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Sustained Cytoplasmic Delivery of Drugs with Intracellular Receptors Using Biodegradable Nanoparticles

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Abstract: Efficient cytoplasmic delivery of therapeutic agents is especially important for drugs with an intracellular site of action for elicitation of a maximal therapeutic effect. In this study, we demonstrate the efficacy of biodegradable nanoparticles for cytoplasmic delivery of dexamethasone, a glucocorticoid, whose site of action is intracellular. Equal doses of two formulations of drug-loaded nanoparticles releasing different doses of the encapsulated drug were tested for antiproliferative activity in vascular smooth muscle cells. The antiproliferative activity of the drug was significantly greater and sustained with nanoparticles that released a higher dose of the drug than with nanoparticles which released a lower dose of the drug. The greater antiproliferative activity of the nanoparticles that released a higher dose of the drug correlated with sustained and higher intracellular drug levels. The antiproliferative activity of the drug in solution was lower and relatively transient compared to that with drug-loaded nanoparticles. The mechanism of inhibition of cell proliferation was mediated through inhibition of cell-cycle progression with a relatively higher percentage of cells in the G0/G1 arrest phase in the group that was treated with drug-loaded nanoparticles compared to that treated with the drug in solution. Results of the study thus suggest that the dose and duration of a drug's availability at the intracellular site of action determine its therapeutic efficacy. In conclusion, biodegradable nanoparticles could be used as an effective delivery mechanism for sustained intracellular delivery of different therapeutic agents.

Keywords: Sustained release; cellular drug delivery; antiproliferative; smooth muscle cells; restenosis

Introduction

Many therapeutic agents have intracellular compartments as their site of action. These compartments could be the nucleus as in the case of anticancer intercalating agents[1] or the cytoplasm as in the case of steroids such as dexamethasone whose receptors are cytoplasmic.[2] The therapeutic efficacy of these drugs would largely depend on the dose and duration of their availability at the intracellular site of action. For many drugs, the intracellular drug levels are maintained transiently when used as solution, and hence, the therapeutic effect is seen only for a shorter duration.[3] Hence, a carrier that could slowly release the drug at the site of action in the intracellular compartment would enhance the therapeutic efficacy of the drug as well as could sustain its

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therapeutic effect. A sustained drug effect is specifically important for treating chronic pathophysiologic conditions which require prolonged drug action for therapeutic efficacy.

Nanoparticles formulated from the biocompatible and biodegradable polymers poly(D,L-lactide-*co*-glycolide) (PLGA) and polylactide (PLA) have shown the potential for various drug delivery applications.[4] Nanoparticles are sustained release colloidal polymeric particles ~100 nm in diameter, with a therapeutic agent of interest entrapped in the polymeric matrix. In our previous studies, we have investigated intracellular trafficking of PLGA nanoparticles and demonstrated their rapid escape from the endolysosomal compartment into the cytosol.[5,6]

We hypothesized that the sustained cytoplasmic release of the drug from nanoparticles could potentiate the therapeutic efficacy of the drugs whose site of action is cytoplasmic. Using dexamethasone as a model drug, we have investigated the therapeutic efficacy (inhibition of vascular smooth muscle cell proliferation) of the drug encapsulated in nanoparticles and compared that with the drug in solution. Dexamethasone is known to mediate its antiproliferative effect by binding to the glucocorticoid receptors present in the cytoplasm. The receptor−drug complex translocates into the nucleus and can bind to either the glucocorticoid responsive element or the nonglucocorticoid responsive element on the genome, resulting in certain genes being turned on or off.[2] Vascular smooth muscle cells (VSMCs) were chosen as a model cell line because of our interest in localized delivery of antiproliferative agents such as dexamethasone[7,8] and genes to the arterial tissue to prevent restenosis, which is the re-obstruction of a blood vessel following balloon angioplasty or stenting, the techniques commonly used to relieve the obstruction. VSMCs are commonly used as a model cell line to study the antiproliferative effect of therapeutic agents.[9]

