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Delivery of calf thymus DNA to tumor by folate receptor targeted cationic liposomes

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Abstract

Calf thymus DNA (ctDNA) has been shown to stimulate macrophages to produce cytokines both in vitro and in vivo when complexed with cationic liposomes. In addition, direct cytotoxicity of ctDNA has been found in tissue culture and in mice. In this study, ctDNA and folate receptor targeted cationic liposome complexes (ctDNA-F-CLs) were prepared and evaluated in FR (+) tumors. In addition, the underlying mechanism for the anti-cancer activity of ctDNA-F-CLs was investigated. Selective uptake of ctDNA-F-CLs was observed in FR (+) KB and L1210JF cells using flow cytometry. In RAW264.7 cells and DBA/2 mice, ctDNA-F-CLs and ctDNA-N-CLs significantly induced TNF- α and IL-6 production compared to free ctDNA. However, no significant difference in cytokine production was observed between ctDNA-N-CLs and ctDNA-F-CLs. In tumor bearing DBA/2 mice, ctDNA-F-CLs significantly increased INF- γ and IL-6 production compared to ctDNA-N-CLs. Furthermore in L1210JF cells, ctDNA-F-CLs had significantly increased cytotoxicity compared to ctDNA-N-CLs. Tumor cell apoptosis was also found in co-culture of RAW264.7 cells and ctDNA-F-CLs treated L1210JF cells. In L1210JF tumor bearing mice, ctDNA-F-CLs were found to significantly inhibit tumor growth and prolong the median survival time (MeST). In contrast, ctDNA-N-CLs and free ctDNA showed similar activities for tumor inhibition and animal survival. Moreover, the anti-cancer effect of ctDNA-F-CL was further enhanced by combination with anti-cancer drug doxorubicin. These results suggest that ctDNA-F-CLs are a promising agent for treatment of FR-positive tumors.

Keywords

Folate receptor targeting; Cationic liposomes; Calf thymus DNA; Anti-tumor activity

1. Introduction

Plasmid DNA (pDNA) and synthetic oligonucleotide share sequence motifs common to bacterial DNA that induce a wide range of immune activities including anti-DNA antibody production, cytokine production, B cell proliferation NK cell activation, and apoptosis suppression [1-5]. These responses are associated with specific DNA sequences and their methylation states. The common sequence known as the unmethylated CpG sequence (CpG motifs) in bacterial DNA and pDNA are recognized by toll-like receptor 9 (TLR 9) and stimulate the production of inflammatory cytokines such as tumor necrosis factor (TNF- α), interleukin-6 (IL-6) and interleukin-12 (IL-12) [2,6,7]. In principle, vertebrate DNA, including calf thymus DNA (ctDNA), should not be recognized by TLR9 because vertebrate DNA either contains methylated CpG or lacks CpG motifs [8,9]. However, Yasuda et al. [9] have shown that methylated pDNA and ctDNA complexed with cationic liposomes (CLs) stimulate murine macrophages and induce TNF- α production. This phenomenon was further confirmed in vivo using TLR9 $^{-/-}$ mice [10]. The cytokine production was observed to be significantly reduced, but not completely abolished in the macrophages from TLR9 $^{-/-}$ mice after treatment with cationic liposomes complexed to ctDNA (ctDNA-CLs), suggesting that the ctDNA-CLs may induce macrophage activation via both TLR9 dependent and independent pathways [10]. In addition, ctDNA-CLs may induce CpG-independent, direct cytotoxic effects in cell culture and in mice [11]. Therefore, ctDNA may be effective as a cancer therapy when complexed with cationic liposomes.

