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Cationic Liposomes Loaded with Proapoptotic Peptide D-(KLAKLAK)$_2$ and Bcl-2 Antisense Oligodeoxynucleotide G3139 for Enhanced Anticancer Therapy

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Abstract: The treatment of cancer using macromolecular therapeutics such as oligonucleotides or peptides requires efficient delivery systems capable of intracellular penetration and may also benefit from use of a combination of therapeutics with different mechanisms of action. With this possibility in mind, we constructed cationic liposome loaded with the proapoptotic peptide, D-(KLAKLAK)$_2$ and the Bcl-2 antisense oligodeoxynucleotide, G3139, and determined whether the combination of the proapoptotic macromolecules in a single cationic liposome can enhance antitumor efficacy. Advantage was taken of alternating charge interaction to entrap macromolecules of opposite charge. The polycationic peptide D-(KLAKLAK)$_2$ was first condensed with the polyanionic oligodeoxynucleotide G3139 to obtain overall negatively charged peptide/oligodeoxynucleotide complexes. The complexes were then entrapped into DOTAP/DOPE cationic liposomes (CL). This sequential charge interaction ensured efficient entrapment of D-(KLAKLAK)$_2$ and G3139 with a high loading efficiency (50%) and capacity (7.5 wt %). *In vitro* treatment of mouse melanoma B16(F10) with CL loaded with D-(KLAKLAK)$_2$/G3139 led to significantly enhanced antitumor efficacy, mediated by stimulated induction of apoptotic (caspase 3/7) activity, when compared to CL loaded with G3139 alone. Intratumoral injection of CL loaded with D-(KLAKLAK)$_2$/G3139 in B16(F10) mice xenograft also led to suppressed tumor growth associated with enhanced apoptotic activity. Thus, the combination of proapoptotic peptide D-(KLAKLAK)$_2$ and antisense oligonucleotide G3139 in a cationic liposome led to enhanced apoptotic/antitumor efficacy and may provide a promising tool for cancer treatment.

Keywords: Cancer gene therapy; cationic liposome (CL); proapoptotic peptide; D-(KLAKLAK)$_2$; proapoptotic Bcl-2 oligodeoxynucleotide; G3139

Introduction

Mitochondria have been prime targets for therapeutic interventions of cell death by apoptosis. The induction of apoptosis can occur either by promotion of mitochondrial outer membrane permeability (MOMP) engaging proapoptotic members of the Bcl-2 family or by the induction of a mitochondrial permeability transition.[1,2] Thus, two separate strategies have been proposed that target mitochondria for induction of apoptosis and antitumor therapy:[3] selective targeting of antiapoptotic proteins with therapeutic agents, such as peptides, genetic materials, and small molecules, or

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disrupting of mitochondrial membranes more directly using the agents with high membrane disrupting capacity.

The 14-amino-acid amphipathic α-helical peptide D-$(KLAKLAK)_2$ has been shown to induce apoptosis in cancer cells.[4] This peptide does not disrupt the zwitterionic plasma membranes of eukaryotic cells but disrupts anionic prokaryotic cytoplasmic membranes and the eukaryotic mitochondrial membranes. When internalized, D-$(KLAKLAK)_2$ can disrupt the negatively charged mitochondrial membrane, resulting in cell death by mitochondrial-dependent apoptosis.[5] The use of this peptide for enhancing the apoptotic activity should involve targeting and internalization strategies. Chemical conjugates of the D-$(KLAKLAK)_2$ with RGD peptide,[5] protein transduction domain,[6] and antibody[7] have been used for intracellular and extracellular targeting.

G3139 (Genasense, Genta Inc., NJ) is an 18-mer phosphorothioate antisense oligodeoxynecleotide (ODN) that selectively targets the initiation codon region of the Bcl-2 mRNA for degradation by RNase H and thereby decreases Bcl-2 protein production. Bcl-2 protein plays a major role in preventing apoptosis[8] and overexpression of the protein was confirmed to be related to the tolerance of various tumors toward chemotherapy.[9] The treatment of cancer cells with G3139 down-regulated Bcl-2 protein and increased the susceptibility toward the apoptosis, enhancing thus the tumor response to combined chemotherapy.[10] This approach is now being evaluated in several clinical trials.[11,12] Despite the high efficacy, the use of G3139 has to overcome several extracellular and intracellular barriers inherent to gene therapeutics, and an efficient delivery system should assist in solving these problems.

