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ORIGINAL ARTICLE

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Efficacy of an intratumoral controlled release formulation of clusterin antisense oligonucleotide complexed with chitosan containing paclitaxel or docetaxel in prostate cancer xenograft models

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Abstract *Purpose*: To develop and evaluate an injectable, controlled release delivery system for a phosphorothioate antisense oligonucleotide (ASO) based on complexed ASO:chitosan dispersed in a biodegradable polymeric paste for intratumoral treatment of solid tumors. *Methods*: Clusterin ASO was complexed with chitosan particles and incorporated into a paste based on a 60:40 blend of methoxy-poly(ethylene glycol) (MePEG) and triblock copolymer of poly(D,L-lactic acid-co-caprolactone)-PEG-(D,L-lactic acid-co-caprolactone). In vitro release profiles of clusterin ASO into phosphate-buffered saline at 37°C were obtained under sink conditions and assayed by anionic exchange high-performance liquid chromatography. In vivo efficacy studies were carried out in human prostate PC-3 and LNCaP tumors grown subcutaneously in mice. Paste formulations of clusterin ASO with or without paclitaxel or docetaxel were injected intratumorally and tumor volumes and serum prostate specific antigen (PSA) levels were measured. *Results*: Controlled release of clusterin ASO was obtained over several weeks. The rate and extent of ASO release was proportional to the ratio of ASO to chitosan in the paste. Treatment of mice bearing PC-3 tumors with clusterin ASO plus paclitaxel or docetaxel paste had reduced mean tumor volume by greater than 50% at 4 weeks. Treatment of mice bearing LNCaP tumors with clusterin ASO plus paclitaxel reduced mean tumor volume and serum PSA level by more than 50% and 70%, respectively. *Conclusions*: Complexation of clusterin ASO with chitosan and incorporation into polymeric paste with paclitaxel or docetaxel produced in vitro controlled release of the ASO and in vivo efficacy over 4 weeks following a single intratumoral injection in solid human prostate tumors in mice.

Keywords Chitosan · Clusterin antisense oligonucleotide · Paclitaxel · Docetaxel · Prostate cancer · Complex

Abbreviations ASO: Phosphorothioate antisense oligonucleotide · CC: Chitosan complexes · CC paste: Formulations of an injectable polymeric paste loaded with chitosan complexes · MePEG: Methoxy-poly(ethylene glycol) · MMO: Mismatch oligonucleotide phosphorothioate · PEG: Poly(ethylene glycol) · PLC: Random poly(D,L-lactic acid-co-caprolactone) copolymer · PLC-PEG-PLC: Triblock copolymer of PLC and PEG in the form of PLC-PEG-PLC · PSA: Prostate specific antigen · Trizma: Tris(hydroxymethyl) aminomethane hydrochloride

Introduction

The clinical application of therapeutic oligonucleotides such as antisense oligonucleotides [1], ribozymes [2], small interfering ribonucleic acid (si RNA) [3], cytosine-phosphate-guanine (CpG) immune modulators [4], aptamers [5] and other oligonucleotides [6] has been hampered by a number of drug delivery issues. In particular, delivery of oligonucleotides to the target site, short tissue half-lives with degradation of oligonucleotides by nucleases, and poor cellular uptake have limited the efficacy of oligonucleotides in vivo [7].

Several strategies have been used to inhibit nuclease degradation and increase transfection of oligonucleotides into cells, including chemical modifications of the

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oligonucleotide [8] or the complexation of polyanionic oligonucleotides with polycations such as polylysine [9] or polyethylenimine [10]. Furthermore, polylysine/oligonucleotide [11] and polyethylenimine/oligonucleotide [12] complexes have been incorporated into polymeric matrices to provide additional protection from nucleases and slow the release of the oligonucleotide from the delivery system. Polyethylenimine/plasmid DNA injected intratumorally has been shown to result in increased expression and efficacy compared to naked plasmid DNA injections [13]. However, there have been few investigations of methods that might more effectively locate and maintain therapeutic concentrations of oligonucleotides at the target disease site.

The role and function of clusterin protein has been reviewed in the literature [14, 15]. Clusterin protein has been shown to inhibit apoptosis [16] and as a result, its overexpression assists in the formation of a prostate cancer phenotype that is resistant to chemotherapy [17], radiation [18] and androgen ablation [19]. siRNA targeted to inhibit the expression of clusterin has been shown to increase the chemosensitivity of PC-3 cells in vitro [20], while antisense oligonucleotide targeted at clusterin has been demonstrated to increase the sensitivity of PC-3 [21] and LNCaP [22] tumors in mice to paclitaxel [17] in vitro and in vivo. Antisense oligonucleotides inhibit gene expression by hybridizing with complementary mRNA regions of a target gene and forming RNA/DNA duplexes [23]. The PC-3 cell line is an androgen-independent human prostate cancer cell line that does not secrete PSA, while the LNCaP cell line is the only androgen-dependent human prostate cancer cell line that is immortalized in vitro and secretes PSA [22]. Since prostate cancer has responded to treatment with the taxanes paclitaxel [24] and docetaxel [25] in the clinic, a suitable therapeutic strategy might be to combine clusterin antisense oligonucleotide treatment with paclitaxel or docetaxel.

