# EGFR-overexpressing tumor cell-specific gene delivery using anti-EGF receptor Fab′-coupled immunoliposomes

Eun-Jung Kim

The Graduate School

Yonsei University

Department of Biomedical Laboratory Science

# EGFR-overexpressing tumor cell-specific gene delivery using anti-EGF receptor Fab′-coupled immunoliposomes

A Master's Thesis submitted to the Department of Biomedical Laboratory Science and the Graduate School of Yonsei University in fulfillment of the requirements for the degree of Master of Science in Medical Technology

Eun-Jung Kim

January 2007

**This certifies that the master's thesis of Eun-Jung Kim is approved.**

---

Thesis Supervisor: Yong Serk Park

---

Jong Bae Kim : Thesis Committee Member

---

Tae Ue Kim: Thesis Committee Member

The Graduate School

Yonsei University

**January 2007**

# CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# ABBREVIATIONS

AAV : adeno-associated virus

BLI : bioluminescence imaging

CMV : cytomegalovirus

DMSO : dimethyl sulfoxide

DMKD : O,O′-dimyristyl-N-lysyl aspartate

DSPE-$PEG_{2000}$ : 1,2-distearoyl-sn-glycero-3-phosphoethanolamine
-N-[methoxy(polyethyleneglycol-2000)]

DSPE-$PEG_{2000}$-MAL : 1,2-distearoyl-sn-glycero-3-phosphoethanol
amine-N-[maleimide(polyethyleneglycol-2000)]

EGFR : epidermal growth factor receptor

ILs : immunoliposomes

MPS : mononuclear phagocytic system

PBS : phosphate-buffered saline

POPC : 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

Rho-DOPE : 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
-N-[lissamine rhodamine B sulfonyl]

RLU : relative light unit

SDS-PAGE : sodium dodecyl sulfate-polyacrylamide-
gel electrophoresis

# EGFR-overexpressing tumor cell -specific gene delivery using anti-EGF receptor Fab′-coupled immunoliposomes

## ABSTRACT

One of the essential constituents in gene therapy is the development of efficient, non-toxic gene carriers that can encapsulate and deliver foreign genetic materials into specific cell types, such as cancerous cells. ErbB family receptors, in particular EGFR (epidermal growth factor receptor), have been described as implicated in immortalization in several solid tumors. Over-activated EGFR can convert a normal to a malignant cell by providing sustained signals for cell proliferation, anti-apoptosis, angiogenesis, and metastasis, which are the basic characteristics of cancer.

Hence, I developed the immunoliposomes specific to EGFR in order to efficiently deliver transgenes to cancer cells overexpressing EFGR. Whole monoclonal antibodies against EGFR (Cetuximab, Erbitux®) were reduced to Fab′ fragments which were then coupled to liposomal surface, to diminish the immunogenicity of the whole antibody.

The liposomes were prepared with POPC (1-palmitoyl-2-oleoyl-*sn*-glycero -3-phosphocholine), DMKD (O,O′-dymyristyl-N-lysylaspartate), DSPE-PEG $_{2000}$(1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethyle neglycol-2000)]), DSPE-PEG$_{2000}$-MAL (1,2-distearoyl-sn-glycero-3-phosphoeth

anolamine-N-[maleimide (polyethyleneglycol-2000)]), and Rho-DOPE (1,2-dio leoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl]). The plasmid DNA encoding a luciferase reporter gene was encapsulated in the liposomes by the freeze-thaw method. The efficiency of plasmid DNA encapsulation was estimated to be approximately 80%. Then, the EGFR-targeted immunoliposomes were constructed by conjugation of Fab′ fragments to termini of PEG coupled to the liposomes encapsulating pDNA. Stability of the plasmid DNA in the immunoliposomes was confirmed by the gel retardation assay.

As a result, the anti-EGFR Fab′-coupled immunoliposomes were able to selectively bind to the various types of EGFR-overexpressing cancer cells and efficiently induce luciferase gene expression. Meanwhile, the same immunoliposomes did not effectively to bind to MCF cells less expressing EGFRs. According to the competitive inhibition assay using free whole antibodies against EGFR, the selective gene transfection and binding were mediated by Fab′ coupled to the liposomes.

Based on these findings, the EGFR-targeted immunoliposomes can be utilized to deliver therapeutic genes to EGFR-expressing cancer cells.

---

Key words : immunoliposomes, epidermal growth factor receptor, Cetuximab, gene delivery

# I. INTRODUCTION

Gene therapy is a form of molecular medicine that has the potential to influence significantly human health in this century. It promises to provide new treatments for a large number of inherited and acquired diseases (1). However, the growing potential of gene therapy for both genetically based and infectious diseases will not achieve its goals until the issue of gene delivery has been resolved. During the past 15 years, more than 400 clinical studies in gene therapy have been evaluated; almost 70% of these studies are in the area of cancer gene therapy (2). The main objective in gene therapy is the development of efficient, non-toxic gene carriers that can encapsulate and deliver foreign genetic materials into specific cell types, such as cancerous cells.

The delivery vectors are broadly categorized: non-viral and viral vectors. It has been known that viral vectors are a very efficient gene delivery system. However, their safety issues, particularly immunogenicity, and their limited capacity of transgenic materials, have encouraged researchers to increasingly focus on non-viral vectors as an alternative to viral vectors (3). Although non-viral vectors are less efficient than viral ones in terms of gene expression, they have advantages of safety, simplicity of preparation and high gene encapsulation capability.

Since their introduction as gene carriers in 1987 (4), cationic liposomes have become one of the most studied non-viral vectors. One of the major drawbacks of classical liposomes was their rapid clearance from blood, due to absorption of plasma proteins (opsonins) to the 'naked' phospholipid membrane, triggering recognition and uptake of the liposomes by the mononuclear phagocytic system (MPS), also referred to as the reticuloendothelial system.

A major advance in the field of liposomes came with the development of

Stealth® liposomes, which utilize surface coating with hydrophilic carbohydrates or polymers, usually a lipid derivative of polyethyleneglycol (PEG), to help evade MPS recognition (5-7). The inclusion of PEG extends the half-life of liposomes from less than a few minutes (classical liposomes) to several hours (Stealth® liposomes) and change the pharmacokinetics of the liposomes from dose-dependent, saturable pharmacokinetics to does-independent pharmacokinetics (8-9). However, they do not actively target the liposome to the tumor.

One effective means of targeting tumors would be via conjugation of antitumor antibodies or portions of antibodies to liposomes (immunoliposomes). In this approach, it has become apparent that many factors must be taken into consideration, including a proper choice of target antigen and reactive antibody against the antigen.

ErbB family receptors, in particular EGFR, have been described as implicated in immortalization of several solid tumors including breast, gastric, ovarian, non-small cell lung cancer, head and neck cancers, prostate cancers and others (10). The epidermal growth factor receptor (EGFR also known as EerB1 or HER-1) was the first identified receptor among the ErbB family receptors (11). Since then, the EerB family proteins have increased to four, including EGFR-1 itself (HER-1, ErbB1), HER-2 (ErbB2), HER-3 (ErbB3), and human HER-4 (ErbB4) (12). The EGFR has been associated with a variety of human malignancies (10, 12-15).

Over-activated EGFR can convert a normal to a malignant cell by providing sustained signals for cell proliferation, anti-apoptosis, angiogenesis, and metastasis, which are the basic characteristics of cancers (16). Both theoretically and practically, it is possible to inhibit EGFR activation by several strategies and if successful, this could selectively eradicate cancer cells (17).

In this study, I have developed EGFR-targeted immunoliposomes by

conjugation of Fab′ of Cetuximab (a potent anti-EGFR antibody) to PEG termini on liposomal surface. The biological functions of the anti-EGFR immunoliposomes were investigated in *in vitro* and *in vivo* systems. According to the experimental results, the immunoliposomes were able efficiently bind to EGFR-overexpressing cells *in vitro* and internalize into the cells. However, at this moment, I found little expression of transgene (luciferase) in EGFR-overexpressing tumors in a mouse model.

# II. MATERIALS AND METHODS

## 1. Materials

POPC (1-palmitonyl-2-oleoyl-*sn*-glycerol-3-phosphocholine), DSPE-$PEG_{2000}$ (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol-2000)]), DSPE-$PEG_{2000}$-MAL (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide (polyethyleneglycol-2000)]), Rho-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[lissamine rhodamine B sulfonyl]) were purchased from Avanti Polar Lipids, Inc. (Alabaster, USA).

DMKD cationic lipid was chemically synthesized by Dr. Jang (Department of chemistry, Yonsei University, Korea). FITC (fluorescein isothiocyanate isomer I) and Bovine Pancreatic DNase I (2000 Kunitz units/mg) were purchased from Sigma Chemical Co. (St. Louis, USA). Centricon of 10,000 MWCO and Amicon ultra-4 of 30,000 MWCO were purchased from Amicon (Beverly, USA). PD-10 columns and Sepharose CL-4B were purchased from Amersham Biosciences (Uppsala, Sweden) were purchased. The DC protein kit and electrophoresis kit were obtained from Bio-Rad (Hercules, USA). Dialysis Cassettes, immobilized pepsin slurry, ImmunoPure protein A column, 2-Mercaptoethylamine HCl (2-MEA) were purchased from Pierce (Rockford, USA).

## 2. Cells and cell culture

Human adenocarcinoma MCF-7, MDA-MB-468 cells, Human colorectal adenocarcinoma HT-29, WiDr cells and Mouse melanoma B16BL6 cells were

purchased from the American Type Culture Collection (Manassas, USA). Human colorectal carcinoma SNU-C2B cells and Human adenocarcinoma SK-Br-3 cells were purchased from Korean Cell Line Bank (Seoul, Korea). MCF-7, HT-29, WiDr cells were maintained as monolayer cultures in DMEM (Gibco, Carlsbad, USA), SNU-C2B cells in RPMI 1640, B16BL6 cells in MEM, SK-Br-3 cells in DMEM/F-12 medium, and MDA-MB-468 cells in Leibovitz L-15 medium (Gibco, Carlsbad, USA) supplemented with 10% fetal bovine serum, 100 units/㎖ penicillin and 100 ㎍/㎖ streptomycin in a humidified atmosphere of 95% air and 5% $CO_2$ at 37℃ (except L-15 medium in 100% air).

