J Huazhong Univ Sci Technol［Med Sci］
DOI 10.1007/s11596-009-0302-2
29 (3): 273-280, 2009

Targeting and Internalization of Sterically Stabilized Liposome Modified with ZCH-4-2E8*

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Summary：Sterically stabilized liposome (SL) modified with 2E8 monoclonal antibody (2E8-SL) was prepared in order to evaluate its targeting ability and internalization efficiency against tumor cells with high expression of CD19. 2E8 was coupled to the surface of SL using post-insertion technique. The shape of liposomes was observed under a transmission electronic microscope. The average size of liposomes was determined by using the Zetasizer instrument. The binding and internalization of 2E8-SL against tumor cells with higher expression of CD19 were tested by flow cytometry and confocal microscopy. The mean diameter of 2E8-SL was 121.25 nm. 2E8-SL was stable after up to 24 days in various buffers. 2E8-SL showed specific binding to tumor cells with high expression of CD19, including B cells in peripheral blood mononuclear cells (PBMCs). 2E8-SL could efficiently internalize into Nalm6 cells with CD19 high expression. It is suggested that 2E8-SL may serve as useful delivery carrier of anti-cancer drugs targeting to hematological malignant tumors with CD19 high expression.
Key words：targeting; liposome; anti-CD19 antibody; hematologic malignant tumors

Cancer chemotherapy is lack of specificity to cancer cells and exerts toxicities to normal cells, so therapeutic index is poor. It is important to increase selective toxicity of anticancer drugs to tumor cells and reduce irritation of anticancer drugs to normal cells. Now, drug carriers may alter pharmacokinetics and blood distribution of anticancer drugs, and increase selective toxicity of anticancer drugs to tumor cells.

Immunoliposome is one of advanced drug carriers and considered to be a mainstream drug delivery technology. As immunoliposome is coupled with monoclonal antibodies (mAbs), antibody fragments or receptor ligands actively recognizing tumor cells, it combines the advantages of colloidal drug delivery systems (liposomes) and biological therapeutics (such as mAbs), holding particular promise for cancer therapy[1]. For hematologic tumor cells, immunolipsome is more accessible to them than to solid tumor cells[2, 3].

CD19 is expressed on a high percentage of malignant B-lymphoid cells and undergoes rapid internalization on antibody binding[4]. And a recent research demonstrated that CD19 is a B-cell lymphoblast leukemia stem cells (LSCs)-specific maker; anti-CD19 antibody may discriminate B-cell lymphoblast LSCs from normal haemopoietic stem cells (HSCs) within the CD34+ CD38- stem population[5]. Consequently, CD19 may be considered to be a promising target site to obtain specific killing CD19 high-expressed malignant cells and B-cell lymphoblast LSCs for immunolipsome. And researches demonstrated CD19-targeted liposomes increased binding efficiency to CD19 high-expressed malignant cells relative to conventional liposomes; thereafter, they rapidly internalized into and exerted specific cytotoxicity to cells. Besides, CD19-targeted liposomes exerted more cytotoxicity to CD19 high-expressed malignant cells than CD20-targeted liposomes (uninternalization epitopse). *In vivo*, CD19-targeted liposomes also significantly prolonged survival time of murine xenograft models of CD19 high-expressed malignancy, compared to free anti-cancer drugs and conventional liposomes. In addition, researchers also discovered that anti-CD19 antibodies with different conformations (such as [IgG] or [Fab'] or [scFv]) may be coupled with liposomes as targeting moieties and remained recognizing ability to CD19 antigens after coupling[6-10].

2E8 is a high-affinity anti-CD19 mouse antibody produced in our laboratory by fusion of immune BALB/c splenocytes with producer myeloma cells[11]. 2E8 can recognize epitopes of CD19 antigen with molecular weight of 95 kD, which are completely different from epitopes that other anti-CD19 Abs binding to[11]. So, 2E8 has approved for an international patent. We demonstrated that the immunotoxin conjugated with 2E8 could selectively recognize and exert toxicity to CD19 high-expressed Nalm6 cells in our previous research[12]. Based on our previous research combined with these unique features of immunolipsome, here we developed a novel sterically stabilized liposome (SL) modified with 2E8 (2E8-SL), and evaluated targeting ability and internalization efficiency of 2E8-SL against hematological malignancies with CD19 high expression.