There is interest in the local delivery of paclitaxel to tumors in order to reduce the systemic side effects of this cytotoxic agent. Paclitaxel has been incorporated into microspheres for intratumoral delivery in prostate cancer [26]. Our group has developed intratumoral biodegradable polymeric pastes for the delivery of paclitaxel and other anticancer agents [27, 28]. Following intratumoral administration of paclitaxel-loaded paste into xenograft LNCaP murine models, decreased tumor volume and serum PSA levels were observed [27]. The paste is based on a biodegradable triblock copolymer of random poly(lactide-co-caprolactone) (PLC) with poly(ethylene glycol) (PEG) in the form of PLC-PEG-PLC, blended with methoxypoly(ethylene glycol) (MePEG) in a 40:60 ratio.

The purpose of this work was to develop an intratumoral polymeric paste delivery system to deliver both clusterin ASO and paclitaxel or docetaxel to the tumor site in a controlled manner. Chitosan is an injectable, biocompatible [29, 30] and biodegradable [31] polycation that has been used to enhance the delivery and cellular uptake [32, 33] of plasmid [high molecular weight (MW)] DNA in vitro and in vivo by condensing the plasmid DNA [34–36] and protecting it from degradation by nucleases [37–39]. The effect of various physicochemical properties of chitosan on plasmid transfection efficiency has been investigated [40]. The objectives of this work were to develop and evaluate the in vivo efficacy of an injectable, controlled release delivery system for ASO based on complexed ASO:chitosan dispersed in a biodegradable polymeric paste for intratumoral treatment of solid tumors. The results from intratumoral injection of the formulations into human PC-3 and LNCaP tumors in mice are presented.

Materials and methods

Materials

Clusterin ASO and mismatch oligonucleotide phosphorothioate (MMO) (used as a control oligonucleotide) were obtained from the Nucleic Acid Protein Service Unit at The University of British Columbia (Vancouver, Canada). The sequence of the clusterin ASO was 5′-CAG CAG CAG AGT CTT CAT CAT-3′. Paclitaxel, sodium perchlorate monohydrate, Trizma base and Trizma hydrochloride were obtained from Sigma-Aldrich Co. (St. Louis, Mo.). Docetaxel was obtained from Aventis Pharmaceuticals (Montreal, Canada). Medical grade chitosan was obtained from Carbomer (Westborough, Mass.). MePEG MW 350 $g \cdot mol^{-1}$ was obtained from Union Carbide (Danbury, Ct.). Biodegradable triblock copolymer with the structure poly(DL-lactide-co-caprolactone)-co-poly(ethylene glycol)-co- poly(DL-lactide-co-caprolactone) was prepared as previously described [28]. Sodium chloride and high-performance liquid chromatography (HPLC) grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, N.J.). Water was distilled and deionized using a Milli-RO Water System obtained from Millipore (Bedford, Mass.). Matrigel was obtained from Becton Dickinson Labware (Bedford, Mass.). RPMI-1640 culture medium was obtained from Terry Fox Laboratory (Vancouver, Canada). Fetal bovine serum and Dulbecco's modified Eagle's medium (DMEM) were obtained from Life Technologies (Gaithersburg, Md.).

Preparation of formulations

Formulations of an injectable polymeric paste loaded with clusterin ASO or mismatch oligonucleotide phosphorothioate (MMO) and chitosan complexes (CCs), referred to as CC paste, were prepared with or without paclitaxel/docetaxel loading. The polymer paste was prepared in 1-g batches by blending 600 mg MePEG and 400 mg PLC-PEG-PLC triblock copolymer in a 20-ml glass scintillation vial at 40°C in a water bath.

The clusterin CCs or MMO CCs were prepared by dissolving the appropriate amount of oligonucleotide in 500 µl water and adding the solution to 40 mg chitosan in a 20-ml glass scintillation vial. The chitosan swelled and the complexes were dried overnight at 37°C. The clusterin CCs or MMO CCs and paclitaxel or docetaxel were added in differing amounts to the polymer paste by blending with a spatula for 10 min at 40°C to achieve homogeneous formulations as summarized in Table 1. The preparations were drawn up into 1-ml plastic syringes, capped and stored at 4°C until use.

In vitro release studies

The formulations used for in vitro release studies are summarized in Table 1. Approximately 100 mg clusterin ASO and paclitaxel- or docetaxel-loaded CC paste was placed in the bottom of 15-ml culture tubes and cooled to 4°C to form solid pellets. Phosphate-buffered saline (15 ml) at 37°C was added to the tubes. The tubes were incubated at 37°C and at various time points the supernatant was removed and replaced with fresh phosphate-buffered saline at 37°C to maintain sink conditions. Clusterin ASO concentrations from formulations 1 through 4, paclitaxel concentrations from formulation 2, and docetaxel concentrations from formulation 5 were determined by HPLC analysis of the withdrawn supernatant. After 35 days, the residual clusterin ASO remaining in the carrier paste was released by adding 3 ml of 50 mmol·l^{-1} phosphate buffer at pH 10 to each replicate, followed by vortexing and sonicating. Four replicates were used per group. Data points and error bars represent the mean and standard deviation of each group, respectively.

