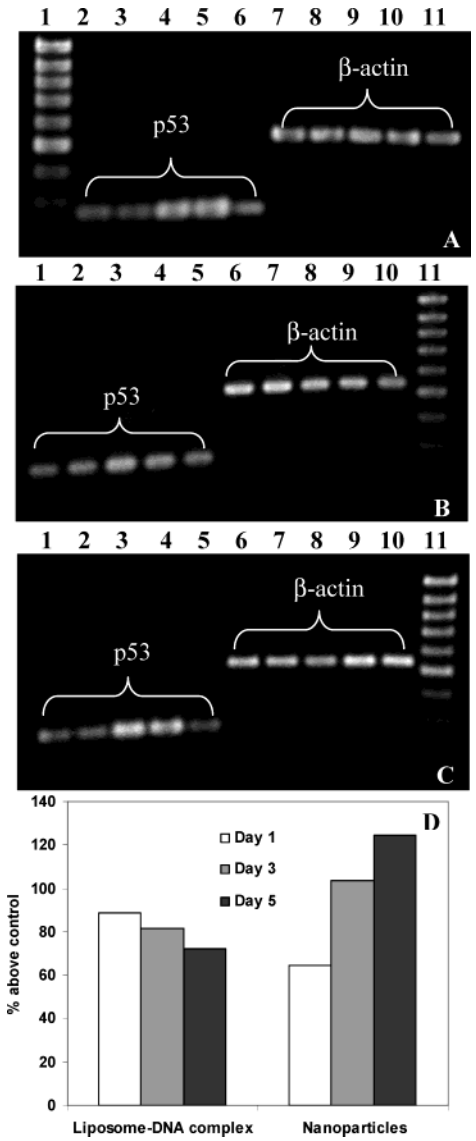

Figure 3. RT-PCR detection of p53 mRNA levels on day (A) 1, (B) 3, and (C) 5 on gel and quantitatively (D) using densitometry. (A) Lane 1: molecular weight markers. Lanes 2−6 (p53) and lanes 7−11 (β-actin): levels in cells transfected with *wt*-p53 DNA, p53(-ve) DNA-loaded nanoparticles, *wt*-p53 DNA-loaded nanoparticles, the *wt*-p53 DNA−Lipofectamine complex, and untreated cells, respectively. (B and C) Lane 11: molecular weight markers. Lanes 1−5 (p53) and lanes 6−10 (β-actin): levels in cells treated with *wt*-p53 DNA, p53-(-ve) DNA-loaded nanoparticles, *wt*-p53 DNA-loaded nanoparticles, the *wt*-p53 DNA−Lipofectamine complex, and untreated cells, respectively. Since untreated cells exhibited some basal levels of p53 mRNA, the quantitative data (D) are expressed as the percentage above the control. Cells transfected with *wt*-p53 DNA-loaded nanoparticles and the *wt*-p53 DNA−Lipofectamine complex exhibited detectable levels of p53 mRNA higher than baseline levels (untransfected cells). Cells transfected with *wt*-p53 DNA-loaded nanoparticles demonstrated sustained and increased p53 mRNA levels with incubation time as opposed to a decrease in mRNA levels in the cells transfected with the *wt*-p53 DNA−Lipofectamine complex.

