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A Combined Chemoimmunotherapy Approach Using a Plasmid−Doxorubicin Complex

Vaishali Bagalkot,[†,‡] In-Hyun Lee,[†,‡] Mi Kyung Yu,[†] Eunhye Lee,[†] Saeho Park,[†] Jae-Hyuk Lee,[§] and Sangyong Jon*[,†]

Cell Dynamics Research Center, Department of Life Science, Gwangju Institute of Science and Technology, 1 Oryoung-dong, Buk-gu, Gwangju, Republic of Korea, and Department of Pathology, Chonnam National University Medical School, Gwangju, Republic of Korea

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Abstract: We report a combined chemoimmunotherapy vehicle consisting of plasmid loaded with doxorubicin and evaluate its efficacy in two different tumor models. A stable complex was formed with a 1300:1 ratio of doxorubicin bound to native plasmid via intercalation. Pharmacokinetics of the complex showed much slower clearance from plasma up to 3 h compared to 10 min for free doxorubicin. In mice bearing NCI-H358 xenografts, lower doses of complex (doxorubicin 0.5 mg/kg, plasmid 4 mg/kg) effectively reduced tumor growth compared to high doses (5 mg/kg) of free doxorubicin (68% versus 77%). Similar results were observed in mice bearing 4T1 murine allografts; the complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) was effective and caused similar reduction of tumor compared to free doxorubicin (4 mg/kg) (47% versus 46%). The complex showed no signs of severe systemic toxicity or cardiotoxicity compared to the free doxorubicin in mice as indicated by body weights and heart tissue histology. Elevated levels of cytokines (IL-12, IL-6, and IFN-γ) were observed in serum as well as in tumor tissue after intravenous injection of complex when compared to plasmid or doxorubicin alone. This approach simultaneously delivers both chemotherapeutic and immunotherapeutic agents without time delay, improves pharmacokinetics of the free drug, lowers drug toxicity, upregulates a variety of cytokines, and is effective against different tumors.

Keywords: Doxorubicin; plasmid; chemoimmunotherapy; adjuvant; complex

Introduction

Antitumor therapy with bacteria and bacterial products dates back to the 1890s with the first use of live cultures of streptococci injected directly into tumor masses that was later on modified and used as Coley's toxin.[1] This early method of immunotherapy resulted in clinical efficacy in 10% of the patients.[2] The immune stimulating component responsible was discovered to be "unmethylated CpG motifs" in bacterial DNA.[3,4] With the discovery of toll-like receptors (TLRs) that detect highly conserved pathogen-expressed molecules, tumor immunology for cancer treatment has recently seen the development of various synthetic agonists for several TLRs.[5−7] For cancer treatment, unmethylated CpG dinucleotides

* Corresponding author: Sangyong Jon, Ph.D., Associate Professor, Department of Life Science, Gwangju Institute of Science and Technology, 1 Oryong-dong, Buk-gu, Gwangju 500-712, South Korea. Phone: (82) 62-970-2504. Fax: (82) 62-970-2484. E-mail: syjon@gist.ac.kr.

[†] Gwangju Institute of Science and Technology.

[‡] Authors have contributed equally.

[§] Chonnam National University Medical School.

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recognized by TLR-9 has shown promise as it elicits the most favored Th1 immune response.[8−10] Short oligonucleotides containing such CpG motifs are used as adjuvants or in combination with cytotoxic chemotherapy, and their antitumor effects are well documented in various experimental animal models.[11−20]

Tumor progression proceeds via immune tolerance and resistance mechanisms.[21−23] The nonspecific nature of conventional chemotherapy giving rise to dose-related toxicities and the lack of success of immunotherapy alone against tumor burden have paved the way for combination chemoimmunotherapy.[24−27] The synergism of combination therapy depends greatly on the right timing and administration schedules.[28] In general, it is desirable to develop carriers that incorporate and simultaneously deliver both immune-stimulating and cytotoxic chemotherapeutic agents and that are active against various tumors with the ability to generate a variety of cytokines rather than a single cytokine-based therapy. To this end, here we report a combined chemoimmunotherapy using a plasmid−doxorubicin complex. Although it is reported that doxorubicin complexed to salmon sperm DNA is less toxic than the free drug and is effective against leukemia,[29] there was no consideration in terms of immune modulation at that time presumably due to lack of immune stimulation by the DNA carrier.[30] Both the plasmid with unmethylated CpG motifs as a TLR9 agonist and doxorubicin as a TLR4 agonist are the subjects of this study.

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Recently, Zitvogel and colleagues reported that chemotherapy can enhance antitumor immune response; specifically, the anthracycline class of compounds can cause immunogenic cell death through the release of HMGB1 protein that can bind to TLR4 receptor on dendritic cells (DCs), leading to efficient DC-mediated antigen cross-presentation to T cells.[31] The plasmid−doxorubicin complex has several favorable characteristics for combined chemoimmunotherapy. First, plasmid containing unmethylated CpG is known for its antitumor activity and compared to CpG oligo itself; plasmid has an abundance of such sequences. Second, a plasmid can carry a number of doxorubicin drugs by intercalation[32,33] and can improve pharmacokinetics of the drug by increasing blood circulation. Third, such intercalation (complexation) can protect the plasmid against nucleases. Lastly, doxorubicin by itself is known to enhance or modulate the immune system.[31,34] We show that combination therapy using the plasmid−doxorubicin complex delivers chemotherapeutic agents simultaneously with immunotherapeutics and is more effective and safer compared to cytotoxic chemotherapy alone.