Experimental Section

Nanoparticle Formulation. Two formulations of dexamethasone-loaded nanoparticles that release the drug at different rates were prepared by an emulsion−solvent evaporation technique.[5] In brief, a dexamethasone solution in methanol (6 mg/240 μL) was mixed with 1 mL of a 30 mg/mL polymer solution in chloroform (formulation A consisted of PLGA with a molecular mass of 140 000 Da and a 50/50 lactide/glycolide ratio and formulation B of PLA with a molecular mass of 88 000 Da and a 100/0 lactide/glycolide ratio), which was then added to 6 mL of a 2.5% (w/v) aqueous polyvinyl alcohol solution (PVA, 30000−70000 kDa, Sigma), and the mixture was sonicated for 5 min. The emulsion that formed was stirred for ~18 h at room temperature followed by 1 h in a desiccator under vacuum to remove the residual organic solvents. Nanoparticles were recovered by ultracentrifugation (35 000 rpm for 30 min at 4 °C, Optima LE-80K, Beckman, Palo Alta, CA), washed two times with distilled water to remove unentrapped drug and PVA, and then lyophilized (−65 °C and <10 μm of mercury pressure, LYPH-LOCK 12, Labconco, Kansas City, MO) for 48 h to obtain a dry powder. Drug loading in the nanoparticles and the entrapment efficiency of the formulation procedure were determined by analyzing the amount of unentrapped dexamethasone and subtracting this from the total amount of dexamethasone added in the formulation. Nanoparticles containing an electron dense dye (osmium tetroxide) were formulated with previously described procedure in an effort to study the cellular uptake of nanoparticles using transmission electron microscopy (TEM).[5]

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

***In Vitro* Release of Dexamethasone from Nanoparticles.** *In vitro* release of the drug from nanoparticles was carried out under sink conditions using side-by-side double-diffusion chambers separated by a Millipore membrane with a porosity of 100 nm (Millipore Co., Bedford, MA).[10] A suspension of drug-loaded nanoparticles in phosphate-buffered saline (PBS, pH 7.4, 0.15 M, 1 mg/2.5 mL) was placed in the donor chamber and plain PBS in the receiver chamber. These nanoparticles contained tritiated dexamethasone. The chambers were then placed in an orbital shaker (Environ orbital

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shaker, Lab Line, Melrose Park, IL) maintained at 37 °C and 100 rpm. At different time intervals, the entire volume of the receiver chambers was removed and replaced with fresh PBS. The amount of drug released was measured by counting the radioactivity in the buffer retrieved from the receiver chambers at different time points using a liquid scintillation counter (Tri-Carb 2500 TR/AB, Packard Instrument Co., Meriden, CT).

Cell Culture. Human vascular smooth muscle cells (VSMCs, Cascade Biologics, Portland, OR) maintained on Medium 231 supplemented with smooth muscle growth supplement (Cascade Biologics) were used in all the studies. All the incubations were performed at 37 °C and 5% CO_2.

***In Vitro* Antiproliferative Studies.** VSMCs were plated overnight in 96-well plates at a density of 5000 cells per well per 0.1 mL. A nanoparticle suspension prepared in regular serum-containing growth medium (600 μg/mL, 0.1 mL) was added to each well. A dexamethasone solution in growth medium (25 μg/mL) was also added to some of the wells. Untreated cells were used as controls. Cell proliferation as a function of time was followed using a standard MTS assay (CellTiter 96 AQ_{ueous}, Promega). The medium was changed on day 2 and every other day thereafter, and no further dose of the drug was added. For the MTS assay, cells were washed twice with PBS and incubated with fresh medium. The MTS reagent (20 μL) was added to each well and 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). Control nanoparticles did not show any inhibition compared to untreated cells (medium) in our previous studies[5] and, hence, were not used in this study.

Intracellular Drug Levels. VSMCs were plated at a density of 100 000 cells per well per 2 mL in six-well plates and allowed to attach overnight. Tritiated dexamethasone encapsulated in nanoparticles (600 μg of nanoparticles/mL, 2 mL) or in solution (25 μg/mL, 2 mL) was added to each well. The medium was changed on day 2 and every other day thereafter, and no further dose of the drug was added. At different time intervals, cells were washed with PBS twice to remove uninternalized nanoparticles and free drug, and then the cells were lysed by incubating them with 0.1 mL of 1× cell culture lysis reagent (Promega, Madison, WI) for 30 min at 37 °C. The total amount of cell protein was determined from the cell lysate using the Bradford protein assay (Bio-Rad, Hercules, CA). The cell lysates were lyophilized (−80 °C and <10 μm of mercury pressure, LYPH-LOCK 12), and the total amount of drug (free and entrapped in nanoparticles) was extracted with dimethyl sulfoxide for 48 h. Radioactivity in the extracts was measured using a liquid scintillation counter. Radioactivity was normalized to the total amount of cell protein.

