Anti-JL1 immunoliposomes encapsulating doxorubicin for leukemia-directed therapy

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ABBREVIATIONS

DEPE-PEG$_{2000}$-Mal: 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)$_{2000}$]

DOX: Doxorubicin

PEG: Polyethylene glycol

POPC: 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphadidylcholine

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

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SILs: Stealth immunoliposomes

SLs: Stealth liposomes

SLs-DOX: Doxorubicin-encapsulating stealth liposomes

SILs-DOX: Doxorubicin-encapsulating stealth immunoliposomes

Anti-JL1 immunoliposomes encapsulating doxorubicin for leukemia-directed therapy

ABSTRACT

Doxorubicin-loaded long-circulating (stealth) liposomes (Doxil®, Alza Pharmaceuticals), PEGylated liposomes encapsulating doxorubicin, are commercially available to treat a variety of cancers. It has been well documented that the liposomal formulation of doxorubicin exhibits reduced side-effects, resulting in enhanced therapeutic efficacy. However, the liposomal doxorubicin still has substantial adverse side-effects due to nonselective toxicity and nonspecific distribution of the drug to normal cells. To address these problems, anti-JL1 immunoliposomes encapsulating doxorubicin were prepared for targeted delivery of doxorubicin to leukemia cells over-expressing JL1 antigens. It was previously reported that JL1 antigens were uniquely expressed on the cell surface of most T leukemias. In this study, stealth immunoliposomes (SILs) were prepared by two different methods, (1) direct coupling of anti-JL1 antibody to 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-[maleimide(polyethylene glycol)$_{2000}$] (DSPE-PEG$_{2000}$-MAL) in

preformed liposomes encapsulating doxorubicin or (2) post-insertion of DSPE-PEG-anti-JL1 antibody conjugates to preformed liposomes encapsulating doxorubicin. The SILs prepared by the two different methods were compared to each other in terms of *in vitro* binding affinity to T-leukemic cells, cellular internalization, and cytotoxicity depending on the presence of JL1 antigens and concentration of anti-JL1 antibody on the liposomal surface. According to FACS analysis and fluorescence microscopy, anti-JL1 SILs were able to specifically bind to JL1-positive CEM and HL60 cells, not to control H9 cells (JL1-negative). With a confocal laser scanning microscope, effective internalization of anti-JL1 SILs into the cytoplasm of CEM and HL60 cells was verified, which was presumably due to JL1 antigen-mediated recognition and endocytosis of the liposomes. As previously shown, doxorubicin encapsulated in liposomes was less cytotoxic than free doxorubicin, regardless presence of JL1 antigen on the cell surface. However, the reduced cytotoxicity was significantly recovered by coupling of anti-JL1 antibodies to the liposomal surface, presumably resulting from JL1-mediated internalization of doxorubicin to tumor cells. Meanwhile, the SIL-DOX formulations prepared by the two different methods did not exhibited significant differences in terms of cell binding, internalization, and cytotoxicity. The experimental results in this thesis provide valuable information that the anti-JL1 SIL-DOX would be applicable as a noble clinical modality for treatment of leukemia expressing JL1 antigens after preclinical and clinical evaluation.

I. INTRODUCTION

Numerous applications of monoclonal antibodies (mAb) have been preposed in cancer diagnosis and therapy. Over the past decades, significant advances have been achieved in the immunodiagnosis and immunotherapy of cancers (1). Ideally, antibodies against tumor-associated antigens for immunodiagnosis and immunotherapy should specifically target tumor cells and have minimal cross reactivity with normal tissues because nonspecific bindings would reduce the diagnostic and therapeutic efficacies (2).

The JL1 molecule is a 120-130 KDa surface molecule uniquely expressed on subpopulation of human thymocytes (3,4). Since the JL1 antigen is selectively expressed on the surface of human leukemic cells, but not on the mature human peripheral blood cells, normal bone marrow cells and various types of normal tissues, it can be an excellent candidate of cancer-targeting molecule for an immunodiagnosis and immunotherapy of hematopoietic malignancies such as leukemia and lymphoma (4).

Despite restricted distribution of the JL1 antigen in normal tissues and cells, anti-JL1 mAb recognizes various types of myeloid leukemia of B-cell origin and T-cell lineage such as human leukemia CEM and HL60 cells. It has been well documented that JL1 antigen/anti-JL1 antibody complexes are readily internalized into the intracellular compartment upon the engagement of JL1 with anti-JL1 mAb. These findings prompted to evaluate the possibility that anti-JL1 mAb can be used as an immunotherapeutic agent for leukemia. The expression of JL1 antigen was observed in 76% of leukemic cases (117 out of 154 leukemic patients tested) by flow cytometric analysis[5]. The percentage of JL1-positive cases of T lineage acute lymphoblastic leukemia (T-ALL) (93%) was higher than that of other leukemia types (75%) (5).

Doxorubicin is the best known and the most widely used anticancer drug of the anthracycline antibiotics. It was first introduced in the 1970, and since that time has become one of the most commonly used drugs for the treatment of both hematological and solid tumors. Its precise mechanism of anticancer is still not fully understood, however it is known to intercalate between DNA base pairs, resulting in inhibition of DNA synthesis and DNA-dependent RNA synthesis due to template disordering and steric obstruction (6). Therefore, doxorubicin has inherent toxicity to most types of dividing cell including cancer cells. Also, its clinical applications, as with other anthracyclines, have been limited by cardiomyopathy (6).

Liposomes are the most advanced particulate drug carriers and now considered to be a mainstream drug delivery technology. The liposomal carrier represents an ideal drug delivery system, as the microvascular in tumors is typically discontinuous, having pore sizes large enough (100-780 nm) for liposomes to move from the blood compartment into the extravascular space surrounding the tumor cells. The primary aim of doxorubicin encapsulation in liposomes has been to decrease nonspecific organ toxicity. Liposomes are able to direct the doxorubicin away from sites with tight capillary junctions such as the heart muscle. Instead, they are distributed in areas where fenestrations or gaps exist in the vasculature (liver, spleen, and bone marrow, areas of inflammation, and neoplasms) (7,8).

However, a major drawback of conventional liposomes in clinical applications is the fast elimination from the blood and capture of the liposomal preparations by the cells of the reticulo-endothelial system (RES), primarily in the liver (9). A number of developments have aimed to resolve this problem. A great deal of effort has been spent to make liposomes to avoid the RES, resulting in prolonged liposome circulation. Liposomal formulations containing polyethylene glycol (PEG)-modified lipids are known to exhibit increased circulation time in

blood. The increased circulation longevity of the PEG-liposomes allows enhanced extravasation across the leaky endothelium of solid tumors (9, 10).