Materials and Methods

Chemicals. Doxorubicin and commercial doxorubicin formula (K.U. doxorubicin HCl for injection; doxorubicin) were purchased from Boryung Pharmaceutical (Seoul, Korea) and Korea United Pharm (K.U. Seoul, Korea), respectively.

Mice. Athymic BALB/c female nude or BALB/c mice were obtained from Orient Bio (Seoul, Korea) and were housed under pathogen-free conditions. Animal care was provided in accordance with the guidelines of the animal care facility at Gwangju Institute of Science and Technology.

Preparation of Plasmid DNA. A 4.7-kbp pEGFP PC-1 vector DNA (Clontech, Palo Alto, CA) was amplified in the *Escherichia coli* strain DH5α and purified using the endofree plasmid giga prep kit (Qiagen, Valencia, CA). Endotoxin levels in the plasmid were below 0.01 EU/μg as determined by the QCL-1000 LAL assay (Cambrex, Walkersville, MD).

Complex Formation. A physical complex between the native plasmid and doxorubicin was generated as described previously.[32] Briefly, increasing picomoles of plasmid were added in a stepwise fashion to a fixed concentration of doxorubicin (3 μM) in PBS buffer and the fluorescence of doxorubicin was then measured by spectroflourophotometer RFPC-100 (Schimadzu, Kyoto, Japan). For in vivo injections, the complex was prepared before injection by mixing plasmid (2−3 μg/μL) with doxorubicin (1−1.5 μg/μL) in sterile 5% glucose solution. Based on fluorescence quenching, the doxorubicin-to-plasmid ratio was 1300:1.

In Vitro Release of Doxorubicin from the Complex. Doxorubicin (125 μg/100 μL) and complex (plasmid 500 μg, doxorubicin 125 μg/100 μL) in sterile 5% glucose were dispensed in 8K membrane dialysis bags (Spectra Por Biotech catalog no. E-02899-32, Cole-Parmer, IL) and dialyzed by placing the bags in conical falcon tubes containing 20 mL of sterile PBS ($n = 2$). Release studies were done in a shaking water bath (40 rpm) maintained at 37 °C. At selected time intervals 2 mL of PBS solution outside the dialysis bags was withdrawn for analysis and replaced with 2 mL of fresh sterile PBS solution. Doxorubicin concentration was calculated by measuring the fluorescence intensity on Spectra Maxplus (Molecular Devices, Sunnyvale, CA) at excitation 480 nm, emission 555 nm, cutoff 530 nm. Doxorubicin in PBS served as standard in the linear range of 5 to 0.1 μg/mL.

In Vitro Serum Stability of Complex. The serum stability assay was performed by incubating 20 μg of plasmid and complex (plasmid 20 μg, doxorubicin 5 μg) in TE buffer with 600 μL of freshly prepared serum and placed at 37 °C shaking water bath. Mouse serum was freshly prepared as follows: 300 μL of blood was collected by retro-orbital bleeding and incubated at 4 °C for 2 to 3 h to allow clotting and then centrifuged (6000*g*, 10 min, 4 °C); approximately 80−150 μL of serum could be obtained per mouse. All procedures were performed as previously described.[35]

Pharmacokinetics. Female BALB/c mice received a single intravenous injection in 5% sterile glucose containing doxorubicin 5 mg/kg or complex (doxorubicin 5 mg/kg, plasmid 20 mg/kg). At given time intervals, 300 μL of blood was collected by retro-orbital puncture and mixed with 3.8% sodium citrate (50 μL). Plasma (100 μL) was obtained by centrifuging the samples at 5000*g* for 10 min and was treated with 2 μL of DNase I (Turbo DNase, Sigma, St. Louis, MO) at 37 °C for 30 min to obtain and ensure a free unbound form of doxorubicin. The amount of doxorubicin in plasma was analyzed by directly measuring the fluorescence of doxorubicin (excitation 480 nm, emission 520−640 nm, 5 mm slit) on a Shimadzu RF-PC100 spectroflourophotometer.

Xenograft/Allograft Tumor Experiments. A human non-small-cell lung cancer xenograft model was established in 7-week-old BALB/c athymic mice by subcutaneous injection of 2×10^7 NCI-H358 cells, purchased from Korea Cell Line Bank (KCLB, Seoul, Korea).When tumors reached at least

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90 to 110 mm^3, the mice were randomly divided into experimental groups (8 mice/group). A suspension of 4T1 murine breast cancer cells (1×10^5cells/mouse), purchased from American Type Culture Collection (ATCC, Manassas, VA), was injected subcutaneously to the dorsal flank of BALB/c female mice (8-week-old) on day 0 and randomly divided according to the experimental groups (10 mice/group).