Cell-Cycle Analysis and Apoptosis. The distribution of DNA in the cell cycle and apoptosis was studied by flow cytometry using Telford's method.[11] VSMCs were seeded at a density of 1×10^6 cells per 10 mL per flask in a 75 cm^2 flask and allowed to attach overnight. Dexamethasone in solution or encapsulated in nanoparticles (the same concentration used for antiproliferative studies) was added to cells. Blank nanoparticle-treated cells and untreated cells were used as controls. The medium was changed on day 2, and no further dose of the drug was added. On day 3, cells were washed twice with PBS, trypsinized, and resuspended in 1.0 mL of Telford's reagent [16.8 mg of EDTA disodium salt, 13.4 mg of ribonuclease A (93 units/mg), 25 mg of propidium iodide, and 0.5 mL of Triton X-100 in 500 mL of PBS]. The cellular DNA content was analyzed with a fluorescent activated cell sorter FACStarPlus flow cytometer operating under Lysis II (Becton Dickinson Immunocytometry Systems, San Jose, CA) to create histograms of cell frequency versus propidium iodide fluorescence intensity.

Intracellular Retention of Nanoparticles by Transmission Electron Microscopy. VSMCs were plated 24 h prior to the experiment in 100 mm tissue culture dishes (Becton Dickinson) at a density of 500 000 cells/dish in 10 mL of growth medium. Cells were incubated with an osmium tetroxide-loaded nanoparticle suspension (100 μg/mL) and were washed twice with PBS 48 h postincubation. Cells were harvested by trypsinization at different time points, then fixed in a 2.5% glutaraldehyde solution in PBS for 1 h, and then postfixed in 1% osmium tetroxide in PBS for 1 h. Fixed cells were processed for TEM studies as described previously.[5]

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

Results

Nanoparticle Formulations. Two nanoparticle formulations that release different amounts of drug were obtained by formulating nanoparticles with polymers with different compositions and molecular masses. Formulation A (600 μg of nanoparticles) released a total of 6 μg of dexamethasone over 14 days, while the same amount of formulation B released a total of 16 μg over 14 days (Figure 1). Formulation A had a lower drug loading level [5.6% (w/w) and 30% entrapment efficiency] than formulation B [9.5% (w/w) and 46% entrapment efficiency]. VSMCs were incubated with DEX in solution (dexamethasone dose of 25 μg/mL) or with drug-loaded nanoparticles (nanoparticle dose of 600 μg/mL). On the basis of the drug loading and *in vitro* release, the estimated dose of the drug is 0.42 μg/day for formulation A and 1.14 μg/day for formulation B. The other physical characteristics such as size and ζ potential of the two nanoparticle formulations were similar (Table 1).

***In Vitro* Antiproliferative Efficacy.** The treatment of cells with dexamethasone in solution demonstrated a transient inhibition of cell proliferation compared to untreated cells. The inhibition was seen up to 5 days following treatment; however, the level of proliferation increased beyond this

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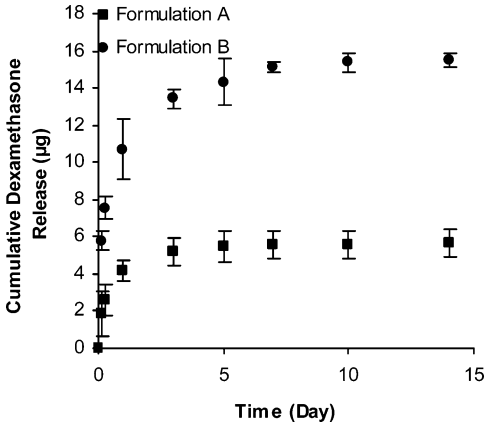

Figure 1. *In vitro* release of dexamethasone from the two nanoparticle formulations used in the study. The release that is shown is from 600 μg of nanoparticles. Data are means $\pm$ the standard deviation ($n = 3$).

Table 1. Nanoparticle Formulations Used in the Study and Their Characterization

formulation	drug loading (%, w/v)	entrapment efficiency (%)	particle size[a] (nm)	ζ potential (mV)
A	5.6	30	260 ± 1	-23.9 ± 3.5
B	9.5	46	271 ± 5	-19.6 ± 1.5

[a] Hydrodynamic diameter.