Despite enhanced accumulation in tumor tissues, PEG-liposome carriers encapsulating anticancer drugs still have a substantial extent of toxicity because of their nonspecific interactions with normal cells. To overcome this limitation, tumor-directed immunoliposomes have been developed adopting varied types of mAb against tumor-associated antigens (11-13). Immunoliposomes have specific antibodies or antibody fragments on their surface to enhance binding to target sites. It has been demonstrated that the immunoliposomes are able to more effectively delivery drugs to target cells than the conventional liposomes lacking antibody (14). General requirements for efficient in vivo targeting of immunoliposomes are a sufficient antibody density and a reduced affinity of the RES.

To construct stealth immunoliposomes, antibodies can be linked to the liposomal surface via noncovalent or covalent coupling. Noncovalent coupling usually is accomplished by using biotinylated antibodies, which are bound to avidin conjugated liposomes (15). Covalent coupling utilizes a variety of bioconjugation techniques, such as forming of thioether, disulfide, or amide bonds between lipids and the antibody molecules (16). In general, antibodies can be directly linked to liposomes through covalent conjugation to functional groups on the liposomal surface (direct coupling) or antibody-lipid conjugates can be inserted into preformed liposomes (post-insertion) (17,18).

It has been generally agreed that coupling of the tumor-specific antibodies to the distal end of the PEG polymer offers a greater advantage for efficient target binding due to less steric hindrance by PEG chains(19, 20). A major parameter governing therapeutic efficacy of immunoliposomal drugs is the antibody characteristics such as cell-specific binding that induces receptor-mediated endocytosis. The cell-specific endocytosis of liposomal drugs

is the most effective way of drug delivery to the cytoplasm. Another advantage of cell-specific endocytic drug delivery specifically to tumor cells is remarkable reduction of systemic cytotoxicity of drugs (21).

In this studies, immunoliposomes were prepared by direct antibody coupling to the distal termini of PEG chains exposed from the liposomal surface. Once the liposomes bind to target cells, the entrapped drugs must be released to the appropriate intracellular compartment. Drugs even extracelluarly released from the immunoliposomes can reach the target cells by free diffusion, thereby producing the localized high concentration needed for treatment (22). Combining the tumor targeting property of antibody with liposomal carriers offers a promise of selective drug delivery to tumor cells, including cell-specific internalization and intracellular drug release within targeted cells.

To proceed to clinical trials, the preparation process for immunoliposomal drugs has to be at most simplified and optimized. There are two different procedures of antibody coupling to the liposomal surface for preparation of immunoliposomes, termed "the post-insertion method" and "the direct coupling method" as described earlier.

The post-insertion involves coupling of ligands to the termini of PEG-lipid derivatives in a micellar phase, followed by time- and temperature-dependent transfer of the ligand-coupled PEG-lipids into the bilayers of preformed, drug-loaded liposomes by a simple incubation step (23,24). This process is appealing from a manufacturing point of view because a wide variety of ligands (including antibodies, antibody fragments, peptides, carbohydrates, etc.) could be easily inserted into liposomes containing any therapeutic drugs. In addition, this method is able to provide an appropriate level of ligand presentation on the liposomal surface by quantitative addition of ligand-coupled micelles, and does not compromise the drug-loading or drug-releasing characteristics of liposomes (25-27).

The direct coupling involves literally direct coupling of ligands to the termini of PEG-lipids in preformed liposomes encapsulating drugs. Ligands can be coupled to the liposomal surface in one step process and therefore, ligand reactivity can be relatively well preserved. Meanwhile, this may have some difficulty in conditioning of ligand concentrations on the liposomal surface.

In this study, anti-JL1 stealth immunoliposomes (SILs) were prepared by the two different methods mentioned above and their binding affinities to leukemia cells (CEM, HL60) expressing the JL1 antigen were compared to each other. The cytotoxicity of doxorubicin encapsulated in the anti-JL1 SILs was also evaluated using the same leukemic cells.

II. MATERIALS AND METHODS

1. Materials

POPC (1-palmitoyl-2-oleoyl-sn-glycero-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 (polyethylene glycol)$_{2000}$]), cholesterol, Rho-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoenthanolamine-N-[lissamine rhodamine B sulfonyl]) were purchased from Avanti Polar Lipid, Inc. (Alabaster, USA). PD-10 column and sepharose CL-4B were purchased from Amersham Bioscience (Uppsala, Sweden). Amicon Ultra-4 30K and 50K MWCO were purchased from Amicon (Beverly, Sweden)

2. Cell lines and cell culture

CEM human leukemia cells were provide by Prof. Jung (Department of Pathology, Seoul National University School of Medicine, Seoul, Korea). HL60 and H9 human leukemia cells were purchased from Korean Cell Line Bank (Seoul, Korea). CEM and HL60 cells express JL1 antigen on their cell surface while H9 cells do not. All the cells were maintained as suspension culture in RPMI 1640 (Gibco, Carlsbad, USA). The culture media were supplemented with 10% fetal bovine serum and 100 units/mℓ streptomycin. The cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37℃.

3. Preparation of immunoliposomes

3-1. Thiolation of anti-JL2 antibody

JL1-targeted humanized minibody (Dinona Inc., Seoul, Korea) were thiolated for 1 hr at room temperature by reacting with Traut's reagent in degassed HEPES buffer (20 mM HEPES, 140 mM NaCl, 2 mM EDTA, pH 8.0). Unreacted Traut's reagent was removed by passing through PD-10 column and elution with the degassed HEPES buffer.

3-2. Preparation of immunoliposomes

3-2-1. Direct coupling of thiolated anti-JL1 minibody to liposomes

POPC (50 mole%), cholesterol (46 mole%), DSPE-PEG$_{2000}$ (3.8 mole%), DSPE-PEG$_{2000}$-MAL (0.2 mole%) and Rho-DOPE (0.1 mole%) were dissolved in the chloroform and methanol mixture (2:1, v/v). The organic solvent was evaporated under a stream of N$_2$ gas. Vacuum desiccation for 2 hr ensured removal of the residual organic solvent. The dried lipid films were hydrated in an appropriate amount of citrate buffer (150 mM, pH 4.0) and then vigorously mixed by vortexing. After hydration, the dispersion was sonicated for 5 min. The resulting suspension was subjected to five cycles of freezing and thawing, and extruded ten times through a polycarbonate membrane with a pore size of 80 nm using a extruder (Avanti polar Lipids, Alabaster, USA).

3-2-2. Post-insertion of anti-JL1 minibody into liposomes

Preparation of liposomes

POPC (50 mole%), cholesterol (47 mole%), DSPE-PEG$_{2000}$ (3 mole%), and Rho-DOPE (0.1 mole%) were also dissolved in the chloroform and methanol mixture (2:1, v/v). The organic solvent was evaporated under a stream of N$_2$ gas. Vacuum desiccation for 2 hr ensured removal of the residual organic solvent. The dried lipid films were hydrated in an appropriate amount of citrate buffer (150 mM, pH 4.0) and then vigorously mixed by vortexing. After hydration, the dispersion was sonicated for 5 min. The resulting suspension was subjected to five cycles of freezing and thawing, and extruded ten times through a polycarbonate membrane with a pore size of 80 nm using a extruder.