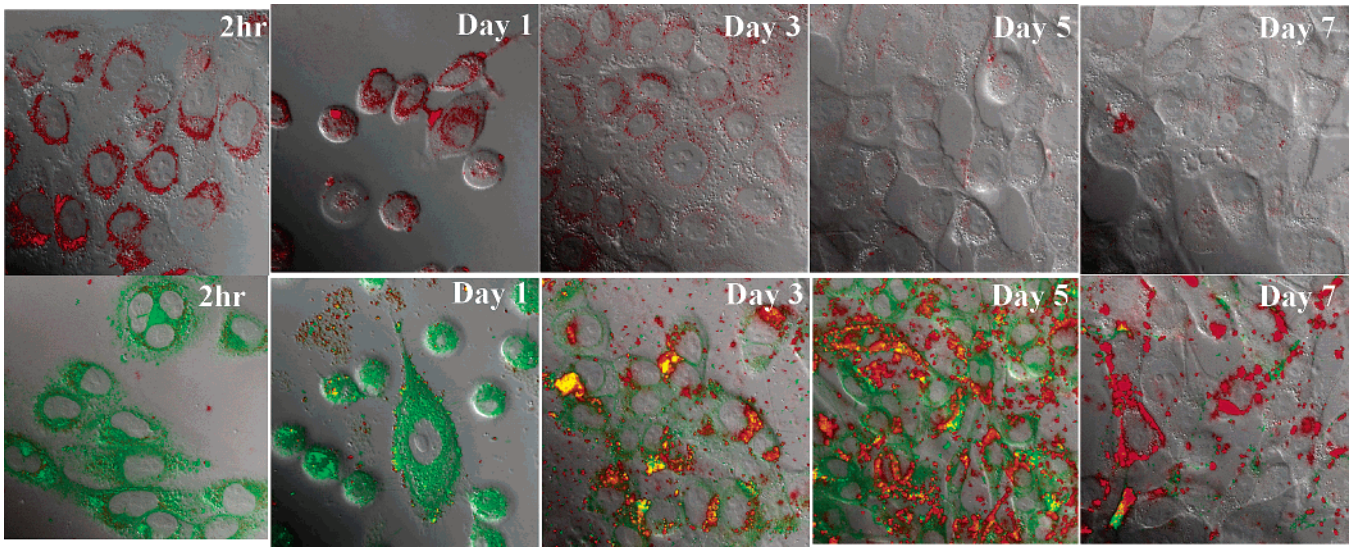

Figure 4. Time course study of intracellular uptake and retention of DNA in MDA-MB-435S cells. Cells were transfected with either naked DNA (red fluorescence, top panel) or nanoparticles (green fluorescence) loaded with DNA (red fluorescence, bottom panel). Cells transfected with nanoparticles demonstrated sustained intracellular DNA localization as opposed to transient localization in the cells transfected with naked DNA.

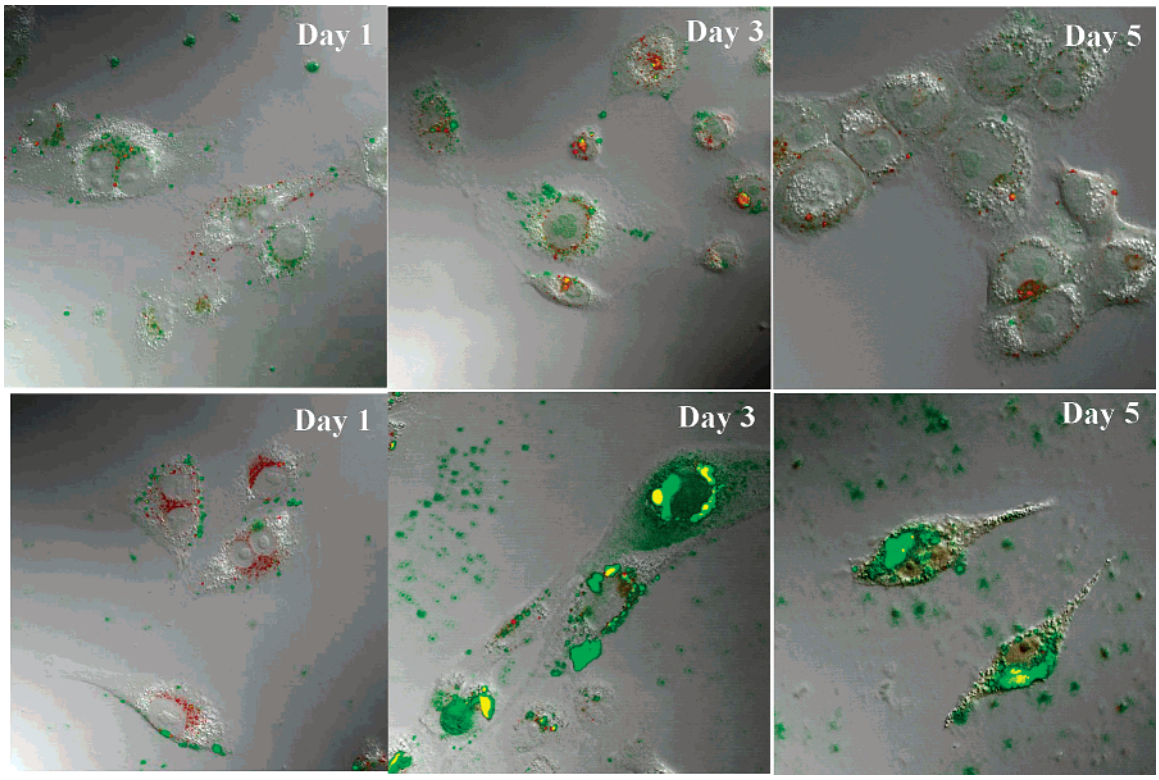

Figure 5. Time course study of the intracellular distribution of DNA. Cells transfected with naked YOYO-labeled DNA (top panel) or encapsulated in nanoparticles (bottom panel). Cells transfected with nanoparticles demonstrated a sustained and significantly greater amount of DNA localization in cytosol (green fluorescence) vs that in endolysosomes (yellow fluorescence due to colocalization of DNA LysoTracker Red, maker for endolysosomes). Cells transfected with naked DNA demonstrated a reduced level of green fluorescence after 3 days.