## 3. Plasmid preparation

The pAAVCMV-Luc plasmid was propagated in DH5α strain of *E. coli* under selective LB media with ampicillin. The plasmid was isolated and purified by a Qiaprep-Spin miniprep kit (Qiagen, Valencia, USA). Purity of the plasmid was confirmed by 0.8% agarose gel electrophoresis and the DNA concentration was measured by UV-spectrophotometry (Amersham Bioscience, Uppsala, Sweden)

## 4. Preparation of F(ab′)$_2$ fragment

Cetuximab (Erbitux®, Imclone) were dialyzed using a dialysis cassettes (Slide-A-Lyzer® 10 K/Pierce, Rockford, USA) against a 20 mM sodium acetate buffer (digestion buffer) at pH 4.5 and then concentrated with a Ultra-Amicon 30 K (Amicon, Beverly, USA). The concentrated antibodies were resuspended in 0.5 ㎖ of the digestion buffer and then added to 0.25 ㎖ of immobilized pepsin slurry (Pierce, Rockford, USA). The solution was incubated in a serum separator tube for 5 h at 37℃ in a shaker water bath. The solution was then

filtrated with 4 ㎖ of ImmunoPure binding buffer (Pierce, Rockford, USA) and $F(ab')_2$ fragments were separated on an ImmunoPure Protein A column (Pierce, Rockford, USA). Fc fragments and undigested IgG were retained in the column whereas $F(ab')_2$ fragments were filtrated. The $F(ab')_2$ fragments (110 KDa) were concentrated using a Centricon-50 (Amicon, Beverly, USA) and then dialyzed against a PBS-EDTA buffer (100 mM sodium phosphate, 150 mM NaCl, and 10 mM EDTA, pH 7.2). The purity of $F(ab')_2$ fragments were assessed by SDS-PAGE.

## 5. Preparation of Fab′ fragment

$F(ab')_2$ fragments were incubated with 50 mM of 2-mercaptoethylamine -HCl (2-MEA) for 90 min at 37℃. 2-MEA is sufficiently mild to cleave disulfides between heavy chains of $F(ab)_2$ molecules while preserving the disulfide linkages between the heavy and light chains. The solution was loaded on a Sephadex G-25 M column (PD-10 column; Amersham Biosciences, Uppsala, Sweden) pre-equilibrated with acetate-EDTA buffer (100 mM anhydrous sodium acetate, 88 mM sodium chloride, and 1 mM EDTA, pH 6.5) and Fab′ fragments were collected in 1 ml fractions. The fractions containing Fab′ fragments (55 KDa) were concentrated using a Centricon-10 (Amicon, Beverly, USA). The purity of Fab′ fragments were assessed by SDS-PAGE.

## 6. Preparation of immunoliposomes and plasmid DNA encapsulation

The lipid composition was 93% neutral POPC, 3% cationic DMKD, 2.8% anionic DSPE-$PEG_{2000}$, 1% anionic DSPE-$PEG_{2000}$-MAL, and 0.2% Rho-DOPE. Appropriate amounts of lipids were mixed in chloroform:methanol (2:1). The organic solvent was evaporated under a stream of $N_2$ gas. Vacuum desiccation

for 2 h ensured removal of the residual organic solvent. The dried lipid films were hydrated in an appropriate amount of HEPES and then vigorously mixed by a vortex mixer for 5 min.

For encapsulation of DNA, plasmid DNA was added to the prepared liposomes (1:10, weight ratio) and repeated freeze/thaw cycles as described elsewhere (18, 19). Following DNA encapsulation in the large lipid vesicles, small 85-100 nm liposomes encapsulating DNA were formed by successive extrusions through 800, 400, 200, and 100 nm pore size of polycarbonate membranes (Avanti Polar Lipids, Alabaster USA), as described elsewhere (18, 19). Then, the liposomes were conjugated with the Fab′ fragments of Cetuximab for overnight. The un-conjugated antibodies and exteriorized DNA were removed from the immunoliposomes by CL-4B gel filtration chromatography. Fab′ quantification by the DC protein assay and fluorescence measurement of liposomal Rhodamine by GENios (TECAN Co., Boston, USA) confirmed the conjugation reaction. The phospholipid concentration was also determined by the phosphorous assay.

The average number of Fab′ molecules per liposome was calculated by (I) the molecular weight of the Fab′ fragment is 46,000, (II) the average number of phospholipid molecules per liposome estimated by the method of Enoch and Strittmatter (20). To control Fab′ molecule number on liposomal surface, the concentration of maleimide lipid (DSPE-PEG-MAL) and cationic lipid (DMKD) were varied.

## 7. SDS-PAGE and DNase I protection assay

To ensure conjugation of liposomes and Fab′ fragments, the reaction samples were analyzed by SDS-PAGE with coomassie blue staining. Then, the same samples were tested by the DNase protection assay. The DNase I (1

unit per μg of DNA) was added to the immunoliposomes which were then incubated at 37℃ for 1 h. EDTA (0.5 M) was immediately added to stop DNase I degradation. The Triton X-100 (1%) was then added and incubated for 30 min at room temperature to release DNA from the complexes. Finally, the samples were run on 1% agarose gel and visualized by UV illumination.

## 8. *In vitro* specific binding of Cetuximab IgG to EGFR -overexpressing cells

The specificity of Cetuximab IgG to EGFR was evaluated in MDA-MB-468, MCF-7, HT-29, B16BL6 cells by immunofluorescence stains. The cells were grown on 13 rmm cover slips (Nunclon, New York, USA) in 24-well plates. Twenty four later, the cells were fixed with 2 % paraformaldehyde. The FITC (fluorescein isothiocyanate)-labeled Cetuximab solution was added to the cells, which were then incubated for 45 min at 4 ℃ under the dark condition. After washing with PBS, the cover slips were immediately examined with a fluorescence microscope.

## 9. *In vitro* cellular binding assay of anti-EGFR immunoliposomes

Binding of the anti-EGFR immunoliposomes contained Rho-DOPE to cell surface was examined with a fluorescence microscope. The immunoliposomes coupled to Fab′ fragments of Cetuximab were added to the MDA-MB-468, SNU-C2B, WiDr, MCF-7 cells in 24-well plates and incubated for 45 min at 4℃. Then, the cells were washed with PBS and observed with a fluorescence microscope.

## 10. *In vitro* transfection by anti-EGFR immunoliposomes

The EGFR-targeted immunoliposomes encapsulating pAAVCMV-Luc plasmid was added to MDA-MB-468, SK-Br-3, MCF-7 cells in 24-well plates. After transfection for 4 h and additional incubation for 24 h, the transfected cells were washed twice with PBS (pH 7.4) and lysed using 200 ㎕ of lysis buffer (1% Triton X-100, 0.1 M Tris, 2 mM EDTA, pH 7.8) for 1 h at room temperature with agitation. The plates were incubated at -20℃ for 30 min and thawed at room temperature.

The cell lysates was then centrifuged for 4 min at 12,000 rpm to pellet debris. Luciferase activities in the supernatant were measured with a luciferase assay kit (Promega Biosciences, San Luis Obispo, USA) and a luminometer (Berthold Technologies, Bad Wildbad, Germany). The protein concentration of the supernatant was measured with a DC protein Assay kit (Bio-Rad, Hercules USA).

## 11. *In vitro* competitive binding assay of anti-EGFR immunoliposomes

To examine specific targeting of the EGFR-targeted immunoliposomes, MDA-MB-468, MCF-7 cells in 24-well plates were treated with the FITC-labelled Cetuximab for 45 min at 4℃ under the dark condition. After the cells were washed with PBS, the immunoliposomes containing Rho-DOPE were added to the cells, which were then incubated for 1 h at 37℃. After washed with PBS, the cells on cover slips were immediately examined with a fluorescence microscope.

## 12. *In vitro* competitive transfection test of anti-EGFR immunoliposomes

The EGFR-targeted immunoliposomes encapsulating pAAVCMV-Luc were added to MDA-MB-468, MCF-7 cells in 24-well plates. Prior to transfection with the immunoliposomes, the cells were pre-treated with Cetuximab whole antibodies for 45 min at 4℃. Then, the cells were transfected with the EGFR-targeted immunoliposomes for 4 h and further incubated for 24 h at 37℃. The transfected cells were washed twice with PBS (pH 7.4) and lysed using 200 ㎕ of lysis buffer (1% Triton X-100, 0.1 M, Tris, 2 mM EDTA, pH 7.8) for 1 h at room temperature with agitation. The plates were incubated at -20℃ for 30 min and thawed at room temperature. And the cell lysates were centrifuged for 4 min at 12,000 rpm to pellet debris. Luciferase activities in the supernatant were measured with a luciferase assay kit from (Promega Biosciences, San Luis Obispo, USA) and a luminometer (Berthold Technologies, Bad Wildbad, Germany). The protein concentration of the supernatant was measured with a DC protein Assay kit (Bio-Rad, Hercules, USA).

## 13. *In vitro* transfection by various types of anti-EGFR immunoliposomes

To verify the effect of molecule number of Fab′ coupled to liposomes on gene transfection, immunoliposomes conjugated with varied numbers of Fab′ were prepared by changing the mol % of coupling lipid, DSPE-PEG-MAL. Transfection was done the same as mentioned above.