In Vivo Dosage and Tumor Measurements. Untreated control groups received sterile 5% glucose solution only. In the case of xenografts, mice received seven intravenous injections every third day with (100 μL) of either plasmid 4 mg/kg or complex (doxorubicin 0.5 mg/kg, plasmid 4 mg/kg) in sterile 5% glucose and five intravenous injections of doxorubicin 5 mg/kg. For allograft experiments mice received six alternate-day intravenous injections (100 μL) of either doxorubicin 2 mg/kg, or plasmid 8 mg/kg, or complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) and four alternate-day injections of 4 mg/kg doxorubicin. At given time intervals mice were weighed and implanted and tumor size was measured with Vernier calipers. Tumor volume was calculated as tumor volume = length × width × height × 0.5236.[36]

Measurement of Serum or Tumor Cytokine Levels. Normal 7-week-old female BALB/c mice or 4T1 tumor bearing BALB/c mice received a single intravenous injection of doxorubicin 2 mg/kg, plasmid 8 mg/kg, or complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) in 100 μL of 5% glucose. For serum cytokine ELISA, at given time intervals 300 μL of blood was collected by retro-orbital bleeding and incubated at 4 °C for 2 to 3 h to allow clotting and then centrifuged (6000*g*, 10 min, 4 °C) to obtain serum. For tumor cytokine assay, the experimental groups of BALB/c mice bearing 4T1 tumors were sacrificed at given time points and tumor tissue were isolated. Isolated tumor tissues were snap-frozen in liquid nitrogen and stored at −70 °C before homogenization. The tumor tissues were homogenized with the use of a high-speed homogenizer (T-25-Ultra-Turrax; Janke & Kunkel GmbH & Co.) and then centrifuged (14000*g*, 20 min, 4 °C) to get the supernatants of the tumor extracts. We assayed the collected sera or the supernatants for IL-6, IL-12, and IFN-γ expression levels with an ELISA kit (Quantikine, R&D Systems, Minneapolis, MN), using a microplate reader to detect the color changes (Molecular Devices, Menlo Park, CA).

Histopathology. Studies were done in 6-week-old female BALB/c mice that received six alternate-day intravenous injections of doxorubicin 2 mg/kg or 4 mg/kg or complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) in 100 μL of 5% sterile glucose solution. On day 13 after drug treatment, mice were humanely killed by cervical vertebra dislocation and heart tissues were harvested, and were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 5 μm thickness and then, stained with hematoxylin and eosin. Histopathologic specimens were examined by light microscopy.

Statistical Analysis. Data are shown as the means ± SE. Statistical significance was determined by ANOVA using SigmaStat 3.0 (Jandel Scientific, San Rafael, CA). $P < 0.001$ was considered to be statistically significant for differences between experimental groups.

Results

Complex Formation, in Vitro Drug Release, Serum Stability, and in Vivo Pharmacokinetics. The combined chemoimmunotherapy vehicle was formulated by complex formation between plasmid and doxorubicin (Figure 1) and monitored by fluorescence spectroscopy as previously reported in our studies with aptamer−Dox complex.[32,33] The native fluorescence spectrum of doxorubicin (3 nmol) was totally quenched at 2.28 pmol of plasmid, with a binding ratio of doxorubicin to plasmid of 1300:1; there was no aggregate formation (Figure 2A). Dox is known to preferentially bind to 5′-GC-3′ or 5′-CG-3′ sequences.[37,38] Since nearly 1300 Dox molecules bind per plasmid DNA, we speculate that one Dox molecule could occupy 3−4 base pairs of DNA. In vitro drug release studies conducted at 37 °C in sterile PBS buffer confirmed that doxorubicin was stably complexed to plasmid and was slowly released (46 ± 0.5%), extending up to 96 h, compared to free doxorubicin (67 ± 15%), which was released rapidly within 6 h (Figure 2B). We next evaluated the stability of drug carrier (plasmid) before or after complexation by an in vitro serum stability test. At initial time points of 0.5 and 1 h, the complex retained both the DNA bands (open circular, supercoiled), while native plasmid showed only one DNA band (open circular form). The complex was stable at least up to 6 h, while native plasmid showed complete degradation at the time (Figure 2C). These results confirm that doxorubicin complexation can partially protect the native plasmid against serum nuclease degradation. Furthermore, we found that the pharmacokinetics of the complex was remarkably changed when intravenously administered (doxorubicin 5 mg/kg, plasmid 20 mg/kg). Doxorubicin levels in serum were much higher and decreased slowly up to 3 h as compared to doxorubicin alone (5 mg/kg), which was cleared within 1 h (0.059 ± 0.0075 μg/mL vs 0.018 ± 0.0058 μg/mL; Figure 2D). The area under curve for doxorubicin (5 mg/kg) was 10.41 μg·min/mL and 186.43 μg·min/mL for complex (doxorubicin 5 mg/kg, plasmid 20 mg/kg) respectively. The prolonged plasma retention of the complex indicates its intact nature and relative resistance to enzymatic degradation.