HPLC of clusterin ASO

Clusterin ASO was assayed at ambient temperature by HPLC using a 600 S controller linked to a 717plus autosampler and a 486 tunable absorbance detector, all from Waters (Milford, Mass.). The injection volume was 20 µl and the detection wavelength was 260 nm. Mobile phase A was prepared by adding 10 ml 1 *M* Tris buffer, pH 8, 5 ml 1 *M* sodium perchlorate, 585 ml filtered water and 400 ml acetonitrile to a 1-l flask. Mobile phase B was prepared by adding 10 ml 1 *M* Tris buffer, pH 8, 300 ml 1 *M* sodium perchlorate, 290 ml filtered water and 400 ml acetonitrile to a 1-l flask. The mobile phase was started with a flow rate of 1 ml·min^{-1} of 100% mobile phase A, and over 10 min using linear gradients was changed to a flow rate of 2 ml·min^{-1} and 100% mobile phase B. Between 11 and 12 min using linear gradients the flow rate was increased to 2.5 ml min^{-1} and the mobile phase to 100% mobile phase A. These conditions were used for the remainder of each run and the total run time was 15 min per sample.

HPLC of paclitaxel and docetaxel

Paclitaxel and docetaxel were assayed by HPLC based on a modification of the procedure used by Liggins and Burt [41]. The modification for paclitaxel concerns the partial conversion of paclitaxel in aqueous media to 7-epi-taxol. Paclitaxel and 7-epi-taxol have similar absorbance values and so the area under the peak of the 7-epi-taxol chromatogram was added to the area of the paclitaxel peak to determine the quantity of paclitaxel being assayed. The modification for docetaxel analysis was the use of 50:50 acetonitrile/water for the mobile phase.

Mice and tumor cell lines

"Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985) were followed or standards equivalent to the UKCCCR Guidelines for the Welfare of Animals in Experimental Neoplasia (Br J Cancer 58:109–113, 1998) were complied with at all times. The health of the animals was observed by monitoring body weight and general appearance (shiny coat, clear nose, gait, etc.). BALB/c strain mice 6–8 weeks old and weighing approximately 25 g were obtained from Charles River Laboratory (Montreal, Canada). PC-3 cells were obtained from the American Type Culture

Table 1 Summary of the constituents in the formulations prepared for in vitro clusterin ASO release and/or in vivo efficacy studies. Formulations 1 through 5 were used in the in vitro release studies. Formulations I through V and I through III were used in the in vivo PC-3 and LNCaP tumor efficacy studies, respectively

Formulation or treatment no.	Constituent (% w/w)					
	Clusterin ASO	Mismatch oligonucleotide	Chitosan	Paclitaxel	Docetaxel	Carrier paste
1	2			1		97
2	3		4	1		92
3 and I	2		4	1		93
4	1		4	1		94
5 and V	2		4		1	93
II		2	4	1		93
III	2		4			94
IV		2	4		1	93

Collection (Rockville, Md.). PC-3 cells were grown in DMEM supplemented with 5% heat-inactivated fetal bovine serum. LNCaP cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum.

Determination of tumor volume and serum PSA levels

Tumor volumes were measured and calculated weekly using the formula length × width × depth × 0.5236. Blood samples were taken weekly from mice with LNCaP tumors via tail vein incision. A kit obtained from Abbott IMX (Montreal, Canada) was used according to the manufacturer's protocol to determine the serum PSA levels. Data were plotted showing the mean and standard deviation for each group.

PC-3 treatment protocols

Clusterin ASO and CCs were loaded into an injectable polymeric paste, CC paste, and formulated with or without paclitaxel/docetaxel loading. Mice were anesthetized with methoxyflurane and injected into the subcutaneous space of the flank region with 1×10^6 PC-3 cells in 100 µl Matrigel (Matrigel was used to assist the growth of the cells in vivo). Mice were randomly assigned into the following treatment groups: (I) clusterin ASO CC paste with paclitaxel, (II) MMO CC paste with paclitaxel, (III) clusterin ASO CC-paste, (IV) MMO CC paste with docetaxel, and (V) clusterin ASO CC paste with docetaxel. Tumors were injected with 100 µl of the appropriate paste after the tumor had reached 1 cm in diameter.