The treatment of cancer using the macromolecular therapeutics, such as oligonucleotides or peptides, requires efficient delivery systems capable of intracellular penetration and may also benefit from use of a combination of therapeutics with different mechanisms of action. With this possibility in mind, we have prepared a cationic liposome-based formulation coloaded with D-$(KLAKLAK)_2$ and G3139 with the aim to enhance the apoptotic antitumor efficacy (i) via the combination of two apoptotic agents with different mechanisms of apoptosis induction, and (ii) by the enhanced intracellular delivery endowed by the cationic nature of the liposomal carrier. The formulation was constructed by precondensation of the cationic peptide with the anionic ODN and subsequent entrapment of these preformed overall negatively charged complexes in CL by lipid membrane rehydration/extrusion techniques. The CL loaded with the D-$(KLAKLAK)_2$ and G3139 were evaluated for their antitumor efficacy following the cytotoxicity and proapoptotic activity toward the mouse melanoma B16(F10) cells.

Materials and Methods

Materials. Phosphorothioate Bcl-2 antisense oligodeoxynucleotides (ODN) G3139 and control ODN G3622 were purchased from Calbiochem (San Diego, CA). Proapoptotic peptide D-$(KLAKLAK)_2$ was purchased from Tufts Core Facility (Boston, MA). Fluorescently labeled 5-FAM-D-$(KLAKLAK)_2$ was purchased from AnaSpec, Inc. (San Jose, CA). l,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and dioleoyl-1,2-diacyl-3-trimethylammonium-propane (DOTAP) were purchased from Avanti Polar Lipids (Alabaster, AL). All other chemicals were of the reagent grade.

Preparation of Cationic Liposomes Loaded with D-$(KLAKLAK)_2$ and G3139. D-$(KLAKLAK)_2$ peptide and G3139 ODN were separately diluted in HEPES-buffered 5% glucose solution (pH 7.4) to a final volume of 250 μL. After 10 min incubation at room temperature, the solutions were rapidly mixed and vortexed immediately, resulting in 500 μL of peptide/ODN complexes at a +/− charge ratio of 1:2. The peptide/ODN complex solution was then added to a cationic lipid film of DOPE and DOTAP (1:1 mol/mol) at a +/−/+ charge ratio of 1:2:6 and incubated for 4 h, followed by 11 times extrusion through a stack of two polycarbonate membranes with a 200 nm pore size using a hand-held extruder (Avanti Polar Lipid, AL). The amounts of peptide and ODN and DOTAP were calculated to obtain the desired charge ratio by assuming that each peptide molecule contains six positive charges from lysine groups, that each ODN molecule contained 18 negative charges from phosphate groups, and that each DOTAP molecule contained one positive charge from an amine group. A formulation loaded with G3139 ODN alone was prepared as a control for *in vitro* and *in vivo* evaluation of antitumor efficacy. The

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attempt to prepare a similar formulation loaded with only peptide was unsuccessful, indication of the strict requirement for the peptide/ODN complex formation for the efficient loading.

The resulting formulations were characterized with respect to complex formation, size distribution, zeta-potential, and loading efficiency. Complex formation was confirmed by the agarose gel electrophoresis. Electrophoresis was performed using the E-Gel electrophoresis system (Invitrogen, Carlsbad, CA). A precast 0.8% E-Gel cartridge was prerun for 2 min at 60 V and 500 mA followed by the loading of 2 μg of ODN. The size distribution and zeta-potential were determined by quasi-elastic light scattering technique using a Zeta Plus analyzer (Brookhaven Instruments, Brookhaven, NY).