1 MATERIALS AND METHODS

1.1 Materials

Soy phosphatidylcholine (PC) was purchased from

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*This Project was supported by the National Basic Research Program of China (No. Z205166).

Tai'wei Corporation (China). Methoxypoly (ethylene glycol) (M_r2000), covalently linked via a carbamate bond to phosphatidylethanolamine (mPEG-PE), Maleimide derivatized PEG_{2000}-distearoylphosphatidylethanolamine (Mal-PEG_{2000}-DSPE) and rhodamine-labeled phosphatidylethanolamine (Rh-PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), 2-iminothiolane (Traut's Reagent) and 4-(2-hydroxyerhyl) piperazine-1- erhanesulfonic acid (HEPES) were obtained from Sigma Chemical Co. (USA). Sephacryls-300-HR and Sepharose CL-4B were purchased from Amersham Biosciences (Canada). Mouse IgG1-PE, mouse IgG1-PerCP, mouse IgG1-APC, mouse anti-human CD20-PerCP or CD4-APC and CD8-PerCP were purchased from Becton Dickinson (USA). DAPI (4,6-diamidino-2-phenylindole) was purchased from Beyotime Institute of Biotechnology (China). All other chemicals were of the highest grade possible.

1.2 Tumor Cell Line and Mice

Nalm6, Raji cells with high CD19 expression and Molt3, K562 cells with low CD19 were purchased from the American Type Culture Collection (USA) and cultured in RPMI 1640 medium (Gibco, USA), supplemented with 10% (v/v) heat-inactivated newborn bovine serum and 100U/ml penicillin G and 0.1 mg /mol streptomycin (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., China) and were grown at 37°C in a 5% CO_2/95% air humidified atmosphere. Nalm6 and Raji cells have been extensively characterized for CD19 expression by flow cytometry. Only cells in the exponential phase of cell growth were used in experiments.

Female, 6-8 week old BALB/C mice were obtained from Health Sciences Lab Animal Services (Zhejiang University, China) and kept in standard housing. They were used for experiments after a minimum acclimation period of 1 week, when they were 7-12 weeks old. All experiments were approved by the Health Sciences Lab Animal Services Animal Policy and Welfare Committee of the University of Zhejiang (China).

1.3 Preparation of 2E8 (IgM) Antibody

BALB/c mice were injected with 2E8 hybridoma cells into abdominal cavity to produce ascites fluid. Then, the collected ascites fluid was loaded to Sephacryl (S-300-HR). The purified mAbs were concentrated by ultrafiltration. The purified mAbs' concentration was determined by a protein Assay (BCA protein Assay Reagent, Pierce Rockford, Ⅲ). All pooled fractions were tested for SDS-PAGE electrophoresis. Sterile-filtered antibodies were stored in aliquots at –70°C.

1.4 Preparation of SL and 2E8-SL

Nontarget SL was composed of PC:CHOL: $mPEG_{2000}$-PE at a 2:1:0.1 molar ratio and prepared by using reverse-phase evaporation followed by passing through 2-stacked polycarbonate membrane filters (0.1 μm pore size; Whatman, Maidstone, UK) for 10 times as described previously[13]. The SL dispersion was stored in tight containers at 4°C for further experiments. 2E8-SL was prepared by coupling 2E8 to the Mal-PEG_{2000}-DSPE through post-insertion technique[14]. Namely, 2E8-Mal- PEG_{2000}-DSPE was formed though reaction of sulfhydryl residues on the antibody with the C-terminal maleimide groups of Mal-PEG_{2000}-DSPE when the initial molar ratio of 2E8 relative to Mal-PEG_{2000}-DSPE was 1:50. Then, 2E8-Mal-PEG_{2000}-DSPE was transferred to pre-formed SL.

The amounts of conjugated 2E8 were measured by a protein assay (BCA protein Assay Reagent, Pierce Rockford, USA)[15]. The phospholipid concentration of liposome was determined using the Stewart method[16]. The final antibody density on the resulting liposomes was calculated on the basis of the molecular mass of 2E8 (900 kD) and the approximate number of phospholipid molecules/liposome (80 000)[17].

Separation of free 2E8 (which might form micelles) from 2E8-SL was confirmed as follows: 0.5 ml 2E8-SL was applied onto a Sepharose CL-4B column (diameter 1.5 cm, bed volume 44 mL) and eluted with HEPES buffer saline (HBS, 25 mmol/L HEPES, 140 mmol/L NaCl, pH 7.4). Fractions (2.0 mL/each) were collected and spectrophotometrically analyzed for 2E8 absorbance (A) at 280 nm or phospholipids A at 486 nm[16].