Preparation of anti-JL1-coupled lipid micelles

DSPE-PEG$_{2000}$ (80 mole%) and DSPE-PEG$_{2000}$-MAL (20 mole%) were dissolved in the chloroform and methanol mixture (2:1, v/v). The organic solvent was evaporated and then vacuum-desiccated for 2 hr. The dried lipid films were hydrated in an appropriate amount of HEPES buffer (25 mM HEPES, 150 mM NaCl, 2 mM EDTA, pH 8.0)

Table 1. **Components of anti-JL1 immunoliposomes**

Ab coupling Methods	Components	
Direct coupling	Liposomes	POPC
		Cholesterol
		DSPE-PEG-Mal
		DSPE-PEG
		Rho-DOPE
Post-insertion	Liposomes	POPC
		Cholesterol
		DSPE-PEG
		Rho-DOPE
	Micelles	DSPE-PEG-Mal
		DSPE-PEG

3-3. Doxorubicin encapsulation by the pH gradient method.

The hydrated liposomes were passed through the sepharose CL-4B column equilibrated with the HEPES-buffered saline (HBS) to replace the extra-liposomal solution. Doxorubicin was added to the liposomal solution(1: 3 weigh ratio) which were then incubated at 60℃ for 10 min with intermittent vortex mixing. The incubation process at 60℃ was necessary for rapid and complete entrapment of doxorubicin inside vesicles containing cholesterol.

The separation of liposomal doxorubicin from free doxorubicin was performed by size exclusion chromatography. Untrapped doxorubicin was separated from the liposomes by passing a sepharose CL-4B column equilibrated with HBS. The doxorubicin concentration was determined by absorbance spectroscopy at 495 nm after lysis of the liposomes with Triton X-100 (final concentration 0.5% v/v).

Table 2. Compositions of liposomal doxorubicin

Component	Amount	Volume
Total lipid (10 mg/mL)	5 mg	500 μl
Doxorubicin (2 mg/mL)	1.67 mg	833 μl

3-4. Anti-JL1 minibody conjugation to liposomes

3-4-1 The direct coupling Method

Liposomes consisting of POPC/Chol/PEG$_{2000}$-DSPE/Mal-PEG$_{2000}$-DSPE/Rho-DOPE (50:46:3.8:0.2:0.1, mol%) were prepared by the lipid extrusion method. The thiolated antibodies were reacted with the maleimide groups on the distal termini of PEG chains on the liposomes. Briefly, the thiolated antibody solution was added to the liposome solution at the two different antibody/lipid ratios (0.2:1, 0.02:1, mAb:Mal-PEG$_{2000}$-DSPE molar ratio) and then incubated for 16 hr at 4°C with continuous stirring. Unconjugated antibodies were separated from the immunoliposomes by chromatography through a Sepharose CL-4B column in HBS (25 mM, pH 7.5).

3-4-2 The post-insertion method

The thiolated antibodies were coupled to Mal-PEG$_{2000}$-DSPE/PEG$_{2000}$-DSPE (1:4, molar ratio) micelles at the molar ratio of 1:20 (mAb:Mal-PEG$_{2000}$-DSPE) by incubation for 16 hr at 4°C with continuous stirring.

For insertion of anti-JL1 antibody-coupled lipids into the outer membrane of liposomes, the micelles of Ab-PEG-DSPE/PEG-DSPE lipid mixture were incubated with preformed liposomes for 4 hr at 37°C. Unconjugated antibodies were removed from the immunoliposomes by chromatography on Sepharose CL-4B columns in HBS (25 mM, pH 7.5).

4. SDS-PAGE

To ensure antibody conjugation to liposomes, the reaction mixtures were analyzed by SDS-PAGE with Coomasssie blue staining.

5. Electron microscopy of immunoliposomes

An aliquot of liposomal solution (10 ul of 2 mg lipid/mL) was placed on a carbonyl-coated 200 mesh copper grid (CF-200Ca) for 15 min. The solution was removed by gentle tapping with a piece of filter paper and then dried out for 10 min at the room temperature. For negative staining of the grid, 10 μl of 2% uranyl acetate was placed on the grid for 10 min, removed, and dried as described above. The negatively stained liposomes on the grid were observed with an electron microscope (Joel 299X-II, Japan) at 30 K of magnification.

6. Size analysis of immunoliposomes

To observed the size changes of liposomal vesicles during doxorubicin encapsulation and antibody-coupling, the liposomal size was measured using a particle analyzer. The liposome samples were diluted with a freshly filtrated isotonic HEPES buffer in order to yield an appropriate counting rate (100 ug lipid/mL). All the samples were placed into the specimen holder of a Zetamaster S (Malvern Instruments Ltd., Malvern, UK) 5 min prior to the measurement in order to allow equilibration to room temperature.

7. In vitro cellular binding of anti-JL1 immunoliposomes

Specific cellular binding affinities of anti-JL1 immunoliposomes were evaluated with in vitro models of leukemia cells (CEM, HL60, and H9) by fluorescence microscopy and FACS (fluorescence-activated cell sorter) analysis.

The immunoliposomes containing lissamine rhodamine B-dioleoyl phosphoenthanolamine (Rho-DOPE, 0.1 mol%) were added to CEM, HL60, or H9 cells (1×10^6 cells each well of 24 well-plate) in 1 mL of culture media without FBS. The treated cells were incubated for 45 min at 4°C with gentle agitation. The cells were washed twice with PBS and then observed under a fluorescence microscope (BX50F4, Olympus, Japan) and Fluorescence-activated cell sorting (FACS) (Beckton Dickinson, USA).

8. Cellular internalization of anti-JL1 immunoliposomes

To monitor the uptake of anti-JL1 immunoliposomes by leukemia cells, the prepared immunoliposomes were incubated with three different types of leukemia cell. The anti-JL1 immunoliposomes were added to CEM, HL60 and H9 cells (1×10^6 cells each well of 24 well-plate) in 1 mL of culture media without FBS. The treated cells were gently agitated for 30 min at room temperature and then incubated for 2 hr at 37°C in a 5% CO_2 incubator. The cells were washed twice with 500 mL PBS and suspended in 100 mL PBS. The washed cells were observed with a FACS.

9. Cytotoxicity analysis of anti-JL1 immunoliposomal doxorubicin

The cytotoxicity of JL1-targeted liposomes containing doxorubicin, prepared either by the post-insertion or the direct coupling method, was determined by direct cell counting. CEM, HL60, and H9 cells were seeded into 24-well plates at 1×10^5 cells/well. Either free doxorubicin or various formulations of liposomal doxorubicin was added to the leukemia cells which were then incubated for 24 hr at 37℃ in an atmosphere of 95% humidity and 5% CO_2. The live cells were directly counted with a microscope after trypan blue staining.