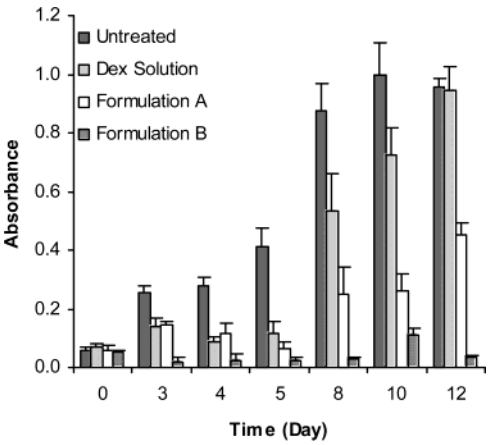

Figure 2. Inhibition of VSMC proliferation with dexamethasone in solution and encapsulated in nanoparticle formulations. Cell growth was followed by an MTS assay, where absorbance is proportional to the number of viable cells. Data are means $\pm$ the standard deviation ($n = 6$).

point, and there was no significant difference in the inhibition between the untreated and treated cells on day 12 of the study. However, significantly higher and more sustained (for up to 12 days) inhibition of cell proliferation was obtained when the cells were treated with dexamethasone-loaded nanoparticles ($p < 0.05$ for formulation B and solution groups for all time points and $p < 0.05$ for formulation A and the solution group from day 8 to day 12) (Figure 2).

Within the two nanoparticle formulations, the inhibition of cell proliferation was found to correlate with the dose of

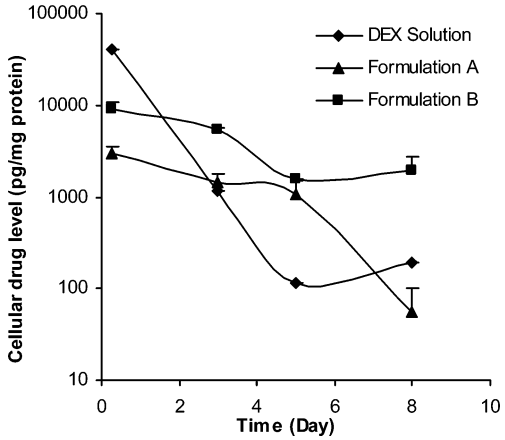

Figure 3. Intracellular dexamethasone levels following treatment with tritiated dexamethasone in solution or in nanoparticles. Data are means $\pm$ the standard error of the mean ($n = 3$).

drug released. Nanoparticles exhibiting a smaller amount of drug release (formulation A) demonstrated a lower level of inhibition of cell proliferation than those with formulation B which exhibited a higher level of drug release (differences significant at $p < 0.05$ after day 5). In the case of formulation A, the level of cell proliferation gradually increased after 5 days, whereas in the case of formulation B, the inhibition of proliferation was maintained over 12 days (Figure 2). Blank nanoparticles (without drug) and medium control had similar growth curves (data not shown), indicating that the inhibitory effect on cell proliferation was due the effect of the drug encapsulated in nanoparticles.

Sustained Intracellular Drug Levels with Nanoparticles. Nanoparticles maintained sustained intracellular drug levels, whereas the levels dropped rapidly in the cells that were treated with the drug in solution once the fresh medium (without drug) was added on day 2 of the study. Of the two nanoparticle formulations, formulation B demonstrated greater and more sustained intracellular drug levels than formulation A. For example, the drug level with formulation B was 35-fold greater than that with formulation A on day 8 (Figure 3). Intracellular drug levels were maintained for only 5 days with formulation A, whereas with formulation B, the drug levels were maintained over 8 days. Thus, intracellular drug levels for the two formulations correlated with their *in vitro* release profiles, and the antiproliferative activity correlated very well with the intracellular drug levels achieved with the two formulations of nanoparticles.

Effect of Dexamethasone-Loaded Nanoparticles on the Cell Cycle and Apoptosis. Cell-cycle analysis demonstrated that the cells treated with dexamethasone-loaded nanoparticles had a higher proportion of cells in the G0/G1 phase and a concurrent lower proportion of cells in the proliferative S phase on day 3 of treatment than the cells treated with the drug in solution (Figure 4). Within the nanoparticle treatment groups, the formulation which demonstrated the greater amount of drug release and higher intracellular drug levels had a relatively lower percentage of cells in the S phase.