## 14. *In vivo* transfection by anti-EGFR immunoliposomes

For *in vivo* gene delivery studies, the 5-6 week old female nude mouse xenografts (BALB/cAnNCrjBgi-nu; Charles River Laboratories, USA) were prepared by subcutaneous inoculation of 1 x $10^7$ WiDr cells on the lower back. The EGFR-targeted immunoliposomes (50 $\mu$g of pDNA encoding luciferase each mouse) were intravenously injected into the tumor-bearing nude mice via tail vein. And the immunoliposomes were intratumorally injected into the tumor-bearing nude mice. At 48 h post injection, the mice were anesthetized with isoflurane and imaged in red fluorescence ($\lambda_{ex}$=500 nm and $\lambda_{em}$=575 nm). Then, the same mice was intraperitoneally injected with D-luciferin substrate dissolved in sterile PBS and imaged using a luminometer. The animals were placed in a light-tight chamber and a gray-scale reference image was obtained under the low-level illumination. The fluorescence and photons emitted from the mice, transmitting through the tissues, were collected with a cooled charge-coupled device (CCD) camera (IVIS™; Xenogen). The acquisition time ranged from 1 to 5 min. The results were analyzed using LivingImage® software. The body temperature was maintained throughout imaging using a 37 ℃ platform in the chamber. After administration of luciferin substrate, the mice were imaged for a period of time ranging from 1 sec to 2 min, depending on signal intensity. Raw values of luciferase expression were recorded as photons of light emitted per second.

# III. RESULTS

## 1. Preparation of Fab′ fragment

The Cetuximab Fab′ fragments were generated by pepsin digestion and treatment with 2-mercaptoethylamine-HCl. The Fab′ fragments were run on 12 % SDS-PAGE to confirm under the reduced condition. The gel was stained with coomassie G250 (Fig. 1).

## 2. Preparation of liposomes encapsulating pDNA

The design for the EGFR-targeted immunoliposomes is summarized in Table I. The mean diameter of the PEGylated liposomes carrying the pAAVCMV-Luc was about 100 nm. To identify efficiency of DNA encapsulation, the liposomes encapsulating pDNA were run on 1% agarose gel and stained with ethium bromide (Fig. 2).

The liposomes containing pDNA were treated with DNase I and Triton X-100 (Fig. 2, lane 3) or treated only with Triton X-100 (Fig. 2, lane 2). The lipoplexes treated only with Triton X-100 showed the undigested pDNA in the exterior and interior of liposomes. Meanwhile, the lipoplexes treated with DNase I and Triton X-100 retained the pDNA only in the interior of liposomes. Because DNase I degraded the exterior plasmid DNA, the interior plasmid DNA were seen by staining with ethium bromide. The liposomes treated with DNase I exhibited successful digestion by DNase I (Figure 2, lane 4). The nuclease completely removed any pDNA bound to the exterior of the liposomes.

Meanwhile, the pDNA encapsulation efficiency was calculated with Quantity One software (Bio-Rad, Hercules, USA). As a result, the encapsulation

efficiency was estimated to be approximately 80%. This value implied that the amount of pDNA added to the liposomes (1:10, wt ratio) was sufficient for maximal pDNA encapsulation into the liposomes.

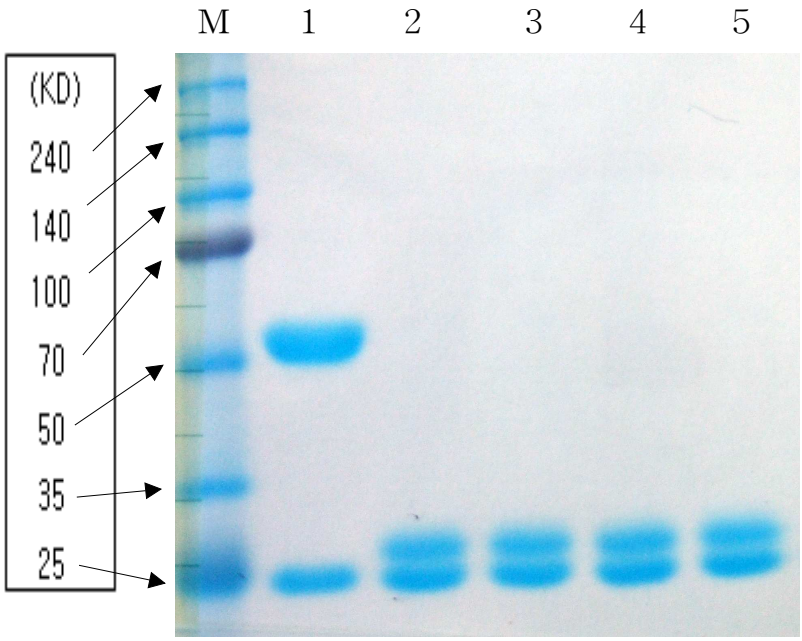



**Figure 1. SDS-PAGE analysis of Cetuximab Fab′ fragments.** Cetuximab whole IgG antibodies and their Fab′ fragments prepared with enzymatic digestion were run on 12 % SDS-PAGE. M, a molecular weight standard marker; lane 1, whole IgG antibodies; lane 2, Fab′ fragments.

## Table I. The design of EGFR-targeted immunoliposomes

| Component | Rationale |
|---|---|
| Antibody<br>: Cetuximab (Erbitux®)-Fab' | Whole Abs (IgG) were reduced to Fab' fragment to diminish clearance and to enhance selective intracellular delivery. |
| Linkage<br>: DSPE-PEG-Mal + Fab' | A single sulfhydryl group (SH-) on Fab' was conjugated to the liposomes via a maleimide group for targeted binding and internalization. |
| Liposomes<br>: Sterically stabilized | The liposomes are prepared as:<br>neutral for *in vivo* stability<br>PEGylated for longer circulation<br>Small diameter for better intratumoral penetration<br>Rho-labeled for intracellular tracing |
| Agent<br>: Nucleic acid | A reporter gene was used to confirm expression of transgene. |

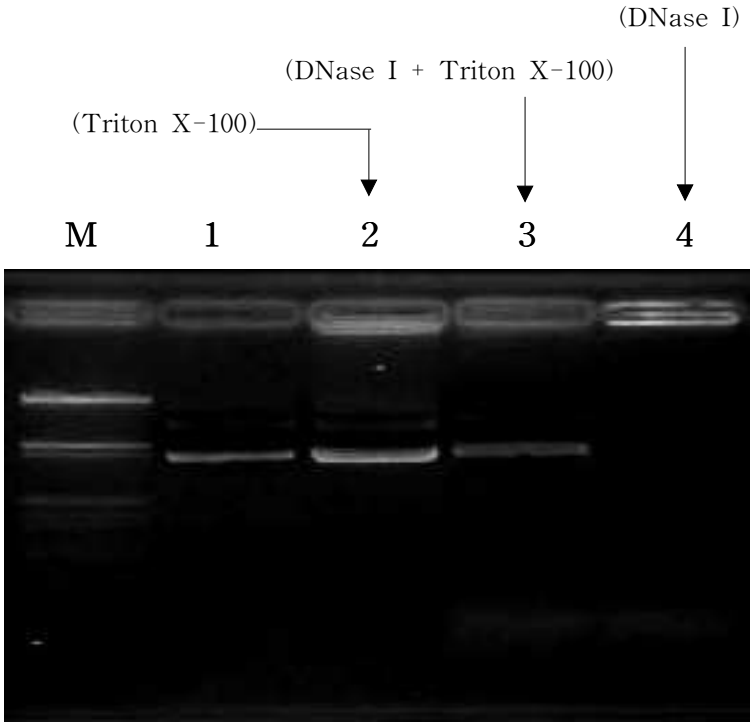



**Figure 2. Encapsulation of plasmid DNA into liposomes.**

The liposomes encapsulating pDNA treated with DNase I (lane 4), treated with DNase I and then Triton X-100 (lane 3), or treated only with Triton X-100 (lane 2) were run on 1% agarose gel and visualized by UV illumination. Lane M, λ/*Hin*d III DNA molecular weight markers; lane 1, untreated naked DNA.

## 3. Conjugation of Fab′ fragments to liposomes

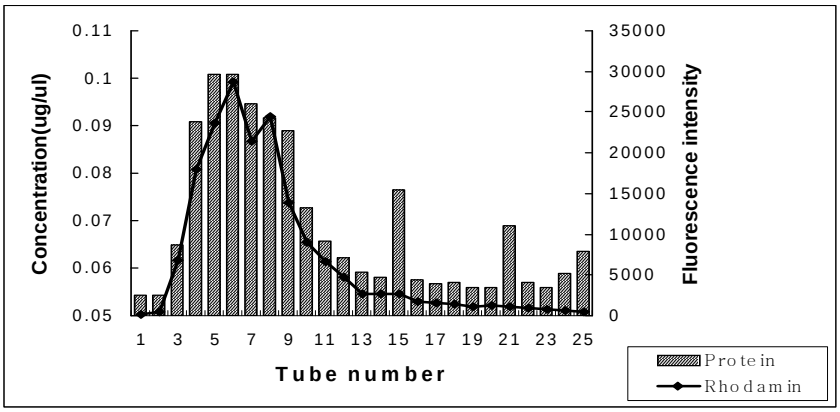
For Fab′ conjugation to the surface of liposomes, the purified Fab′ fragments were added to the prepared liposomes exposing functional maleimide groups at 1:2.5 (molar ratio of protein and lipid). The reaction mixture was incubated at 4℃ for overnight in mild agitation. The un-conjugated Fab′ fragments and un-encapsulated pDNA were removed via CL-4B gel chromatography. The fractions of Fab′ fragments were coincided with the fractions of liposomes coupled to rhodamine (Fig. 3A).

Also, in order to calculate the average number of Fab′ molecules per liposome, the lipid concentration and the amount of Fab′-conjugated liposomes were determined by the phosphorous assay and DC protein assay, respectively. According to Enoch and Strittmatter method, the average number of Fab′ fragments per liposome vesicle were 8-12 molecules. At the same time, the conjugated antibodies were identified by SDS-PAGE (Fig. 3B).

## 4. Stability of the pDNA encapsulated in anti-EGFR immunoliposomes

The *in vitro* DNase I protection assay was performed to assess the role of liposomal encapsulation in protecting DNA molecules from attack of degrading enzymes. The free pDNA was completely degraded by digestion with DNase I (Fig. 4, lane 6). However, the pDNA in the immunoliposomes were still intact without any degradation by nuclease (Fig. 4, lane 4, 5).