III. RESULTS

1. Preparation of anti-JL1 stealth immunoliposomes (SILs) by the direct coupling or the post-insertion method

A conceptual illustration of the stealth (PEG-stabilized) immunoliposomes (SILs) is shown in Figure 1. The illustration shows two different methods for the modification of liposomal surfaces.

The conventional methods of direct ligand coupling to liposomal surface is based on the principle of integrating an active anchor into the lipid mixture, and thus into the liposomal bilayers at the production stage. In the second step, the anchor reacts with the corresponding ligand via varied reactive groups such as amine or thiol groups. However, a serious disadvantage of this method is that this approach does not allow controlling selective integration of the active anchor into only the outer layer of vesicles. As a consequence of this, it is possible for the still reactive anchors to interact with encapsulated materials. Another disadvantage of the direct coupling is the difficulty in quantitative coupling of ligand molecules to the liposomal surface.

Meanwhile, the so-called post-insertion method requires coupling of the active anchors to ligands first. The resulting conjugates is then selectively inserted into the outer bilayers upon incubation with plain liposomes at 60℃ for 1 hr or at 37℃ for 4 hr.

After direct coupling of anti-JL1 antibodies or post-insertion of the antibody conjugates, the SIL solutions were passed through a Sepharose CL-4B gel filtration column (Figure 2). The fractions 5-7 represents the SILs and the

fraction 9-14 are free Ab. According to protein quantification by the Lowry protein assay, a similar pattern of antibody conjugation at the high and low concentration of antibody added (0.2, 0.02; Ab:Mal-PEG-DSPE molar ratios). However, at the both concentrations, the direct coupling method exhibited higher Ab coupling efficiency than the post-insertion method.

The eluted immunoliposomes were run on 12% SDS-PAGE to verify the coupling of anti-JL1 antibody to the liposomal surface (Figure 3). Antibody molecules were effectively coupled to the surface of liposomes by the both methods. A higher amount of antibody molecules added yielded a higher antibody density on the liposomal surface.

(a)

(b)
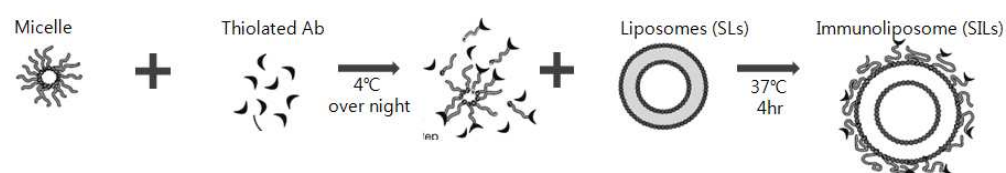
Figure 1. A schematic illustration of anti-JL1 immunoliposomes coupled to JL1 targeted minibodies.

(a) the direct coupling method: Thiolated antibodies was coupled to the Mal-PEG-DSPE already incorporated in the bilayers for overnight at 4℃ (b) the post-insertion method: thiolated antibodies were coupled to the MAL-PEG-DSPE micelle (PEG-DSPE:MAL-PEG-DSPE=4:1 molar ratio) and the Ab-lipid conjugates were subsequently inserted into the liposomal membrane.
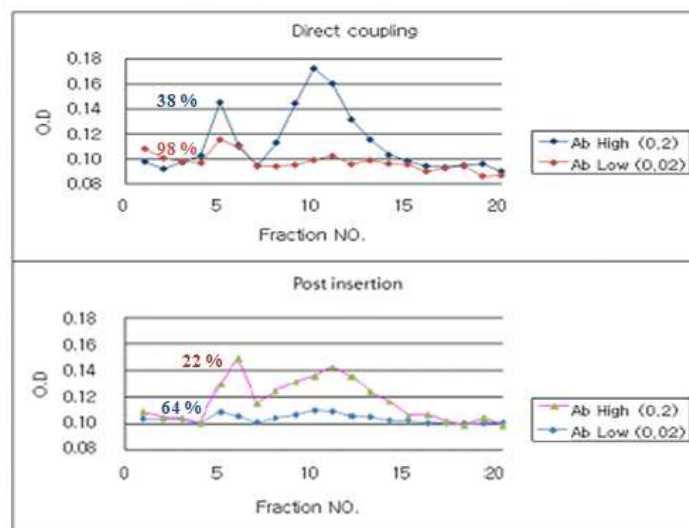
Figure 2. Elution profiles of stealth immunoliposomes and unbound antibodies.

The liposome mixtures were loaded onto a Sepharose CL-4B gel filtration column and eluted with HBS (pH 7.5). The fractions 5-7 represents the SILs and the fraction 9-14 were free Ab (each fraction 1 mL). The protein content was quantified by the Lowry protein assay, absorbance measurement at 750 nm.

SDS-PAGE (Reducing)

M. Marker
Lane 1. mAb (JL1 minibody)
Lane 2. SILs(by Direct coupling, High Ab)
Lane 3. SILs(by Direct coupling, Low Ab)
Lane 4. SILs(by Post-insertion, High Ab)
Lane 5. SILs(by Post-insertion, Low Ab)
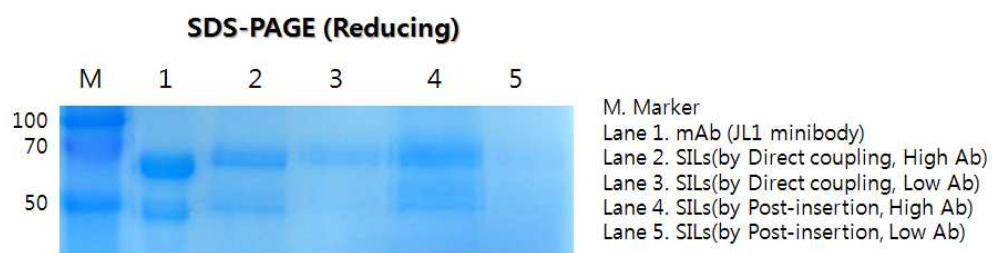
**Figure 3. SDS-PAGE analysis of antibody-coupling to liposomes.**

SILs were made by direct coupling or post-insertion at two different concentration of antibody (0.02, 0.2; Ab:Mal-PEG-DSPE molar ratios) and then separated by gel filtration. The eluted SILs were run on 12% SDS-PAGE. M; molecular weight markers, lane 1; free antibody, lane 2, 3; SILs made by direct coupling, lane 4, 5; SILs made by post-insertion.