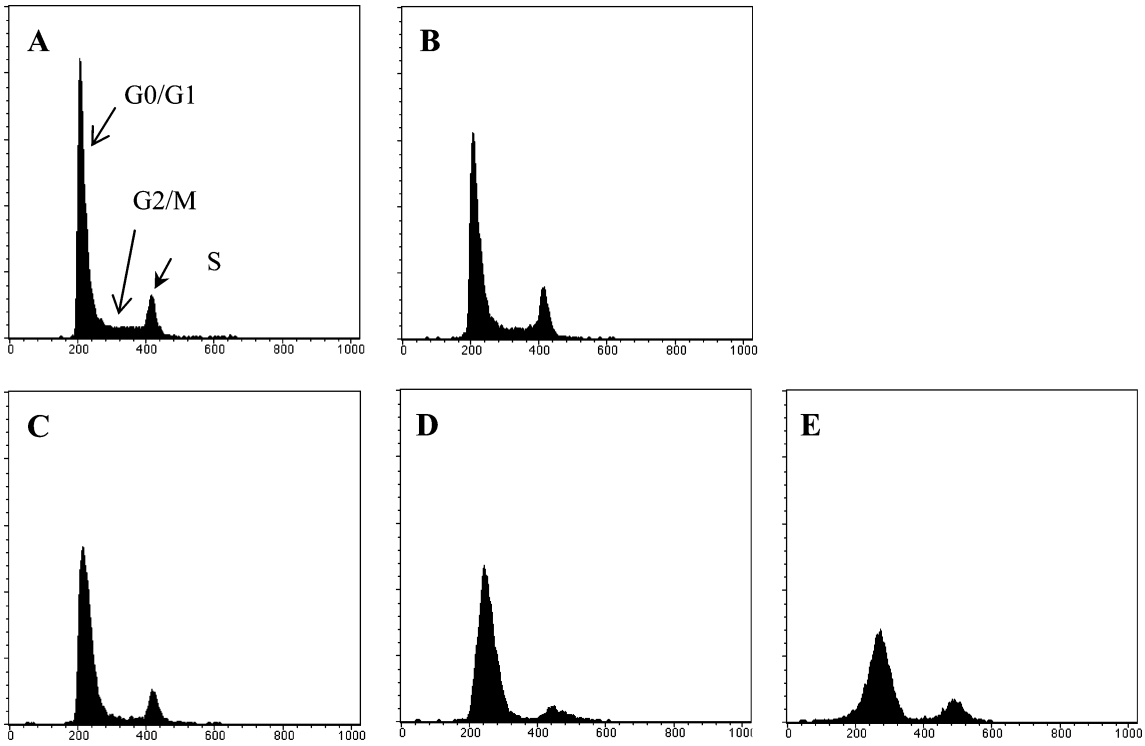

Figure 4. Cell-cycle analyses of VSMCs following treatment with (A) medium (untreated control), (B) blank nanoparticles, (C) dexamethasone in solution, (D) formulation A, and (E) formulation B. Cells were washed after being treated for 48 h and incubated with fresh medium. Cell-cycle perturbations were analyzed by flow cytometry 72 h after the beginning of the treatment. The profiles are from one representative experiment.

Table 2. Effect of Dexamethasone Treatments on the Cell-Cycle Distribution in VSMCs

treatment[a]	estimated dose of Dex (μg/mL)[b]	total dose of Dex (μg/mL)[c]	G0/G1	G2/M	S	% apoptotic cells
untreated	–	–	62.0 ± 0.4	9.8 ± 0.6	28.0 ± 0.2	0.1 ± 0.0
blank NP	–	–	60.3 ± 1.9	12.9 ± 0.6	26.7 ± 2.5	0.6 ± 0.5
Dex solution	25	25	75.7 ± 2.3	9.8 ± 1.0	14.5 ± 1.4	1.7 ± 0.5
formulation A	5.6	33.3	83.6 ± 0.0	8.4 ± 0.5	8.1 ± 0.5	0.5 ± 0.5
formulation B	15.5	57.0	82.9 ± 1.5	17.2 ± 1.5	0.0 ± 0.0	0.3 ± 0.0

[a] NP, nanoparticles; Dex, dexamethasone. [b] Dose based on the amount of drug released over the experimental time period. [c] Dose based on the total drug loading in nanoparticles.

