

Anti-EGFR immunonanoparticles containing IL12 and salmosin genes for targeted cancer gene therapy

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Abstract. Tumor-directed gene delivery is of major interest in the field of cancer gene therapy. Varied functionalizations of non-viral vectors have been suggested to enhance tumor targetability. In the present study, we prepared two different types of anti-EGF receptor (EGFR) immunonanoparticles containing pDNA, neutrally charged liposomes and cationic lipoplexes, for tumor-directed transfection of cancer therapeutic genes. Even though both anti-EGFR immunonanoparticles had a high binding affinity to the EGFR-positive cancer cells, the anti-EGFR immunolipoplex formulation exhibited approximately 100-fold higher transfection to the target cells than anti-EGFR immunoliposomes. The lipoplex formulation also showed a higher transfection to SK-OV-3 tumor xenografts in mice. Thus, IL12 and/or salmosin genes were loaded in the anti-EGFR immunolipoplexes and intravenously administered to mice carrying SK-OV-3 tumors. Co-transfection of IL12 and salmosin genes using anti-EGFR immunolipoplexes significantly reduced tumor growth and pulmonary metastasis. Furthermore, combinatorial treatment with doxorubicin synergistically inhibited tumor growth. These results suggest that anti-EGFR immunolipoplexes containing pDNA encoding therapeutic genes could be utilized as a gene-transfer modality for cancer gene therapy.

Introduction

Gene therapy is thought to have great potential for the treatment of intractable diseases, including cancer. This approach largely

depends on the effective transfection of genetic materials such as plasmid DNA (pDNA) (1) and antisense oligonucleotides (2) to control gene expression in the cytoplasm. For successful gene therapy, transgenes must reach the functional location of target cell nuclei without any form of biodegradation (3). Therefore, effective gene delivery systems are an essential component in the development of gene medicines.

Among non-viral gene delivery systems, lipid nanoparticles such as liposomes and lipoplexes are one of the most efficient carriers for delivering genetic materials to target cells (4). Compared to viral vectors, lipidic nanoparticles have the advantages of low immunogenicity, large loading capacity, biocompatibility and easy functionalization (5). Among the lipidic formulations, cationic liposomes have been most widely utilized as a carrier of genetic materials for gene therapy. Their physicochemical properties provide them with easy complexation via charge interactions and high transfection efficiency (6,7). However, their positive charge can be a double-edged sword, because positively charged nanoparticles are not only easily aggregated under aqueous conditions, but also interact with serum proteins or non-target cells *in vivo* (8), resulting in rapid uptake by the reticuloendothelial system (RES) and inefficient transfection to target cells (9).

A great deal of effort has been spent on enhancing the transfection efficiency of lipidic vectors *in vivo*. Adding a polyethylene glycol (PEG) coating to the surface of liposomes is a typical process for avoiding RES uptake and increasing serum stability (10). The hydrated PEG layer on the surface of the liposomes suppresses their interaction with serum proteins and RES, especially in liver Kupffer cells (11). Therefore, PEGylated liposomes have a longer circulation in the bloodstream and a higher likelihood of reaching the target cells (12). In addition, modification of the PEG termini with tumor-specific ligands makes lipidic vectors a more useful cancer-directed therapeutic gene delivery system (13).

Targeting specific cancer cells is another pivotal process for successful cancer gene therapy. Many different types of ligands such as antibodies (14), aptamers (15), and peptides (16) have been adopted for cancer targeting. Theoretically, cancer targeting can enhance transfection to cancer cells and reduce the off-target delivery of therapeutic genes, thereby minimizing aberrant side effects. For example, the epidermal

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growth factor receptor (EGFR) is a typical target molecule for cancer-directed drug delivery. EGFR is known to be overexpressed in many types of cancers such as breast, ovarian, lung, head and neck, prostate and colorectal cancers (17). Antibodies against EGFR such as cetuximab and panitumumab have been approved for cancer therapy via EGFR-mediated binding and signal blocking (18). In addition to these direct therapeutic applications, they can also be used as EGFR-specific ligands for cancer-directed delivery systems.

In the present study, we developed two different types of EGFR-directed lipidic nanoparticles: neutrally charged pDNA-encapsulating immunoliposomes and cationic immunoliposome complexes with pDNA (immunolipoplexes). Their EGFR-directed gene delivery efficiencies were compared in target cancer cells and in a mouse tumor model. In order to verify their clinical applicability for cancer gene therapy, anticancer therapeutic genes encoding salmosin and/or IL12 were encapsulated and administered to mice carrying target tumors. Studies have shown that salmosin, a novel disintegrin-derived snake (*Gloydius saxatilis*) venom, is a single-chain polypeptide composed of 73 amino acids, including 12 cysteines and an RGD (Arg-Gly-Asp) sequence (19), and has a potent anti-angiogenic function resulting in inhibition of tumor progression (20). IL12 is a well-known immunostimulatory cytokine that activates NK cells and cytotoxic T cells (21). IL12 is also known to be anti-angiogenic (22). The antitumoral efficacies of salmosin and/or IL12 gene delivery by anti-EGFR immunoliposomes and immunolipoplexes were compared in mice. This study suggests an appropriate gene delivery formulation that can be applied to the targeted treatment of cancer.

Materials and methods

Materials. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (DSPE-PEG2000), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[maleimide(polyethyleneglycol)-2000] (DSPE-PEG2000-MAL), cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (Rho-DOPE) were purchased from Avanti Polar Lipid, Inc. (Alabaster, AL, USA). 0,0'-dimyristyl-*N*-lysyl-glutamate (DMKE) cationic lipid was chemically synthesized by Dr D.O. Jang (Yonsei University, Wonju, Korea).