2. Preparation of stealth liposomes encapsulating doxorubicin by the pH gradient method

Encapsulation of doxorubicin into liposomes was the first important step for preparation of anti-JL1 liposomal doxorubicin. Doxorubicin was effectively encapsulated into the preformed liposomes by the pH gradient method (Figure 4). The concentration of doxorubicin encapsulated in the liposomes was determined by spectrophotometric measurement (λ= 490 nm). The encapsulation efficiency was greater than 90% regardless the antibody coupling procedure and the antibody concentration on the liposomal surface (Table 3).
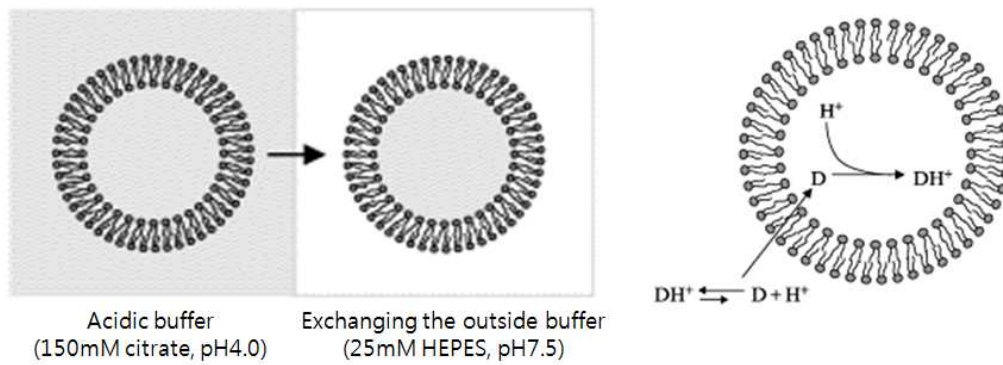
Figure 4. A illustration of doxorubicin uptake in response to transmembrane pH gradients. Prior to drug loading, the primary pH gradient (intravesiclular pH 4 and extravesicular pH 7.5) is necessary.

Table 3. Doxorubicin encapsulation efficiency of stealth anti-JL1 immunoliposomes

Liposomes	Ab coupling	Ab concentration	Doxorubicin encapsulation %[*,**]
SLs (no Ab)			98.2
SILs	direct coupling	high Ab	92.5
		low Ab	95.1
	post insertion	high Ab	99.1
		low Ab	97.7

[*]%=final doxorubicin concentration/starting doxorubicin concentration×100

[**]Percent doxorubicin encapsulation efficiency of SILs made by the direct coupling or post-insertion method was determined after spectrophotometric measurement (λ=490 nm) using doxorubicin solutions as standard controls. The samples or standards were adjusted to 100 µl with the extravesicular buffer, to which 900 µl of 1% Triton X-100 was added.

3. Changes of vesicular size and surface charge of liposomal vesicles during doxorubicin encapsulation and antibody-coupling

The vesicular size of liposomes is a major parameter governing in vivo targeting of liposomes to intended cells or tissues. It is well documented that smaller particles exhibit more enhanced permeation and retention (EPR) in inflammation sites and solid tumors. In addition, To exploit the EPR effect for passive targeting, the particles should have neutral or negative surface charge.

According to the vesicular size measurement by the particle analyzer, bare PEG-liposomes (SLs, 102.2, 144.4 nm) extruded through an 80 nm pore size of polycarbonate membrane filter became slightly larger (184.4, 158.5 nm) after doxorubicin encapsulation. Also, the immunoliposomes became even larger during the precessed of direct antibody coupling (227.8 nm) and Ab-lipid insertion (198.2 nm). However, the surface charge of PEG-liposomes remained unchanged during the processes of doxorubicin encapsulation and antibody conjugation.

The same pattern of liposome size changes during doxorubicin encapsulation and antibody conjugation was monitored with an electron microscope. The electron microscope photographs also showed that the liposomal sizes became larger after the precesses. The final anti-JL1 stealth immunoliposomes encapsulating doxorubicin made by the two methods appeared to be less than 200 nm of average vesicular size.

Table 4. Changes of vesicular size and surface charge of liposomal vesicles during doxorubicin encapsulation and antibody-coupling.

Liposomes	Ab coupling	Size* (nm)	Zeta potential* (mV)
SLs	for direct coupling	102.2	-4.9
	for post-insertion	144.4	-4.4
SLs-DOX	for direct coupling	184.4	-4.5
	for post-insertion	158.5	-3.9
SILs-DOX	direct coupling	227.8	-4.2
	post-insertion	198.2	-4.7

*Vesicular sizes and surface charge of liposomal vesicles was measured with a particle analyzer.

A. Direct coupling

SLs-empty   SLs-DOX   SILs-DOX
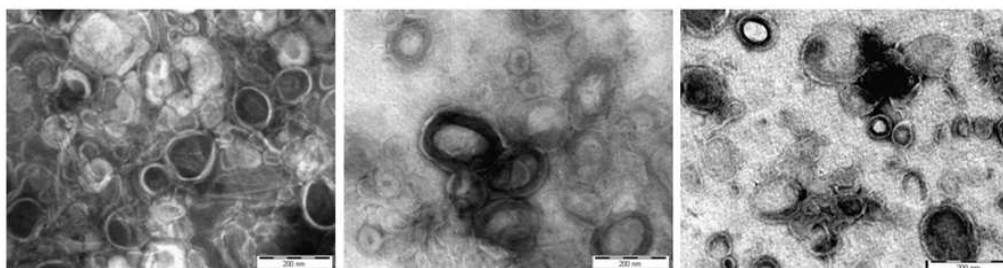
B. Post-insertion

SLs-empty   SLs-DOX   SILs-DOX

Figure 5. Electron microscope photographs of anti-JL1 stealth immunoliposomes.

The PEG-liposomes (SLs), PEG-liposomes encapsulating doxorubicin (SLs-DOX), and Ab-PEG-liposomes (SILs-DOX) stained with 2% unranyl acetate were observed with an electron microscope at 30 K of magnification.

4. Specific cellular binding of anti-JL1 immunoliposomes to leukemia cells

Specific cellular binding of anti-JL1 immunoliposomes encapsulating doxorubicin was examined by fluorescence microscopy and FACS analysis (Figure 6). The rhodamine-labeled lipid were included in the imm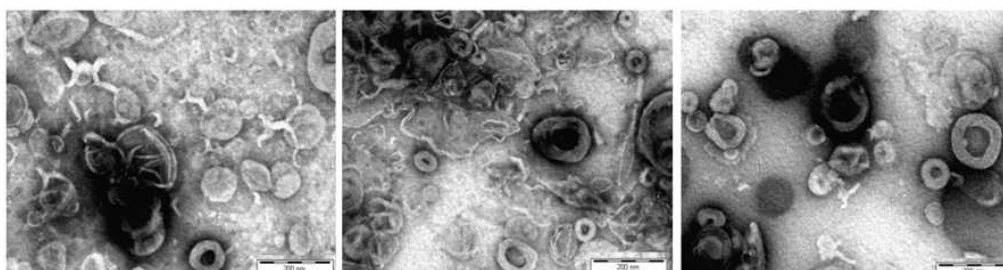unoliposomes (Rho-DOPE, 0.1 mole%) to monitor their cell-specific binding capabilities. The rhodamine-labeled SILs and SLs were incubated with three different leukemia cells (CEM, HL60, and H9). The SILs were able to specifically bind to JL1-expressing cells (CEM and HL60), but not to H9 cells, a negative control cell line. In addition, nontargeted PEG-stabilized liposomes (PLs) did not show any specific cellular binding to all the three cell lines.

