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# Systemic Gene Therapy in Human Xenograft Tumor Models by Liposomal Delivery of the *E1A* Gene[1]

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## ABSTRACT

**The adenovirus type 5 E1A protein has been demonstrated to elicit antitumor effects through the induction of apoptosis, inhibition of cell cycle progression, induction of differentiated epithelial phenotypes, repression of oncogene expression and function, and sensitization to chemotherapeutic agents and radiation. These unique properties have led to use of the *E1A* gene in adenoviral and lipid-based gene therapy systems, and it has demonstrated antitumor effects in tumor xenograft model systems. However, the delivery systems used in those studies are best suited for local or intratumoral delivery rather than systemic delivery. Because the effective treatment of many primary tumors as well as metastatic disease requires systemic delivery systems, a novel gene delivery system composed of liposome/protamine/DNA (LPD) was investigated for systemic delivery of the *E1A* gene. Athymic nude mice bearing human breast (MDA-MB-361) or head and neck (WSUHN-31) tumor xenografts were treated i.v. with LPD-*E1A*, and the expression of E1A protein and effects on tumor growth were assessed. In the MDA-MB-361 breast model, expression of E1A protein was detected in the tumors after LPD-*E1A* treatment, which was associated with down-regulation of HER-2/*neu* protein expression and the presence of apoptotic cells. Tumor volume was also smaller in mice treated with LPD-*E1A* than in controls in both of the xenograft models. Lastly, LPD-*E1A* in combination with paclitaxel was more effective than LPD-*E1A* or paclitaxel alone in the MDA-MB-361 model. Additional preclinical and clinical development of LPD-*E1A* is warranted for the treatment of advanced or metastatic cancer.**

## INTRODUCTION

The adenovirus type 5 E1A protein, which is transcribed and translated early after adenoviral infection, is a potent tumor inhibitor in certain types of human cancer (1). E1A protein inhibits tumor growth by inducing apoptosis (2), affecting cell cycle progression, inducing differentiated epithelial phenotypes, repressing HER-2/*neu* overexpression, inhibiting angiogenesis (3), and sensitizing cells to chemotherapeutic agents and radiation (4, 5). Because E1A can elicit antitumor effects regardless of the genetic background of the tumor and in a variety of tumor types, it is an ideal candidate for cancer gene therapy regimens, used either as a single agent or in combination with chemotherapy or radiation.

Several preclinical studies in which the *E1A* gene, delivered by either adenoviral vectors or cationic liposomes, has been used to treat localized tumors have demonstrated suppression of tumor growth (2, 4, 5). In these studies, E1A evoked strong proapoptotic responses within the tumor that ultimately led to tumor growth delays and regression. On the basis of these findings, a Phase I clinical trial was initiated in which the *E1A* gene was delivered by lipoplex, a cationic DC-Chol:DOPE liposome-based delivery system (DCC-*E1A*[3]). In that trial, patients with breast or ovarian cancer were treated with DCC-*E1A* liposome complexes administered into the thoracic or peritoneal cavity. Results demonstrated successful transfection and expression of E1A, decreased expression of the HER-2/*neu* oncogene, and the presence of apoptotic cells within the tumors (6). Similar findings were observed in a Phase I clinical trial of DCC-*E1A* liposome complexes administered directly into tumors of the head and neck (7).

Although these results are encouraging, gene delivery via a lipoplex system such as the DCC liposome-based system has thus far been limited to either intratumoral or regional intracavitary delivery (6–8). Systemic gene delivery using DCC liposomes may be of limited effectiveness because of the inherent sensitivity of these complexes to serum, which may reduce transfection efficiency (9). Furthermore, mass production of a liposome/DNA complex (such as would be required for development as a pharmaceutical product) would require that the complex be chemically stable. Also, lipoplex are relatively unstable, and transfection reagents need to be freshly prepared before use. Therefore, we have developed a new lipid formulation based on a lipopolyplex, LPD, which demonstrated improved stability and enhanced transfection efficiency (9–14). Early studies with the LPD complexes demonstrated that i.v. administration through the lateral tail vein of nude mice facilitated the delivery of the DNA to distant organ sites such as lung or liver (9, 10). Thus, the LPD system is an attractive candidate for the systemic delivery of therapeutic genes to treat advanced or metastatic cancer. In the current study, we examined the transfection capabilities and antitumor effects of systemically delivered LPD-*E1A* in tumor xenograft models of breast cancer and head and neck cancer. Our results demonstrate that LPD led to effective delivery and subsequent expression of E1A at the tumor site, as well as reducing HER-2/*neu* protein expression and inducing apoptosis. Suppressed tumor growth and increased survival were also observed in the animals treated with LPD-*E1A*, either alone or in combination with paclitaxel. These results provide justification for continuing the preclinical and clinical development of LPD-*E1A* as a novel therapeutic approach for the treatment of both primary and metastatic cancer.