LNCaP treatment protocols

Mice were anesthetized with methoxyflurane and injected into the subcutaneous space of the flank region with 2×10^6 LNCaP cells in 75 µl Matrigel and 75 µl RPMI-1640 and 5% fetal bovine serum. In LNCaP tumors, serum PSA levels correlate with tumor progression [22]. When the serum PSA levels increased above 10 $ng{\cdot}ml^{-1}$ and the tumor volume reached 200–300 mm^3, the mice were anesthetized with methoxyflurane and castrated. Mice were randomly assigned into treatment groups 1–3 as per the PC-3 treatment protocols. After the serum PSA levels had decreased, tumors were injected with 100 µl of the appropriate paste after the serum PSA levels had increased above 60 $ng{\cdot}ml^{-1}$ and tumor volumes had reached 150–650 mm^3. Mean tumor volumes and serum PSA levels were similar at the beginning of treatment in each group.

Statistical analysis

Statistical analysis was completed using Microsoft Excel software. The in vivo effects of clusterin ASO and paclitaxel or docetaxel were analyzed using analysis of variance (ANOVA).

Results

Release studies

The pastes set to a semisolid mass in less than an hour following injection into aqueous medium. Figure 1 shows the in vitro release profile for paclitaxel and docetaxel. Paclitaxel released in a controlled, approximately linear manner from the paste. After 5 weeks, a total of 6% of the loaded paclitaxel had been released. Docetaxel rapidly released in a short burst phase during the first day followed by a slower release phase over the following 22 days. After 23 days, a total of 40% of the loaded docetaxel had been released. Figure 2 shows the in vitro clusterin ASO release profiles for formulations 1 through 4 (formulation constituents are summarized in Table 1). The formulation without chitosan, formulation 1, rapidly released the clusterin ASO in a short burst phase in the first 3 days followed by a slow phase of release over the following 31 days. The formulations containing ASO:chitosan ratios of 3:4 and 2:4 (formulations 2 and 3) released the clusterin ASO in a biphasic pattern. Over the first 11 days there was a faster release of ASO, followed by a slower phase of release over the following 23 days. There was little release of ASO for the formulation containing an ASO:chitosan ratio of 1:4 (formulation 4) and in 34 days only 1% of the loaded ASO was released. Hence, the clusterin ASO was released more slowly from the preparations as the ratio of clusterin ASO to chitosan was decreased. Analysis of residual clusterin ASO at 34 days showed that the formulation without chitosan contained no remaining oligonucleotide, while the preparations with chitosan contained intact oligonucleotide. In mass balance determinations, when the amount of clusterin ASO released by 34 days was added to the amount of residual clusterin ASO for the formulations with chitosan, the amount totaled approximately 100% of the loaded clusterin ASO, whereas the total for the formulation without chitosan was only 70% of the loaded clusterin ASO.

Effect of intratumoral injection of paste on PC-3 tumor growth in vivo

The pastes were easily injected through a 20-gauge needle, and could be injected through a 22-gauge needle less readily, into PC-3 and LNCaP tumors. The pastes set to a waxy solid implant in approximately 1 h at the injection site following injection into a tumor and left a palpable semisolid implant in the tumor for approximately 3–4 weeks. As shown in Figs. 3 and 4, groups treated with clusterin ASO alone (treatment group III) had a rapid growth of tumors such that by 4 weeks the mean tumor volume was approximately 3000 mm^3.

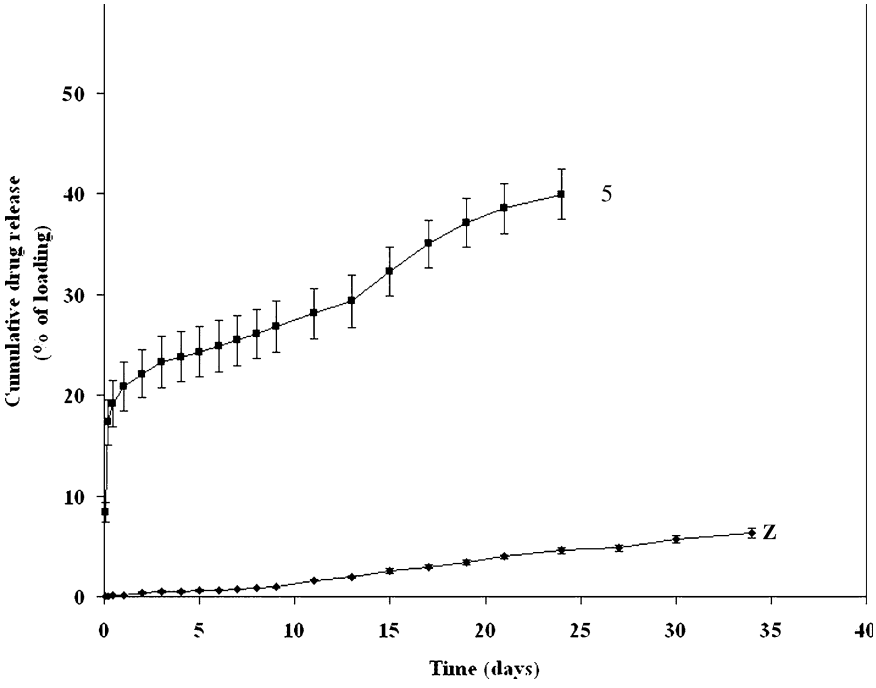

Fig. 1 In vitro release profile of paclitaxel and docetaxel from clusterin ASO CC paste. Formulation constituents are summarized in Table 1. Formulations 2 and 5 each contained 3% and 2% w/w clusterin ASO, respectively, and 4% w/w chitosan. *Filled diamonds* 1% w/w paclitaxel (formulation 2), *filled squares* 1% w/w docetaxel (formulation 5). Data points and error bars represent means ± SD ($n = 4$)