The cellular binding intensity of SILs was varied depending on the anti-JL1 antibody concentration on the liposomal surface. The SILs with the higher amount of anti-JL1 antibody exhibited more efficient binding to JL1-expressing leukemia cells than those with the lower amount of antibody. These results indicate that the JL1 molecule is an appropriate ligand for leukemia-specific targeting and the anti-JL1 immunoliposomes are an efficient delivery system directed leukemia cells expressing JL1.

(A) Fluorescence microscopy

	A	B	C	D	E
CEM					
HL60					
H9					
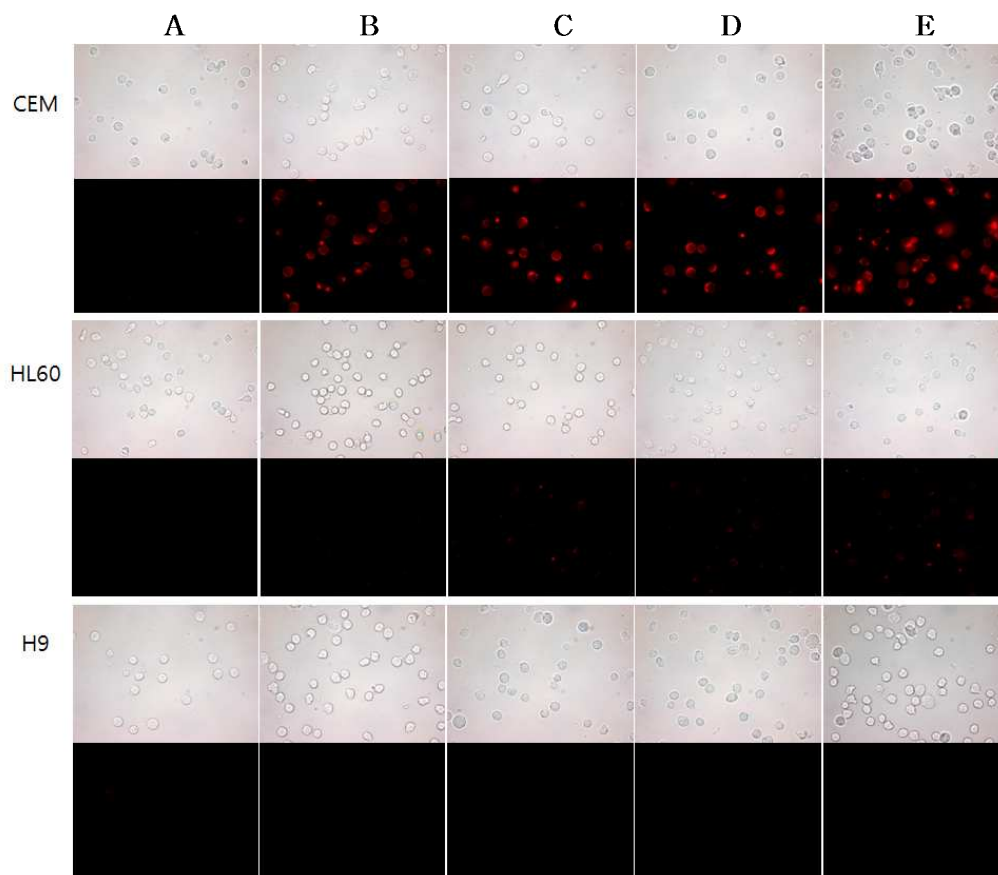
A; SLs

B; SILs made by post-insertion (Low concentration of Ab)

C; SILs made by post-insertion (High concentration of Ab)

D; SILs made by direct coupling (Low concentration of Ab)

E; SILs made by direct coupling (High concentration of Ab)
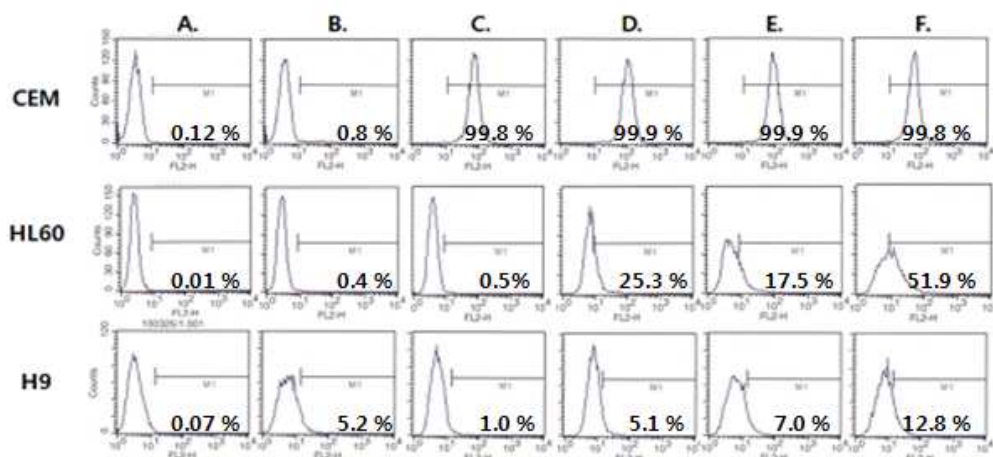
(B) FACS analysis

A; no treatment

B; SLs

C; SILs made by post-insertion (Low concentration of Ab)

D; SILs made by post-insertion (High concentration of Ab)

E; SILs made by direct coupling (Low concentration of Ab)

F; SILs made by direct coupling (High concentration of Ab)

Figure 6. Specific cellular binding of anti-JL1 immunoliposomes to leukemia cells.

CEM, HL60, and H9 (1×10^5 cells each well) were incubated with rhodamine-labeled SILs made by the direct coupling method or the post-insertion method at two different concentrations of anti-JL1 antibody for 30 min at room temperature. The treated cells were washed with PBS and then observed by fluorescence microscopy (A) and FACS analysis (B).