The peptide loading into CL was analyzed by size exclusion chromatography (SEC) using the fluorescently labeled peptide as a tracer. We attempted to prepare the formulations with 5-FAM-D-(KLAKLAK)$_2$, and the fractions of the Sepharose CL4B column eluent were checked for the fluorescence intensity using a fluorescence plate reader (Synergy HT multimode microplate reader, BioTek Instrument, USA). The entrapment efficiency of the peptide was calculated as the percentage of total area found in the first peak. A formulation without ODN was used as a control to demonstrate the effect of complexation of peptide by ODN on the encapsulation efficiency.

Cytotoxicity Assay. The mouse melanoma cell line B16(F10) was grown in DMEM supplemented with 10% fetal bovine serum (FBS) in 96-well plates to ~50% confluency. The cells were treated by replacement of the media with serum-free media (100 μL) containing a serial dilution of each formulation up to 1 μM of ODN (corresponding to 1.5 μM of D-(KLAKLAK)$_2$ peptide and 50 μM of DOTAP). After 24 h incubation, the cells were washed twice with PBS and returned to fresh complete media. Then, 20 μL of CellTiter-Blue cell viability assay reagent (Promega, Madison, WI) was added to each well, and the plates were reincubated for 2 h. The fluorescence at 490 nm was measured for each well using a 96-well fluorescence plate reader (Synergy HT multimode microplate reader, BioTek Instrument). Relative cell viability was calculated with cells treated with medium only as a control. The assay was carried out in triplicate.

Caspase 3/7 Activity. The B16(F10) cells were grown and treated as above for 4 h. After washing the cells and adding fresh media, 20 μL of Apo-ONE caspase 3/7 reagent (Promega, Madison, WI) was added to each well and the plates were reincubated for 2 h. The fluorescence at 490 nm was measured for each well using a 96-well plate reader (Synergy HT multimode microplate reader, BioTek Instrument). Relative caspase 3/7 activity was calculated by comparing the results from the cells treated with medium only.

Antitumor Activity in Tumor-Bearing Mice. Male C57BL/6 mice (Charles River Laboratories) were inoculated subcutaneously in the left flank with 1×10^6 B16(F10) melanoma tumor cells 14 days before the treatment according to a protocol approved by the Institutional Animal Care and Use Committee at Northeastern University. When tumors reached a volume of approximately 200 mm^3 ([length $\times$ width^2]/2), a dose of 20 μL of CL formulation loaded with peptide/G3139 or G3139 alone was administered in mice twice daily by intratumoral injection (each dose corresponds to 4 μg of G3139 and 1.52 μg of D-(KLAKLAK)$_2$ peptide). Saline-injected mice with similar-sized tumors were used as controls. Five days after the second injection when the control group's mean tumor volume reached approximately 1000 mm^3, the mice were sacrificed by CO_2, and tumors were excised. Percent tumor growth inhibition (% TGI) was calculated by comparing the treatment group's tumor volume (T) to the control group's tumor volume (C) at that time ([1 $-$ (T/C) $\times$ 100]).

TUNEL Assay. Tumor-bearing mice were administered a single dose of 60 μL of the CL formulation loaded with peptide/G3139 when tumor volume reached approximately 600 mm^3 by the intratumoral injection. Nontreated mice with similar-sized tumors were used as controls. After 24 h, the mice were sacrificed by CO_2, excised tumors were immediately frozen in Tissue-Tek OCT 4583 compound (Sakura Finetek, CA) without fixation, and 8 μm thick sections were prepared with a cryostat. A terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed with a TdT-FragEL DNA fragmentation detection kit (Calbiochem, NJ), and the sections were examined by fluorescence microscopy (Olympus BX51).

Results

Cationic Liposomes Loaded with D-(KLAKLAK)$_2$ Peptide and G3139 ODN. Alternating charge interaction was employed to load the polycationic peptide in CL. The polycationic D-(KLAKLAK)$_2$ peptide was first condensed with the polyanionic ODN to obtain overall negatively charged peptide/oligodeoxynucleotide complexes, and then the complexes were entrapped into CL made of DOTAP/DOPE. The sequential charge interaction ensured efficient entrapment of D-(KLAKLAK)$_2$ and G3139 with high loading efficiency (50%) and capacity (7.5 wt %).