Groups treated with MMO/paclitaxel and MMO/docetaxel (treatment groups II and IV, respectively) at 4 weeks had mean tumor volumes of approximately 1500 and 500 mm^2, respectively. Intratumoral formulations containing clusterin ASO/paclitaxel and clusterin ASO/docetaxel (treatment groups I and V, respectively) significantly reduced mean tumor volume by more than 50% 4 weeks after treatment, compared to

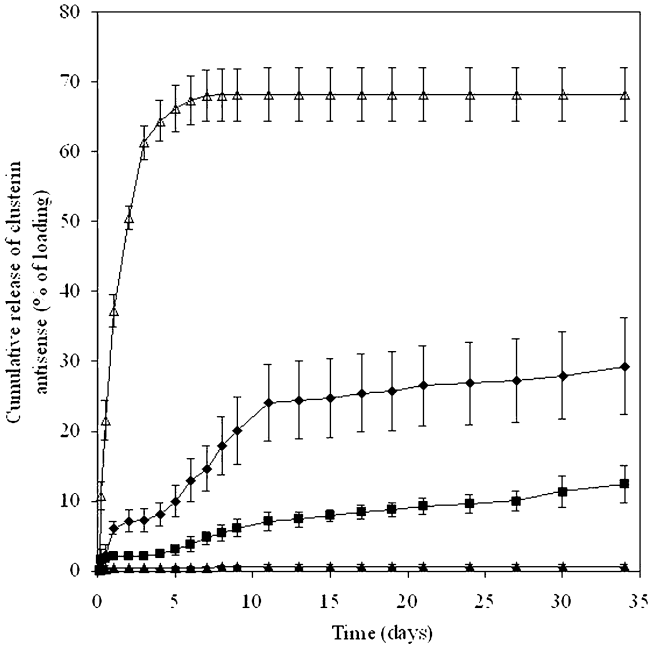

Fig. 2 Effect of clusterin ASO to chitosan ratio on in vitro clusterin ASO release from paste loaded with clusterin ASO and paclitaxel. Formulation constituents are summarized in Table 1. Formulations 1–4 all contained 1% w/w paclitaxel. *Open triangles* ASO alone (no chitosan) (formulation 1), *filled diamonds* ASO:chitosan 3:4 (formulation 2), *filled squares* ASO:chitosan 2:4 (formulation 3), *filled triangles* ASO:chitosan 1:4 (formulation 4). Data points and error bars represent means ± SD ($n = 4$)

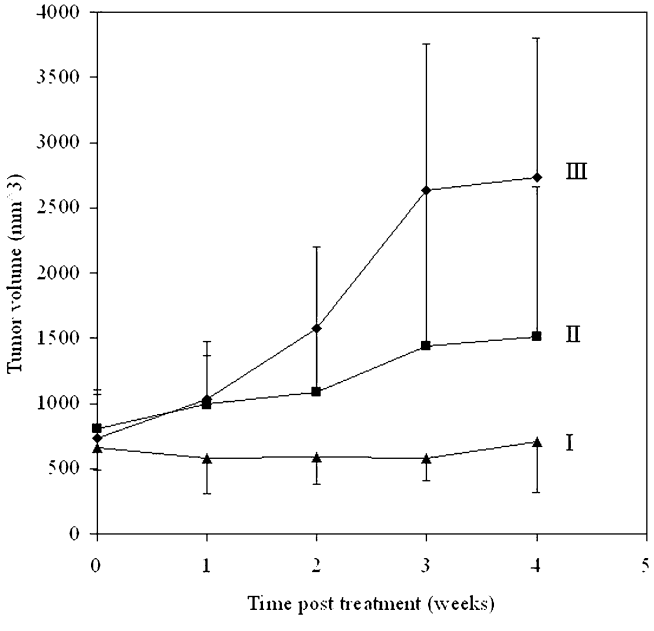

Fig. 3 Effect of CC paste loaded with clusterin ASO and paclitaxel on tumor volume in mice with PC-3 tumors following intratumoral administration. Treatment protocols are summarized in Table 1. *Filled diamonds* 2% w/w clusterin ASO CC paste alone (no paclitaxel) (treatment III), *filled squares* 2% w/w MMO CC paste with 1% w/w paclitaxel (treatment II), *filled triangles* 2% w/w clusterin ASO CC paste with 1% w/w paclitaxel (treatment I). Data points and error bars represent the means ± SD. Results are shown for a minimum of four mice (if fewer than four mice remained in any group, then the data are not shown)