CLs are used in gene delivery both in vivo and in vitro. Features of CLs that have made them versatile and desirable for DNA delivery include lack of immunogenicity and cytotoxic response. The transfection efficiency of CLs may be dramatically increased when they bear a ligand recognized by a cell surface receptor, such as folate, which facilitates entry of DNA into cells through internalization of the complex via receptor-mediated endocytosis [12,13]. Folate receptor (FR) expression is elevated in various types of tumors, such as those of ovary, lung, kidney, mesothelium, head and neck, and brain [14-17]. Therefore, FR is a promising target for tumor-specific drug delivery. Studies using folate receptor targeted cationic liposomes as tumor-targeted delivery vehicles for p53 gene therapy enhanced anti-tumor efficacy of conventional chemo- and radiotherapy treatments [18]. Furthermore, folate receptor targeted cationic liposomes can enhance transfection efficiency of plasmid DNA and antisense oligodeoxynucleotide in FR-overexpressing tumor cells [19-21].

In the present study, folate receptor targeted cationic liposomes and ctDNA complexes (ctDNA-F-CLs) were prepared and the cytokine production of ctDNA-F-CLs was measured both in vitro and in vivo. In addition, cytotoxicity was measured in L1210JF murine leukemia cells. Tumor cell apoptosis was measured in co-culture of RAW264.7 and L1210JF cells by flow cytometry. Anti-tumor activity of ctDNA-F-CLs was evaluated in L1210JF tumor bearing DBA/2 mice.

2. Materials and methods

2.1. Materials

Calf thymus DNA (ctDNA) and doxorubicin hydrochloride (DOX) was purchased from Sigma-Aldrich (St. Louis, MO). Egg phosphatidylcholine (EPC), 3-[N-(N', N'-dimethylaminoethane)-carbamoyl] cholesterol hydrochloride (DC-Chol), and methoxy-polyethylene glycol (MW = 2000) distearoyl phosphatidylethanolamine (mPEG-DSPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Folate-polyethylene glycol (MW = 3350)-cholesterol hemisuccinate (F-PEG-CHEMS) was synthesized as described previously [22]. All chemicals used were of analytical grade.

2.2. Cell lines

KB human carcinoma, L1210JF murine lymphocytic leukemia, and RAW264.7 murine macrophages were purchased from American Type Culture Collection (Rockville, MD). KB and L1210JF cells were grown in folate-free RPMI 1640 medium (Life Technologies Inc., Bethesda, MD) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin at 37 °C in a 5% CO₂/95% air humidified atmosphere.

2.3. Preparation of folate receptor targeted cationic liposomes

Folate Receptor Targeted cationic liposomes (F-CLs) and non-targeted cationic liposomes (N-CLs) were prepared by polycarbonate membrane extrusion method. The lipid composition of N-CLs was EPC/DC-CHOL/mPEG-DSPE at molar ratios of 65:30:5. For F-CLs, the composition was EPC/DC-CHOL/mPEG-DSPE/f-PEG-CHEMS at molar ratios of 65:30:4.5:0.5. Briefly, lipids were dissolved in chloroform and dried in a round-bottom flask on a rotary evaporator under reduced pressure. The resulting lipid film was hydrated with PBS solution (pH 7.4). The lipid suspension was then serially passed through 0.2 and 0.1 µm pore size polycarbonate membranes on a nitrogen-driven Lipex™ extruder (Northern Lipid Inc., Canada). F-CLs and N-CLs were then complexed with ctDNA. The final product was stored at 4 °C before use.

2.4. Size and zeta potential measurement

The particle size of ctDNA-F-CLs was measured by Nicomp model 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA). Zeta potential () was measured by dynamic light scattering instrument (Zeta-plus, Brookhaven Instrument Co., NY).

2.5. Cellular uptake determined by flow cytometry

Cellular uptake of DNA complexes was determined in folate receptor positive KB and L1210JF cells using Gwiz-GFP plasmid DNA complexed F-CLs and N-CLs. Approximately 1×10^6 cells were treated with Gwiz-GFP plasmid complexed CLs for 4 h at 37 °C. After incubation, liposomes were removed and fresh culture medium was added to the cells and further cultured for 48 h. The cells were then washed twice with PBS and then analyzed by flow cytometry (FACScan, Becton & Dickinson).