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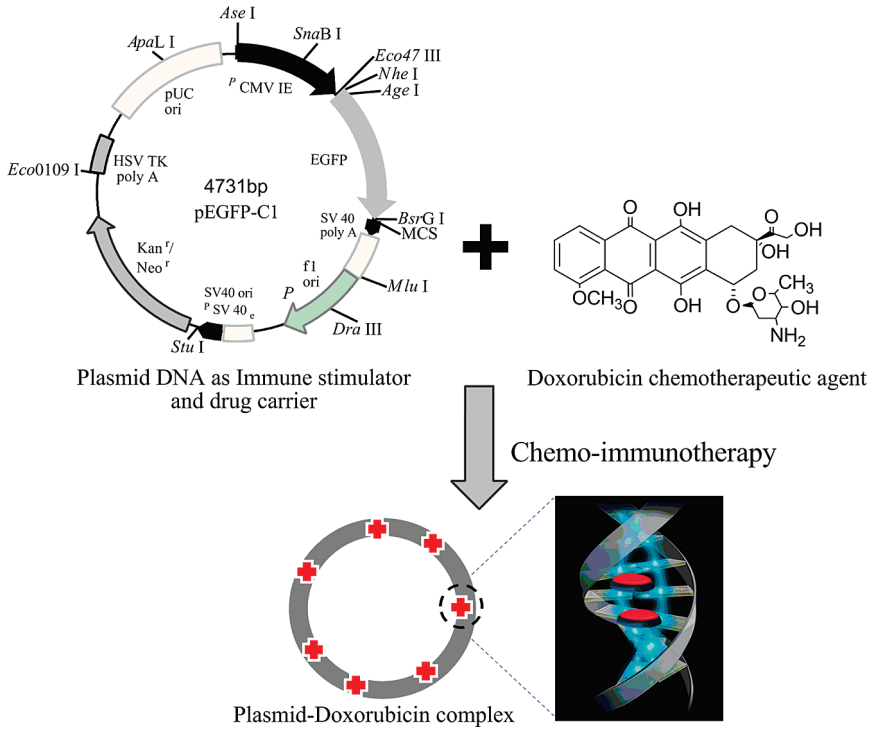

Figure 1. Graphic representation of plasmid−doxorubicin complex as a vehicle for chemoimmunotherapy-based cancer treatment.

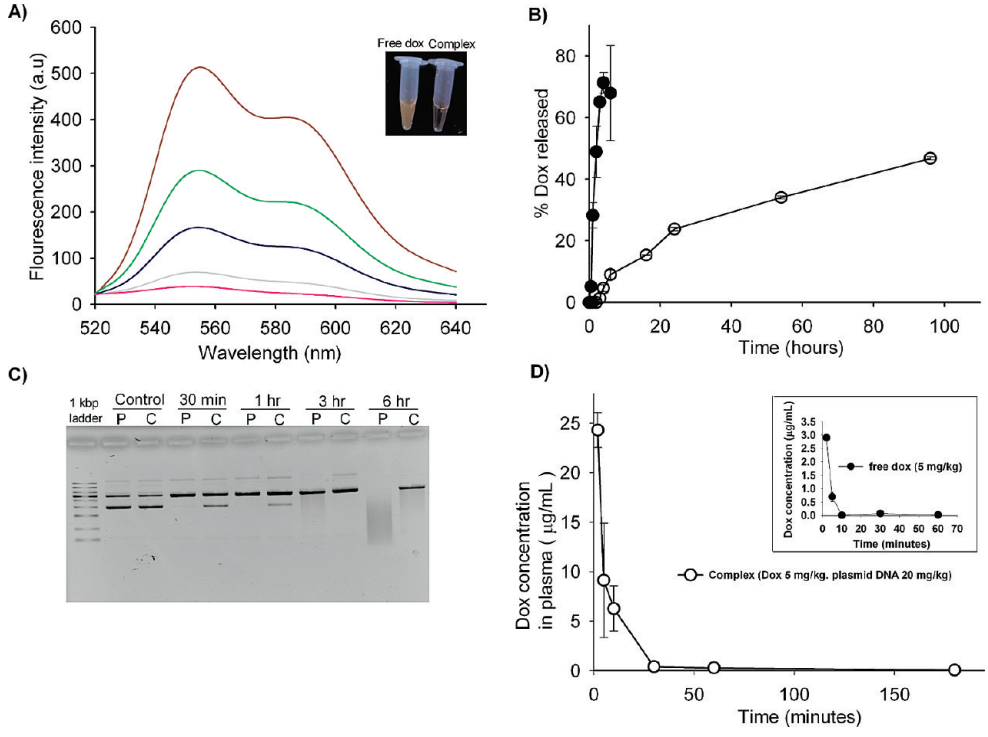

Figure 2. (A) Fluorescence spectra of doxorubicin (3 nmol) with increasing picomoles of plasmid (from top to bottom: 0, 0.57, 1.14, 1.71, and 2.28.); the inset shows flourescence quenching of plasmid−doxorubicin complex after excitation with a hand-held filtered UV lamp (VL-6LC, Vilber Laurmat, France). (B) Time-dependent in vitro release profile of free doxorubicin 125 μg (●; $n = 2$) and complex (plasmid 500 μg, doxorubicin 125 μg [○; $n = 2$]). (C) Serum stability of plasmid 20 μg and complex (plasmid 20 μg and doxorubicin 5 μg), incubated with mouse serum at 37 °C and analyzed at various time points by gel electrophoresis as described in Materials and Methods, P represents plasmid and C for complex. (D) Doxorubicin concentration−time profile in plasma of intravenously injected free doxorubicin 5 mg/kg (●; $n = 3$) and complex (plasmid 20 mg/kg, doxorubicin 5 mg/kg [○; $n = 3$]) in female BALB/c mice.