## MATERIALS AND METHODS

**Cell Lines and Culture Conditions.** The human breast adenocarcinoma cell line MDA-MB-361 was obtained from American Type Culture Collection (Manassas, VA), grown in DMEM/F12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum and penicillin-streptomycin, and maintained in a humidified incubator at 37°C containing 5%

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[3] The abbreviations used are: DCC, 3$\beta$-[*N*-(*N*′,*N*′dimethylaminoethyl) carbamoyl] cholesterol; LPD, liposome/protamine/DNA; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; DOTAP, *N*-[1-(2,3-dioleoyloxyl)propyl]-*N*,*N*,*N*-trimethylammoniummethyl sulfate.

$CO_2$. The human head and neck squamous carcinoma cell line WSUHN-31 was generously provided by Dr. John Ensley (Wayne State University, Detroit, MI), grown in DMEM medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and penicillin/streptomycin, and maintained in a humidified incubator at 37°C containing 10% $CO_2$. The MDA-MB-361 cell line expresses high levels of HER-2/*neu* (15), whereas the WSUHN-31 cell line expresses minimal levels of HER-2/*neu* (data not shown).

**Paclitaxel.** A stock solution of paclitaxel (Bristol-Myers Squibb Co., Wallingford, CT) was stored at −80°C before use. At the time of its use, paclitaxel was diluted in PBS to administer three doses of 15 mg/kg in 250 μl, given i.p. or i.v., once every 3 weeks.

**Preparation of Cationic Liposomes and LPD.** Liposomes containing DOTAP (Avanti Polar Lipids Inc., Alabaster, AL) in a 1:1 molar ratio with cholesterol (Sigma Chemical Co., St. Louis, MO or Avanti Polar Lipids Inc.) were prepared in chloroform. The lipid mixture was then dried as a thin layer in a 100-ml round-bottomed flask or glass bottle under a stream of $N_2$. The resulting lipid film was hydrated in 5% dextrose, and briefly heated and sonicated until solubilized completely. The lipid solution was extensively vortexed, incubated at 50°C for 10 min, and then sequentially extruded through polycarbonate membranes with pore sizes of 1.0, 0.6, and 0.1 μm to generate small unilaminar vesicles. Alternatively, liposomes were prepared by sonication. To prepare the LPD, the E1A expression vector pE1A-k2 (16) was combined with protamine sulfate dropwise. The *E1A*-protamine mixture was then added to the DOTAP-cholesterol liposomes to yield a final ratio of 1.0 μg protamine sulfate:12 nmol DOTAP:1 μg DNA (9, 10). LPD size was determined to be in the range of 150–250 nm.

**Athymic Nude Mice.** Four to 6-week-old female athymic BALB/c-*nu/nu* mice were purchased from the Animal Production Facility at the National Cancer Institute-Frederick Cancer Research Center (Frederick, MD), from B&K Universal Inc. (Kent, WA), or from Harlan Sprague Dawley (Indianapolis, IN). The animals were allowed to acclimate for 7 days before the study initiation. All of the animals were housed under pathogen-free conditions, and were given water and chow *ad libitum*. Animal care and use were in accordance with Institutional and NIH guidelines.