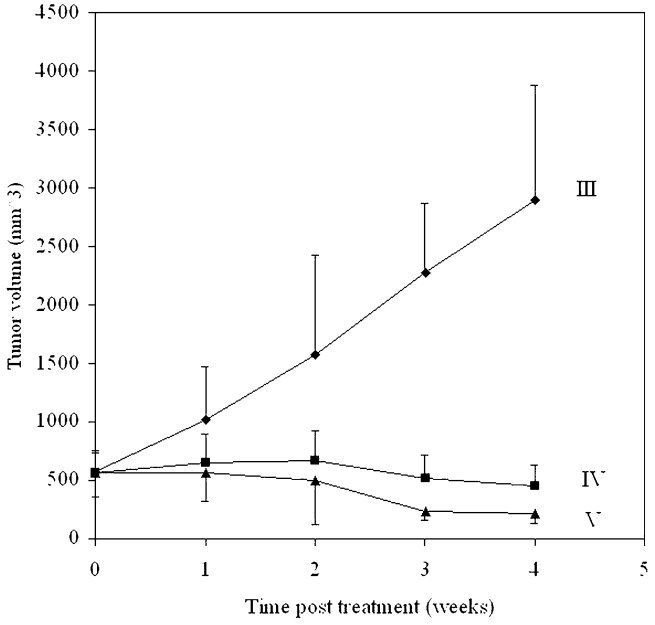

Fig. 4 Effect of CC paste loaded with clusterin ASO and docetaxel on tumor volume in mice with PC-3 tumors following intratumoral administration. Treatment protocols are summarized in Table 1. *Filled diamonds* 2% w/w clusterin ASO CC paste alone (no docetaxel) (treatment III), *filled squares* 2% w/w MMO CC paste with 1% w/w docetaxel (treatment IV), *filled triangles* 2% w/w clusterin ASO CC paste with 1% w/w docetaxel (treatment V). Data points and error bars represent the means ± SD. Results are shown for a minimum of four mice (if fewer than four mice remained in any group, then the data are not shown)

MMO/paclitaxel and MMO/docetaxel, respectively ($P = 0.06$ for both comparisons).

Effect of intratumoral injection of paste on LNCaP tumor growth and PSA levels in vivo

As shown in Figs. 5 and 6, the group treated with clusterin ASO alone (treatment group III) showed rapid tumor growth such that by 4 weeks the mean tumor volume and serum PSA level were approximately 1370 mm^3 and 500 ng ml^{-1}, respectively. The group treated with MMO/paclitaxel (treatment group II) at 4 weeks had a mean tumor volume and serum PSA level of approximately 1370 mm^3 and 500 $ng{\cdot}ml^{-1}$, respectively. Intratumoral formulations containing both clusterin ASO/paclitaxel (treatment group I) significantly reduced mean tumor volume ($P < 0.001$) and serum PSA level ($P = 0.08$) by more than 50% and 70%, respectively, 4 weeks after treatment compared to MMO/paclitaxel.

Discussion

The polymer formulations used in this work were pastes at room temperature that could be injected through a 22-gauge needle. Following injection into aqueous

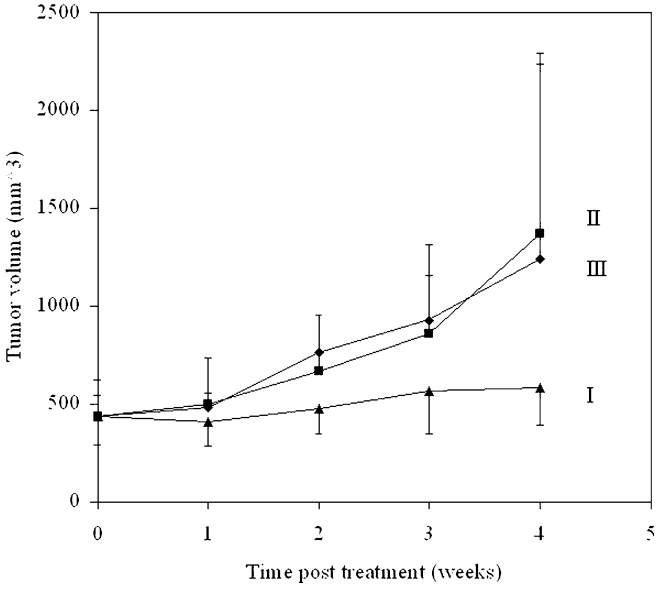

Fig. 5 Effect of CC paste loaded with clusterin ASO and paclitaxel on tumor volume in mice with LNCaP tumors following intratumoral administration. Treatment protocols are summarized in Table 1. *Filled diamonds* 2% w/w clusterin ASO CC paste alone (no paclitaxel) (treatment III), *filled squares* 2% w/w MMO CC paste with 1% w/w paclitaxel (treatment II), *filled triangles* 2% w/w clusterin ASO CC paste with 1% w/w paclitaxel (treatment I). Data points and error bars represent the means ± SD. Results are shown for a minimum of four mice (if fewer than four mice remained in any group, then the data are not shown)