Blank nanoparticles had no effect on the cell-cycle distribution, and the results were similar to the results with the medium-treated control. The treatment of cells with drug-loaded nanoparticles did not result in significant apoptosis in the cell population; however, a marginally higher number of apoptotic cells were seen in the cells treated with the drug in solution (Table 2).

Sustained Intracellular Retention of Nanoparticles. To study the intracellular retention of nanoparticles, the formulation containing osmium tetroxide as an electron dense dye was used. The suitability of the above formulation for studying the uptake of nanoparticles by cells and/or tissue using TEM was reported in our previous studies.[12] The results of this study demonstrated that the nanoparticles were retained intracellularly and were detected in the cytoplasm even after postincubation for 14 days (Figure 5).

Discussion

Intracellular delivery could be critical for a number of different types of therapeutic agents. For example, endolysosomal escape of a gene expression vector into the cytoplasm followed by nuclear translocation of the DNA is an important step governing the efficiency of gene transfection.[13] Similarly, many anti-cancer agents such as doxorubicin and cisplatin, which act by intercalating with the genomic DNA, require their nuclear localization to be effective.[1] Dexamethasone, a model drug used in this study, exerts several of its pharmacological actions by binding to the

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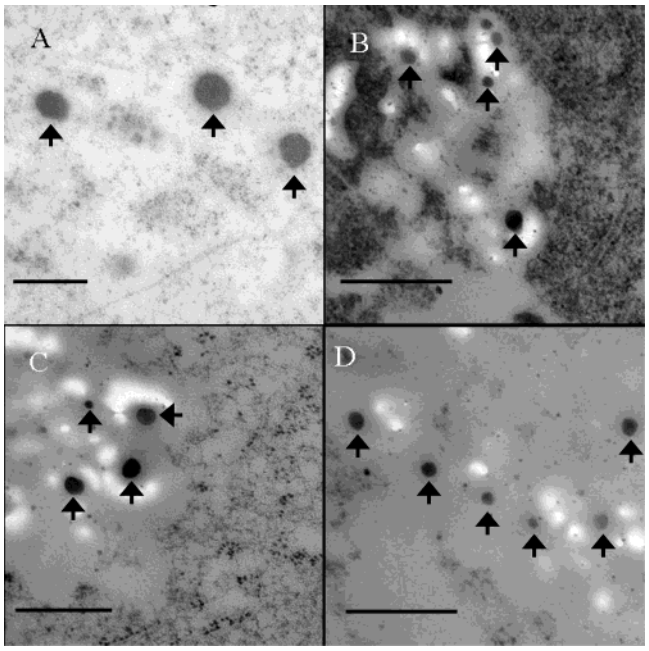

Figure 5. TEM pictures demonstrating the presence of nanoparticles in the VSMCs on day 1 (A), 3 (B), 10 (C), and 14 (D) postincubation. The bar is 500 nm long. Nanoparticles are indicated by arrowheads.

glucocorticoid receptors present in the cytoplasmic compartment.[2] Thus, a specific intracellular compartment is the site of action for many therapeutic agents.

Significant attention has been focused recently on intracellular delivery of different classes of therapeutic agents such as peptides, proteins, and DNA using lipid- and peptide-based delivery systems.[14,15] While these systems result in relatively higher intracellular delivery efficiency, the duration of drug retention inside the cells with these systems is transient and is often less than 24 h.[15] Further, the effectiveness of these systems for delivering low-molecular mass compounds has not yet been demonstrated.

Polymeric nanoparticles formulated from PLGA and PLA have been found to be effective for the delivery of both low-molecular mass and macromolecular therapeutics.[4] In our previous studies, PLGA nanoparticles have been demonstrated to escape the endolysosomes through the mechanism of surface charge reversal (from anionic to cationic) selectively in the acidic endolysosomal compartment, causing nanoparticles to interact with the endolysosomal membrane and escape into the cytoplasm.[5] Other polymeric systems such as polyethylenimines and poly-L-lysines escape the endolysosomal compartment, but they disrupt the endolysosomal vesicles and hence could induce cell toxicity.[16] In our studies, we did not observe nanoparticle-associated cell toxicity in the 1−1000 μg/mL range in different cell lines.[12] Once inside the cytoplasm, nanoparticles are hypothesized to release the drug slowly, resulting in a sustained therapeutic effect of the encapsulated agent. On the basis of this model, the therapeutic efficacy of the nanoparticle-encapsulated drug would depend on nanoparticle uptake, their intracellular distribution, and more importantly on the dose of the drug that is released from the internalized nanoparticles inside the cell.