(A)




(B)

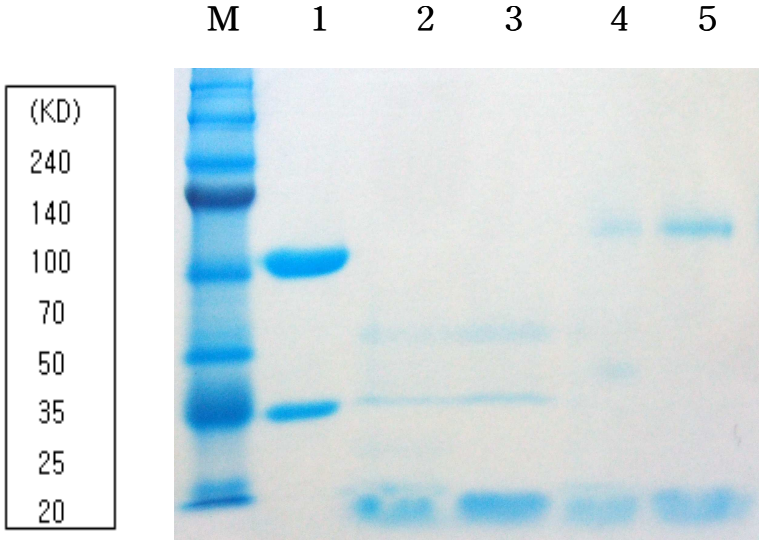



**Figure 3. Conjugation of Fab′ fragments to liposomes.**

Each sample collected from CL-4B gel chromatography was quantified by measurement of rhodamine fluorescence and the protein assay (A). The samples in the fractions containing proteins were run on 12% SDS-PAGE. M, molecular weight marker; lane 1, whole IgG; lane 2 and 3, reduced; lane 4-5, unreduced.

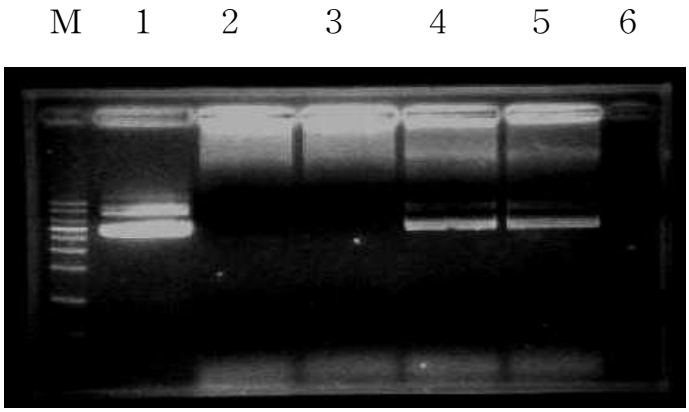



**Figure 4. Stability of the encapsulated DNA in anti-EGFR immunoliposomes.** The DNase I protection assay was performed to verify protection of DNA molecules from attack of degrading enzymes. Lane M, λ/*Hin*d III DNA molecular weight markers; lane 1, untreated naked DNA; lane 2 and 3, immunoliposomes treated with DNase I; lane 4 and 5, immunoliposomes treated with DNase I and Triton X-100.

## 5. *In vitro* specific binding of Cetuximab IgG to EGFR-overexpressing cells

Specificity of Cetuximab (whole IgG) to EGFR was determined in many types of cells by immunofluorescence staining. MDA-MB-468 cells overexpressing EGFR were stained by the FITC-labelled whole IgG. However, MCF-7, HT-29, B16Bl16 cell lines less expressing EGFR were not stained by the FITC-labeled whole IgG (Fig. 5).

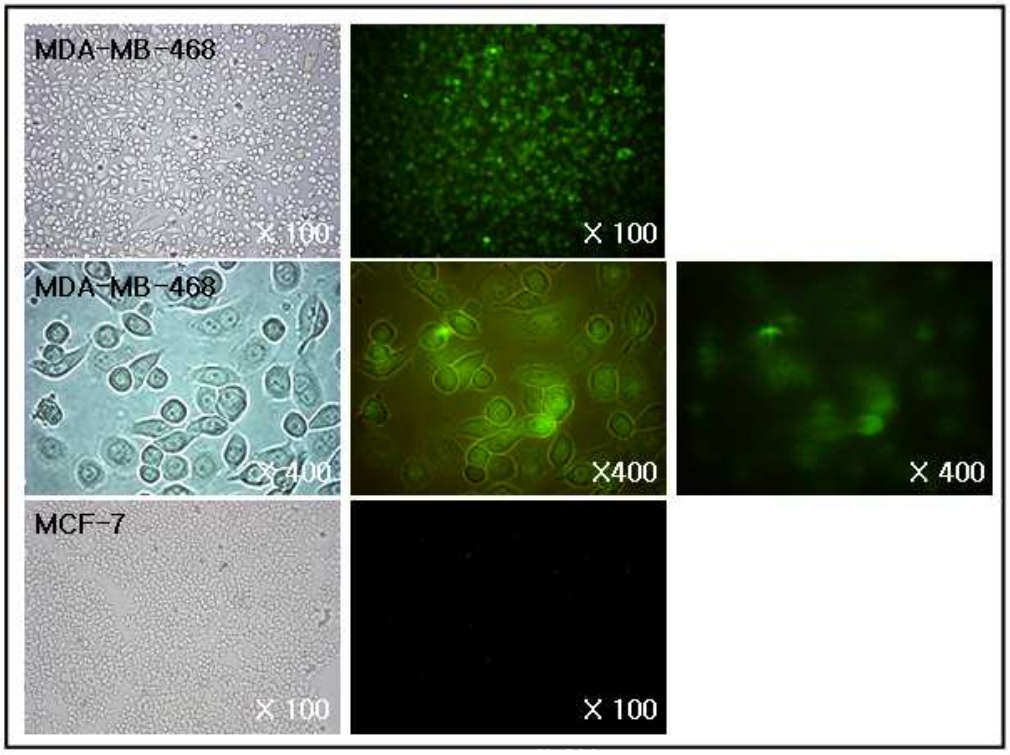
## 6. *In vitro* cellular binding of anti-EGFR immunoliposomes

Specific binding of the EGFR-targeted immunoliposomes was evaluated in various cell lines. The immunoliposomes were able to bind to the cell lines overexpressing EGFR (MDA-MB-468, SNU-C2B, and WiDr), but not to the cells less expressing EGFR (Fig. 6).

## 7. *In vitro* gene transfection by anti-EGFR immunoliposomes

*In vitro* transfection mediated by the EGFR-targeted immunoliposomes were compared in the EGFR-overexpressing cell lines (MDA-MB-468 and SK-Br-3) and the cell lines less expressing EGFR (MCF-7). The EGFR-targeted immunoliposomes exhibited more efficient transfection in the EGFR-overexpressing cells than EGFR less expressing cells.

(A) (B)



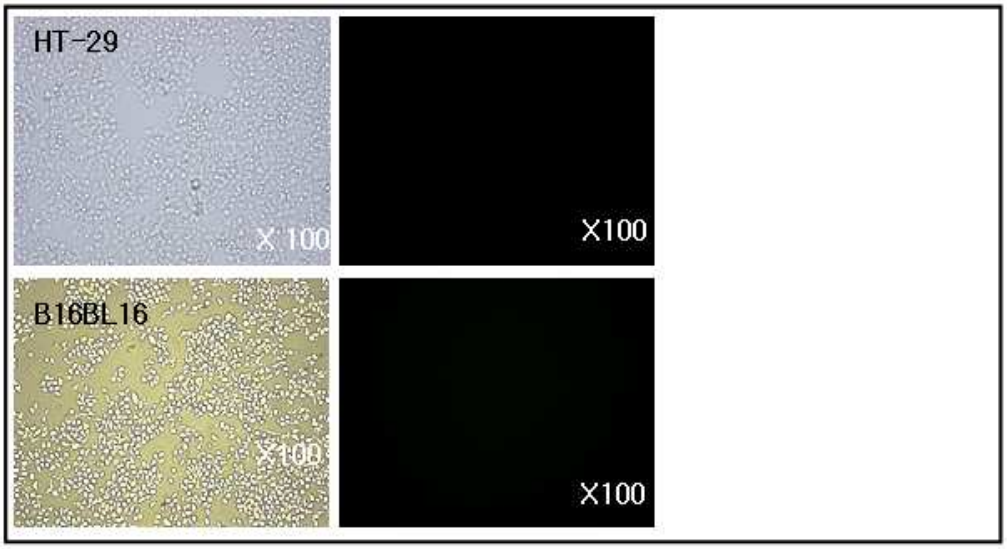
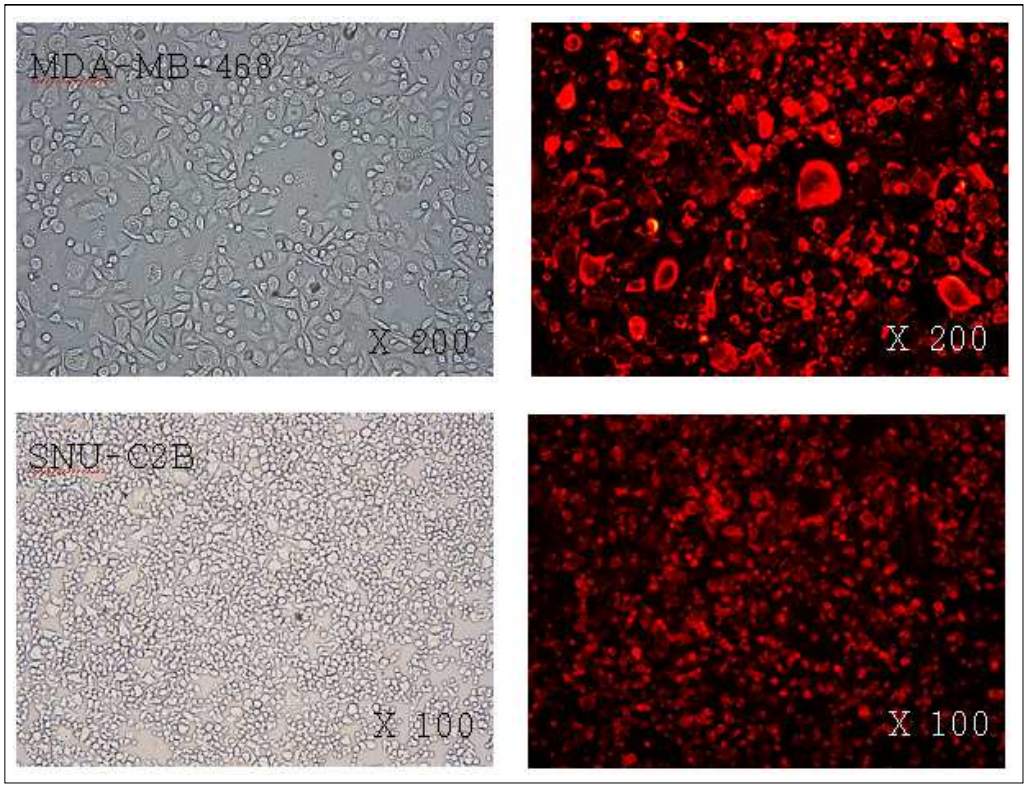