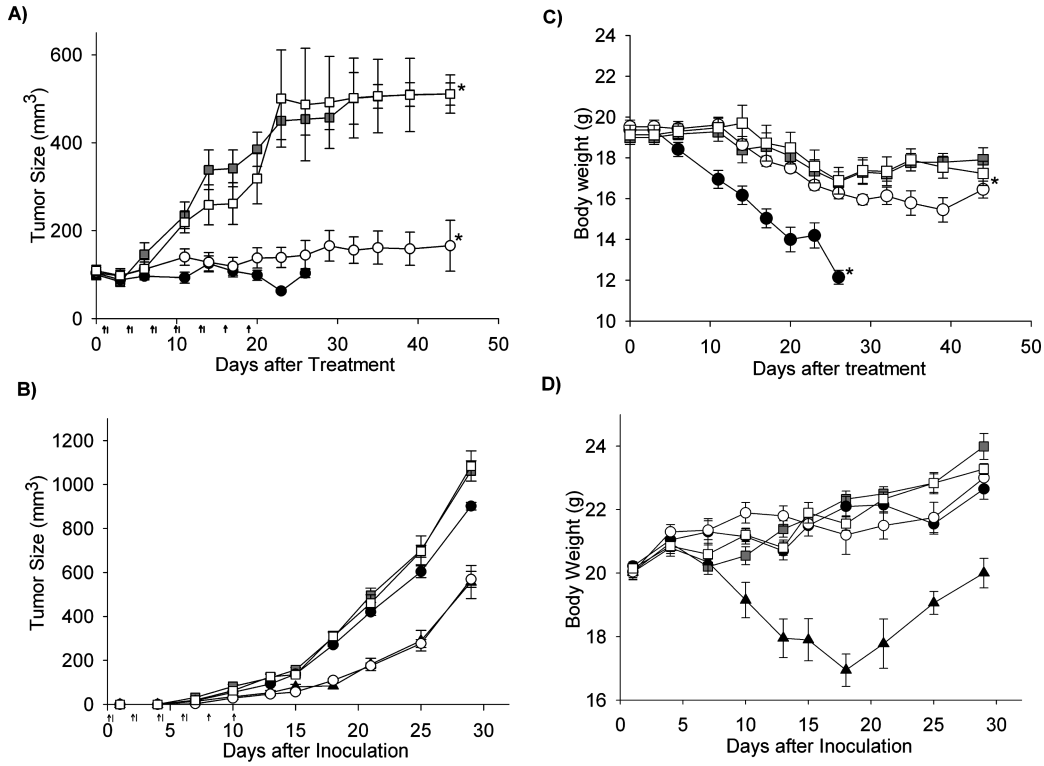

Figure 3. Tumor xenograft and allograft treatments and body weight measurements in mice. (A) Mice bearing NCI-H358 xenografts (8/group) were treated with seven intravenous injections every 3rd day of either plasmid 4 mg/kg (□) or complex (plasmid 4 mg/kg, doxorubicin 0.5 mg/kg) (○) or doxorubicin 5 mg/kg (●) (received five injections) in different groups as shown. Control animals were treated with 5% sterile glucose (gray boxes); arrows represent injection schedule for complex (↑) and doxorubicin 5 mg/kg (|) groups. Inhibition of tumor growth was significant with complex (* indicates $P < 0.001$ versus untreated control or plasmid alone) without any loss of mice as compared to free doxorubicin treatment that resulted in loss of 4 mice by day 26, and the remaining mice were sacrificed on day 27. (B) Mice (10/group) bearing 4T1 allografts were treated with six alternate-day intravenous injections of either doxorubicin 2 mg/kg (●) or plasmid 8 mg/kg (□) or complex (plasmid 8 mg/kg, doxorubicin 2 mg/kg) (○) and control (gray boxes), whereas the doxorubicin 4 mg/kg (▲) group received four alternate day injections. Arrows represent injection schedule for complex (↑) and doxorubicin 4 mg/kg (|) groups. (C) Body weight change in mice over 45 days after tumor inoculation treated with either doxorubicin 5 mg/kg or plasmid 4 mg/kg or complex (plasmid 4 mg/kg, doxorubicin 0.5 mg/kg) and control. Mice treated with doxorubicin 5 mg/kg showed significant body weight loss (* indicates $P < 0.001$ versus complex or plasmid), and all mice in the group were dead by day 27. (D) Body weight change in mice over 29 days after tumor inoculation treated with either doxorubicin 2 mg/kg or 4 mg/kg or plasmid 8 mg/kg or complex (plasmid 8 mg/kg, doxorubicin 2 mg/kg) and control.

Combined Chemoimmunotherapy in Mice Bearing NCI-H358 Xenograft Tumors. To test the efficacy of the present combination therapy, we chose two different tumor models for studying the effect of plasmid−Dox complex: a xenograft (NCI-H358 non-small-cell lung cancer) tumor model (immunocompromised) and 4T1 murine breast cancer model (immunocompetent). Xenograft models are frequently used to test the efficacy of TLR-9 agonists such as immunomodulatory oligonucleotides, and they do possess normal B cells, natural killer cells and macrophages to elicit immune responses.[39,40] In the case of mice bearing NCI-H358 xenografts at the end of the treatment period by day 45, animals that had received complex (doxorubicin 0.5 mg/kg, plasmid 4 mg/kg) and doxorubicin (5 mg/kg) had tumor volumes that were 68% and 77% smaller than the controls ($n = 8$; mean tumor volume ± SE: 144 ± 32.86 mm^3, 103 ± 10.21 mm^3 versus 453 ± 36.65 mm^3; $P < 0.001$; Figure 3A). Animals that received plasmid alone (4 mg/kg) and 5% sterile glucose-treated controls had similar tumor volumes (453 ± 36.65 mm^3 versus 486 ± 127.96 mm^3; Figure 3A). The antitumoral effect from the plasmid (4 mg/kg) alone was not observed. This could be attributed to result from lowered stability of plasmid alone in blood serum as observed in the pharmacokinetics of complex versus free Dox. Our results