**WSUHN-31 Head and Neck Cancer Xenograft Model.** WSUHN-31 cells, in log-phase growth, were trypsinized and washed twice with PBS. Cell viability was determined by trypan blue exclusion and cells resuspended in PBS to a final concentration of $2.5 \times 10^7$ cells/ml. A total of $5 \times 10^6$ cells in 0.2 ml of PBS were injected s.c. above the scapula of nude mice under aseptic conditions. Six days after inoculation of the tumor cells, nude mice bearing tumors were treated weekly with vehicle only (DOTAP-cholesterol/protamine sulfate liposome, Fig. 2, *triangles*), with liposome-*E1A* (20 μg DNA, Fig. 2, *squares*), or with LPD-*E1A* (20 μg DNA, Fig. 2, *circles*; 10 mice/group), and i.v. administration of the different formulations was initiated. The formulations were injected once weekly for 11 weeks. Tumor volume was measured weekly over the course of the experiment. The tumor volume ($mm^3$) was calculated by multiplying the length, width, and depth of each tumor, and then dividing by 2 [(L × W × D)/2]. When tumor weight exceeded 10% of the body weight, the mice were euthanized by overdose with $CO_2$. Statistical analysis to compare tumor sizes was performed with Student's two-tailed *t* test.

**MDA-MB-361 Breast Cancer Xenograft Model.** MDA-MB-361 cells, in log-phase growth, were trypsinized and washed twice with PBS. Cell viability was determined by trypan blue exclusion and cells resuspended in PBS to a final concentration of $1–2 \times 10^7$ cells/ml. A total of $2–4 \times 10^6$ cells in 0.2–0.5 ml of PBS were injected under aseptic conditions into the mammary fat pad of nude mice. After tumors reached ≥0.5 mm in diameter, the tumor-bearing mice were randomly assigned to treatment groups and treatment initiated. In the first experiment, vehicle, plasmid, or LPD complexes were administered once weekly for 7 weeks, and chemotherapy (three 15-mg/kg doses of paclitaxel) was administered i.p. once every 3 weeks. In the second experiment, vehicle or LPD complexes were administered once weekly for 9 weeks, and chemotherapy (three doses of paclitaxel, at a dose of 15 mg/kg) was administered i.v. once every 3 weeks. In both studies, tumor size was measured once weekly. The tumor ratio was calculated by computing the original (pretreatment) size and comparing it to tumor size measured at various times after treatment (Fig. 3). Tumor volume ($mm^3$) was calculated as described above. Changes in tumor ratios and volumes were tested for statistical significance with the Mann-Whitney test or Student's two-tailed *t* test.

**Immunohistochemical Analysis.** Expression of HER-2/*neu* and E1A protein levels in established MDA-MB-361 tumors was evaluated by immunohistochemical staining after treatment with LPD-*E1A*, paclitaxel, or both. Briefly, tumors were harvested 18–48 h after LPD-*E1A* administration or, in the case of combination therapy, LPD-*E1A* treatment was followed 24 h later by i.p. administration of paclitaxel and tumors harvested 18–48 h after the paclitaxel treatment. The sections were incubated with c-*erbB2* polyclonal antibody (DAKO Corp., Carpinteria, CA) diluted 1:300 or E1A M73 monoclonal antibody (Oncogene Science, Inc., Cambridge, MA) diluted 1:20. The slides were then incubated with biotinylated goat antirabbit IgG (Vector Laboratories) or biotinylated horse antimouse IgG (Vector Laboratories) diluted 1:200 in PBS. The slides were then incubated with an avidin-biotin-peroxidase complex (Vector Laboratories), and the peroxidase-catalyzed product was visualized with 0.125% aminoethyl carbazole chromogen buffer (Sigma Chemical Co.). To maintain interassay and intra-assay consistency among each batch of stained slides, a negative control (in which the primary antibody was replaced with an isotype-match-irrelevant IgG) and a positive control (a slide identified previously as having intense tumor cell staining) were stained alongside the experimental samples. Intensity of E1A and HER-2/*neu* staining were determined as described previously (6). Data are presented from 3 animals/group.

**Apoptosis Detection by the TUNEL Assay.** The presence of apoptotic cells within the tumor sections was evaluated by TUNEL assay as described previously (6). Percent apoptosis was determined by counting the number of apoptotic cells and dividing by the total number of cells in the field (9 high power fields/slide). Data are shown for 3 animals/group.