**Figure 5. Specific binging of IgG to EGFR-overexpressing cells.** The FITC-labelled Cetuximab whole IgG was added to MDA-MB-468, MCF-7, HT-29, and Bl6BL6 cells and those cells were then examined under a light microscopy (A) and a fluorescence microscopy (B).

(A) (B)



(A) (B)

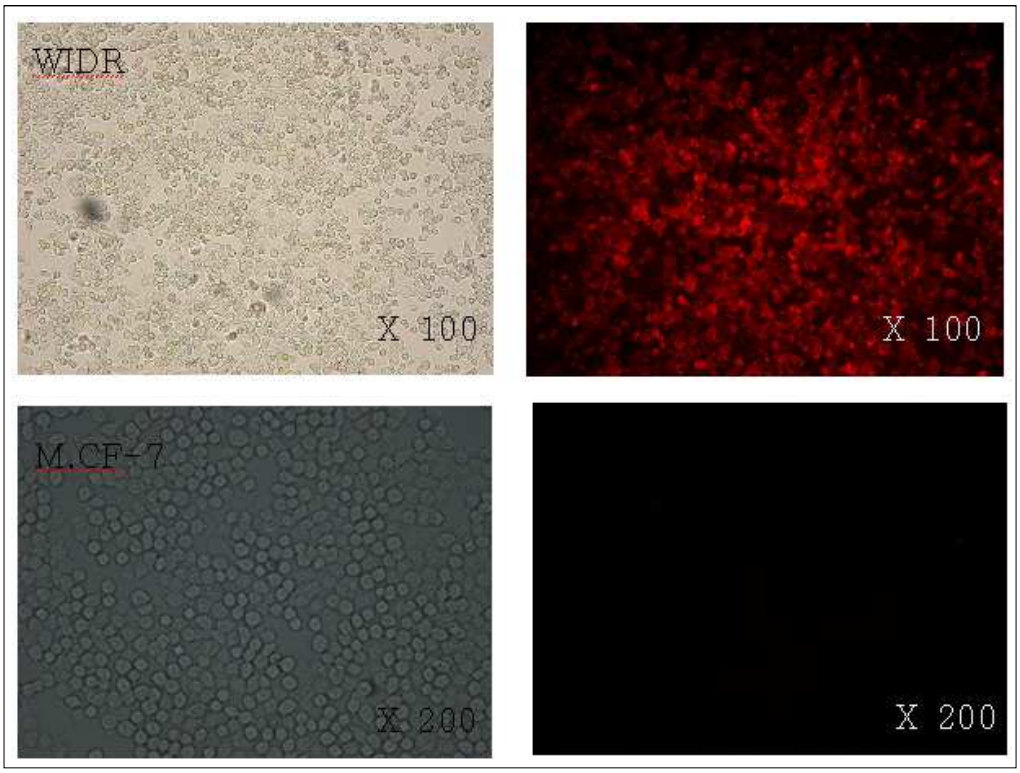



**Figure 6. *In vitro* cellular binding of the anti-EGFR immunoliposomes.** MDA-MB-468, SNU-C2B, WiDr, and MCF-7 cells were treated with the EGFR-targeted immunoliposomes containing 0.2 mol % Rho-DOPE and examined with a light microscopy (A) and a fluorescence microscopy (B).

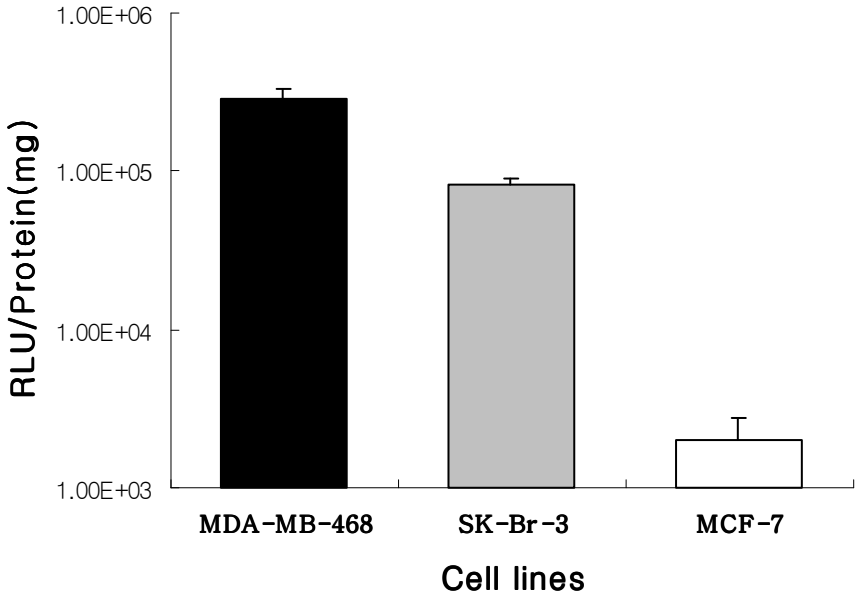



**Figure 7. *In vitro* gene transfection by the anti-EGFR immunoliposomes.** MDA-MB-468, SK-Br-3, and MCF-7 cells were transfected with the EGFR-targeted immunoliposomes containing pAAV-CMV-Luc in the absence of fetal bovine serum for 4 h. Then, the transfected cells were further incubated for 24 h. Each bar represents the mean ± S. D. for three separate experiments of the luciferase assay.

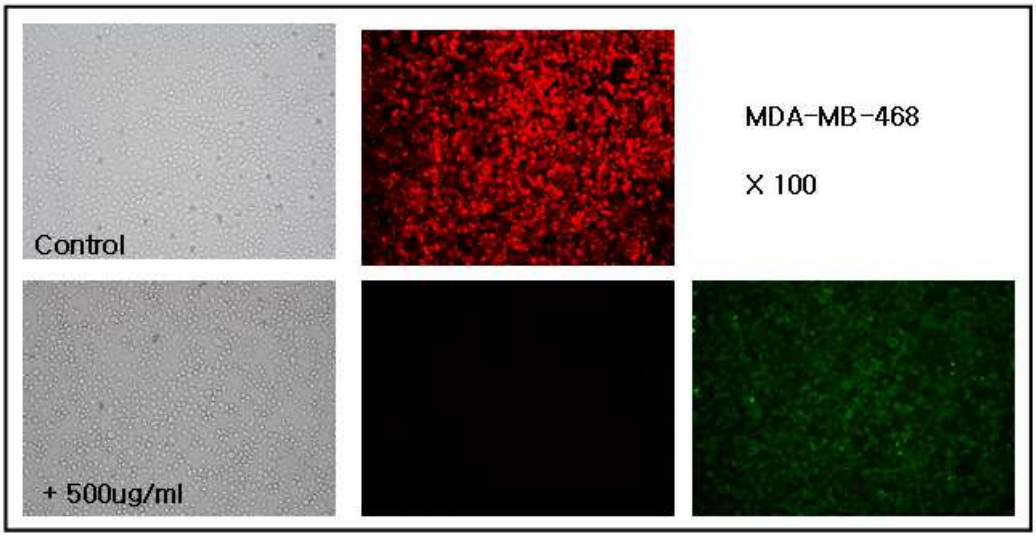
## 8. *In vitro* competitive binding assay of anti-EGFR immunoliposomes

To verify Fab′-mediated specific binding of the immunoliposomes, the cells were pre-treated with FITC-labeled whole antibodies for 45 min before treated with immunoliposomes. The cells were treated with the EGFR-targeted immunoliposomes containing Rho-DOPE for 1 h. Binding of the immunoliposomes to MDA-MB-468 cells (EGFR-overexpressing) were inhibited by pre-treatment with FITC-labelled antibodies (Fig. 8). Meanwhile, the same immunoliposomes nor the FITC-labelled antibodies did not bind to MCF-7 cells (EGFR less expressing). These results implied that the specific binding of the immunoliposomes to the EGFR-overexpressing cells was due to the Fab′ fragments conjugated to the liposomes.

## 9. *In vitro* competitive transfection by anti-EGFR immunoliposomes

Also, to verify Fab′-mediated specific transfection with the immunoliposomes, the cells were pre-treated with Cetuximab whole antibodies before transfection. Then, the pre-treated cells were transfected with the EGFR-targeted immunoliposomes or non-targeted bare liposomes containing luciferase genes. Expression of the trans-gene in MDA-MB-468 cells were significantly inhibited by pre-treatment with whole antibodies while that in MCF-7 was not. The gene expression by the non-targeted bare liposomes was lower than that by the EGFR-targeted immunoliposomes and not inhibited by pre-treatment with the whole antibodies. There results implied that the efficient expression by the immunoliposomes in the EFGR-overexpressing cells (MDA-MB-468) was due to specific recognition of EGFRs on tumor cell surface by the Fab′ fragments coupled to the immunoliposomes (Fig. 9).