Cells and cell culture. Human ovarian adenocarcinoma SK-OV-3 (no. HTB-77), lung adenocarcinoma A549 (no. CCL-185), and breast carcinoma MCF-7 cells (no. HTB-22) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Mouse melanoma B16BL6 cells were developed by Dr I.J. Fidler at MD Anderson Cancer Center (Houston, TX, USA). SK-OV-3 cells were maintained as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM)/F12 (Gibco, Carlsbad, CA, USA), A549 cells in RPMI-1640, MCF-7 cells in DMEM and B16BL6 cells in minimum essential medium (MEM). The culture media were supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco), 100 units/ml penicillin and 100 mg/ml streptomycin (Gibco). The cancer cells were cultured in a humidified atmosphere of 95% air and 5% CO₂ at 37°C.

Preparation of immunonanoparticles. For preparation of the immunoliposomes encapsulating pDNA, DMKE (5 mol%), POPC (91 mol%), DSPE-PEG2000 (3.8 mol%), DSPE-PEG2000-MAL (0.2 mol%), and Rho-DOPE (0.1 mol%) were dissolved in a chloroform and methanol mixture (2:1, v/v). The organic solvent was evaporated under a stream of N₂ gas. Vacuum desiccation for 2 h ensured removal of the residual organic solvent. The dried films of 2 mg lipids were hydrated in 1 ml of 0.1 M phosphate buffer (pH 5.5) containing pDNA (lipid:pDNA = 10:1 weight ratio) and then vigorously mixed in a vortex mixer for 5 min. After hydration, the liposomes were subjected to 10 cycles of freezing and thawing and extruded 10 times through a polycarbonate membrane with a pore size from 800 to 80 nm using an extruder (Avanti Polar Lipids). Cetuximab (Erbix[®]; ImClone Systems, Bridgewater, NJ, USA) was thiolated for 1 h at room temperature by reacting with Traut's reagent in degassed phosphate buffer (0.1 M, 2 mM EDTA, pH 8.0). The thiolated antibodies were added to the liposomes (0.2:1, molar ratio of Ab and maleimide) and then incubated for 20 h at 4°C. Unconjugated antibodies were removed using a Sepharose CL-4B column in phosphate buffer (0.1 M, pH 7.2). Antibody unconjugated liposomes loading plasmid DNA were referred to as PEG-liposomes.

For preparation of immunolipoplexes containing pDNA, DMKE (48 mol%), cholesterol (48 mol%), DSPE-PEG2000 (3.8 mol%), DSPE-PEG2000-MAL (0.2 mol%), and Rho-DOPE (0.1 mol%) were dissolved in the chloroform and methanol mixture (2:1, v/v). The liposomes were then prepared as described above. Plasmid DNA was pre-condensed in the presence of protamine sulfate (PS) (1:1, wt ratio of pDNA and PS) for 30 min at room temperature. Then the pre-condensed pDNA was gently mixed with liposome solution at an appropriate N/P ratio of DNA/lipid for 30 min at room temperature. Cetuximab was also conjugated to the surface of lipoplexes as described above. Antibody unconjugated lipoplexes were referred to as PEG-lipoplexes.

Gel retardation and enzyme protection assay. To examine the extent of pDNA encapsulation into neutral liposomes or pDNA complexation with cationic liposomes, the prepared immunoliposomes or immunolipoplexes were run on agarose gel. Briefly, 2 μ l of DNase I was added to the liposomes containing pDNA (1 μ g) and then incubated for 2 h at 37°C. The reaction was stopped by the addition of 2 μ l stopping solution (0.5 M EDTA) and then further incubated for 45 min at room temperature. The reaction solutions were treated with 2 μ l of 10% Triton X-100 and incubated for an additional 2 h at room temperature to release the pDNA from the liposomes. Finally, the reaction mixtures were run on 1% agarose gel and pDNA bands visualized by UV illumination.

Assay of EGFR-specific cell binding. SK-OV-3, A549, MCF-7 and B16BL6 cell lines (4x10⁵/well) were cultured on 6-well plates (Nunclon, New York, NY, USA). After 24 h, the cells were treated with Rhodamine B-labeled immunonanoparticles for 30 min at room temperature. After being washed twice with PBS, the cells were trypsinized, washed with PBS and fixed with 0.2% paraformaldehyde for 5 min at room temperature in the dark. Anti-EGFR immunonanoparticle binding to the

cells was counted by a flow cytometer (FACSCalibur; Becton-Dickinson, San Jose, CA, USA).

In vitro transfection. The SK-OV-3, A549, MCF-7 and B16BL6 cells were treated with prepared anti-EGFR immunonanoparticles (1 μ g of pDNA) containing pDNA encoding luciferase (pLuc) in 24-well plates (4×10^4 /well). After transfection for 4 h, the treated cells were additionally incubated in fresh 10% FBS-containing media for 24 h at 37°C. The transfected cells were harvested and then lysed with 200 μ l of lysis buffer (1% Triton X-100, 1 mM dithiothreitol and 2 mM EDTA, pH 7.8) for 2 h at room temperature with gentle agitation. Luciferase activity in the lysates was measured with a luciferase assay kit (Promega Bio Sciences LLC, San Luis Obispo, CA, USA) and a MiniLumat LB 9506 luminometer (Berthold Technologies, Bad Wildbad, Germany). The protein concentration of the supernatant was measured with a DC Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The data were expressed as relative light units (RLU) of luciferase/mg of total proteins.

For verification of EGFR-mediated transfection, the same cancer cells were pretreated with free cetuximab (1 μ g each well) for 30 min, and then treated with the anti-EGFR immunonanoparticles containing pLuc (1 μ g pDNA). Finally, the cells were transfected and assayed as described above.