## RESULTS

**Expression and Function of E1A Protein after Systemic Administration of LPD-*E1A*.** We first tested whether *E1A* can be delivered by the LPD formulation. Immunohistochemical analysis was performed on tumor tissue sections obtained from mice bearing MDA-MB-361 tumors 48 h after treatment. Animals treated i.v. with LPD-*E1A* (20 μg DNA) demonstrated expression of E1A protein within the peritumoral areas, whereas in the control-treated animals, no E1A expression was observed (Fig. 1). Because E1A functions to down-regulate HER-2/*neu* oncogene expression and induce apoptosis, additional sections and variables were assessed (17). Coinciding with the expression of E1A was a down-regulation of HER-2/*neu* protein expression within the peritumoral area in the LPD-*E1A*-treated samples, whereas intense staining was still present in the control-treated samples (Fig. 1, *bottom panels*). Sections from the LPD-*E1A*-treated animals also demonstrated the presence of TUNEL-positive, apoptotic tumor cells in the E1A-transfected area (Fig. 1, *bottom panels*). Again, in tumor tissue sections obtained from control mice treated with LPD-*luciferase* (control DNA), no E1A expression, down-regulation of HER-2/*neu* expression, or apoptosis induction were observed (Fig. 1, *top panels*). These results clearly demonstrate the expression and function of E1A delivered to the distant tumor site by systemic administration of LPD.

***In Vivo* Growth Suppression of Head and Neck Tumors by LPD-*E1A*.** After we confirmed that E1A protein could be expressed in xenografted MDA-MB-361 tumors after i.v administration of LPD-*E1A*, we examined potential antitumor effects of E1A in a WSUHN-31 head and neck xenograft model. This model was specifically used to demonstrate that the effects of E1A do not depend on HER-2/*neu* expression levels, because this cell line expresses only minimal levels of HER-2/*neu in vitro* (data not shown). LPD-*E1A* (20 μg DNA) elicited a strong antitumor effect, significantly suppressing tumor growth after 11 weekly injections as compared with the control vehicle (Fig. 2; $P = 0.005$). Moreover, 8 of 10 mice treated with LPD-*E1A* were tumor-free at week 11, whereas only 1 of 10 vehicle-treated mice remained tumor-free. Administration of liposomal *E1A* also inhibited tumor growth, but only 6 of 10 mice remained tumor-free.