For cell flow cytometry and laser scanning confocal microscopy, 0.1 mol% of Rh-PE relative to the total lipids was added to liposomes.

1.5 Characterization of 2E8-SL

The shape of SL was observed using a Jem-100sx from Japan electron (Japan). The mean particle size and particle size distribution of 2E8-SL and SL were determined using Zetasizer 3000 HSa instrument (Worcestershire, UK) after diluting with HBS (pH 7.4).

The stability of 2E8-SL and SL was observed by investigating changes of the mean particle size of liposomes kept under a nitrogen atmosphere in HBS, HBS with Tween 80 (3%, v/v), or HBS with 50% newborn bovine serum at 4°C on the day 1, 2, 4, 6, 8, 16, 24 in duplicates for each sample.

Integrity of 2E8 during the preparation of the 2E8-SL was investigated by reducing the 2E8 with 5% (v/v) 2-mercaptoethanol in each step, followed by the SDS-PAGE on 12% Tris-HCl minigel[18].

1.6 Cellular Binding and Internalization Study

Binding and internalization of 2E8-SL labeled with PE (2E8-SL-PE) was analyzed by flow cytometry. 1×10^6 cells were incubated with SL-PE and 2E8-SL-PE for 30 min at 37°C, and cells were pelleted by centrifugation. To observe internalization of 2E8-SL-PE in Nalm6 cells, cell surface-bound liposomes were removed by incubation with 2 mg/ml papain (Roche Diagnostics, Switzerland) for 20 min at 37°C, and then cells were fixed with 1% PFA in PBS. To examine the specificity of 2E8-SL-PE binding, Nalm6 cells were treated with 100 μg/mL 2E8 monoclonal antibody for 30 min at 4°C before incubation with liposomes. Thereafter, cells were analyzed by FCM with use of a fluorescence activated cell sorting (FACS) Calibur flow cytometer (Becton Dickinson, USA).

To examine internalization of 2E8-SL-PE in Nalm6 cells by laser scanning confocal microscopy, cells were incubated with 2E8-SL-PE at 37°C for 30 min, 4 h, and 8 h. After incubation, cells were rinsed three times with cold PBS, followed by fixation with 1% paraformaldehyde (PFA) for 15 min. Thereafter, cells were incubated with 1 U of DAPI for 10 mins at room temperature (RT) to stain DNA. Finally, these cells were attached on a slide glass using Cytospin 3 (Shandon, Roncorn, UK). Red fluorescence of rhodamine and blue fluorescence of DAPI were analyzed, by LSM 510 META Confocal and

Multi-photon Imaging Systems and Laser Sharp 2000 software (Bio-Rad Laboratories, Hercules, CA).

1.7 Interaction of 2E8-SL with Peripheral Blood Mononuclear Cells

To determine whether 2E8-SL-PE specifically bound B cells in heterogeneous populations of PBMCs from healthy donors, we used three-color experiments. Cells were incubated with, in addition to 2E8-SL-PE, either anti-CD20-PerCP (B cell marker) or a combination of anti-CD4-APC and anti-CD8-PerCP (T cell markers), which allowed us to identify the individual lymphocyte populations. The negative control group was incubated with mouse IgG1-PE, mouse IgG1-APC and mouse IgG1-PerCP. Thereafter, cells were analyzed by FCM with use of a FACS Calibur flow cytometer.

1.8 Statistical Analyses

One way ANOVA analyses (SPSS software, version 16.0) were carried out to measure statistical significance at $P<0.05$. Data were expressed as $\bar{x}\pm s$.

2 RESULTS

2.1 Separation of 2E8-SL from Free 2E8

Conjugation was performed by transferring 2E8 micelles to SL. Finally, about 58.70%±3.32 of 2E8 was attached to liposomes, corresponding to approximate 9 IgM molecules/liposome. Free 2E8 and 2E8-SL were separated on the Sepharose CL-4B column by spectrophotometric analysis. In the part of 2E8-SL, the peaks of phospholipids and antibodies *A* were detected at the same time, while in the part of 2E8, only the peak of antibody *A* was detected. And the two parts were completely resolved (fig. 1), which implied that 2E8-SL could be separated from free 2E8.