In vivo gene transfection. Five-week-old BALB/c nude mice (Orient, Co., Ltd., Seongnam, Korea) were subcutaneously injected with 1×10^7 SK-OV-3 cells in the right abdominal quadrant. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Yonsei University at Wonju (YWC-100323-1). After the tumors grew to ~ 200 mm² (length \times width²/2), the mice (n=3) were injected with the Rhodamine B-labeled anti-EGFR immunonanoparticles containing pLuc (40 μ g DNA in 200 μ l) via the tail vein. The transfected tumor tissues were excised 24 h post-administration and were immediately frozen. The frozen tumor tissues were transversally sectioned (3 μ m) using a Leica CM1510 cryostat (Leica Microsystems, Wetzlar, Germany). The sections were examined by fluorescence microscopy (magnification, $\times 100$).

Two days post-administration, the major organs, including tumors, were excised and homogenized in a lysis buffer. Luciferase activities in the lysates were measured with a luciferase assay kit and a luminometer as described above. Transfection efficiency was also expressed as RLU per mg of proteins.

Transfection of anticancer genes by anti-EGFR immunonanoparticles. In order to verify the expression of anticancer genes, anti-EGFR immunonanoparticles containing pAAVCMV-mIL12 (pIL12) and/or pFLAG-Sal (pSal) were intravenously administered to mice carrying SK-OV-3 tumors (10 μ g pDNA in 200 μ l PBS per mouse). The tumor tissues were excised 2 days post-administration and transversally sectioned (3 μ m). The tumor sections were fixed in 4% paraformaldehyde solution for 6 h and stained with hematoxylin for counterstaining. The tumor sections were additionally stained with anti-FLAG antibody (1/10; Abcam, Cambridge, UK) and anti-mouse IL12 antibody (1/10; Abcam) for examination of

salmosin and IL12 expression, respectively. The stained tissues were then examined under a microscope (magnification, $\times 100$).

For cancer treatment, when the tumors reached a volume of ~ 50 mm², the mice (n=5) were intravenously injected with anti-EGFR immunolipoplexes containing anticancer genes at a dose of 0.5 mg/kg (10 μ g DNA, 4 injections at intervals of 3 days). For combinatorial treatment with anticancer genes and chemical drugs, doxorubicin was also administered intravenously at a dose of 15 mg/kg (4 times at intervals of 3 days). Tumor growth in the treated mice was monitored until mouse sacrifice on day 43, followed by the counting of tumor colonies in the lungs.

Statistical analysis. Statistical analysis was performed using ANOVA. P<0.05 was considered statistically significant. P<0.05, P<0.01 and P<0.001 vs. control or between experimental groups, respectively. Error bars represent the standard deviation.

Results

Characterization of anti-EGFR immunonanoparticles containing pDNA. Two different types of anti-EGFR immunonanoparticles (immunoliposomes and immunolipoplexes) containing pDNA were prepared and carefully characterized. To enhance pDNA encapsulation into liposomal vesicles, 4 mol% of DMKE was added to the neutral lipid components, followed by freezing and thawing. According to the gel retardation assay (Fig. 1A), $\sim 70\%$ of the pDNA was encapsulated in the liposome formulation and well protected from DNase treatment.

Lipoplexes were also prepared by pre-condensation of pDNA in the presence of protamine sulfate (PS), followed by complexation with DMKE cationic liposomes via electrostatic interaction. According to a gel retardation test (Fig. 1B), the pDNA was completely complexed with DMKE cationic liposomes at a 3:1 N/P ratio. Therefore, all lipoplex formulations utilized in this study were prepared at a 3:1 N/P ratio, unless otherwise specified.

The size of the liposomes containing pDNA was slightly larger than that of the empty liposomes (Table I). The zeta-potential of liposomes consisting 4 mol% DMKE also shifted from weakly positive to weakly negative due to the encapsulation of negatively charged pDNA. Moreover, the complexation of pDNA with cationic DMKE liposomes significantly increased their vesicle size, but not their surface charge. However, the conjugation of anti-EGFR antibody to the liposomes or lipoplexes increased their vesicle size and reduced their surface charge. The sizes of the immunoliposomes (173.1 nm) and immunolipoplexes (153.1 nm) containing pDNA were suitable for *in vivo* tumor targeting via an enhanced permeability and retention (EPR) effect.

Tumor-directed cellular binding and transfection by anti-EGFR immunonanoparticles. The target-specific cellular binding of the anti-EGFR immunonanoparticles was analyzed by flow cytometry (Fig. 2). Both the anti-EGFR immunoliposomes and immunolipoplexes showed higher cellular binding to EGFR-positive cells (A549 and SK-OV-3) than to EGFR-negative cells (MCF-7 and B16BL6). The conventional