(A) (B) (C)



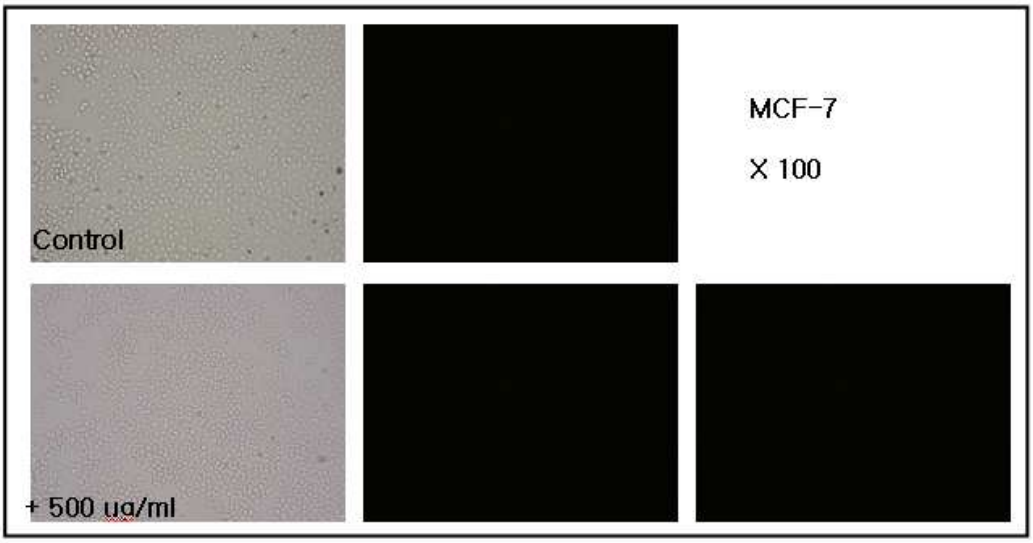



**Figure 8. *In vitro* competitive binding assay of the anti-EGFR immunoliposomes.** FITC-labeled anti-EGFR whole antibodies were added to MDA-MB-468 and MCF-7 cells before addition of immunoliposomes containing Rho-DOPE. The treated cells were examined with a light microscopy (A) and a fluorescence microscopy (B, C).

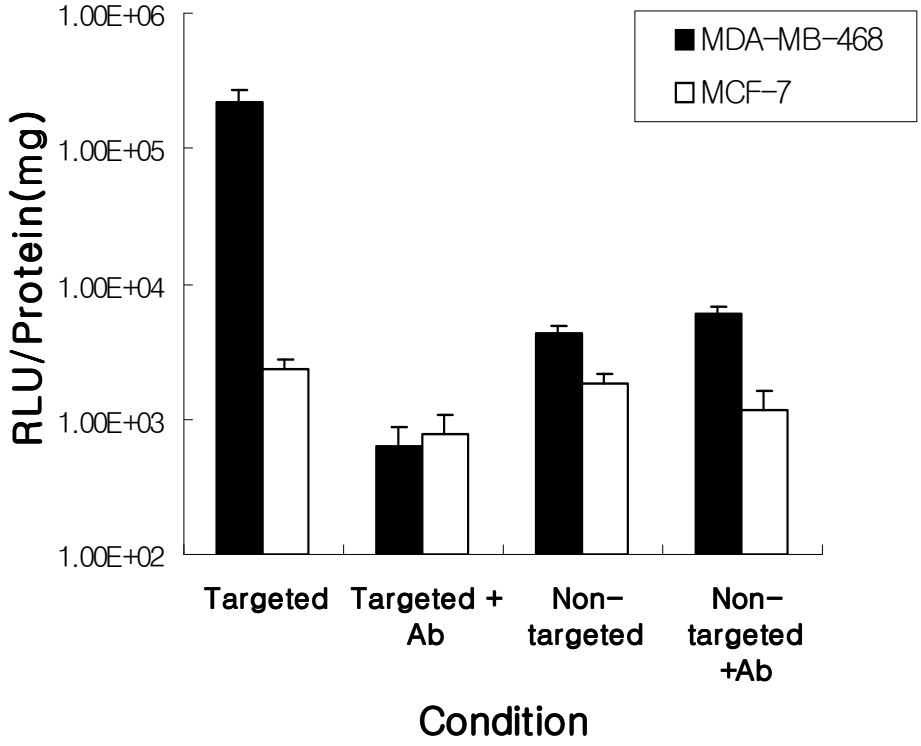



**Figure 9. *In vitro* competitive transfection of the anti-EGFR immunoliposomes.** The MDA-MB-468 and MCF-7 cells pre-treated with anti-EGFR antibodies were transfected with the EGFR-targeted immunoliposomes or non-targeted bare liposomes.

## 10. *In vitro* transfection by various types of anti-EGFR immunoliposomes

To evaluate the influence of Fab′ density and lipid composition in the immunoliposomes on gene transfection, various types of immunoliposomes were preparated as described in Table II and III. Basically, mole% of the coupling lipid (DSPE-PEG-MAL) and the other lipids were varied appropriately to change the Fab′ density on the liposomal surface. The amount of cationic lipid (DMKD) was balanced with that of anionic lipid (DSPE derivatives) (Table I). The changes of lipid composition resulted in changing the amounts of encapsulated pDNA in the prepared immunoliposomes (O, A, B, C, D) (Table III). The cells were transfected with the equimolar concentration of immunoliposomes. According to the experimental results, the "O" type of immunoliposomes exhibited the best transfection in SK-Br-3 cells overexpressing EGFRs. Generally, the all immununoliposomes showed higher gene expression in SK-Br-3 cells than MCF-7 cells expressing less EGFRs.

## 11. *In vivo* transfection by anti-EGFR immunoliposomes

To examine tumor-specific localization of the anti-EGFR immunoliposomes, resulting in tumor-specific gene expression, the immunoliposomes were intravenously injected into WiDr tumor-bearing nude mice. And the immunoliposomes were intratumorally injected into WiDr tumor-bearing nude mice. At 48 h post injection, the mice were anesthetized with isoflurane and imaged in red fluorescence ($\lambda_{ex}$=500 nm and $\lambda_{em}$=575 nm). Then, the same mice was intraperitoneally injected with D-luciferin substrate dissolved in sterile PBS and imaged using a luminometer. The fluorescence and photons emitted from the mice, transmitting through the tissues, were collected with a cooled charge-coupled device (CCD) camera (IVIS$^{TM}$; Xenogen). As a result, the immunoliposomes were predominantly localized in tumor tissues (Fig. 10A). This data clearly showed that the anti-EGFR Fab′ immunoliposomes were able to recognize EGFR-expressing tumor cells in vivo. All mice exhibited certain amounts of autofluorescence regardless of administration of the immunoliposomes (Fig. 10B). However, unexpectedly, expression of the luciferase gene delivered via the immunoliposomes was not monitored by this method (Fig. 11). The bioluminescence signal was represented as photon/sec/㎠/steradian (p/sec/㎠/sr).

Table II. Compositions of various types of immunoliposomes

| Type \ Lipid | POPC | DMKD | DSPE-PEG | DSPE-PEG -MAL | Rho-DOPE |
|---|---|---|---|---|---|
| O | 93 | 3 | 2.8 | 1 | 0.2 |
| A | 92 | 4 | 2.8 | 1 | 0.2 |
| B | 92 | 4 | 1.8 | 2 | 0.2 |
| C | 90 | 5 | 0.8 | 4 | 0.2 |
| D | 86 | 7 | 0.8 | 6 | 0.2 |

*The mole of cationic lipid (DMKE) was balanced with that of anionic lipids (DSPE-PEG-MAL and DSPE).

Table III. The number of Fab′ molecules and the amount pDNA in the prepared immunoliposomes

| Type | O | A | B | C | D |
|---|---|---|---|---|---|
| Ab molecules/vesicle | 10.5 | 8.9 | 23(22.948) | 33.5 | 23.5 |
| Encapsulation DNA/0.4uM | 723.9 | 1197.7 | 1018.6 | 972.2 | 1081.7 |

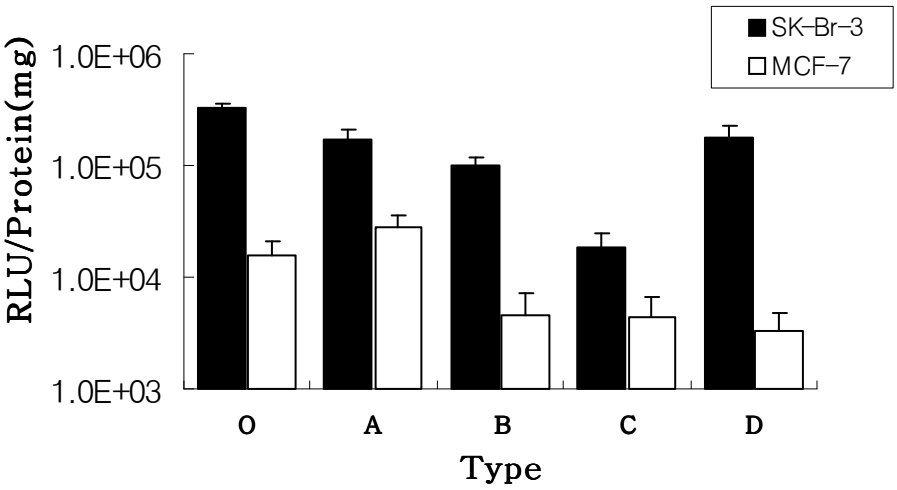



**Figure 10. *In vitro* gene transfection efficiencies of the immunoliposomes having varied amounts of Fab′ and pDNA.** SK-Br-3 and MCF-7 cells were transfected with the various types of immunoliposomes. They have varied numbers of anti-EGFR Fab' molecule their surfaces and are encapsulating varied amounts of pDNA.