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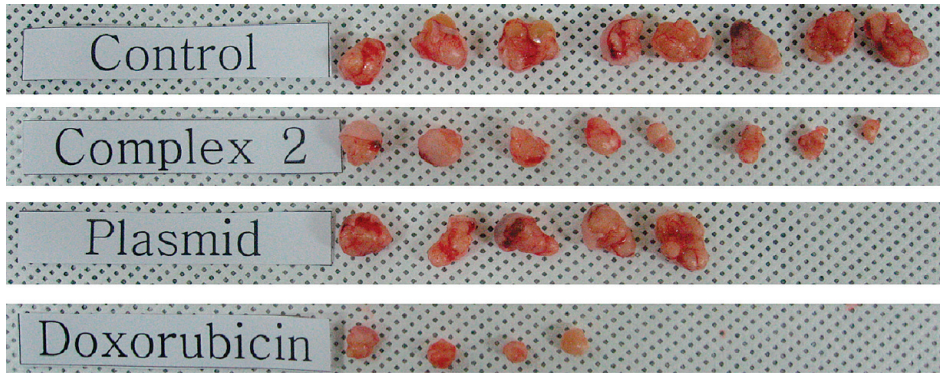

Figure 4. Excised tumors from NCI-H358 xenograft tumor bearing mice at 27th day that received seven intravenous injections every 3rd day of either plasmid 4 mg/kg or complex 2 (plasmid 4 mg/kg, doxorubicin 0.5 mg/kg) or doxorubicin (5 mg/kg group received five injections, tumors excised were stored at −70 °C and were imaged together with all other groups at end of study) in different groups as shown. Control animals were treated with 5% sterile glucose.

agree well with published data that supports the inefficacy of CpG alone against reducing tumor volume. Balsari et al. reported that indeed intravenous injection of CpG oligonucleotide (200 μg) did cause an elevation of interleukin-12 and interferon-γ but only results in 50% tumor volume reduction as compared to CpG plus topetecan (chemotherapeutic) treatment that resulted in 97% tumor reduction, and it was also shown in a syngeneic rat bifocal liver metastasis model that plasmid DNA injection without prodrug treatment was not sufficient to induce therapeutic effect in this model.[26,41]Body weights at the end of the study for the treatment groups varied between doxorubicin (5 mg/kg) and complex (doxorubicin 0.5 mg/kg, plasmid 4 mg/kg) compared to control or plasmid alone (4 mg/kg; $n = 8$; 12 ± 0.33 g, 16 ± 0.56 g versus 17 ± 0.58 g, 17 ± 0.59 g). Because the weight loss for mice in the free doxorubicin group was high, they underwent a five intravenous injection schedule instead of seven as for all other groups, with loss of all mice in the doxorubicin (5 mg/kg) group by day 26 (Figure 3C). Inhibition of tumor growth was significant with complex ($P < 0.001$ versus untreated control or plasmid alone) without any loss of mice as compared to free doxorubicin treatment (5 mg/kg) that resulted in loss of 4 mice by day 26. For ethical reasons, we humanely killed the remaining mice in the doxorubicin-treated group because of the severe toxicity (25% weight loss) at day 27 (Figure 4).

Combined Chemoimmunotherapy in Mice Bearing 4T1 Allograft Tumors. We then investigated whether the combination therapy was effective against 4T1 murine allograft tumors. By day 29, mice in the complex and doxorubicin (4 mg/kg) treatment groups showed similar reductions with tumor volumes, which were 46% and 47% smaller than the controls ($n = 10$; mean tumor volume ± SE: 556 ± 75.59 mm^3, 568 ± 36.06 mm^3 versus 1062 ± 46.22 mm^3; $P < 0.001$); however, at similar dose, doxorubicin (2 mg/kg) treatment groups showed only a 15% reduction ($n = 10$; 901 ± 17.32 mm^3 Figure 3B). This result indicates that the complex was more effective in reducing the growth of 4T1 murine tumors when compared to free drug at a similar dose (Figure 3B). Body weights varied between experimental groups free doxorubicin (4 mg/kg or 2 mg/kg), complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg), plasmid 8 mg/kg, and controls ($n = 10$; 20 ± 0.46 g, 22 ± 0.32 g, 23 ± 0.39 g, 23 ± 0.16 g, and 24 ± 0.40 g, respectively) (Figure 3D).