To investigate the relationship between the amount of drug released and therapeutic efficacy, dexamethasone-loaded nanoparticle formulations that released different amounts of drug were formulated (Figure 1). However, to study the effect of drug release on the therapeutic efficacy, it is important to have nanoparticle formulations that would have similar cellular uptake and distribution. Previous studies have suggested that particle size is an important parameter that determines cellular nanoparticle uptake.[17] Similarly, endolysosomal escape of nanoparticles has been shown to depend on the ζ potential of nanoparticles.[5] Thus, particle size and ζ potential are considered important parameters that influence nanoparticle uptake and intracellular distribution. As is evident from Table 1, the particle size and ζ potential of the two nanoparticle formulations used in our study are almost similar, and it is therefore expected that both nanoparticle formulations would have similar cellular uptakes and intracellular distributions. Therefore, the antiproliferative activity of the two formulations of nanoparticles should be the function of the dose of the drug that is released in the cytoplasmic compartment.

In this study, dexamethasone in solution demonstrated only a transient inhibition of proliferation as compared to dexamethasone encapsulated in nanoparticles (Figure 2). This could be explained on the basis of the differences in the intracellular drug levels obtained with the drug in solution and encapsulated in nanoparticles. From Figure 3, it can be seen that drug levels in the cells treated with the drug in solution fall rapidly as compared to the drug levels in the cells treated with nanoparticles. The intracellular drug levels shown with nanoparticles are the total drug levels (free + entrapped in nanoparticle). Thus, a small fraction of free drug that is available in the cytoplasmic compartment is responsible for eliciting the antiproliferative effect of dexamethasone. Free drug levels in the cell lysate could not be detected since the levels were lower than the limit of detection (~40 pg). The marginal increase in the intracellular drug levels at later time points (day 8) seen in the dexamethasone solution group is considered within the experimental variation since

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the levels are close to the lower limit of detection (40 pg) and the difference is not statistically significant compared to the earlier time point (114 ± 38 pg for day 5 vs 190 ± 76 pg for day 8).

TEM results suggest that nanoparticles are retained in the cytoplasm for a sustained period of time (Figure 5), which probably resulted in sustained intracellular drug levels (Figure 3). There seems to be no apparent change in the morphology of nanoparticles found in the cytoplasm of the cells. Our recent study with protein-loaded nanoparticles demonstrated that although the molecular mass of the polymer is reduced during the initial phase of the *in vitro* release study (∼20 days), the morphology of nanoparticles was maintained, and only beyond ∼30 days of the release study were the nanoparticles seen to lose their structure.[18] Nanoparticles probably maintain their morphology until there is significant bulk erosion of the nanoparticle polymer matrix which occurs only after considerable degradation of the polymer.

Sustained cytoplasmic delivery of the drug from nanoparticles coupled with the fact that the receptors for the drug are cytoplasmic could have resulted in the enhanced therapeutic efficacy of the nanoparticle-encapsulated dexamethasone. It is interesting to note that in many of the *in vitro* antiproliferative studies with dexamethasone, the drug is required to be in contact with cells to elicit prolonged inhibition of proliferation of VSMCs.[9,19,20] This effect has been noted for other antiproliferative drugs also. Suh *et al*.[3] demonstrated that the antiproliferative effect of paclitaxel in VSMCs was observed as long as the drug was in contact with the cells. The reversal of the antiproliferative effect after the removal of drug was attributed to the steep drop in the intracellular taxol levels once it was removed from the medium (>80% of the internalized drug eliminated from the cells within 60 min). Kawai *et al.*[19] in their studies have made an interesting observation about the antiproliferative effect of dexamethasone. It was demonstrated that while prolonged contact of the drug with VSMCs resulted in growth inhibition, its short-term exposure resulted in stimulation of proliferation. Thus, a continuous exposure of dexamethasone to the cells is probably necessary for sustained inhibition of proliferation.