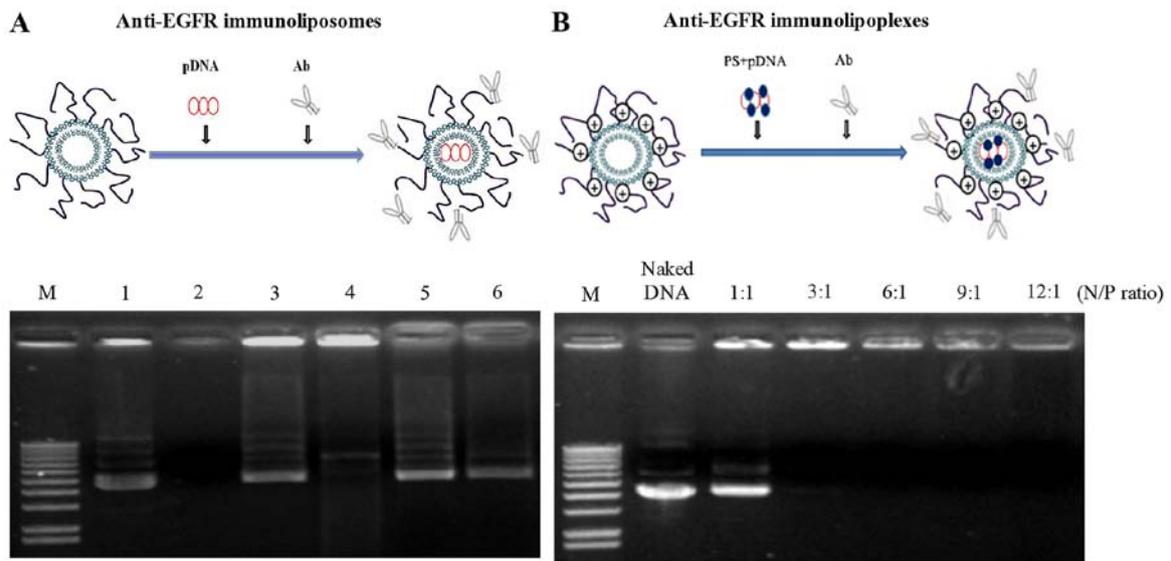


Figure 1. pDNA encapsulation in anti-EGFR immunoliposomes and immunolipoplexes. (A) The prepared immunoliposomes containing pDNA, run on 1% agarose gel and visualized by UV illumination. Lane M, λ /*Hind* III DNA molecular weight markers; lane 1, naked pDNA; lane 2, naked pDNA treated with DNase I; lane 3, liposomes containing pDNA; lane 4, DNase I-treated liposomes containing pDNA; lane 5, Triton X-100-treated liposomes containing pDNA; lane 6, DNase I/Triton X-100-treated liposomes containing pDNA. (B) The lipoplexes of pDNA prepared at various N/P ratios, run on 1% agarose gel and visualized by UV illumination.

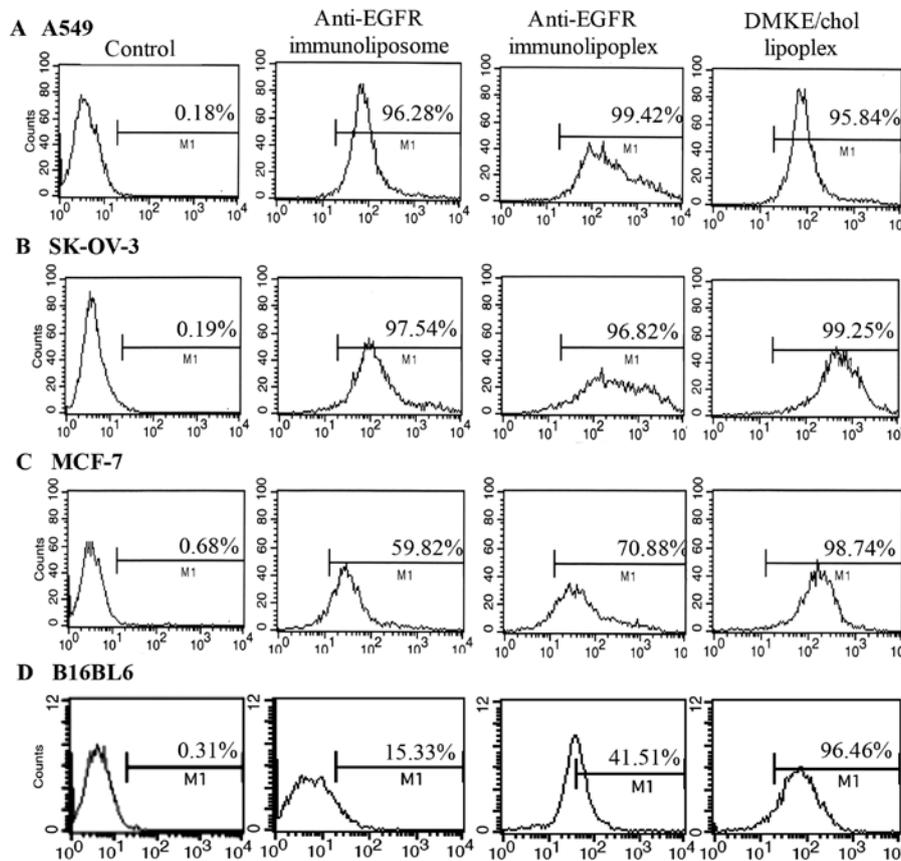


Figure 2. Cellular binding of anti-EGFR immunonanoparticles. EGFR-positive (A, A549; B, SK-OV-3) and EGFR-negative (C, MCF-7; D, B16BL6) cancer cells were incubated with rhodamine B-labeled anti-EGFR immunonanoparticles for 30 min at 4°C. The treated cells were then analyzed by flow cytometry.

DMKE lipoplexes showed a high binding affinity to all types of cell lines, regardless of the EGFR expression level on the cell surface.

The *in vitro* gene transfection efficiencies of the anti-EGFR immunonanoparticles (Fig. 3) were comparable to their cellular binding affinities. According to the results of *in vitro*

Table I. Sizes and ζ -potentials of anti-EGFR immunonanoparticles.

Immunonanoparticles	Size ^a (nm)	Zeta-potential ^a (mV)
Liposome formulation		
Neutral liposomes	130.8±3.0 ^b	8.2±1.0 ^b
Liposomes with pDNA (PEG-liposomes)	151.8±8.9	-3.3±0.2
Anti-EGFR immunoliposomes	173.1±7.5	-4.9±0.5
Lipoplex formulation		
Cationic DMKE liposomes	98.6±3.0	56.7±6.0
Lipoplexes (PEG-lipoplexes)	135.6±1.0	50.9±0.3
Anti-EGFR immunolipoplexes	153.1±4.2	25.4±1.2

^aThe particle size and ζ -potentials were measured 4 times using a zetasizer. ^bParticle size (nm), average particle size ± SD; z-potential (mV), average ζ -potential ± SD.