2.6. In vitro cytokine production by RAW264.7 cells

In vitro cytokine production was determined in RAW264.7 macrophages. Briefly, 5×10^5 cells were seeded in 24 well plates and cultured overnight. Macrophages were treated with ctDNA-F-CLs and ctDNA-N-CLs for 2 h at 37 °C. After incubation, the liposomes were removed and fresh culture medium was added to the macrophages. The supernatant were collected 24 h later. The content of TNF- α and IL-6 was measured by specific ELISA Ready-Set-Go kits (eBioscience Inc., San Diego, USA).

2.7. In vivo assessment of serum cytokine level

The serum cytokine level was investigated in L1210JF tumor bearing DBA/2 mice (Charles River, Wilmington, MA). Female mice (18–22 g) were injected intravenously with free ctDNA, ctDNA-F-CLs or ctDNA-N-CLs at a dose of 2.5 mg/kg via the tail vein. Before treatment, mice were placed on a folate-deficient diet (AIN-90G, Dyets, Bethlehem, PA) for at least 1 week. Normal rodent chow was not used because it contains approximately 3.19 mg/kg folic acid, which leads to supra-physiological serum folate concentration. Blood samples were collected at 2 and 6 h after injection. Serum was obtained by centrifugation of blood samples at 7000 $\times$ g for 10 min and stored at –20 °C until the time of assay. INF- γ and IL-6 were determined by ELISA Ready-Set-Go kits.

2.8. In vitro cytotoxicity of ctDNA-F-CLs in L1210JF cells

In vitro cytotoxicity was determined by MTT assay in L1210JF cells. Briefly, 5000 cells/well were seeded in 96 well plates and cultured overnight. Cells were incubated with 1:4 serially diluted free ctDNA, ctDNA-N-CLs, and ctDNA-F-CLs for 4 h at 37 °C. After incubation, cells were washed 2 times with cold PBS and cultured for an additional 72 h in fresh medium. For cell viability determination, 20 μ L of 5 mg/ml MTT was added to each well, and incubated for 4 h at 37 °C. Cells were then sedimented by centrifugation at 1000 $\times$ g for 8 min, and the formazan crystals were dissolved in 200 μ L of DMSO. The absorbance was determined at 570 nm on a Dynatech MR-600 microplate reader.

2.9. Tumor cell apoptosis in co-culture of RAW264.7 and L1210JF cells

Tumor cell apoptosis in co-culture of RAW264.7 and L1210JF cells was investigated by Annexin V/PI staining assay. Briefly, 4×10^5 RAW264.7 cells were seeded in 24 well plates and cultured overnight. Meanwhile, 5×10^5 L1210JF cells were treated with 100 μ g/ml of ctDNA-F-CLs, ctDNA-N-CLs, or free ctDNA for 2 h at 37 °C. After washing, L1210JF cells were co-cultured with RAW264.7 cells for 24 h at 37 °C. L1210JF cells containing supernatant were collected and harvested by centrifugation. After washing two times with cold PBS, cells were resuspended in 1 $\times$ binding buffer. 5 μ L Annexin V-FITC (50 μ g/ml) and 5 μ L propidium iodide (50 μ g/ml) were added to the suspension. L1210JF cell viability was determined by flow cytometry.

2.10. Therapeutic efficacy in murine leukemia models

Therapeutic efficacy was evaluated in DBA/2 mice (Charles River, Wilmington, MA). Female mice (18–22 g) were inoculated subcutaneously on day 0 with 5×10^6 L1210JF cells and then randomized into different treatment groups (5 mice per group) to avoid cage effects. Before leukemia cell inoculation, mice were placed on a folate-deficient diet (AIN-90G, Dyets, Bethlehem, PA) for at least 1 week. When the tumor reached a volume of 50–100 mm³, the mice were injected intravenously (i.v.) with various ctDNA formulation or PBS on days 1, 5, 9 via tail vein. For combination therapy, 5 mg/kg of doxorubicin and various ctDNA formulations were injected intravenously on days 1, 5, 9 via tail vein. Tumor dimensions were determined by measurement with calipers and tumor volume (mm³) was calculated by $0.5 \times (\text{length} \times \text{width} \times \text{height})$. Animal survival was evaluated by Kaplan–Meier analysis. Mice were sacrificed once the tumor size exceeded 1500 mm³.