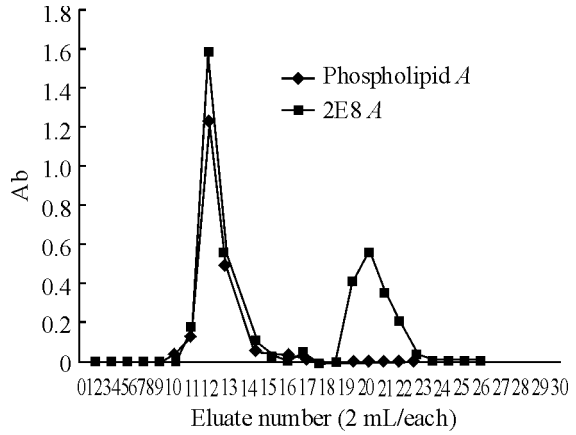

Fig. 1 SL was applied onto a Sepharose CL-4B column and eluted with HBS. Fractions were collected and detected for phospholipids absorbance (*A*) at 486 nm or Abs *A* at 280 nm by spectrophotometer.

2.2 Characterization of 2E8-SL

2.2.1 Shape and Size of SL and 2E8-SL The shape of SL was assessed using transmission electron microscopy. SL was not aggregated and stable (fig. 2). The diameters of SL and 2E8-SL averaged 106.32±3.21 and 121.25±4.32 nm, respectively (fig. 3).

2.2.2 Stability of 2E8-SL The mean particle size within SL and 2E8-SL suspensions did not significantly change when SL and 2E8-SL suspensions were kept in HBS or in HBS including 3% Tween 80, or in the solution composed of equal volume of newborn bovine serum and HBS at 4°C after up to 24 days (fig. 4).

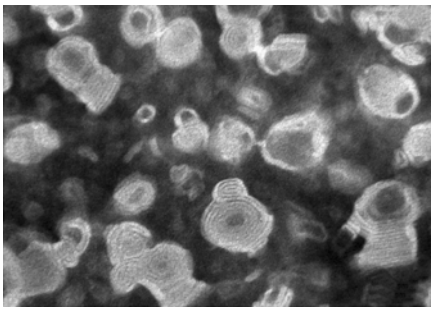

Fig. 2 View of the uranyl acetate stained blank liposomes under the transmission electron microscopy.

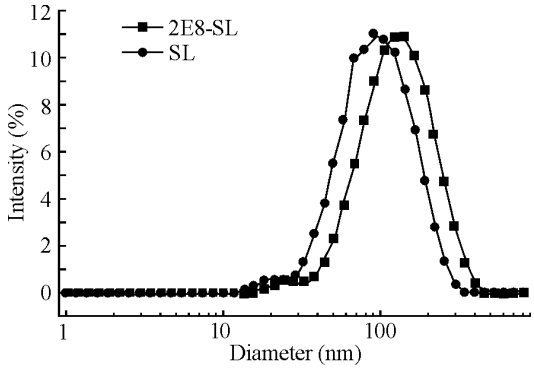

Fig. 3 The mean particle size and particle size distribution of 2E8-SL and SL were determined using Zetasizer 3000 HSa instrument after diluting with HBS.

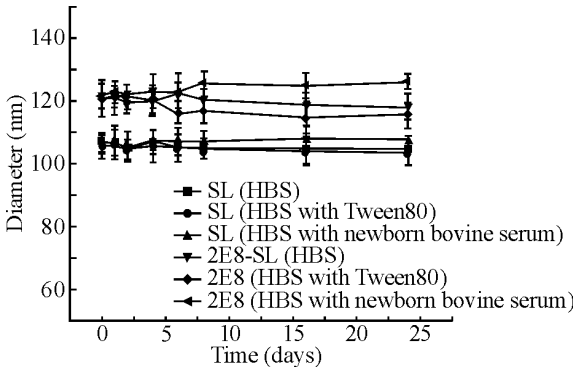

Fig. 4 The mean diameters of SL and 2E8-SL remained stable for up to 3 weeks after synthesis after storage at 4°C in HBS (pH 7.4), or in HBS including 3% Tween 80, or in the solution composed of equal volume of newborn bovine serum and HBS at 4°C. Mean particle sizes were measured over time using Zetasizer 3000 HSa instrument. Values were expressed as $\bar{x}\pm s$ of at least three measurements

2.2.3 The Integrity of 2E8 Before and after 2E8 was attached to SL, the integrity of 2E8 in each step was confirmed by the SDS-PAGE under reduced condition. The two distinct bands at 70 and 23 kD, which represented the intact heavy chain and light chain, respectively, demonstrated that 2E8 was incorporated into the SL in intact form (fig. 5).