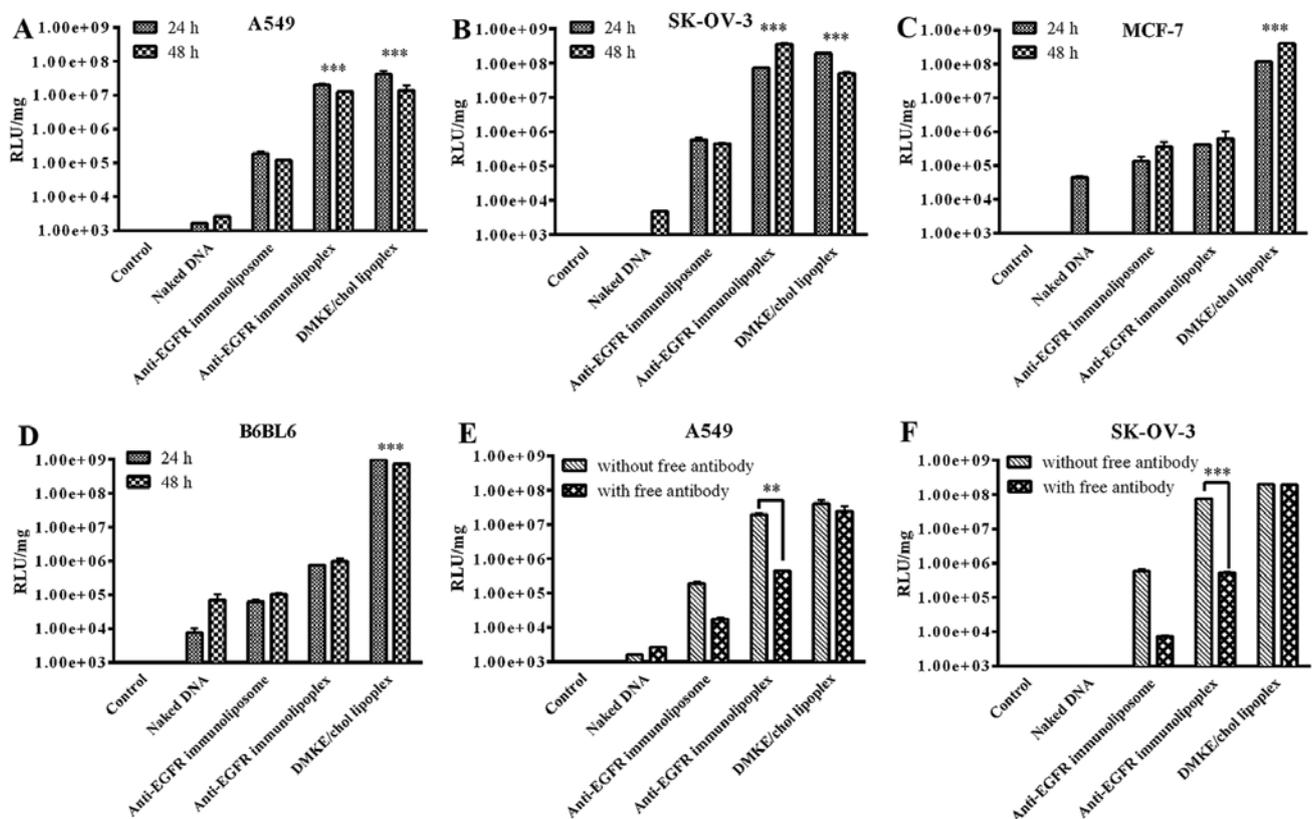


Figure 3. *In vitro* gene transfection using anti-EGFR immunonanoparticles containing pDNA. EGFR-positive (A, A549; B, SK-OV-3) and EGFR-negative (C, MCF-7; D, B6BL6) cancer cells were treated with anti-EGFR immunonanoparticles containing pLuc. Another set of EGFR-positive cells (E, A549; F, SK-OV-3) was pre-treated with free cetuximab and then treated with anti-EGFR immunonanoparticles containing pLuc. The levels of luciferase activities in the transfected cells were calculated to RLU per mg of proteins. **P<0.01 and ***P<0.001 vs. control (A-D) or between experimental groups (E and F), respectively. Each bar represents the mean ± SD for three separate luciferase assay experiments.

pLuc transfection, EGFR-positive cells were effectively transfected by anti-EGFR immunolipoplexes, but EGFR-negative cells were not. Surprisingly, the neutrally charged immunoliposomes were far less efficient than the cationic immunolipoplexes under the same transfection conditions. Similar to the cellular binding results, the conventional DMKE lipoplexes were effective at transfection to all types of cells, regardless of their EGFR expression level. The addition of free anti-EGFR antibodies significantly reduced the levels of

luciferase expression by the anti-EGFR immunolipoplexes in EGFR-positive SK-OV-3 and A549 cells. The addition of free cetuximab did not interfere with the transfection efficiency of DMKE lipoplexes in the cancer cells.

In vivo gene transfection by anti-EGFR immunonanoparticles. To investigate the *in vivo* tumor-targeting capabilities and biodistribution patterns of anti-EGFR immunonanoparticles, immunoliposomes and immunolipoplexes containing pLuc