2.11. Statistical analysis

Statistical analysis of survival data was performed using the log-rank test. Comparisons between groups were made by Student's *t* tests using the MiniTAB software (Minitab Inc., State College, PA). Difference in survival or tumor growth were considered significant when the *p* value was <0.05.

3. Results

3.1. Characterization of ctDNA-F-CLs

The size and zeta potential were monitored by Nicomp Submicron Particle Sizer and dynamic light scattering instrument. To optimize ctDNA to lipid ratio, ctDNA was complexed with F-CLs at different weight ratios and their gel retardation activity was determined. As shown in Fig. 1A, the particle size of ctDNA-F-CLs was consistently found to be ~150 nm at different weight ratios of ctDNA and F-CLs, indicating that the formulated ctDNA-F-CLs did not aggregate. However, the zeta potential of F-CLs increased from –12.03 to 10.05 mV with increasing weight ratios of CLs/ctDNA from 1 to 10, but remained constant when the weight ratio was >10. The data suggests that negatively charged ctDNA

gradually interacts with cationic liposomes up to the weight ratio of 10, above which the charge interaction is likely saturated. Similarly, gel retardation results show that cationic liposome and ctDNA are fully retarded at a weight ratio of 15:1 (data not shown). Both zeta potential and gel retardation data reveal that complete complexation can be attained above the weight ratio of 15.

3.2. Folate receptor targeted GFP delivery to L1210JF cells

To evaluate folate receptor targeting efficiency, GFP expression was determined by flow cytometry after treatment with Gwiz-GFP plasmid DNA (pDNA). In FR-positive L1210JF cells, GFP expression was significantly increased by treatment of Gwiz-GFP-pDNA/F-CLs complexes compared to Gwiz-GFP-pDNA/N-CLs complexes. Moreover, the GFP expression was inhibited by 1 mM of free folate (Fig. 1B). Similar results were observed in FR-positive KB cells (Fig. 1C). These results indicate that F-CLs can be used to target FR-positive tumor cells.

3.3. In vitro cytokine production from RAW264.7 cells

Previous studies have shown that ctDNA induces cytokine production when complexed with cationic liposomes [9]. Here, the cytokine production was determined in RAW264.7 macrophages. In RAW264.7 cells, free ctDNA alone cannot produce significant levels of TNF- α and IL-6 (Fig. 4), confirming the necessity of immunostimulatory CpG motifs in cytokine production. Similar to free ctDNA, N-CLs and F-CLs without ctDNA did not induce TNF- α and IL-6 production in RAW264.7 cells. In contrast, ctDNA-N-CLs and ctDNA-F-CLs significantly increased TNF- α and IL-6 production in RAW264.7 cells (Fig. 2). However, no significant difference was observed between ctDNA-N-CLs and ctDNA-F-CLs.

3.4. In vivo serum cytokine production in tumor bearing DBA/2 mice

In vivo cytokine production was determined in tumor bearing DBA/2 mice after administration of free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs. As shown in Fig. 3, free ctDNA alone did not induce INF- γ and IL-6 production. In contrast, ctDNA-N-CLs and ctDNA-F-CLs significantly increased cytokine production. These results are consistent with the results obtained in the assay of in vitro cytokine production (Fig. 2). Levels of INF- γ and IL-6 peaked at 2 h and then decreased after 6 h. Interesting results were observed with treatment of ctDNA-F-CLs. ctDNA-F-CLs significantly increased INF- γ and IL-6 production compared to ctDNA-N-CLs (Fig. 3). In addition, we confirmed that N-CLs and F-CLs alone couldn't induce cytokine release (data not shown). These results imply that enhanced INF- γ and IL-6 production in tumor bearing DBA/2 mice might be due to the folate receptor targeting.