used, or they could be due to the quenching of the fluorescence due to complexation of the DNA with Lipofectamine. A larger dose of the DNA−Lipofectamine complex could not be used because of cell toxicity.

Discussion

Several recent studies have shown gene expression with biodegradable nano- and microparticles,[8,11−13] but their

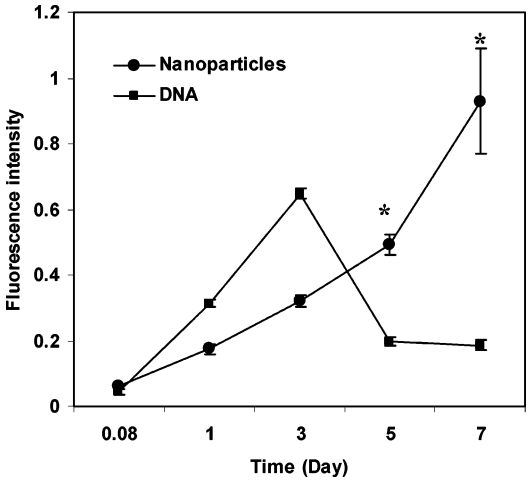

Figure 6. Quantitative determination of intracellular DNA levels. Cells transfected with YOYO-labeled DNA-loaded nanoparticles demonstrated sustained and increased intra hfcellular DNA levels as opposed to transient DNA levels in the cells transfected with naked DNA. Data are represented as the mean ± the standard error of the mean ($n = 6$; $p < 0.001$ for points marked with asterisks).

mechanism of gene expression at the cellular level and the efficacy using a therapeutic gene have not been investigated. In this study, we observed sustained retention of DNA with nanoparticles which resulted in sustained p53 mRNA levels and greater and sustained inhibition of cell proliferation compared to that with plasmid DNA. As demonstrated previously, a fraction of nanoparticles following intracellular uptake escape the endolysosomal compartment and are localized in the cytosolic compartment.[11,12] Thus, the DNA is released slowly from the nanoparticles localized in the cytoplasmic compartment, resulting in sustained intracellular gene delivery. It is interesting to note that the red color of DNA in the cells transfected with nanoparticles was seen predominantly ~3 days post-transfection. This could be because of the retarded release of DNA from the nanoparticles and its slow diffusion into the viscous cytosolic fluid.[25] The release of DNA from nanoparticles under *in vitro* conditions in Tris-EDTA buffer is immediate, with ~10% of the encapsulated DNA release occurring within 1 day.[12]

The slow intracellular release of DNA from nanoparticles also explains the gradual increase in the level of maker gene (luciferase) expression with incubation time observed in our previous studies with nanoparticles as compared to that with a commercially available transfecting agent (FuGene 6, Roche Diagnostics, Indianapolis, IN).[11] With transfecting agents, most of the DNA probably dissociates from the complex following its internalization and, hence, is available for nuclear transport, whereas with nanoparticles, the DNA is released slowly.

In this study, the cells transfected with *wt*-p53 DNA-loaded nanoparticles demonstrated significant and sustained inhibition of cell proliferation (Figure 1). The slow intracellular release of the entrapped DNA could have resulted in sustained p53 mRNA levels observed in the RT-PCR studies and hence is the sustained antiproliferative effect with nanoparticles. The DNA−Lipofectamine complex exhibited only transient inhibition of cell proliferation which was not significantly different from the medium control at 3 days (Figure 2). While DNA−Lipofectamine complexes resulted in p53 mRNA levels similar to that observed with nanoparticles at the earlier time points, mRNA levels dropped significantly with time in the case of the DNA−Lipofectamine complex. However, since the dose of DNA (as suggested by the manufacturer) used with DNA−Lipofectamine complexes was smaller than that used in nanoparticles because of the toxicity of the DNA−lipid complex, it was not possible to directly compare the inhibition efficiency of the two vectors at the same DNA dose. The increase in the dose of *wt*-p53 DNA-loaded nanoparticles did not significantly increase the antiproliferative effect, which could be because of the saturation uptake of nanoparticles by the cells at the smallest nanoparticle dose that was studied.[26]