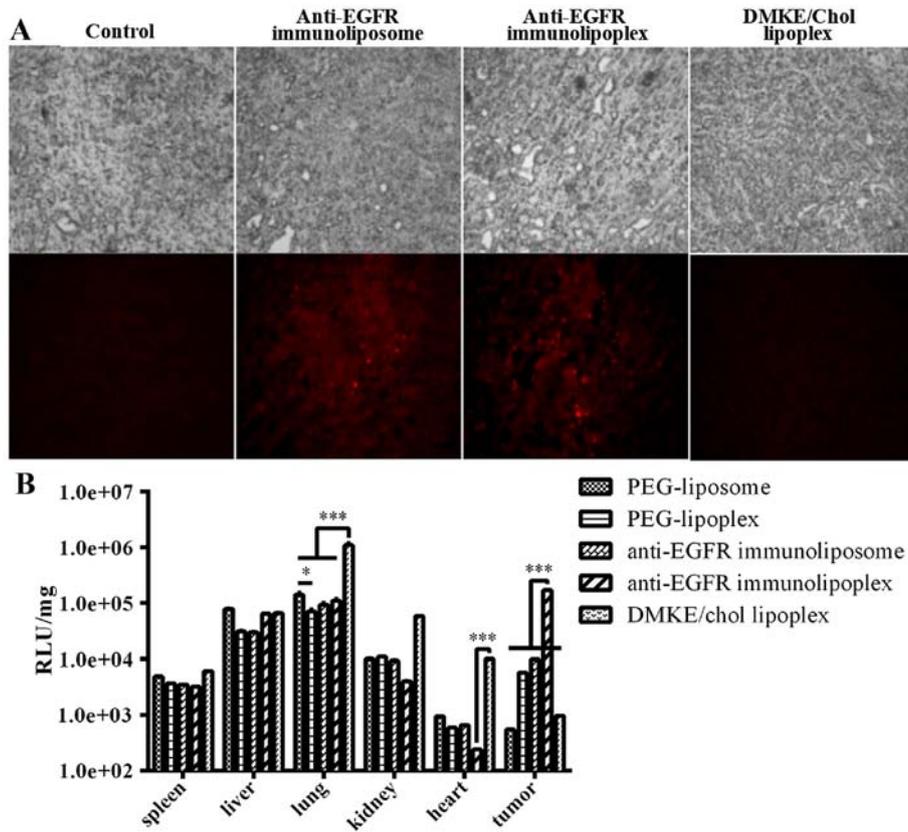


Figure 4. *In vivo* transfection by anti-EGFR immunonanoparticles. Rhodamine B-labeled anti-EGFR immunonanoparticles containing pLuc were intravenously administered to SK-OV-3-xenografted nude mice. (A) After 24 h post-injection, anti-EGFR immunonanoparticles localized in tumor tissues were observed under a fluorescence microscope (magnification, x100). (B) Other groups of treated mice were sacrificed 24 h post-injection and major organs were collected. The luciferase expression in the spleen, liver, lungs, kidneys, heart and tumor tissues was calculated to RLU per milligram of proteins. *P<0.05 and ***P<0.001 between experimental groups, respectively. Each bar represents the mean ± SD for three separate luciferase assay experiments.

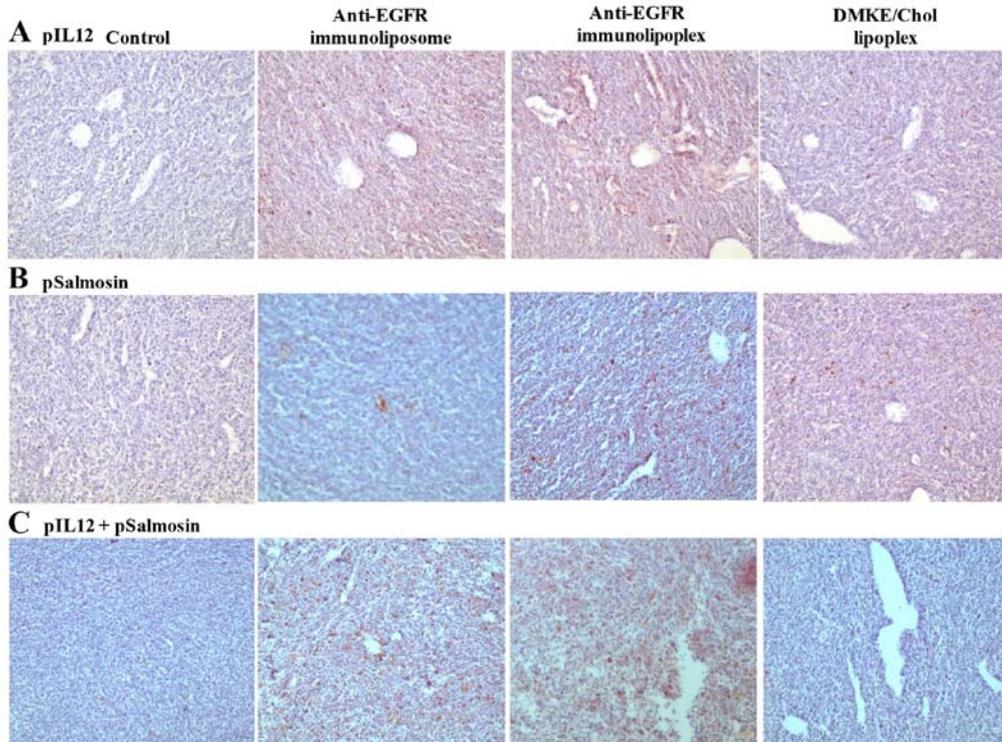


Figure 5. *In vivo* expression of IL12 and salmosin genes transferred by anti-EGFR immunonanoparticles. Anti-EGFR immunonanoparticles containing anti-cancer genes (pIL12 and/or pSal) were intravenously administered to SK-OV-3-xenografted nude mice. The tumor tissues were dissected 24 h post-injection. The tumor sections were immunostained and observed under a microscope (magnification, x100).