Dexamethasone is known to have a variety of pharmacological actions, including its effect on genes controlling the cell cycle, induction of pro- and antiapoptotic genes, and inhibition of secretion of various growth factors and cytokine-promoting inflammation.[2,21] Dexamethasone inhibits cell growth and proliferation primarily through its effect on the cell cycle[20] and induction of apoptosis.[22] It has been shown that in many cell lines, including in smooth muscle cells, dexamethasone induces cell-cycle arrest in the late G1 phase of the cell cycle. While the mechanism of cell-cycle arrest in VSMCs is still not clear and could proceed through inhibition of phosphorylation of retinoblastoma protein, in lymphocytes, cell-cycle arrest proceeds through the induction of the *myc* pathway and the downregulation of the *Bcl* gene.[22] In our studies, treatment with dexamethasone resulted in a greater proportion of cells in the G0/G1 phase, compared to the proportion for cells left untreated or treated with the blank nanoparticle (Table 2). A higher proportion of cells were in the G0/G1 arrest phase in the nanoparticle treatment groups than in the group treated with the drug in solution. On the basis of the intracellular drug levels, it could be concluded that more drug is available at the site of action for a sustained period of time in the case of nanoparticles than in the case of the drug in solution, resulting in greater antiproliferative activity with drug-loaded nanoparticles.

Induction of apoptosis by dexamethasone is a complex phenomenon as the drug is known to have both pro- and antiapoptotic effects.[22,23] While dexamethasone-induced apoptosis has been unequivocally demonstrated in immune cells,[22] there are contradictory reports about dexamethasone-induced apoptosis in VSMCs. Chen *et al.*,[24] in their studies on rat aortic smooth muscle cells, have reported the presence of significant early apoptotic and advanced apoptotic and necrotic cell populations following the treatment with 10^{-6} M dexamethasone. However, Pross *et al.*[25,26] have reported the lack of apoptosis and necrosis in both rat and human

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aortic cells following treatment with dexamethasone in the concentration range of $10^{-9}-10^{-5}$ M. In our studies, we found that there was not a significant induction of apoptosis following treatment with dexamethasone. The fraction of apoptotic cells was marginally higher in the cells treated with the drug in solution than in the cells treated with drug-loaded nanoparticles (Table 2). In the case of the drug in solution, the entire dose of the drug is available all at once, and this might have contributed to the slightly higher observed level of apoptosis.

While induction of apoptosis and/or necrosis is probably the main mechanism of growth inhibition in many proliferative disorders such as cancer, it is debatable whether it is beneficial in vessel wall pathologies such as the inhibition of restenosis following balloon angioplasty. Induction of apoptosis immediately following mechanical interventions in the medial VSMCs is thought to contribute to vessel wall remodeling and development of restenosis.[27] Thus, inhibition of proliferation through cell-cycle arrest with a lower extent of apoptosis obtained with nanoparticles might be a better therapeutic approach for treating vessel wall pathologies such as restenosis.

Although we have demonstrated the efficacy of nanoparticles for sustained cytoplasmic delivery using dexamethasone as a model drug in this study, a similar strategy could be used for other therapeutic agents whose target compartment is cytoplasm, for example, intracellular delivery of prolidase using biodegradable nanoparticles in case of prolidase deficiency, a rare autosomal recessive inherited disorder, whose main manifestations are chronic, intractable ulcerations of the skin, particularly of lower limbs.[28] Similarly, introduction of pro-apoptotic proteins into the cytoplasm of tumor cells with mutated or missing apoptotic proteins[15] and inhibition of ischemia-induced apoptosis by delivering anti-apoptotic proteins[29] represent some of the methods by which therapeutic agents can be delivered into the cytoplasmic compartment using biodegradable nanoparticles.

Conclusion

In this study, sustained intracellular delivery of dexamethasone using biodegradable nanoparticles resulting in a prolonged antiproliferative effect was demonstrated. Biodegradable nanoparticles thus could offer a potentially useful delivery mechanism for achieving sustained cytoplasmic delivery of other therapeutic agents such as proteins, peptides, and DNA. Results of this study suggest that the dose of the drug and its retention at the intracellular site of action (cytoplasm in this case) determine the magnitude and duration of the therapeutic effect of the drug. The dose of the drug required in the cytoplasmic compartment to achieve the optimal therapeutic effect would depend on the drug and its potency. Therefore, nanoparticle formulation based on the drug response could be a more effective approach in achieving the optimal therapeutic efficacy using nanoparticles.

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