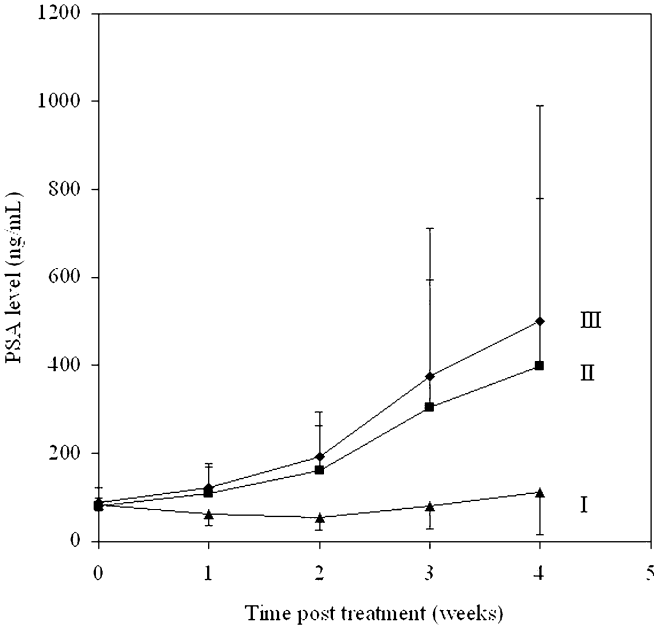

Fig. 6 Effect of CC paste loaded with clusterin ASO and paclitaxel on serum PSA levels in mice with LNCaP tumors following intratumoral administration. Treatment protocols are summarized in Table 1. *Filled diamonds* 2% w/w clusterin ASO CC paste alone (no paclitaxel) (treatment III), *filled squares* 2% w/w MMO CC paste with 1% w/w paclitaxel (treatment II), *filled triangles* 2% w/w clusterin ASO CC paste with 1% w/w paclitaxel (treatment I). Data points and error bars represent the means ± SD. Results are shown for a minimum of four mice (if fewer than four mice remained in any group, then the data are not shown)

medium in vitro the pastes formed semisolid implants in several minutes. Implants loaded with 1% w/w paclitaxel or docetaxel released the cytotoxic agent in vitro in a controlled manner. In previous studies, a similar paste formulation loaded with 10% w/w paclitaxel but without chitosan produced a slow release of paclitaxel over 35 days, such that approximately 40% of the loaded dose was released [27, 28]. In another study, a paste based on polycaprolactone or a polycaprolactone/MePEG blend loaded with 1–30% w/w paclitaxel showed very slow release of paclitaxel over 20 days, such that approximately 5–20% of the loaded dose was released [42]. It is anticipated that the remaining paclitaxel or docetaxel will be released as the biodegradable polymer undergoes degradation.

The paste formulations in this work were composed of a 60/40 MePEG/PLC-PEG-PLC blend. It has been shown that following injection of the paste into aqueous medium or into a tumor, the hydrophilic MePEG rapidly partitions and diffuses out from the paste into the surrounding aqueous compartment or tissue fluids. The remaining hydrophobic PLC-PEG-PLC polymer and paclitaxel or docetaxel then solidifies [27] due to an increased melting temperature and precipitation of drug [28].

Clusterin ASO was rapidly released from MePEG/PLC-PEG-PLC and paclitaxel paste (no chitosan) into an aqueous release buffer. The hydrophilic oligonucleotide probably released from the paste via rapid dissolution and diffusion into the surrounding aqueous compartment. However, formulations of clusterin ASO and CCs loaded into injectable polymeric paste (CC paste) with paclitaxel, slowly released the ASO. During the preparation of the clusterin ASO CC pastes, clusterin ASO CC particles were incorporated into the MePEG/PLC-PEG-PLC and paclitaxel paste. We believe that when the pastes solidified in the aqueous medium the solid clusterin ASO CC particles remained within the semisolid implant. The polyanionic oligonucleotide forms electrostatic complexes [43] with the polycationic chitosan, somewhat similar to precipitation complexes formed between chitosan and plasmid DNA [34, 35, 37], and the same as complexes reported by others between chitosan and oligonucleotides [44]. Generally a minimum of only six to ten salt bonds are required to form a cooperative system [43], so it is likely that the chitosan amine groups formed a coordinated system of salt bonds with the ASO thiophosphate groups [45] resulting in strong complexes [46]. However, as the ratio of ASO to chitosan is increased, the number of positively charged amine sites available to form electrostatic bonds with negatively charged thiophosphate groups would be decreased. This would leave a portion of the ASO molecules forming too few salt bonds with the chitosan to form a coordinated complex. Indeed, we observed that as the ratio of ASO to chitosan was increased from 1:4 to 3:4 in drug release studies, the amount of ASO released during the burst phase increased. In addition, it is likely that the coordinated complexes can dissociate into free ASO and ''free'' chitosan and these species are in equilibrium. Some of the free ASO would be released from the paste via diffusion and re-equilibration would lead to the formation of more free ASO, resulting in the controlled release of ASO that we observed from our formulations over 5 weeks. Mass balance determinations showed that only 70% of the loaded clusterin ASO was accounted for from the formulation without chitosan, whereas approximately 100% of the loaded clusterin ASO was accounted for from the formulations with chitosan. These results suggest that chitosan protected the ASO from degradation as has been observed in studies using chitosan and plasmid DNA [37].