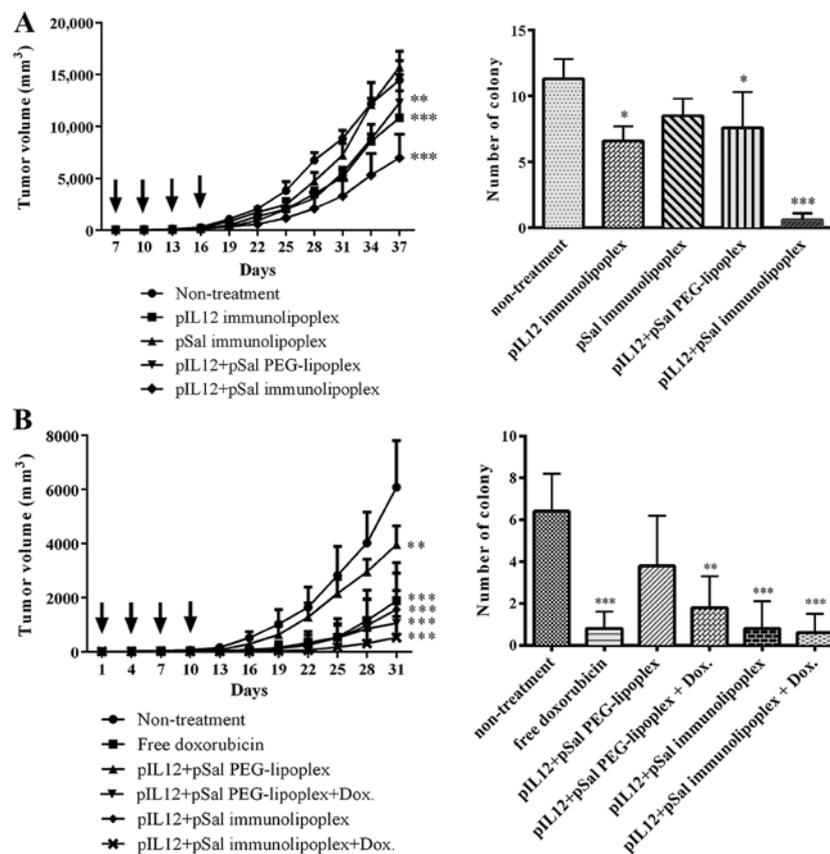


Figure 6. Inhibition of tumor progression by anti-EGFR immunonanoparticles containing IL12 and/or salmosin genes. (A) Anti-EGFR immunonanoparticles containing pIL12 or/and pSal were intravenously administered to mice carrying SK-OV-3 tumors. (B) Other sets of mice were intravenously co-treated with doxorubicin and anti-EGFR immunonanoparticles containing pIL12 and pSal. Their tumor growth was measured for 37 days, and thereafter, colony numbers in the lungs were counted. Solid arrows indicate administrations of the anti-EGFR immunonanoparticles. Each group consisted of 5 mice. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. control, respectively.

were intravenously injected into BALB/c nude mice carrying SK-OV-3 tumors. Localization of the anti-EGFR immunonanoparticles in the tumor tissues was verified by microscopic examination, and the luciferase expression levels in the collected internal organs (spleen, liver, lungs, kidneys, heart and tumor) were compared. Both types of immunonanoparticles showed relatively higher localization in the tumor tissues than did the undirected DMKE lipoplexes (Fig. 4A). However, luciferase expression in the tumor tissues by the anti-EGFR immunoliposomes was far lower than that of the anti-EGFR immunolipoplexes (Fig. 4B). Generally, as compared to cationic DMKE lipoplexes, the PEGylated nanoparticles exhibited lower transfection to lungs, kidney and heart, but higher transfection to tumors. PEGylation and tumor targeting provided the lipidic nanoparticles with at least 250-fold higher transfection to tumors than that of the unPEGylated and untargeted DMKE lipoplexes.

Cancer therapy with anti-EGFR immunolipoplexes containing pIL12 and/or pSal. The expression of therapeutic genes must be verified before the therapeutic administration of the genes to mice carrying tumors. When prepared vehicles containing pIL12 and/or pSal were intravenously administered to SK-OV-3-xenografted mice via the tail vein, the immunonanoparticles were definitely more effective at expressing the genes in the tumor tissues than the DMKE lipoplexes were

(Fig. 5). Among the immunonanoparticles, the anti-EGFR immunolipoplexes showed the best transgene expression in the tumors. Therefore, the anti-EGFR immunolipoplex formulation was adopted for therapeutic transfection of pIL12 and/or pSal to a mouse tumor model.

For cancer treatment, the anti-EGFR immunolipoplexes containing pIL12 and/or pSal were intravenously injected into three different sets of mice carrying SK-OV-3 tumors, four times, at 3-day intervals. According to the measurement of tumor growth, co-transfection of pIL12 and pSal was most effective at inhibition of tumor growth and pulmonary metastasis among the formulations (Fig. 6A). The tumor-targeted transfection of the therapeutic genes by the anti-EGFR immunolipoplexes was also more effective than untargeted transfection by the PEG-lipoplexes (Fig. 6A). Combinatorial treatment with the anti-EGFR immunolipoplexes containing pIL12/pILSal and doxorubicin resulted in the least tumor growth among the tested treatments (Fig. 6B).

On 37th day, all the treated mice were sacrificed and the number of metastatic colonies in the lungs was counted. The pattern of metastasis inhibition by the anti-EGFR immunolipoplexes containing pIL12 and/or pSal was similar to that of tumor growth inhibition. Co-administration of the two genes was more effective for the inhibition of pulmonary metastasis than treatment with either gene alone. In addition, the mice treated with anti-EGFR immunolipoplexes showed

fewer pulmonary colonies than those treated with untargeted PEG-lipoplexes. Combinatorial treatment with anti-EGFR immunolipoplexes containing pIL12/pSal and doxorubicin treatment appeared to better inhibit the pulmonary metastasis of tumors.