The 18-mer single stranded phosphorothioate ODN was first complexed with cationic peptide D-(KLAKLAK)$_2$ and then entrapped into cationic liposome by hydrating the cationic lipid film with the aqueous buffer containing the negatively charged peptide/ODN complexes followed by the membrane extrusion. The charge ratio of (+) in the peptide to (−) in ODN to (+) in DOTAP was 1:2:6, i.e., positive charges were in excess.

The complexation of the polycationic peptide with polyanionic ODN led to nanoscale complexes with a narrow size distribution and negative zeta potential (235.7 $\pm$ 20.6 nm, -28.9 ± 3.5 mV, $n = 3$). The suspension obtained after the hydration of the cationic lipid film showed a multimodal size distribution with a mean diameter greater than 1300 nm and positive zeta potential (1436 $\pm$ 183.4 nm, 58.1 $\pm$ 3.8 mV,

Table 1. Size Distribution and Zeta Potential of Peptide/ODN Complexes and Cationic Liposomes Loaded with Peptide/ODN Complexes[a]

	size distribution (nm)	zeta potential (mV)
peptide/ODN complex	235.7 ± 20.6	−28.9 ± 3.5
CL(peptide/ODN)	194.7 ± 2.6	43.15 ± 3.6

[a] CL(peptide/ODN) were determined by QELS. Data represent mean ± SEM ($n \geq 3$).

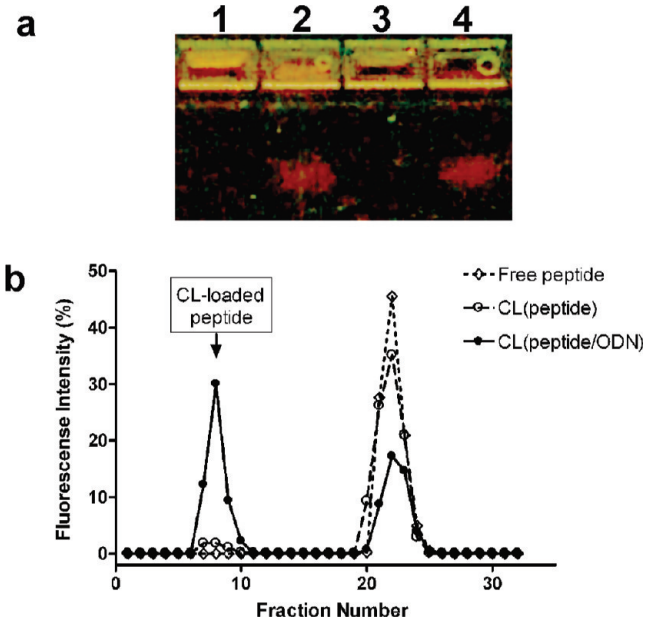

Figure 1. Preparation of cationic liposome (CL) loaded with D-(KLAKLAK)$_2$ peptide and G3139 ODN. (a) Agarose gel electrophoresis confirmed complex formation between peptide and ODN and subsequent entrapment of the complex with cationic liposome. Lane 1: naked peptide/ODN complexes, nontreated. Lane 2: naked peptide/ODN complexes, treated with heparin. Lane 3: CL loaded with peptide/ODN complexes, nontreated. Lane 4: CL loaded with peptide/ODN complexes, treated with heparin and Triton X-100. (b) Size exclusion chromatography (SEC) using FAM-D-(KLAKLAK)$_2$ tracer revealed that about 54% of peptide was loaded in CL. Free peptide was separated from CL-loaded peptide on a Sepharose CL4B column. Entrapped D-(KLAKLAK)$_2$ eluted with CL at void volume. Without precomplexation, the loading of peptide into CL was insignificant.

$n = 3$). After the extrusion, the suspension was formed with a narrow and unimodal size distribution (mean diameter of 194.7 ± 2.6 nm). Zeta potential measurement revealed that the negative charge of the peptide/ODN complexes (−28.9 ± 3.5 mV) was completely shielded by the cationic lipid membrane, resulting in positively charged particles (43.2 ± 3.6 mV) (Table 1). The liposome formulations remained colloidally stable in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) for at least 3 weeks.