Administration of Complex Upregulated Th1 Cytokine Response. Both plasmid and doxorubicin used in this combination therapy are known to stimulate the production of cytokines such as IL-12 and IL-6.[14,34,42,43] We monitored the time-dependent level of cytokines (IL-12, IL-6, and IFN-γ) after a single intravenous administration of doxorubicin (2 mg/kg), plasmid (8 mg/kg), or complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) in the serum of female BALB/c mice. The levels of IL-12 and IFN-γ in the serum were significantly increased at 4 and 8 h for complex group as compared to doxorubicin alone that did not induce the production of both cytokines ($n = 3$; 39.7 ± 14.2 pg/mL versus 4.2 ± 1.4 pg/mL for IL-12 and 19 ± 3.89 pg/mL versus 0.82 ± 0.93 pg/mL for IFN-γ; Figure 5A,B). As reported in previous literature, doxorubicin alone (2 mg/kg) indeed induced a high level of IL-6 in serum as observed at 12 h; however, an additional effect was seen from the complex group containing both plasmid and doxorubicin ($n = 3$; 111 ± 19.03 pg/mL versus 196 ± 19.79 pg/mL; Figure

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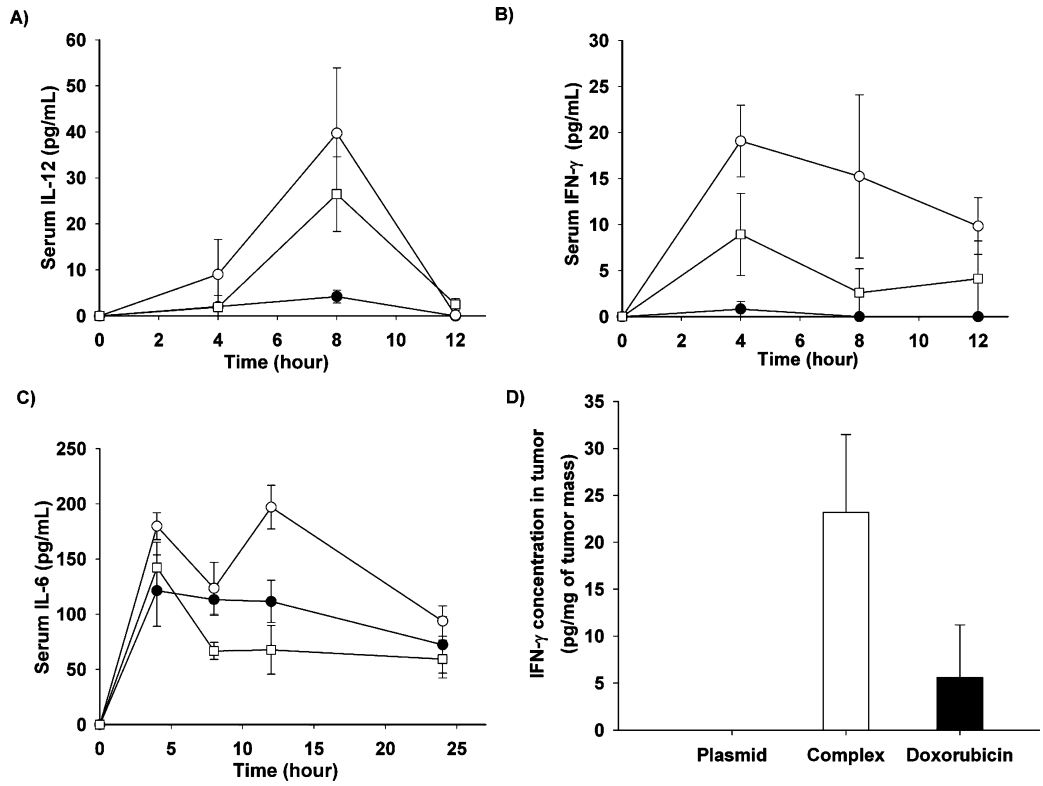

Figure 5. Time-dependent production of serum cytokine levels of (A) IL-12, (B) IFN-γ, and (C) IL-6 after treatment with single intravenous injection of doxorubicin 2 mg/kg (●), plasmid 8 mg/kg (□), or complex (plasmid 8 mg/kg, doxorubicin 2 mg/kg) (○); 3 mice/group. (D) Tumor cytokine level of IFN-γ measured at 24 h after treatment with doxorubicin, plasmid, and complex.

5C). Since IFN- γ is known to cause immune mediated tumor suppression when expressed in tumors,[44] we were interested in monitoring the levels of this cytokine in the tumors and in its role in causing antitumor efficacy. Indeed, the amounts of IFN-γ in tumor tissue were significantly increased at 24 h after complex (doxorubicin 2 mg/kg, plasmid 8 mg/kg) injection as compared to doxorubicin (2 mg/kg) alone ($n = 5$; 23.18 ± 8.2 pg/mg tumor versus $n = 3$; 5.5 ± 5.5 pg/mg tumor; Figure 5D). In contrast, we could not observe any detectable amounts of IFN-γ in the tumors of mice that received naked plasmid (8 mg/kg). Although the tumor cytokine assay was carried out for early time points of 7 h, 12 h, after treatment (data not shown), we could not observe any significant change of IFN-γ level in tumor tissue that was blanked with control tumor tissue. Taken together, effective antitumor effect of complex seems to be attributed to synergistic effect of chemotherapeutic doxorubicin and immunostimulant plasmid.