3.5. Cytotoxicity of ctDNA-F-CL in L1210JF cells

Cell toxicity was determined by MTT assay after treatment of ctDNA-F-CLs, ctDNA-N-CLs and free ctDNA. As shown in Fig. 4, free ctDNA did not exhibit cytotoxicity at tested concentrations (1000–0.25 μ g/ml). In contrast, ctDNA-N-CLs and ctDNA-F-CLs showed cytotoxicity with IC₅₀ of 615.2 $\pm$ 11.8 and 101.6 $\pm$ 5.7 μ g/ml respectively. These results suggest that folate receptor-mediated targeting may increase cytotoxicity of ctDNA-F-CLs.

3.6. Tumor cell apoptosis in co-culture of RAW264.7 and L1210JF cells

To further investigate tumor cell apoptosis, ctDNA treated L1210JF cells were co-cultured with RAW264.7 cells for 24 h and analyzed by Annexin V/PI double staining method. Annexin V, a calcium-dependent phospholipid-binding protein with a high affinity for phosphatidylserine (PS), may be used as a marker for pre-apoptosis. Early in the apoptosis,

the membrane PS is translocated from the inner to the outer leaflet of plasma membrane, thereby exposing PS to the external cellular environment [23]. Propidium iodide (PI) is a non-specific DNA intercalating agent, which is excluded by the plasma membrane of living cells, and thus can be used to distinguish necrotic cells from apoptotic and living cells by supravital staining without prior permeabilization [24]. As shown in Fig. 5, almost no pre-apoptotic cells were detected in the control (3.6%), free ctDNA (2.5%), ctDNA-N-CLs (2.3%) and ctDNA-F-CLs (2.9%) groups. However, a large number of apoptotic cells (20.1%) were found in the ctDNA-F-CLs group (Fig. 5). These results further establish the high cytotoxicity of ctDNA-F-CLs in L1210JF cells.

3.7. Anti-tumor activity of ctDNA-F-CLs in murine leukemia model

In vivo anti-tumor efficacy of ctDNA-F-CLs was evaluated in L1210JF bearing DBA/2 mice. All of the control mice died approximately 24 days after tumor inoculation (Fig. 6A). In contrast, survival time was significantly prolonged by treatment of free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs ($p < 0.01$), indicating that ctDNA has anti-tumor activity when complexed with CLs or alone (Fig. 6A). The mean survival time of free ctDNA, ctDNA-F-CLs and ctDNA-N-CLs treated samples were 26, 26, 31 days, respectively (Table 1). No significant difference was observed between free ctDNA and ctDNA-N-CLs. However, the survival time of ctDNA-F-CLs was significantly prolonged compared to free ctDNA or ctDNA-N-CLs ($p < 0.01$). Percentage ILS values are 123.8% for both of free ctDNA and ctDNA-N-CLs and 147.6% for ctDNA-F-CLs (Table 1). The tumor growth inhibition ratio (T/C %) of free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs were 58.5, 55.1 and 71.3%, respectively. These results indicate potent anti-tumor efficacy for ctDNA-F-CL treatment.