The complex formation between the peptide and ODN was confirmed by electrophoresis on a 0.8% agarose gel (Figure 1a). When complexed with the peptides, ODN could not enter the gel and is retained in wells because of the size restriction. All ODN was retained in wells without the migration of free ODN (lane 1), indicating complete complexation. The ODN in the complex was released by the treatment with heparin (lane 2). The ODN in the CL formulation also cannot enter the gel, and ODN in the CL was also retained in wells (lane 3). The ODN was released from CL after treatment with Triton X-100 followed by heparin and could enter the gel after that (lane 4).

Size exclusion chromatography showed that about 54% of FAM-D-(KLAKLAK)$_2$ peptide was entrapped into the CL (eluted in the void volume), but only when it was first complexed with the ODN (Figure 1b). Peptide alone cannot be entrapped into the CL (compare respective curves). Thus, the precomplexation of the peptide by ODN is an absolute request for the preparation of peptide/ODN-coloaded CL.

***In Vitro* Antitumor Activity.** The *in vitro* cytotoxicity of CL loaded with D-(KLAKLAK)$_2$/G3139 complexes was assessed using the B16(F10) melanoma cells. The cell line and drug concentration ranges were chosen based on previous results.[13−16] The formulations loaded either with G3139 alone or with the complex of the peptide with a reverse sequence ODN G3622 were used as controls. About 20% reduction in cell viability was observed with the liposome formulation loaded with both D-(KLAKLAK)$_2$ peptide and G3139 ODN at 125 nM ODN concentration, while no cytotoxicity was observed with control formulations. Only ODN concentration in control preparation as high as 250 nM resulted in ca. 10% decrease in cell viability, while at the same ODN concentration the CL preparation loaded with both D-(KLAKLAK)$_2$ and G3139 killed about 40% of all cells. At 1 μM ODN concentration, however, the control formulations led to cytotoxicity comparable to the formulation with D-(KLAKLAK)$_2$ and G3139 indicating non-antisense mediated cell death (Figure 2).

***In Vitro* Apoptosis Assay (Caspase 3/7 Activity).** The CL loaded with D-(KLAKLAK)$_2$/G3139 complexes were also assessed for the induction of apoptosis in B16(F10) melanoma cells. Caspase 3/7 activity was determined after 4 h treatment with different formulations. About a 40−50% increase in caspase 3/7 activity was observed over 15−500

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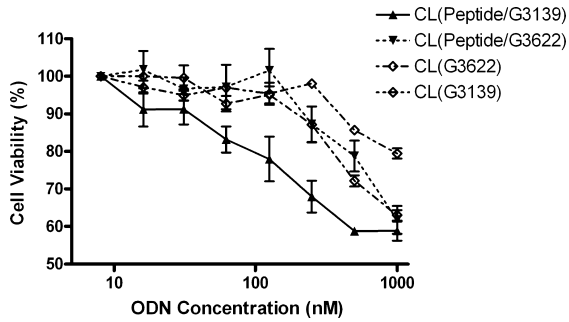

Figure 2. *In vitro* antitumor activity of CL loaded with D-(KLAKLAK)$_2$/G3139 toward B16(F10) melanoma cells. The cells were treated for 24 h before detection of redox enzymatic activity. CL loaded with G3139 only, complexes of the D-(KLAKLAK)$_2$ peptide with a reverse sequence ODN G3622, and G3622 only were used as controls. Relative cell viability was expressed as a percentage of control cells treated with the medium. Reduced cell viability was observed in the cells treated with CL loaded with the D-(KLAKLAK)$_2$ peptide and G3139 ODN at a relatively low ODN concentration, demonstrating the advantage of the combined apoptotic therapeutic.