5. JL1-specific Internalization of Anti-JL1 Immunoliposomes into Leukemia Cells

The cellular association of anti-JL1 immunoliposomes with CEM, HL60, and H9 cells was further investigated using two-color fluorescent confocal imaging. After 30 min incubation, red dots representing rhodamine-labeled anti-JL1 immunoliposomes began to spread out into the cytosol (data was not shown). Two hrs later they were evenly distributed inside CEM and HL60 cells, but not in H9 cells (Figure 7). The red spots in CEM cells appeared to be brighter then those in HL60 cells, which was presumably due to higher expression of JL1 on the surface of CEM cells. The H9 cells show no accumulation of the anti-JL1 immunoliposomes within the cytosol at the same time point.
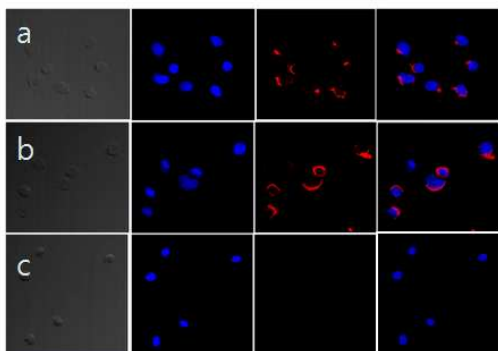
(A) CEM   (B) HL60
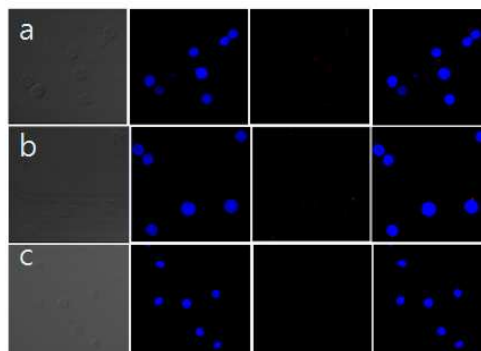
(C) H9

a. SILs made by direct coupling
b. SILs made by post-insertion
c. SLs

Figure 7. JL-specific internalization of anti-JL1 immunoliposomes into leukemia cells.

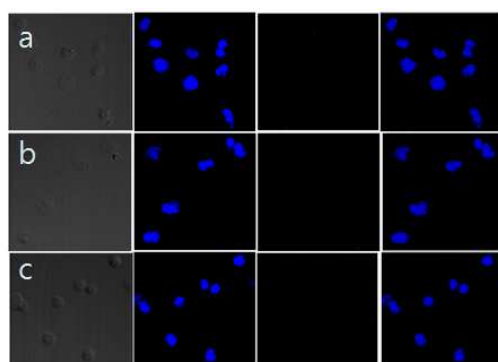Cellular internalization of the anti-JL1 immunoliposomes was investigated using a confocal microscope after incubation of the immunoliposomes with CEM, HL60, and H9 cells for 2 hr.

6. Cytotoxicity of anti-JL1 immunoliposomes encapsulating doxorubicin to leukemia cells.

In vitro cytotoxicities of doxorubicin-loaded liposomal formulations were determined after treatment for 24 hr (Table 5). When the doxorubicin was loaded in the plain stealth liposomes (SL-DOX), its cytotoxicity was decreased regardless the presence of JL1 antigens on the surface of leukemia cell. However, the reduced cytotoxicity was significantly recovered by coupling of anti-JL1 antibodies to the liposomal surface (SIL-DOX). Moreover, the cytotoxicity depends on not only the JL1 antigen density on the cancer cell surface, but also the concentration of anti-JL1 antibody on the immunoliposomal surface.

There were no significant differences found between the SIL-DOX formulations prepared by the two different methods in terms of cytotoxicity measurement. However, the immunoliposomal doxorubicin made by post-insertion of lower concentration of antibodies was less cytotoxic than that by direct coupling under the same condition.

Table 5. Cytotoxicity of anti-JL1 immunoliposomal doxorubicin to leukemia cells

DOX formulations	Ab-coupling conditions	IC_{50} (nM of DOX)[*]		
		CEM	HL60	H9
Free DOX		94.0 (±2.2)	124.0 (±7.1)	136.1 (±6.7)
SL-DOX		646.0 (±69.9)	292.4 (±25.6)	443.1 (±16.2)
SIL-DOX	Direct coupling, High Ab	139.6 (±21.3)	173.2 (±8.73)	408.4 (±5.5)
	Direct coupling, Low Ab	289.3 (±21.7)	237.3 (±15.8)	475.1 (±54.1)
	Post-insertion, High Ab	269.6 (±8.4)	188.3 (±14.1)	419.6 (±14.4)
	Post-insertion, Low Ab	391.7 (±13.9)	253.3 (±39.3)	449.1 (±47.5)

[*]In vitro cytotoxicity was determined by directed cell counting after trypan blue staining (cell viability=number of live cells treated with DOX formulations/number of untreated live cells×100). CEM, HL60 or H9 cells were incubated with free DOX, SL-DOX or SIL-DOX in media for 24 hr at 37℃ in an atmosphere of 95% humidity and 5% CO_2. Data are expressed as concentration of DOX that gives a 50% inhibition of cell growth compared to untreated control cells (IC_{50}).

Discussion

It has been well-documented that liposomal formulations enhances therapeutic efficacy of doxorubicin (28). However, the liposomal doxorubicin still has substantial adverse side-effects because of its non-specific distribution *in vivo.* To address these issues, the anti-JL1 stealth immunoliposomes encapsulating doxorubicin were prepared for targeted delivery of doxorubicin to leukemia cells over-expressing JL1 antigens. The JL1 antigens, uniquely expressed on the cell surface of most T leukemias, have been suggested as an appropriate antigen for targeted delivery (29).

Tumor-specific targeting is the most concerned research area in the field of liposomal drug delivery. In order to develop a tumor-specific delivery system for doxorubicin, there are a few critical considerations which have to be kept in mind. First of all, the immunoliposomes should encapsulate doxorubicin completely and stably. Although recently a number of doxorubicin-encapsulating methods have been reported, the pH gradient method is still the most efficient method so far. In this study, doxorubicin molecules were effectively encapsulated into the anti-JL1 immunoliposomes by the pH gradient method. Moreover, the antibody-coupling procedures did not affect the integrity of the immunoliposomal doxorubicin.

The primary aim of doxorubicin encapsulation in liposomes is to decrease the nonspecific organ toxicity of the drug. Liposomal formulations are able to direct the doxorubicin away from the sites with tight capillary junctions such as the heart muscle. Instead, they are effectively distributed in the areas where fenestrations or gaps exist in the vasculature such as solid tumors. Adopted in this study, the liposomal formulation containing polyethylene glycol(PEG)-modified lipids are known to exhibit increased circulation time.

The increased circulation longevity of the liposomal drug allows enhanced extravasation across the leaky endothelium of solid tumors, rendering elevated in vivo therapeutic efficacy.

The second critical point concerned was that the liposomal drugs should be able to direct to the intended cells or tissues specifically. In this study, JL1-targeting antibodies were adopted for targeted delivery of doxorubicin to leukemia cells over-expressing JL1 antigens. The antibodies were thiolated by the traut's reagent for effective coupling and than conjugated to the maleimide groups at the termini of PEG-lipids. In order to suggest a convenient procedure to prepare the anti-JL1 immunoliposomal doxorubicin, the antibody was coupled to the liposomal surface by two different methods, direct coupling or post-insertion.