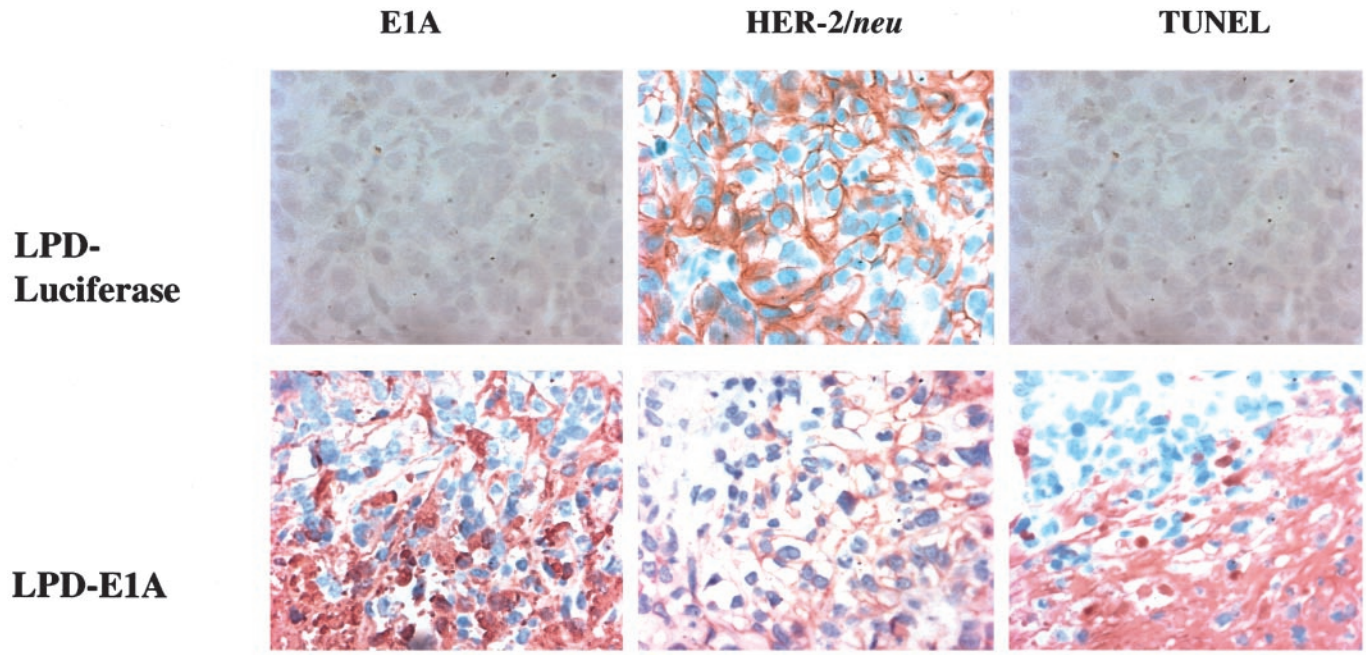


Fig. 1. Immunohistochemical analysis of MDA-MB-361 tumor tissue from mice treated with LPD-*E1A*. Tumor-bearing mice were treated with i.v. LPD-*E1A* or LPD-*luciferase* (20 $\mu$g DNA), and tumor tissues were harvested 48 h later. Expression of E1A protein was associated with down-regulation of HER-2/*neu* protein expression and the presence of TUNEL-positive apoptotic cells. *Top panels*, LPD-*luciferase* samples; *bottom panels*, LPD-*E1A*-treated samples.

**LPD-*E1A* Enhances the *in Vivo* Chemosensitivity of Breast Cancer Xenografts to Paclitaxel.** Previous preclinical experiments have shown that HER-2/*neu*-overexpressing breast cancer cell lines are resistant to paclitaxel (18–21). Previous results from our laboratory also demonstrated that E1A expression can overcome this resistance and sensitize HER-2/*neu*-overexpressing cancer cells by HER-2/*neu* down-regulation (4, 18). On the basis of these *in vitro* findings, we examined the possibility that systemic *E1A* gene therapy could enhance the efficacy of paclitaxel in the MDA-MB-361 breast cancer xenograft model. Treatment with 20 $\mu$g naked E1A DNA, vehicle, or LPD-*luciferase* (20 $\mu$g DNA) did not suppress tumor growth (Fig. 3). However, treatment with either LPD-*E1A* (20 $\mu$g DNA; $P = 0.0253$) or paclitaxel (15 mg/kg, i.p.; $P = 0.0644$) decreased tumor growth compared with LPD-*luciferase* (20 $\mu$g DNA). The combination of LPD-*E1A* and paclitaxel elicited the greatest antitumor effect compared with LPD-*luciferase* ($P = 0.0071$), paclitaxel ($P = 0.0104$), or LPD-*E1A* ($P = 0.0490$). To verify these observations, an additional experiment was performed to assess the effects of LPD-*E1A* alone and in combination with i.p. paclitaxel in the MDA-MB-361 xenograft model. Again, paclitaxel (15 mg/kg), LPD-*E1A* (20 $\mu$g DNA), and the combination of LPD-*E1A* (20 $\mu$g DNA) with paclitaxel (15 mg/kg) suppressed tumor growth compared with the vehicle control group ($P < 0.04$; data not shown). Finally, significantly fewer tumors were present in the animals treated with both paclitaxel and LPD-*E1A* as compared with the other treatment groups ($P = 0.0106$). The combination of LPD-*E1A* and paclitaxel clearly enhanced the suppression of tumor growth over that of either agent alone, demonstrating robust additive effects between the two agents.