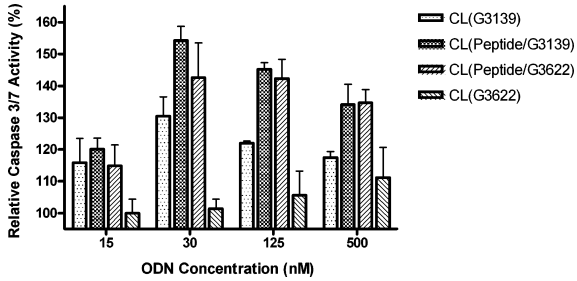

Figure 3. Caspase 3/7 activity in B16(F10) melanoma cells treated with CL loaded with D-(KLAKLAK)$_2$/G3139. Cells were treated for 4 h followed by 24 h incubation before detection of the enzymatic activity. CL loaded with G3139 only, complexes of D-(KLAKLAK)$_2$ peptide with a control ODN G3622, and G3622 only served as controls. Significantly increased caspase 3/7 activity was observed in the cells treated with CL loaded with D-(KLAKLAK)$_2$ and G3139, demonstrating the advantages of combining the apoptotic therapeutics for enhanced antitumor efficacy through increased apoptotic activity.

nM ODN concentration range in cells treated with formulations loaded with both, D-(KLAKLAK)$_2$ and ODN. At the same range of ODN concentration, only about 15−25% increase in caspase 3/7 activity was observed in the cells treated with formulations loaded with antisense ODN G3139 alone. The formulation loaded only with control ODN G3622 alone led to 10−15% increase only at high ODN concentration (125 and 500 nM) (Figure 3). Somewhat apparently reduced activity of CL coloaded with the peptide and G3139 after the maximum reached at 30 nM concentration could be explained by the fact that at the same time points we actually observe different stages of apoptosis for different concentrations of the drug: the caspase 3/7 activity shows maximum at the early stage of apoptosis and higher concentrations of the drug provide the maximal apoptosis at earlier times, so we "miss" the highest caspase activity at the selected time point.

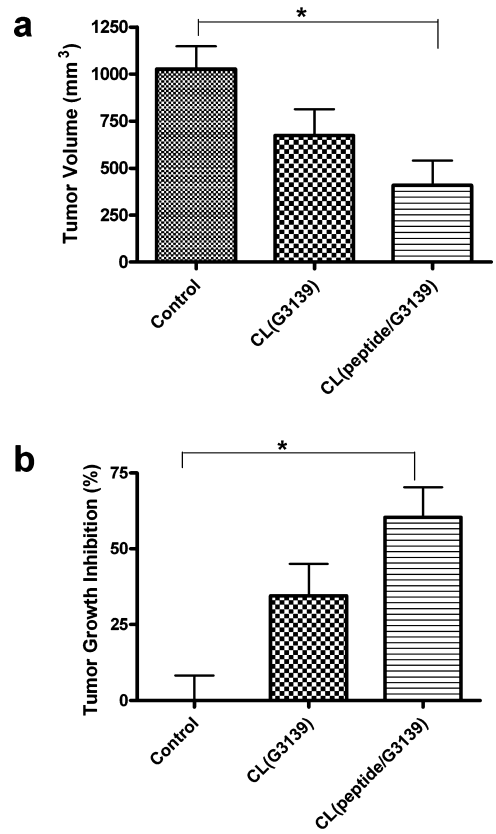

Figure 4. *In vivo* antitumor efficacy of CL loaded with D-(KLAKLAK)$_2$/G3139 in the tumor-bearing mice. The mice bearing B16(F10) tumors were administered via intratumoral injection with CL loaded with D-(KLAKLAK)$_2$/G3139 or G3139 alone, CL(peptide/G3139) and CL(G3139) respectively. After 4 days postinjection, (a) tumor volumes were measured and (b) relative inhibition of tumor growth was assessed by comparing tumor volumes of the treated animals to saline-treated controls. Intratumoral administration of CL(peptide/G3139) led to significantly decreased tumor volumes whereas CL(G3139) alone showed no significant decrease (n = 4, one-way ANOVA followed by Tukey posthoc test).