Cells transfected with naked DNA demonstrated intracellular localization of DNA within 2 h post-transfection, but its retention was transient, suggesting that the free DNA either was degraded due DNAse or undergoes exocytosis.[27] Lechardeur et al.[28] have shown the apparent half-life of 50−90 min of plasmid DNA in the cytoplasm of HeLa and COS cells. Rapid degradation of DNA in the cytosol is also considered one of the limiting factors in gene delivery using a nonviral gene expression system. Nanoparticles have the advantage because the encapsulated DNA is protected, and hence can sustain gene expression as is evident from the increase in mRNA levels and the antiproliferative activity of the encapsulated *wt*-p53 gene with incubation time (Figures 1−3).

The DNA in the transfected cells was seen mostly in the cytosolic compartment but not in the nucleus (Figures 4 and 5), indicating that the nuclear envelope is a barrier for the delivery of DNA to the nucleus, as has been originally reported by Capecchi.[29] Nanoparticles that are ~200 nm in diameter are not expected to carry the DNA directly to the nucleus through the nuclear pore that is ~25 nm in

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diameter.[30,31] Most lipid−DNA complexes have also demonstrated cytoplasmic delivery of DNA but not nuclear delivery.[32] However, Pollard et al.[33] reported, although now debated, that polyethylenimine can enhance the movement of exogenous DNA into the nucleus.

Thus, the gene expression with nanoparticles probably occurs through interaction of the released DNA in cytosol with certain cellular proteins that carry DNA to the nucleus,[34−36] or the DNA is transported to the nucleus during cell division when the nuclear envelop is more permeable.[27] While DNA delivery across the cell membrane is important and a significant emphasis is placed on developing such vectors, relatively little attention is focused on developing ways to deliver DNA into the nucleus once it is localized inside the cells.[37] One could probably couple NLS (nuclear localization signal) peptides to DNA prior to encapsulation into nanoparticles to help facilitate nuclear targeting of the DNA once it is released into the cytosolic compartment.[38,39]

One of the important considerations in p53 gene delivery for tumor growth inhibition would be the sustained expression of the p53 protein in the target cells. Takenobu et al.[40] reported that when the p53 protein was delivered to oral cancer cells using the protein transduction approach, it was necessary to maintain p53 protein levels by providing multiple doses to obtain inhibition of cell proliferation comparable to that with viral vectors. A single-dose regimen resulted in only a weak and transient inhibition of cell proliferation. Thus, the sustained presence of the p53 protein via gene transfection might be an important consideration for tumor inhibition. Several mechanisms have been attributed to *wt*-p53 gene-mediated cancer therapy such as apoptosis of cancer cells, cell cycle arrest, and/or the antiangiogenic effect of the protein.[20] Gene delivery with nanoparticles would require direct intratumoral injection in the case of a solid tumor or delivery via a catheter to an accessible diseased tissue. However, tumor targeting via intravascular administration would require nanoparticle surface modification to avoid extravasation by the reticuloendothelial system.[41]

The success of the gene therapy for clinical applications, in part, would depend on the efficiency of the expression vector as determined by the level as well as the duration of gene expression.[3] Although various cationic polymers and lipid-based systems are being investigated, most of these systems exhibit higher-level but transient gene expression.[42] Most often, the emphasis is on the level of gene expression rather than on the duration of gene expression.[4] In certain disease conditions, a relatively low level of gene expression (therapeutic level) but for a sustained duration may be more effective than higher-level but transient gene expression.[43] Therefore, a gene expression system that can modulate the level as well as the duration of gene expression in the target tissue is desirable. Polymer-based sustained release formulations such as nanoparticles have the potential of developing into such a system.

Conclusion

PLGA nanoparticles loaded with *wt*-p53 DNA demonstrated a sustained antiproliferative effect whose magnitude increased with incubation time in a breast cancer cell line. Inhibition of cell proliferation was found to be due to sustained gene expression following slow intracellular release of the encapsulated DNA from nanoparticles. The results of the study suggest that *wt*-p53 DNA-loaded nanoparticles could be potentially useful in the therapy of breast and other cancers which are ascribed to mutation in the p53 gene.

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