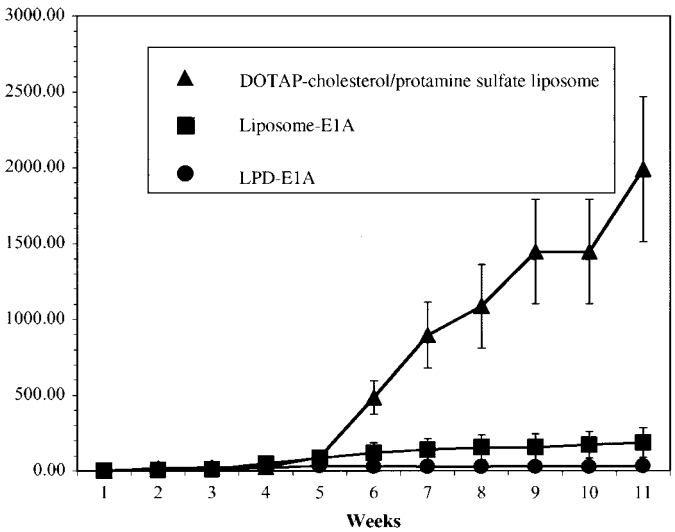


Fig. 2. Suppression of tumor growth in the head and neck xenograft model. Nude mice bearing WSUHN-31 low HER-2/*neu*-expressing tumor xenografts were treated weekly with vehicle only (DOTAP-cholesterol/protamine sulfate liposome, ▲), with liposome-*E1A* (20 $\mu$g DNA, ■), or with LPD-*E1A* (20 $\mu$g DNA, ●). Tumors were measured weekly and represented as average tumor volume ($mm^3$; $P = 0.005$); *bars*, ±SE.

**The Additive Effects between LPD-*E1A* and Paclitaxel Occur through Apoptosis Induction.** Previous preclinical experiments demonstrated that HER-2/*neu*-overexpressing breast cancer cell lines are resistant to paclitaxel and that *E1A* sensitized HER-2/*neu*-overexpressing cancer cells by HER-2/*neu* down-regulation (4, 18). Therefore, to assess the cause of the additive effects of LPD-*E1A* and paclitaxel, we evaluated apoptosis induction in the tumor sections 36 h after combined treatment, where i.p. paclitaxel was administered 24 h after the i.v. injection of LPD-*E1A*. Quantification of apoptotic cells within the tumor sections revealed a significant increase in apoptosis in the tumors treated with both LPD-*E1A* and paclitaxel (mean 26.3%) compared with tumors treated with either LPD-*E1A* (mean 8.7%; $P = 0.0065$) or paclitaxel (mean 6.3%; $P = 0.0037$; Fig. 4). These results suggest that LPD-*E1A* enhanced the sensitivity of HER-2/*neu*-overexpressing human breast cancer cells to paclitaxel through apoptotic mechanisms.

## DISCUSSION

The antitumor effects of the *E1A* gene have been demonstrated in many studies using different cells lines from numerous tumor back-