In Vivo **Antitumor Efficacy.** The enhanced antitumor efficacy *in vivo* was demonstrated in mice bearing the B16(F10) tumor. First, the inhibition of tumor growth was assessed by measuring tumor volumes after intratumoral administration of the CL preparation loaded with the peptide/G3139 complex (Figure 4). The average mouse weight at the beginning of the treatment was 24.4 ± 0.35 g and increased by 7.6% by the end of the study, i.e., day 4 postinjection. The average tumor volume at the beginning of the treatment was 218 ± 72 mm^3. The percentage change in tumor volume by day 4 postinjection was 470 ± 95% for the control group, whereas 308 ± 128% and 186 ± 120% of tumor volume increases were observed for the groups

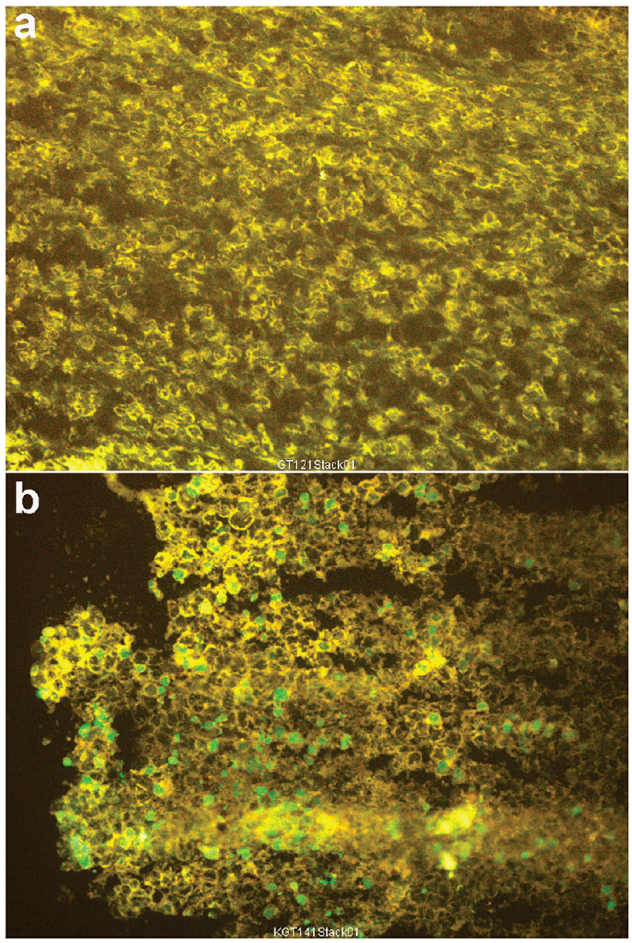

Figure 5. *In vivo* induction of apoptosis by CL loaded with D-(KLAKLAK)$_2$/G3139 in the tumor-bearing mice. The mice bearing B16(F10) tumors were administered CL loaded with the peptide/G3139 via intratumoral injection. At 24 h postinjection, apoptotic activity in tumors was assessed by DNA fragmentation with TUNEL staining. Dual channel fluorescence microscopy of frozen tumor sections from *in vivo* grown-B16(F10) tumors is shown. (a) Tumor section from a nontreated animal (background pattern). (b) Tumor section from an animal injected with CL loaded with D-(KLAKLAK)$_2$/G3139. Intratumoral injection of CL loaded with D-(KLAKLAK)$_2$/G3139 led to bright green staining of apoptotic nuclei.

treated with the CL loaded with G3139 alone or with peptide/G3139 complex, respectively.

Thus, treatment of tumor-bearing mice with the CL loaded with the peptide/G3139 led to significant tumor growth inhibition of 60 ± 25% as compared to the untreated control group ($p < 0.05$, $n = 4$). The treatment with the CL loaded with G3139 alone showed much smaller inhibition (34 ± 27%, $n = 4$). The apoptotic activity of the combined treatment was clearly confirmed by the TUNEL staining of the tumor sections from the animals treated with the CL loaded with the peptide/G3139 complex (Figure 5). The brightly stained apoptotic nuclei were readily found in the sections from the tumor treated with this preparation.

Discussion

The results of the current study are compatible with the following conclusions: first, the suggested method provides a promising way to formulate CL for intracellular delivery of cationic peptide therapeutics, while previously, the application of the CL was limited to the delivery of anionic gene therapeutics; second, the CL loaded with proapoptotic peptide D-(KLAKLAK)$_2$ and Bcl-2 antisense ODN G3139 exerts enhanced antitumor efficacy *in vitro* and *in vivo* toward B16(F10) melanoma.