Discussion

Lipidic nanoparticles such as liposomes and lipoplexes are considered versatile delivery systems for anticancer genes due to their biocompatibility, high loading capacity, and convenient scale-up and functionalization. However, lipidic delivery systems still have some limitations that need to be overcome for wide clinical application. The *in vivo* transfection efficiency of liposomal vectors, which is generally lower than that of most viral vectors, must be improved to a clinically meaningful level (23). Therefore, a great deal of effort has been spent on enhancing the *in vivo* gene-transferring capability of lipidic nanoparticles. Some groups have suggested novel lipidic structures for efficient formulation (24-26), and others have provided effective functions for longer circulation, target recognition, cellular uptake (27-29). Among these trials, the targeting of lipidic nanoparticles to specific cells or tissues by conjugation of targeting ligands to their surface has been suggested as a pivotal process for the improvement of *in vivo* transfection, as well as the reduction of off-target effect. For target cell recognition, a variety of targeting ligands such as antibodies, aptamers, and peptides have been adopted for the development of target-directed lipidic nanoparticles (30-32).

In the present study, we prepared two different types of lipidic nanoparticles, liposomes and lipoplexes, containing anticancer genes. POPC-based liposomes (33) encapsulate pDNA, and a small amount of cationic DMKE was added to attract anionic pDNA near the lipid bilayers. DMKE-based lipoplexes are complex structures of cationic liposomes and anionic pDNA (34). Both types of lipid nanoparticles were PEGylated to increase their blood circulation time. At the same time, tumor-recognizing ligands, i.e., cetuximab and anti-EGF receptor antibody, were conjugated to the surface of the lipidic nanoparticles for tumor-targeted gene delivery. These are referred to as anti-EGFR immunoliposomes and anti-EGFR immunolipoplexes.

When both types of tumor-directed lipidic nanoparticles were intravenously administered to mice carrying SK-OV-3 tumors, their localization in the tumor tissues was apparent and similar, according to microscopic examination. However, as expected, the untargeted DMKE lipoplexes were not efficiently transported to the tumors, presumably due to their non-specific deposition in the lung endothelium (35). This implies that both of the vehicles were stably small (<200 nm) enough to travel inside tumor tissues by the EPR effect. Even though the anti-EGFR immunolipoplexes exhibited a certain level of cationic surface charge, shielding by heavy PEGylation (4 mol%) may have protected them from the binding of serum proteins and enhanced their circulation in the blood.

Once the gene-containing lipidic nanoparticles arrived at the cell surface, the type and extent of interaction between the vesicular and plasma membranes may have affected gene transfection to the tumor cells. In general, compared to the cationic DMKE lipoplexes, the PEGylated nanoparticles

showed lower transfection efficiency in the lungs, kidney and heart, but higher transfection efficiency in the tumors. This result implied that the renal clearance rates of the PEGylated nanoparticles were reduced and its blood circulation time was prolonged. PEGylation and tumor targeting provided the lipidic nanoparticles with at least 250-fold higher transgene expression in the tumors. This implies that attachment of the nanoparticles to the plasma membranes of tumor cells via EGF receptors may play a beneficial role in transfection. In fact, transfection inhibition by the addition of free cetuximab may explain the significance of the EGFR-mediated interactions between the nanoparticles and cancer cells.

Even though both types of anti-EGFR immunonanoparticles were highly accumulated in SK-OV-3 tumor tissues, their accumulation was not directly translated into transgene expression. The cellular binding affinity of the anti-EGFR immunoliposomes was similar to that of the anti-EGFR immunolipoplexes. However, interestingly, the FACS analysis was not directly related to the luciferase expression levels in the tumors. The *in vitro* and *in vivo* luciferase expressions by the immunolipoplex formulations were approximately 100- and 10-fold greater than those of the immunoliposome formulation, respectively. These results imply that cellular recognition and endocytosis of the nanoparticles via EGF receptors may not be able to translocate enough nanoparticles carrying transgenes into the tumor cells. The cationic charge on the surface of immunolipoplexes clearly aided transgene expression, presumably due to the effective condensation of pDNA into the carriers and enhanced cytoplasmic release of pDNA from the endosomal vesicles (36). Therefore, it appears that the transfection of pDNA can be facilitated by cellular recognition via EGF receptors as well as via electrostatic interactions between the liposomal bilayers and plasma membranes.

In addition, the *in vivo* transfection results for anticancer genes, i.e., IL12 and salmosin genes, also showed that anti-EGFR immunolipoplexes were a better gene delivery system. Therefore, pIL12 and/or pSal were loaded into the anti-EGFR immunolipoplexes for the formulation of anticancer gene medicines and then administered into mice carrying SK-OV-3 tumors. We previously reported that local administration (37) or hydrodynamic transfection (38) of pIL12 and pSal was effective in the inhibition of tumor progression. There have been few reports regarding the tumor-targeted systemic transfection of genes. According to the cancer treatment results in this study, the anticancer genes were efficiently transferred by intravenous administration of the anti-EGFR lipoplexes and effectively expressed in the target tumors grown in mice. In fact, IL12 and salmosin genes simultaneously delivered by the anti-EGFR immunolipoplexes were the most effective at inhibition of SK-OV-3 tumor growth and pulmonary metastasis. In addition to gene transfection, the intravenous administration of doxorubicin contributed an additive inhibition of tumor progression. The mice co-treated with anti-EGFR immunolipoplexes containing pIL12/pSal and doxorubicin showed the highest reduction in tumor growth and metastasis. This implies that the anti-EGFR immunolipoplexes containing pIL12/pSal could be supportively combined with conventional chemotherapy to elicit a better cancer treatment outcome.

In conclusion, the anti-EGFR immunonanoparticles prepared in this study were able to specifically recognize

the EGF receptor-overexpressing cancer cells. Among the nanoparticles examined, anti-EGFR immunolipoplexes are a better formulation for the *in vivo* transfection and expression of therapeutic genes in the target tumor tissues. Anti-EGFR immunolipoplexes containing pIL12/pSal appear to be good candidates for anticancer gene medicine. This formulation of anticancer genes could be clinically adopted as a supportive combinatorial treatment with conventional chemotherapy to elicit the best cancer treatment outcome.

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