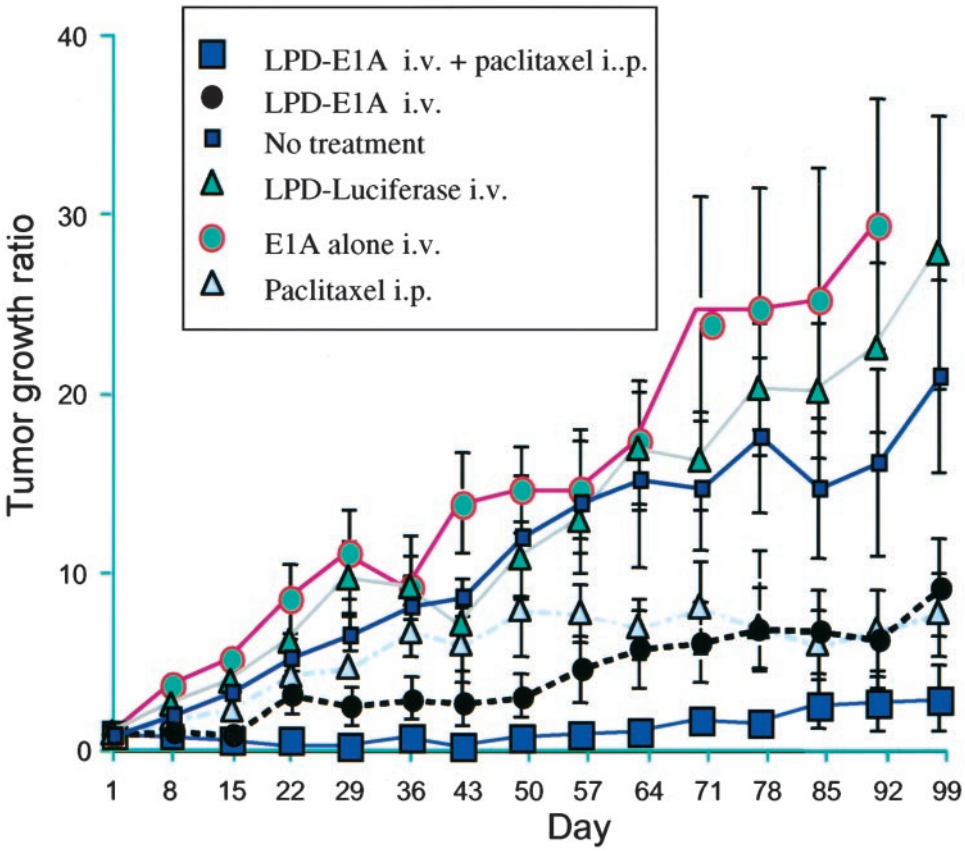


Fig. 3. LPD-*E1A*, given alone or in combination with paclitaxel, decreased tumor growth in HER-2/*neu*-overexpressing tumor xenografts. Female nude mice were inoculated with HER-2/*neu*-overexpressing MDA-MB-361 cells in the mammary fat pad. After the formation of 8–10 tumors/group (5 animals/group, ~2 tumors/animal), mice were given one of the following treatments: naked *E1A* DNA (20 μg DNA, *light circles*), vehicle only (*small squares*), LPD-*luciferase* (20 μg DNA, *dark triangles*), LPD-*E1A* (20 μg DNA, *dark circles*), paclitaxel (15 mg/kg, *light triangles*), or LPD-*E1A* (20 μg DNA) plus paclitaxel (15 mg/kg; *large squares*). The tumor growth ratio was determined by comparing the size of the treated tumors at various times with the size of the original tumor before any treatment; *bars*, ±SE.

grounds (1, 16, 22, 23). This unique ability to elicit a generalized antitumor response has attracted attention on the potential of *E1A* for cancer gene therapy. Delivery of *E1A* by cationic liposomes suppressed the growth of HER-2/*neu* overexpressing ovarian and breast tumor xenografts in nude mice, in part through the down-regulation of the HER-2/*neu* oncogene (16, 24). The validity of lipid-based gene delivery is also supported by previous studies using DOTAP:cholesterol liposomes to deliver tumor suppressor genes (*e.g.*, *p53*) and inhibit tumorigenicity (25, 26).

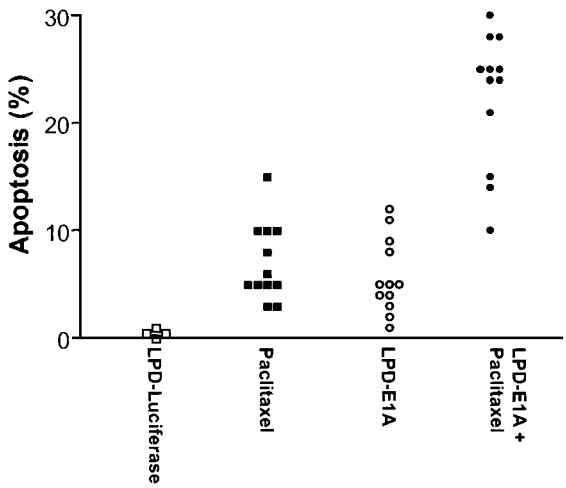


Fig. 4. Apoptosis induction in tumor sections after treatment. Tumor-bearing animals were treated as described in "Materials and Methods," and tumors were harvested and prepared for TUNEL analysis. The number of apoptotic cells was determined by counting nine fields per slide from 3 animals/group. The combination of LPD-*E1A* plus paclitaxel increased the number of apoptotic cells in breast tumor sections relative to either LPD-*E1A* or paclitaxel alone.