Earlier, it was shown that approximately 400 μM cationic peptide D-(KLAKLAK)$_2$ is required to kill 50% of eukaryotic cells in the monolayer (LC_{50}).[5] In a cell-free system, however, the peptide showed 0.44 μM and 0.4 μM of the half-maximal effect (ED_{50}) in a mitochondrial swelling and mitochondrial membrane potential ($\Delta\Psi$m) loss assay,[3] i.e., only 0.1% the LC_{50} for eukaryotic cells. This indicated that the peptide, with efficient means of intracellular delivery, could be used for antitumor treatment via the induction of apoptosis. As with other gene therapeutics of poor *in vivo* stability during extracellular phase, G3139 is distributed and eliminated rapidly after iv injection with 5 and 37 min of α and β half-lives, respectively, and demonstrates an elevated renal accumulation.[17] In addition to the unfavorable pharmacokinetics, G3139 has to overcome intracellular barriers inherent to gene therapeutics to be effective. All said clearly indicates that an efficient delivery system for the intracellular delivery of the peptide and antisense ODN G3139 is required for effective antitumor therapy.

In this study, we constructed and assessed CL coloaded with the D-(KLAKLAK)$_2$ peptide and antisense ODN G3139 with the aim of solving the delivery problems for both drugs and producing an enhanced antitumor efficacy through the combination of two different strategies for induction of apoptosis. Cationic liposomes have provided an efficient tool for enhanced intracellular delivery of antisense gene therapeutics. Complexation with cationic lipids, such as oligofectamine and DDAB/DOPE, resulted in a 25- and 50-fold increase, respectively, in antisense cellular uptake.[18] In general, the use of CL for the delivery of cationic peptide would not be practical due to the lack of charge interaction that provides the mechanism for an efficient loading and intracellular delivery of anionic gene therapeutics. However, we proposed that the CL can be used for delivery of cationic peptides by employing a two-step alternating charge interaction, i.e., by first precomplexing a cationic peptide with

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anionic ODN into a complex with an overall negative charge, and then entrapping such complexes within CL with high efficacy due to charge interaction. This procedure would produce a CL formulation simultaneously loaded with cationic peptides and anionic ODN, and provide an efficient means of intracellular delivery of two apoptotic agents to reach a therapeutic intracellular level.

The combination of a simple lipid film hydration and extrusion procedure with precomplexation of the oppositely charged polyelectrolytes (polycationic peptide and polyanionic ODN) is a modification of the previously described procedure for liposome encapsulation of PEI/ODN polyplexes,[19] and can find analogues in procedures which generate "pre-condensed stable plasmid lipid particles" (pSPLP)[20] or a "multifunctional envelope-type nano device" (MEND).[21] Consistent with the previous reports, a high encapsulation efficiency of 54% was achieved with precondensation while just a negligible quantity of the positively charged peptide could be achieved without precondensation.

The promise of CL coloaded with D-(KLAKLAK)$_2$ and G3139 for antitumor therapy was clearly confirmed by the enhanced cytotoxicity in cell culture and inhibition of tumor growth in the mouse model. In the subsequent apoptotic assay, the enhanced antitumor efficacies were proven to be the results of increased apoptotic activity in the cultured tumor cells and *in vivo* grown tumor tissues. Thus, these *in vitro* and *in vivo* experiments clearly demonstrated a positive apoptotic effect obtained by loading two proapoptotic agents into CL. In our *in vivo* study, the formulation was directly injected into tumor tissues to ensure the maximal exposure of the tumor tissues to the formulation and thus demonstrate the feasibility of *in vivo* use of the formulation. However, although the intratumoral drug administration attracts increasing attention,[22−24] the use of the intratumoral injection could in some cases be limited to more easily accessible tumors. This is why in our future experiments we plan to investigate the *in vivo* therapeutic efficacy of the formulation on internal or disseminated tumors other than melanoma following iv administration.

Concluding, the CL coloaded with both proapoptotic peptide and ODN led to significantly enhanced antitumor efficacy and suggests a promising approach to cancer combining proapoptotic/gene therapy.

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