Discussion

The present study demonstrates the proof of concept for a combined chemoimmunotherapy approach. Entrapping or conjugating drugs to synthetic biocompatible polymeric carriers or natural polymers such as RNA ensures efficient drug delivery and, in turn, minimizes the adverse toxicity of drugs.[45−47] In this regard, we used a plasmid as a drug carrier in that it can load large amounts of doxorubicin without need of chemical conjugations or treatments and form a stable complex. We found that the stability of naked plasmid was markedly enhanced in the form of complex with doxorubicin as revealed in the in vitro serum stability assay and in the in vivo pharmacokinetics data. Immunotherapy approaches actively use synthetic CpG oligonucleotides that can activate DCs; such activated DCs interact with T cells and result in priming an efficient cytotoxic T-cell response.[5,48] Since both plasmid that contains 27 unmethylated CpG sequences and doxorubicin are known to stimulate the production of several cytokines in parallel, we expected that the complex could be used for immunotherapy as well as chemotherapy owing to doxorubicin.[34,42] Indeed, systemic

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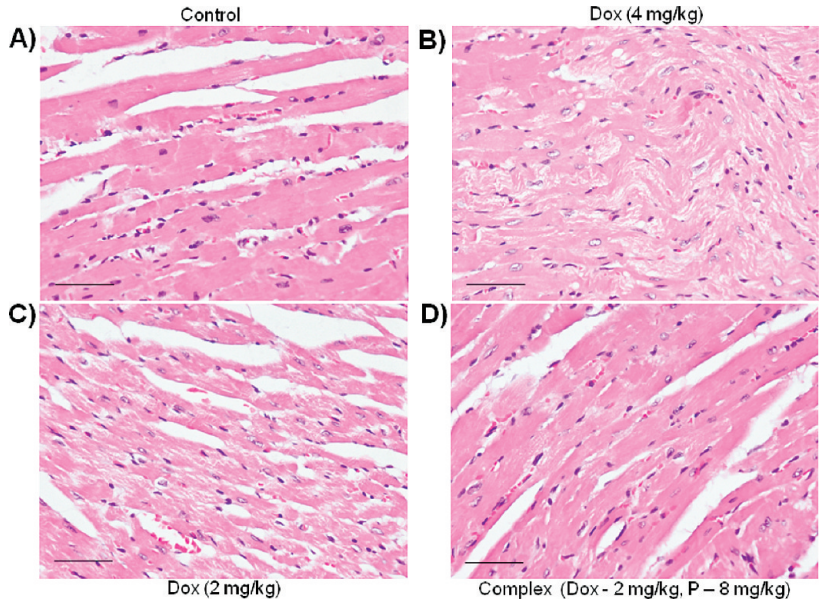

Figure 6. Histologic appearance of heart tissues (n = 10/group) by light microscopy isolated on day 13 after a single intravenous injection of sterile 5% glucose (control; A), doxorubicin (4 mg/kg; B), doxorubicin (2 mg/kg; C), and complex (plasmid 8 mg/kg, doxorubicin 2 mg/kg; D). Hematoxylin and Eosin stain; magnification ×400; scale bar = 50 μm; Dox (doxorubicin); P (plasmid).

administration of the complex delivering both agents (chemo- and immunotherapeutics) simultaneously caused effective tumor reduction in both NCI-H358 xenografts and 4T1 murine allografts. The higher antitumor effect of the complex, on one hand, may be attributed to its higher stability in plasma as compared to naked plasmid alone, which was found to be ineffective, similar to reports where nonviral gene delivery vehicles such as cationic lipid−DNA complexes, liposome that both protect and prolong circulation time of DNA can increase interactions of DNA with antigen presenting cells such as macrophages and promote potent antitumor responses through activation of cytotoxic host effector cells.[49,50] The cytokines such as IL-12 and IFN-γ have shown antitumor activity in various animal tumor models and can cause increased proliferation of natural killer cells and T cells.[51,52] We observed elevated cytokine levels of IL-12 and IFN-γ after administration of the complex, which clearly indicates its effect on immunostimulation as compared to naked plasmid alone.[53,54] The main goals of such combined chemoimmunotherapy approaches are to integrate tumor cell killing and antigen release with an activated immune system that, by default, is tolerant.[55] By combining such chemotherapy (doxorubicin) with immunostimulatory plasmid, we observed reduced tumor growth in both xenograft and allograft tumor models but not a complete tumor reduction. Most importantly, we were able to substantially reduce the effective chemotherapy dose, an important factor in the context of dose-dependent cytotoxicity. Doxorubicin is widely used to treat a variety of neoplasms, such as breast, ovarian, prostate, bladder, gastric, and bronchogenic carcinomas.[56] However, it is also associated with acute as well as cumulative dose-dependent cardiotox-

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icities, and this limits its clinical efficacy.[57−59] Doxorubicin cardiotoxicity causes myocyte apoptosis and was shown to be linked to intracellular hydrogen peroxide formation.[60] Therefore we have evaluated the safety of our plasmid−doxorubicin complex in this context. Because doxorubicin is released from the complex at a slower rate than its free form as confirmed by the in vitro drug release, such acute toxicities to tissues (e.g., heart) might be reduced as compared to free doxorubicin (2 mg/kg and 4 mg/kg). Indeed, histopathologic analysis after administration of complex resulted in no visible loss of myocardial tissue (Figure 6). Degenerative changes indicated by myofibrillary loss, disarray, and cytoplasmic vacuolization are seen in doxorubicin-treated groups (Figures 6B and 6C), compared to the control and complex groups (Figure 6A and 6D).

In conclusion, we have shown that the combined chemoimmunotherapy approach through systemic administration of the bare plasmid−doxorubicin complex is safe and effectively reduces tumor growth.

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