It is expected that chitosan's amine groups bind strongly to polyanionic oligonucleotides in the acidic extracellular environment of a tumor, which is believed to be between pH 6.7 and 7.0 [47–49]. The pKa of chitosan has been reported to increase with the degree of deacetylation and varies between 6.4 and 7.1 [50]. Thus, the degree of ionization of chitosan in the acidic extracellular tumor fluids may be as high as approximately 70%.

Targeting the oligonucleotide/CCs into the tumor via direct injection is an advantage compared to other routes of administration, since this route bypasses the healthy tissue where the basic extracellular environment may otherwise provide for the release of the oligonucleotide from the complex before reaching the tumor site. Indeed, the uptake and expression of plasmid DNA in vitro has been shown to be dependent on the pH of the cell medium, with the greatest uptake and expression resulting from a medium at pH 7.0 [35].

In an effort to decrease systemic drug levels and corresponding systemic side effects, paclitaxel was incorporated into injectable sustained release formulations consisting of biodegradable polymeric microspheres [26] or paste [27] for local delivery. Clusterin has been shown to be an antiapoptotic gene that confers resistance to androgen ablation, increases the rate of progression to androgen independence [16] and provides resistance to cytotoxic agents in PC-3 [21] and LNCaP [22] prostate cancer cells. Miyake et al. used cultured PC-3 cells and a PC-3 tumor model in mice to show that clusterin ASO reduces the expression of clusterin mRNA and clusterin protein, but fails to reduce PC-3 growth [21]. However, the combination of systemic clusterin ASO with paclitaxel enhances apoptosis and reduces PC-3 [17, 21] and LNCaP [22] cell growth and tumor volume. Hence, our approach in this work was the targeting of clusterin ASO and paclitaxel or docetaxel directly into tumors by first incorporating the ASO and cytotoxic agents into an injectable paste. Following injection into tumors in vivo the pastes formed semisolid implants in less than an hour. Intratumoral injection of clusterin ASO CC paste with paclitaxel or docetaxel into PC-3 tumors grown in mice showed a synergistic effect on tumor volume compared to paste with clusterin ASO CCs, paclitaxel, or docetaxel alone. Similarly, intratumoral injection of clusterin ASO CC paste with paclitaxel into LNCaP tumors grown in mice resulted in a

synergistic effect on tumor volume and serum PSA levels compared to clusterin ASO CCs or paclitaxel alone.

In this work, efficacy was achieved for 4 weeks against PC-3 and LNCaP tumors grown in mice following a single, low-dose intratumoral injection of biodegradable triblock polymeric paste loaded with 2 mg clusterin ASO, complexed with chitosan particles and combined with 1 mg paclitaxel or docetaxel. This new formulation required only one injection over 4 weeks and used less than one-tenth the total clusterin ASO dose and less than one-fifth the total paclitaxel dose while attaining similar efficacy compared to the previous work of Miyake et al. using intraperitoneal injections of clusterin ASO solution and multiple intravenous injections of micellar paclitaxel over approximately 1 month against PC-3 [21] and LNCaP [22] tumors in mice.

Paclitaxel has been targeted to tumors using several different formulations. Following intratumoral administration in LNCaP tumors grown in mice, Lapidus et al. [26] saw a decrease in tumor volume and serum PSA levels with 40% w/w paclitaxel-loaded microspheres dosed at 240 mg of microspheres per kilogram of body weight [26] (estimated at 2.4 mg paclitaxel for a 25-g mouse) and our group observed similar efficacy with a 10% w/w paclitaxel-loaded triblock copolymer paste [27] equivalent to 10 mg paclitaxel per 25-g mouse. However, intratumoral administration of the 40% w/w paclitaxel-loaded microspheres resulted in detectable levels of paclitaxel in the serum [26], while intratumoral administration of the 10% w/w paclitaxel-loaded paste produced areas of redness close to the injection site and evidence of inhibition of wound healing [27]. After incorporating clusterin ASO into our triblock copolymer paste and using one-tenth the dose of paclitaxel in 25-g mice as used in our previous studies, we observed similar efficacy and no toxicity.

The complexation of clusterin ASO with chitosan and incorporation into polymeric paste with paclitaxel or docetaxel produced in vitro controlled release of the ASO and in vivo efficacy over 4 weeks following a single intratumoral injection in solid human prostate tumors in mice. This study provides proof of principle data for an intratumorally administered sustained release formulation of clusterin ASO CCs and paclitaxel or docetaxel as a treatment for solid prostate tumors.

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