3.8. Therapeutic efficacy of ctDNA-F-CLs combined with doxorubicin in murine leukemia model

Studies involving combinations of doxorubicin with IL-2 have been shown to be effective against a variety of murine tumors, including renal cell carcinoma [25], colon and mammary adenocarcinoma [26], lung carcinoma [27] and EL4 lymphoma [28]. We hypothesized that the anti-cancer efficacy of doxorubicin would be enhanced when combined with ctDNA-N-CLs or ctDNA-F-CLs, which induce cytokine production. Thus, antitumor activity of doxorubicin in combination with free ctDNA, ctDNA-N-CLs or ctDNA-F-CLs was evaluated in L1210JF bearing DBA/2 mice (Fig. 7). Consistent with our previous findings [29], doxorubicin had significant anti-tumor activity compared with PBS control (Fig. 7). In combination with free ctDNA or ctDNA-N-CLs, anti-cancer efficacy of doxorubicin did not prolong mean survival time compared with doxorubicin alone (Fig. 7B). However, tumor inhibition ratio (T/C %) increased by treatment of doxorubicin in combination with free ctDNA (75.1%) or ctDNA-N-CLs (75.2%) compared with doxorubicin alone (69.2%) (Fig. 7A). In contrast, anti-tumor activity of doxorubicin significantly prolonged mean survival time when combined with ctDNA-F-CLs (Fig. 7B). The percentage ILS value of doxorubicin in combination with ctDNA-F-CLs was 195.2%, significantly higher than that of doxorubicin alone (152.4%) or doxorubicin combined with free ctDNA (152.4%) or ctDNA-N-CLs (152.4%) (Table 1). Tumor inhibition ratio significantly increased by treatment of doxorubicin combined with ctDNA-F-CLs (86.0%). These results indicate that the anti-tumor efficacy of doxorubicin is significantly enhanced by combination with ctDNA-F-CLs.

4. Discussion

DNA containing methylated CpG or without CpG dinucleotide motifs such as ctDNA cannot activate immune cells to induce cytokine production. However, ctDNA can stimulate macrophages to induce potent cytokine production both in vivo and in vitro when

complexed with cationic liposomes [9,10]. The cytotoxic effect of ctDNA was previously found in cell culture and in mice when complexed with cationic liposomes [11]. In this study, the effect of ctDNA-F-CLs on the anti-tumor activity was evaluated in tumor bearing DBA/2 mice. In addition, the underlying mechanism for the anti-cancer activity of ctDNA-F-CLs was investigated.

ctDNA-F-CLs were successfully prepared in this study. The liposomes were composed of EPC, DC-Chol, PEG-DSPE and F-PEG-CHEMS. DC-Chol, a cationic lipid, was a major contributor to the surface charge observed in our study. The charge potential fostered complete complexation with ctDNA. Furthermore, DC-Chol is considered to be less toxic than other cationic lipids. PEG tethered to liposome surfaces can decrease uptake of particles by the reticuloendothelial system (RES), resulting in longer in vivo circulation. F-PEG-CHEMS was previously applied to folate receptor targeting in vitro. We have demonstrated that F-PEG-CHEMS better retains its FR-targeting activity during prolonged storage compared to folate-PEG-cholesterol and folate-PEG-DSPE systems [22]. Selective FR targeting was observed in L1210JF and KB cells by treatment of ctDNA-F-CLs (Fig. 1B and C). The potent anti-cancer activity was observed by treatment of ctDNA-F-CLs in L1210JF tumor bearing mice. ctDNA-F-CLs were effective in inhibiting tumor growth and prolonging animal survival in tumor bearing mice (Fig. 6). Moreover, the anti-cancer activity was significantly enhanced when ctDNA-F-CLs were combined with doxorubicin (Fig. 7). In contrast, ctDNA-N-CLs and free ctDNA showed less potent anti-tumor activity than ctDNA-F-CLs (Fig. 6). However, similar anti-cancer activity was observed between ctDNA-N-CLs and free ctDNA.

Previous data showed that ctDNA significantly induces cytokine production both in vitro and in vivo when complexed with cationic liposomes. Therefore, the effect of cytokine production on the antitumor activity was investigated. Consistent with previous reports, ctDNA alone did not induce cytokine (TNF- α and IL-6) production in cultured RAW264.7 cells (Fig. 4) and DBA2 mice (data not shown) due to the presence of methylated CpG or absence of CpG dinucleotide motifs in the structure [8,9]. In contrast, ctDNA-N-CLs or ctDNA-F-CLs could stimulate macrophages to increase TNF- α and IL-6 production and release in RAW264.7 cells (Fig. 4) and DBA/2 mice (data not shown). However, no difference was found between ctDNA-N-CLs and ctDNA-F-CLs, indicating folate targeting does not play a role in cytokine production under normal conditions (Fig. 4). Recently, Turk and Low have demonstrated that the folate receptor is overexpressed in tumor associated macrophages [30]. It is conceivable that the observed anti-tumor activity is partially due to the targeted elimination of FR-expressing tumor associated macrophages. Consistent with our expectation, ctDNA-F-CLs significantly increased INF- γ and IL-6 production in L1210JF tumor bearing DBA/2 mice compared with ctDNA-N-CLs or free ctDNA (Fig. 5). However, increased INF- γ and IL-6 was also observed by treatment of ctDNA-N-CLs compared with free ctDNA (Fig. 5). These results indicate that tumor associated macrophage-induced cytokine production does not contribute to the observed in vivo anti-tumor activity of ctDNA-N-CLs and free ctDNA.