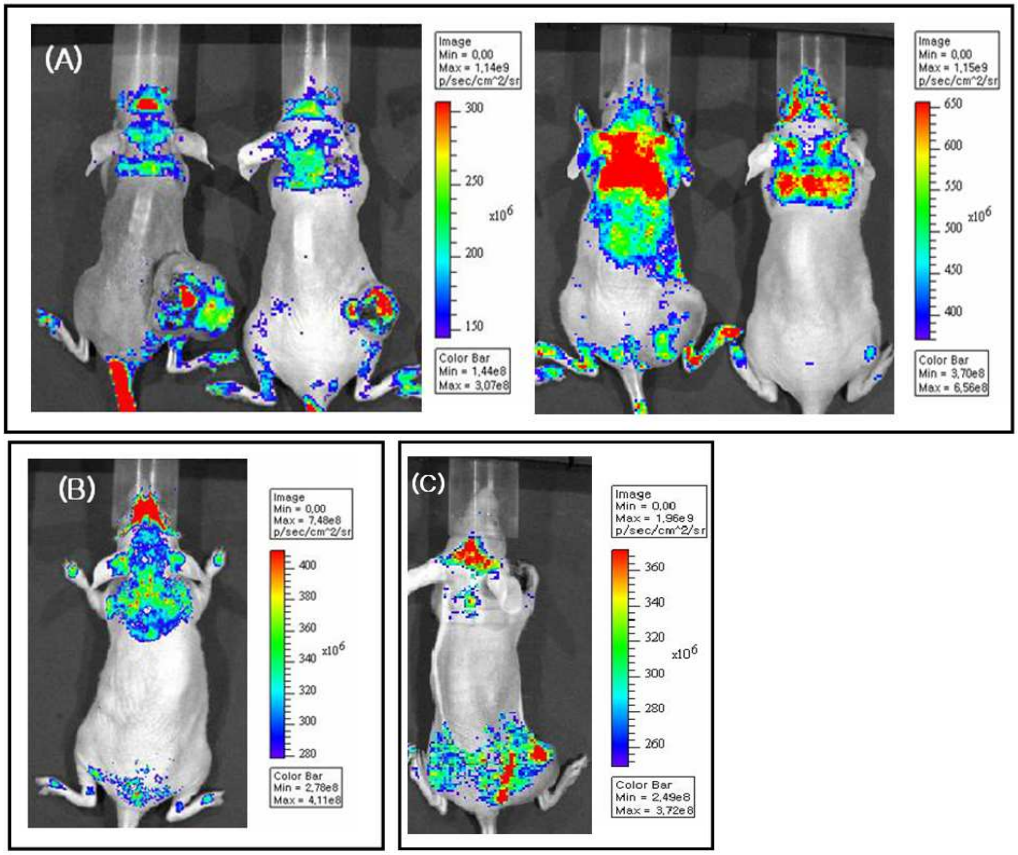



**Figure 11. *In vivo* biodistribution and transfection by the anti-EGFR immunoliposomes.** The O type of immunoliposomes were intravenously injected into the WiDr tumor-bearing nude mice via tail vein. And the immunoliposomes were intratumorally injected into the WiDr tumor-bearing nude mice. At 48 h post injection, the immunoliposome intravenoualy treated mice (A), untreated mice (B) and immunoliposomes intratumorally treated mice were anesthetized and imaged in red fluorescence with CCD IVIS$^{TM}$ system.

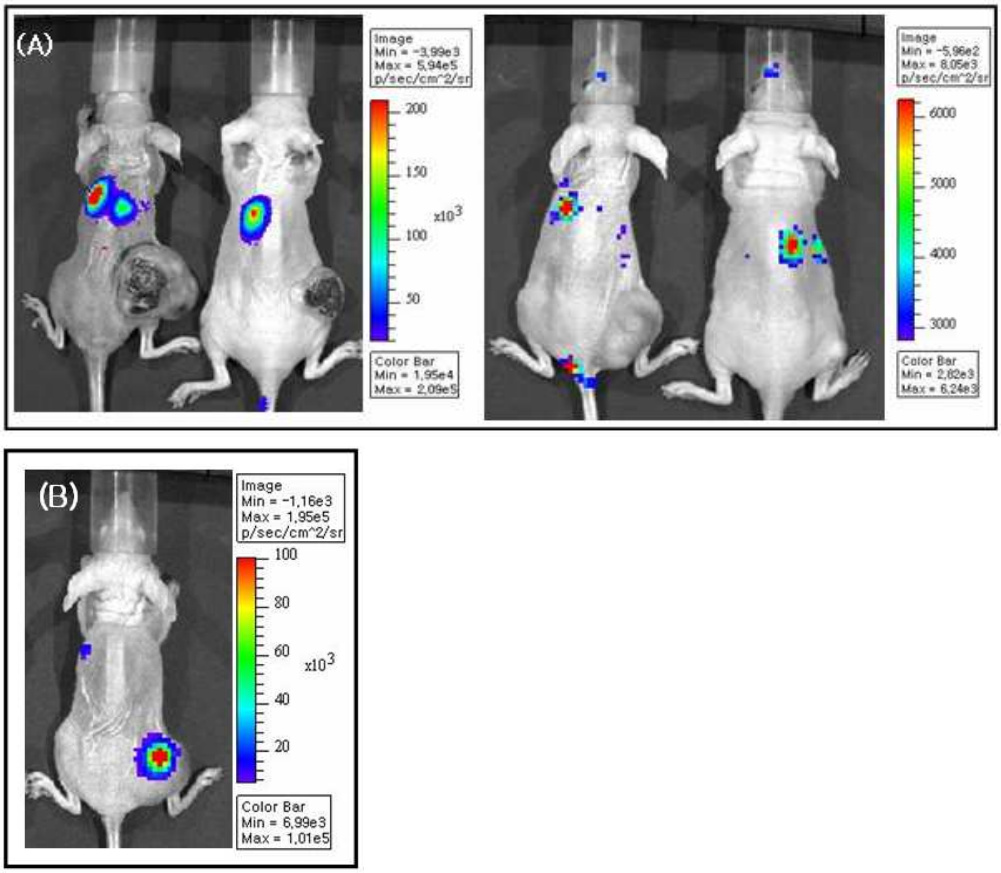



**Figure 12. *In vivo* transfection by the anti-EGFR immunoliposomes.** The O type of immunoliposomes were intraveneously injected into the WiDr tumor-bearing nude mice via tail vein. And the immunoliposomes were intratumorally injected into the WiDr tumor-bearing nude mice. At 48 h post injection, the mice were intraperitoneally injected with D-luciferin (3 mg/mouse) 10 min before imaging. The immunoliposome intravenoualy treated mice (A) and immunoliposomes intratumorally treated mice (B) were anesthetized and imaged with CCD IVIS$^{TM}$ system.

# IV. DISCUSSION

Broadly defined, the concept of gene therapy involve the transfer of genetic materials into a cell, or whole organ, with the goal of curing a disease or at least improving the clinical status of patient. A key factor in the success of gene therapy is the development of delivery systems that are capable of efficient gene transfer in a variety of tissues, without causing any associated pathogenic effects.

In this study, anti-EGF receptor Fab′-coupled immunoliposomes were developed for gene delivery specific to EGFR-overexpressing tumor cells. When any types of immunoliposomes are designed for target-directed gene delivery, a number of aspects have to be considered in terms of target specificity, gene expression efficiency, and so on.

Firstly, the immunoliposomes should encapsulate pDNA completely and stably, rendering protection from degradation by nuclease (Fig. 2). Although recently a number of pDNA-encapsulating methods have been reported (21), the freeze-thaw method is still the best method to encapsulate pDNA into liposomes easily and rapidly.

Secondly, the immunoliposomes should be able to direct to the intended cells or tissues specifically. In this study, Fab′ fragments were prepared from the whole antibodies against EGFR (Cetuximab, Erbitux ®) (Fig. 1). When the whole antibodies are conjugated to the immunoliposomes, a number of thioether bonds are formed between the liposomes and the whole antibody molecules having multiple thiolation sites. Therefore, the antibody molecules are randomly and irregularly oriented on the liposome surface, which undermines the target-specificity of immunoliposomes. In addition, the Fc regions of antibodies may be available to bind to the Fc receptors, resulting in rapid clearance of

the immunoliposomes by the immune cells. Based on this consideration, the Fab′ fragments, which have only thiol group, conjugation to the liposomes will give longer circulation in blood and more effective targeting.

Thirdly, an simple and efficient conjugation reaction is necessary to preserve the vesicular size of liposomes. The conjugation of Fab′ fragments should not increase the vesicular size. It is well known the smaller immunoliposomes (less than 150 nm) exhibits more specific targeting than the bigger ones. Considering the all aspects mentioned above, a small sized anti-EFGR Fab′ fragments-conjugated immunoliposomes encapsulating pDNA were prepared as a gene delivery system specific to EGFR-overexpressing tumor cells.

Lastly, the liposomal composition is a critical parameter governing gene delivery and expression mediated by the immunoliposomes. The balance between the cationic and the anionic lipid is a very important factor. Addition of too much anionic lipids induced aggregation of the liposomes and made it difficult to encapsulate the negatively charged pDNA (data not shown). Addition of too much cationic lipids induced non-specific interaction with negatively charged cell surface, resulting in non-specific transfection (Fig. 10).

Previously, anti-EGFR Fab′ fragment conjugated to liposomes could bind specifically to MDA-MB-468 cells over-expressing EGFR (22). The total cellular uptake of the EGFR-targeted immunoliposomes was up to 3 orders of magnitude higher in MDA-MB-468 cells than in the cells less expressing EGFRs, indicating their substantial specificity for target versus non-target cells.