Compared with lipoplexes (DOTAP:cholesterol or liposomes), lipopolyplexes such as the LPD-based gene delivery system are well suited for systemic delivery. In the study reported here, *E1A* liposome (lipoplexes) treatment in the head and neck xenograft model did suppress tumor growth. Although the extent of suppression was not significantly different from that produced by LPD-*E1A*, greater heterogeneity in the antitumor effects was observed, and greater numbers of mice possessed tumors (40% for *E1A* liposomes *versus* 20% for LPD-*E1A*). This finding of equivalent tumor suppression may be related to the E1A bystander effect (3) despite of possible lower transfection efficiency by the lipoplex (9). Lipoplexes are heterogeneous in size and tend to aggregate over time. In contrast, LPD are well-defined particles that are homogenous in size. They contain a core of protamine-condensed DNA coated with lipid bilayer. They can be kept at 4°C for >1 month or lyophilized and kept at room temperature for more than 6 months without any obvious changes in biophysical and biological properties (27). This may account for a higher and more consistent *in vivo* transfection efficiency for LPD compared with lipoplex.

Preliminary studies using LPD-based gene delivery have demonstrated effective transduction and gene product expression *in vivo* (9, 10). For instance, previous studies demonstrated that i.v. administration of LPD-*luciferase* to mice produced the highest luciferase activity in lung, followed by activity in the liver and spleen, indicating that gene delivery is depends on the vascular density of the target tissue (9, 10). With regard to gene expression within tumors, spontaneously arising lung tumors in retinoblastoma-deficient mice have been transfected efficiently after i.v. administration of an LPD-*retinoblastoma* complex (12). In our study, luciferase expression was detected in s.c. tumors after i.v. administration of LPD-*luciferase* with maximal expression at 6 h after administration (data not shown). Also in the present study, LPD-*E1A*, given either alone or in combination with

paclitaxel, resulted in tumor suppression and apoptosis induction, which is in accordance with results published previously (4, 18–20). However, treatment with LPD-*E1A* alone did not completely eradicate the tumors in the xenograft models examined and suggests the possibility of limited transfection efficiency at the tumor sites. Because it is well known that human tumor xenografts in immunodeficient mice have limited vasculature at the surface of the engrafted tumor and because luciferase expression was observed predominantly within highly vascularized tissues in previous studies, the delivery of LPD-*E1A* to the tumor site in the nonxenograft tumor model may be much higher, but additional study is needed (9, 10).

One potential concern regarding systemic gene therapy with lipid-based formulations, including LPD, is the associated cytokine immunotoxicity. Several reports have described the transient activation of nonspecific immune responses, such as production of the proinflammatory cytokines IFN-$\gamma$ and tumor necrosis factor $\alpha$, from bacterially derived DNA containing unmethylated CpG motifs (11, 28, 29). When the plasmid DNA was methylated and then complexed with cationic lipids, the associated toxicity was significantly reduced (29). However, the magnitude of the cytokine response can be modulated by varying the DNA dose, and a moderate immune response has been shown to be beneficial for the tumor treatment in a synergeneic model (30). Indeed, when 20 $\mu$g of DNA was administered into each mouse for 11 consecutive weeks (Figs. 2 and 3), no obvious toxicity was seen in our study. Additional studies are planned to test the induction of cytokine profiles and safety endpoints after LPD-*E1A* administration at various doses.

In summary, current cancer gene therapy protocols and agents are restricted to local or regional delivery to specific tumor sites because of either lack of serum stability or toxicity of the agent when delivered systemically. Adequate treatment of cancer requires a systemic delivery system such as LPD. The results in this report clearly demonstrate the efficacy and future promise of systemically delivered LPD-*E1A* in the treatment of breast, and head and neck cancer. Certainly these results provide ample justification for the continued preclinical and clinical development of LPD-*E1A* as a novel therapeutic agent for primary and metastatic cancer treatment, given either alone or in combination with chemotherapy and radiation.

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