In addition to cytokine production, in vitro cytotoxicity and cell apoptosis were determined by MTT assay and flow cytometry, respectively. ctDNA-F-CLs showed higher cytotoxicity with an IC₅₀ value of 101.6 ± 5.7 μ g/ml in L1210JF cells, possibly due to folate receptor targeting. In contrast, ctDNA-N-CLs showed 6 times lower cytotoxicity (Fig. 2) than ctDNA-F-CLs. Similar results were obtained by tumor cell apoptosis assay. Consistent with our results, direct cytotoxicity of nonbacterial DNA and cationic liposome complex against C-26 and M-109 tumor cells have been reported [11]. Tousignant et al. have also demonstrated that methylated mammalian CpG sequences are able to induce systemic toxicity through non-immunological effects when systemically administered in cationic

lipid-DNA complexes [31]. Therefore, the DNA taken up by the tumor cells is responsible for the cytotoxicity and folate-mediated receptor targeting [32,33] increased tumor cell uptake of this DNA. In contrast, plasma ctDNA concentration cannot reach 100 µg/ml after i.v. administration of 2.5 mg/kg ctDNA-N-CLs, indicating that cell cytotoxicity is not involved in the anti-cancer activity of ctDNA-N-CLs. These results suggest that a direct tumor suppressive effect, rather than the cytokine production, is responsible for the anti-cancer activity of ctDNA-F-CLs, ctDNA-N-CLs and ctDNA.

5. Conclusion

In summary, ctDNA-F-CLs were shown to exhibit potent antitumor activity in vivo. This seems to occur by two major mechanisms, increased cytotoxicity and elevated cytokine production. Therefore, ctDNA-F-CLs alone or combined with anti-cancer drugs is a promising strategy for treatment of FR-positive cancers.

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Fig. 1. Characterization of ctDNA-F-CLs. (A) Measurement of particle size and zeta potential. (B, C) Uptake of F-CLs by flow cytometry in L1210JF and KB cells. 10^6 cells were incubated with Gwiz-GFP plasmid DNA complexed F-CLs or N-CLs at 37 °C for 4 h. After treatment, cells were cultured in the fresh culture medium at 37 °C for 48 h and the uptake was measured by flow cytometry. For the receptor inhibition study, 1 mM of free folate was added to the Gwiz-GFP plasmid DNA complexed F-CLs liposomes. (1) Untreated cells, (2) Cells treated with non-targeted calcein liposome, (3) Cells treated with Folate Receptor Targeted calcein liposome, (4) Cells treated with Folate Receptor Targeted calcein liposome and free folate.

Fig. 2.

In vitro cytokine production in RAW264.7 cells. Cells were treated with 100 μ g/ml of free ctDNA, ctDNA-F-CLS, ctDNA-N-CLS, F-CLS or N-CLS for 2 h at 37 °C. After incubation, Cells were washed with fresh growth medium and cultured 24 h. The supernatant were collected and analyzed for TNF- α and IL-6 content by ELISA. Each results represents the mean $\pm$ SD ($n = 4$).

Fig. 3.

In vivo serum cytokine levels in L1210JF bearing mice. L1210JF bearing mice (4 per group) were injected intravenously with free ctDNA, ctDNA-F-CLs or ctDNA-N-CLs at a dose of 2.5 mg/kg via the tail vein. Blood samples were collected at 2 and 6 h after injection. Serum cytokine content (A, INF- γ , B, IL-6) was determined by specific ELISA. (* $p < 0.05$ compared with free ctDNA).