Characteristics of the immunoliposomal doxorubicin prepared by the two different methods were compared to each other in terms of *in vitro* binding affinity to T-leukemic cells, cellular internalization, and cytotoxicity depending on the presence of JL1 antigen on the cell surface and the anti-JL1 antibody concentration on the liposomal surface. According to the experimental results, there was little to choose between the two methods for immunoliposome preparation. However, in terms of their easiness and convenience of preparation and their flexibility, the post-insertion procedure would be the favorite for use in manufacturing immunoliposomes for clinical applications. Because the post-insertion methods involves exposing the antibody to an elevated temperature for a certain period of incubation time, for example 60℃ for 1 hr or 37℃ for much longer times, the insertion procedure may harm its binding affinity and/or avidity for its target antigen.

In this study, the anti-JL1 antibody appears to be unstable at 60℃ for 1 hr. Moreover, antibody coupling to termini of PEG-DSPE micelles was less efficient because of steric hinderance between long PEG arms. Consequently,

the concentration of anti-JL1 antibody on the liposomal surface made by direct coupling was higher than the post-insertion method. Therefore, the immunoliposomes prepared by the direct coupling method exhibited relatively more effective cell binding and internalizations, and higher cytotoxicity than those by the post-insertion method under the same preparation condition. It was presumably due to the a higher concentration of less damaged anti-JL1 antibody provided on the liposomal surface. Nevertheless, the results of cell binding and internalization experiments support a conclusion that the JL1 antigen may be an appropriate tumor target and anti-JL1 immunoliposomes are an effective system doxorubicin delivery.

The last point concerned was the internalization capability of immunoliposomes into the cytoplasm. If non-internalizing ligands are coupled to liposomes, liposomal contents will be released over time at or near the cell surface, and the released drug has to enter the cell by passive diffusion or other transport mechanisms to show its therapeutic effectiveness. Although increased concentrations of drug may be achieved at the cell surface by specific cell binding, the released drugs may diffuse away from the area in the dynamic in vivo environment. However, targeting to non-internalizing epitopes might be still efficacious in solid tumors through the bystander effect, in which untargeted cells also can be killed by drugs released extracelluarly.

Liposomes targeting to non-internalizing epitopes did not improve therapeutic outcome over non-targeted liposomes which have substantial therapeutic efficacy via an EPR (enhanced permeation and retention) effect. Meanwhile, liposomes directed against internalizing receptors have demonstrated increased therapeutic activity in some tumor models (30,31). Internalization of ligand-targeted therapeutics depends on various factors such as type of receptor, receptor density, antibody avidity, and rate of internalization and recycling. In this study, the cells with a higher density of JL1 epitope (CEM

cells) showed more effective internalization of anti-JL1 immunoliposomes than the cells with a lower density of the epitope (HL60).

All the experimental results in this study provide valuable information that the JL1 molecule expressed on the surface of most leukemia cells is an appropriate target for tumor-directed drug delivery. The results also supported that the anti-JL1 immunoliposomal doxorubicin would be applicable as a noble clinical modality for treatment of leukemia expressing JL1 antigens after further preclinical and clinical evaluation.

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국문요약

Doxorubicin을 포획하고 있는 항 JL1 면역리포솜을 이용한 leukemia 치료

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백 진이

Doxorubicin을 포획하고 있는 리포솜(Doxil®, Alza Pharmaceuticals)은 다양한 항암치료에 사용하고 있다. 리포솜에 doxorubicin을 포획하게 되면 doxorubicin이 갖고 있는 부작용을 줄이고 치료효과를 높일 수 있다고 알려져 있다. 그러나 doxorubicin을 포획 하고 있는 리포솜을 체내에 투여했을 때 doxorubicin이 암세포뿐만 아니라 정상세포에도 미치는 영향은 여전히 존재한다. 이를 해결하기위해서 본 연구에서는 암세포에만 특이적으로 전달하여 doxorubicin이 갖고 있는 부작용을 줄이며 효율적으로 암을 치료 할 수 있는 새로운 면역리포솜 전달체를 만들고자 하였다.

JL1 항원은 대부분의 T 세포성 혈액암의 세포 표면에 특이적으로 발현된다고 보고되어있다. 이를 이용하여 본 연구에서는 doxorubicin을 포획하고 있으며 JL1을 표적하여 혈액암세포를 특이적으로 치료하는 면역리포솜을 만들고자 하였다. 이러한 관점에서 본 연구에서는 암세포에만 특이적으로 항암제 doxorubicin을 전달하여 doxorubicin이 갖고 있는 부작용을 줄이며 효율적으로 암을 치료 할 수 있는 새로운 면역리포솜 전달체를 만들고자 하였다. 본 연구에서는 면역리포솜을 direct coupling 방법과 post-insertion방법을 이용하여 만들었다. Direct coupling 방법은 doxorubicin을 포획하고 있는 리포솜에 JL1을 표적하는 항체를 직접 붙이는 방법이다. 이 리포솜에는 1,2-distearoyl-sn-glycero-3-phosphoethanol-amine-N-

[maleimide (polyethylene glycol)$_{2000}$] (DSPE-PEG$_{2000}$-MAL)가 있어서 여기에 항체가 붙을 수 있다. 반면, post-insertion 방법은 항체가 붙을 수 있는 지질 (DSPE-PEG$_{2000}$-MAL)로 이루어진 micelle에 항체를 먼저 붙이고 미리 만들어 두었던 리포솜의 인지질 이중층에 끼워 넣는 방법이다. 이렇게 두 가지 방법으로 만든 리포솜의 형태와 크기는 차이를 보이지 않았고 세포실험 결과에서도 차이가 없었다.

두 가지 방법으로 만든 면역리포솜을 가지고 각각 세포실험을 하였다. 형광현미경과 유세포 분석결과에 따르면 JL1을 발현하는 세포(CEM, HL60)에만 특이적으로 친화성을 갖으며 JL1을 발현하지 않는 세포(H9)에서는 친화성을 보이지 않았다. 공초점 현미경에서도 역시 JL1을 발현하는 세포(CEM, HL60)에 특이적으로 면역리포솜이 세포 안으로 유입이 되었고, JL1을 발현하지 않는 세포(H9)에서는 그렇지 않음을 알 수 있었다. 이것은 리포솜이 세포표면의 JL1항원에 인식되어 endocytosis된 결과이다. 세포독성 실험결과, doxorubicin을 리포솜에 포획하면 doxorubicin이 갖고 있는 세포독성이 낮아지는데 여기에 항체를 붙이면 JL1을 발현하는 세포의 경우 다시 독성이 증가하는 양상을 보였다. 그러나 JL1을 발현하지 않는 세포의 경우 항체의 유무가 세포독성에 크게 영향을 미치지 않았다. 또한 이러한 결과들은 리포솜 표면에 붙어있는 항체의 양에도 영향을 받았다. 면역리포솜 표면의 항체가 많을수록 암세포에 대한 친화성과 doxorubicin의 세포독성이 증가했다.

이러한 연구들을 종합하여 볼 때, 본 연구를 통해 제시된 항 JL1 면역리포솜은 암세포에 항암제를 특이적으로 전달 할 수 있는 전달체로 향후 항암제 개발에 있어 디딤돌이 될 수 있을 것이다.