Another important feature of targeting with the immunoliposomes is the threshold concentration of epitopes on the target cell surface. The EGFR-targeted immunoliposomes were effectively internalized in EGFR-overexpressing cells ($10^5$~$10^7$ EGFRs/cell) such as MDA-MB-468, but not in the cells less expressing EGFRs ($10^4$ EGFRs/cell), such as MCF-7. This

result implies that non-specific delivery of immunoliposomes unlikely occurs in normal tissues, including epithelial cells expressing $10^3$~$10^4$ EGFRs/cell (22). This study also supported the idea that the specific targeting of immunoliposomes is dependant upon the epitope concentration on the cell surface (Fig. 6).

The specific targeting of immunoliposomes reflected specific gene expression in the target cells. The transgene expression mediated by the anti-EGFR Fab′-conjugated immunoliposomes was at least two order higher in the cell over-expressing EGFRs (MDA-MB-468 and SK-Br-3) than the cells less expressing EGFRs (MCF-7) (Fig. 7). Meanwhile, the non-targeted liposomes exhibited a similar level of gene expression in MDA-MB-468 and MCF-7 cells. The competitive inhibition using free antibodies against EGFR suggested that the selective gene transfection was solely mediated by the Fab′ coupled to the liposomes (Fig. 8, 9).

The previous study using a tumor-xenograft model showed that the target-specific delivery mediated by the immunoliposomes is far more efficient than that by the conventional liposomes lacking of antibodies (23). The immunoliposomes could deliver therapeutic drugs to a specific target cell population in a mouse (24). In this study, the anti-EGFR Fab′-conjugated immunoliposomes were administered to the WiDr-xenografted animal model, and their target-specific delivery and target-specific expression of the delivered transgene (luciferase) was analyzed using the CCD IVIS$^{TM}$ system. The anti-EGFR Fab′-conjugated immunoliposomes were specifically delivered to xenografted tumor cells. Unfortunately, the gene expression was little monitored in the same mice (Fig. 11). It may be resulted from inefficient encapsulation of pDNA into liposomal vesicles or inefficient delivery into the tumor tissues due to rapid clearance of the liposomes.

In summary, the anti-EGFR Fab′-coupled immunoliposomes was prepared

for specific gene delivery to the tumor cells over-expressing EGFRs. The immunoliposomes was able to induce transgene expression specific in the tumor cells over-expressing EGFRs *in vitro*, but not *in vivo*. Before preclinical and clinical applications of the anti-EGFR Fab′-coupled immunoliposomes for anti-cancer gene therapy, the major obstacle, inefficient internalization of the immunoliposomes into the target cells, has to be resolved in future.

# REFERENCES


1. Friedman T. (1999). The development of human gene therapy. *Cold Spring Harbor, New York : Cold Spring Harbor Lab. Press.*
2. Breyer, B, Jiang, W, Cheng, H, Zhou, L, Paul, R, Feng, T, He, T. C. (2001). Adenoviral vector-mediated gene transfer for human gene therapy. *Curr. Gene Ther.,* 1, 149-162.
3. El-Aneed A. (2004). An overview of current delivery systems in cancer gene therapy. *J. Control. Release*, 94, 1-14.
4. Felgner, P. L., Gadek, T. R., Holm, M, Roman, R, Chan, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., Danielsen, M. (1987). Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc. Natl. Acad. Sci. U. S. A.,* 84, 7413-7417.
5. Allen, TM, Chonn, A. (1987). Large unilamellar liposomes with low uptake into the reticuloendothelial system. *FEBS Lett.,* 223, 42-46.
6. Gabizon, A, Papahadjopoulos, D. (1988). Liposome formulations with uptake by tumors. *Proc. Natl. Acad. Sci. U.S.A.,* 85, 6949-6953.
7. Papahadjopoulos, D, Gabizon, A. (1990). Liposomes designed to avoid the reticuloendothelial system. *Prog. Clin. Biol. Res.*, 343, 85-93.
8. Allen, T. M., Hansen, C. (1991). Pharmacokinetics of stealth versus conventional liposomes : effect of dose. *Biochim. Biophys. Acta,* 1068, 133-141.
9. Allen, T. M., Stuart, D. Janoff, A. S. (1998). Liposomes Rational Design. New York: Marcel Dekker, Inc. 63 - 97.
10. Salomon, D. S., Brandt, R, Ciardiello, F, Normanno, N. (1995). Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.,* 19, 183 - 232.

11. Sato, J. D., Kawamoto, T., Le, A. D., Mendelsohn, J., Polikoff J., Sato G. H. (1983). Biological effects in vitro of monoclonal antibodies to human epidermal growth factor receptors. *Mol. Biol. Med.*, 1, 511 - 529.
12. Carpenter, G. (1987). Receptors for epidermal growth factor and other polypeptide mitogens. *Annul. Rev. Biochem.,* 56, 881 - 914.
13. Harris, A. L., Nicholson, S., Sainsbury, R., Wright, C., Farndon, J. (1992). Epidermal growth factor receptor and other oncogenes as prognostic markers. *J. Natl. Cancer Inst. Monogr.*, 11, 181 - 187.
14. Wong, A. J., Bigner, S. H., Bigner, D. D., Kinzler, K. W., Hamilton, S. R., Vogelstein, B. (1987). Increased expression of the epidermal growth factor receptor gene in malignant gliomas is invariably associated with gene amplification. *Proc. Natl. Acad. Sci. U. S. A.,* 84, 6899-6903.
15. Yamanaka, Y., Friess, H., Kobrin, M. S., Buchler, M., Kunz, J., Beger, H. G., Korc, M. (1993). Overexpression of HER2/neu oncogene in human pancreatic carcinoma. *Hum. Pathol.,* 24, 1127 - 1134.
16. Arteaga, C. L. (2002). Epidermal growth factor receptor dependence in human tumors: more than just expression. *Oncologist*, 4, 31 - 39.
17. Arteaga, C. L. (2002). Overview of epidermal growth factor receptor biology and its role as a therapeutic target in human neoplasia. *Semin. Oncol.,. 5,* (Suppl. 14), 3 - 9.
18. Shi N, Zhang Y, Zhu C, Boado RJ, Pardridge WM. (2001). Brain-specific expression of an exogenous gene afer i.v. administration. Proc. *Natl. .Acad. Sci..U.S.A.,* 98, 12754-12759.
19. Zhang, Y., Zhu, C., Pardridge, W. M. (2002). Antisense gene therapy of brain cancer with an artificial virus gene delivery system. *Mol. Ther.,* 6, 67-72.
20. Enoch, H. G., Stfittmatter, P. (1979). Formation and properties of 1000 A diameter, single-bilayer phospholipid vesicles. *Proc. Natl. Acad. Sci. U.*

*S. A,* 76, 145-149.

21. Semple, S. C., Klimuk, S. K., Harasym, T. O., Dos, Santos N., Ansell, S. M., Wong, K. F., Maurer, N., Stark, H., Cullis, P. R., Hope, M. J., Scherrer, P. (2001). Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. *Biochim. Biophys. Acta,* 1510,152-166.
22. Mamot, C., Drummond, D. C., Greiser, U., Hong, K., Kirpotin, D. B., Marks, J. D., Park, J. W. (2003). Epidermal Growth Factor Receptor (EGFR)-targeted Immunoliposomes mediate specific and efficient drug delivery to EGFR- and EGFRvIII-overexpressing Tumor cells. *Cancer Res.*, 63, 3154 - 3161.
23. Holmberg, E., Maruyama, K., Litzinger, D. C., Wright, S., Davis, M., Kabalka, G. W., Kennel, S. J., Huang, L. (1989). Highly efficient immunoliposomes prepared with a method which is compatible with various lipid compositions. *Biochem. Biophys. Res. Commun.,* 165, 1272-1278.
24. Iden, D. L., Allen, T. M. (2001). In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach., *Biochim.. Biophys.. Acta.* 1513, 207-216.

## 국문 요약

# 상피세포 수용체 표적 면역리포좀을 이용한 암세포 선택적 유전자 전달

연세대학교 대학원
임상병리학과
김은정

효율적인 유전자 전달체는 유전자 치료에 있어서 매우 중요한 요소이다. 본 논문에서는 정상세포에는 접근하지 않고, 암세포에만 특이적으로 항암유전자를 전달하여 효율적으로 암을 치료할 수 있는 유전자 전달체를 만들고자 하였다. 상피세포 성장인자 수용체는 세포막에 존재하는 단백질로, 상피세포 성장인자와 결합하면 성장신호가 전달되어 세포의 증식이 촉진된다고 알려져 있다. 이러한 상피세포 성장인자는 대부분의 암세포에 과량으로 발현하고 있어, 이를 표적으로 하여 암세포에만 특이적으로 유전자를 전달하는 면역리포솜을 만들고자 하였다. 상피세포 성장인자 수용체에 특이적으로 결합하는 항체는 전항체를 대신하여 Fab' 절편을 이용하였다. 우선 대식 세포에 탐식되지 않고, 혈중에 오랫동안 순환할 수 있는 조성의 리포좀을 제조한 후 표지유전자를 봉입하였다. 그리고 여기에 항체의 Fab' 절편을 결합시켜 면역리포솜을 제조하였다. 이러한 면역리포솜은 상피세포 수용체

를 과량으로 발현하는 암세포에만 특이적으로 반응하여 세포내로 표지 유전자를 전달하였고, 또한 전달된 유전자는 효율적으로 발현되었다. 항체경쟁 실험을 통해 표적암세포에의 보다 효율적인 유전자발현이 면역리포솜에 결합된 항체의 Fab' 절편에 기인함을 알 수 있었다. 그러나 면역리포솜을 암세포가 이식된 누드마우스 모델에 정맥 투여한 결과 종양조직에 분포하는 것은 확인이 되었으나, 유전자발현은 관찰되지 않았다. 본 논문에서 제조한 면역리포솜의 세포내 이입활성을 향상시킬 수 있다면 항암 치료유전자를 표적암세포에 선택적으로 전달하는 수단으로 이용할 수 있을 것이다.

주요 단어 : 면역리포좀, 상피세포 수용체, Cetuximab, 유전자 전달체