Fig. 4.

Cytotoxicity of ctDNA-F-DNA in L1210JF cells. Cells were incubated with 1:4 serially diluted free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs for 4 h at 37 °C. After incubation, cells were washed 2 times with cold PBS and cultured for an additional 72 h in fresh medium.

Fig. 5.

Tumor cell apoptosis in co-culture of RAW264.7 and L1210JF cells. RAW264.7 cells were seeded in 24 well plates and cultured overnight. Meanwhile, 5×10^5 L1210JF cells were treated with 100 $\mu\text{g}/\text{ml}$ of ctDNA-F-CLs, ctDNA-N-CLs and free ctDNA for 2 h at 37 °C. After washing off unbound of liposomes, L1210JF cells were co-cultured with RAW264.7 cells for 24hr at 37 °C. L1210JF cells containing supernatant were collected and L1210JF cells were harvested by centrifugation. After washing two times with cold PBS, cells were resuspended in 1 $\times$ binding buffer and then stained with Annexin V-FITC and propidium iodide. L1210JF cell apoptosis was determined by flow cytometry.

Fig. 6.

In vivo anti-tumor efficacy of ctDNA-F-CLs in L1210JF bearing DBA/2 mice. Mice (5 per group) were implanted subcutaneously on day 0 with 5×10^6 L1210JF cells. On day 5, animals were treated with 2.5 mg/kg of free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs. The treatment was continued every 4 days for 3 times. (A) Inhibition of tumor growth. Tumor dimensions were measured every 4 days using a caliper. (B) Survival of L1210JF bearing mice after treatment with ctDNA-F-CLs. PBS was injected as a vehicle control. (* $p < 0.05$ compared with PBS group).

Fig. 7.

In vivo anti-tumor efficacy of ctDNA-F-CLs combined with doxorubicin in L1210JF bearing DBA/2 mice. Mice (5 per group) were implanted subcutaneously on day 0 with 5×10^6 L1210JF cells. On day 5, animals were treated with 5 mg/kg of doxorubicin combined with 2.5 mg/kg of free ctDNA, ctDNA-N-CLs and ctDNA-F-CLs. (A) Inhibition of tumor growth. Tumor dimensions were measured every 4 days using a caliper. (B) Survival of L1210JF bearing mice after treatment with ctDNA-F-CLs. (* $p < 0.05$ compared with doxorubicin group).

Table 1

The anti-tumor activity of ctDNA-F-CLs in L1210JF tumor bearing mice.

Treatment	Tumor inhibition ratio ^a (%)	MeST (days)	ILS ^b (%)	Log-rank <i>p</i> value ^c
PBS	–	21	0	–
Free ctDNA	58.5	26	123.8	0.00835
ctDNA-N-CLs	55.1	26	123.8	0.00264 (0.800) ^d
ctDNA-F-CLs	71.3	31	147.6	0.00264 (0.0309) ^d
DOX	69.2	32	152.4	0.00771
DOX + free ctDNA	75.1	32	152.4	0.00202 (0.962) ^e
DOX + ctDNA-N-CLs	75.2	32	152.4	0.00202 (0.581) ^e
DOX + ctDNA-F-CLs	86.0	41	195.2	0.00202 (0.00885) ^e

The mice were treated with free or liposomal ctDNA, alone or in combination with DOX (*n* = 5 for each treatment Group).

^aTumor inhibition ratio was calculated by $(C-T)/C \times 100\%$, where T and C represent the mean tumor volume of the treated and control mice, respectively.

^bILS(%) was calculated by $(MeST \text{ of the treated mice} / MeST \text{ of control mice}) \times 100\%$. Where MeST is represent the mean survival time.

^cThe *p* value was calculated compared to PBS, free ctDNA and DOX, respectively.

^dThe *p* value was calculated compared to PBS, free ctDNA and DOX, respectively.

^eThe *p* value was calculated compared to PBS, free ctDNA and DOX, respectively.