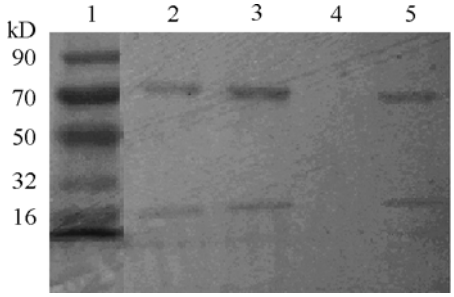

Fig. 5 SDS-PAGE analysis of free 2E8, 2E8 micelles and 2E8-SL
1: Protein marker: 2: Free 2E8; 3: 2E8 micelles; 4: Blank; 5: 2E8 after incorporation into SL and purification by gel filtration on Sepharose CL-4B column

2.3 Targeting Ability and Internalization Effect of 2E8-SL

2.3.1 Binding and Internalization of 2E8-SL against Various Cells To determine the binding ability of 2E8-SL against tumor cells, we tested a number of cell lines including Nalm6, Raji cell lines and Molt3, K562 as control cell lines. The positive rate of cells for PE fluorescence reflected the binding ability of liposomes to cells. As in fig. 6, 2E8-SL could selectively recognize Nalm6 cells .The similar results were not obtained in SL. Almost no Molt3 cells bound 2E8-SL and SL-PE. Fig. 7 showed the positive rate of cells for liposomal PE fluorescence. The result implied binding ability of 2E8-SL-PE was higher than that of nontarget SL-PE to Nalm6 and Raji cells. In contrast, binding abilities of both 2E8-SL-PE

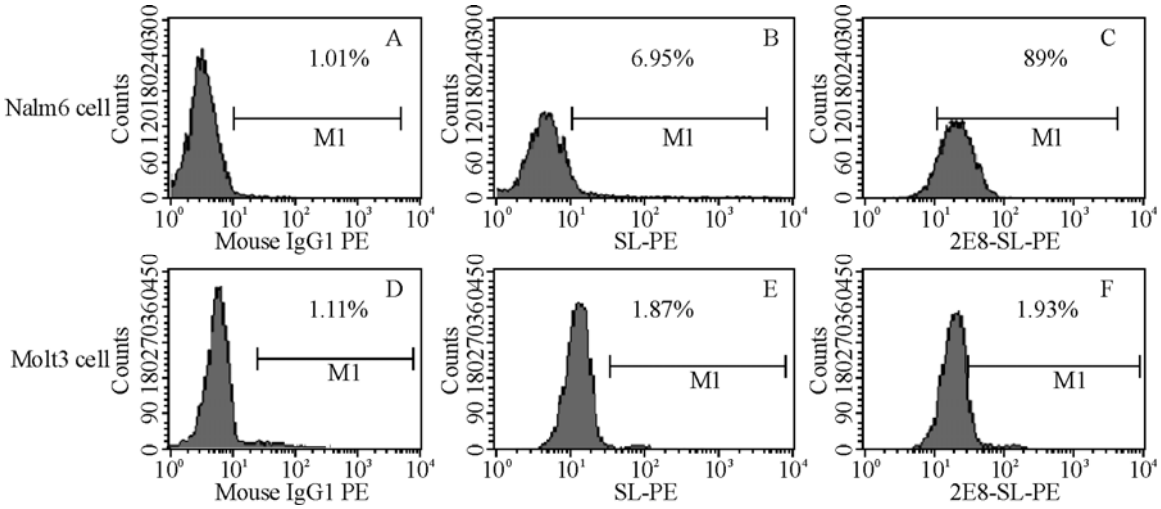

Fig. 6 Targeting binding of 2E8-SL-PE and SL-PE to Nalm6 cells and Molt3 cells
(A) Nalm6 cells were incubated with mouse IgG1 PE; (B) Nalm6 cells were incubated with SL-PE; (C) Nalm6 cells were incubated with 2E8-SL-PE; (D) Molt3 cells were incubated with mouse IgG1 PE; (E) Molt3 cells were incubated with SL-PE; (F) Molt3 cells were incubated with 2E8-SL-PE

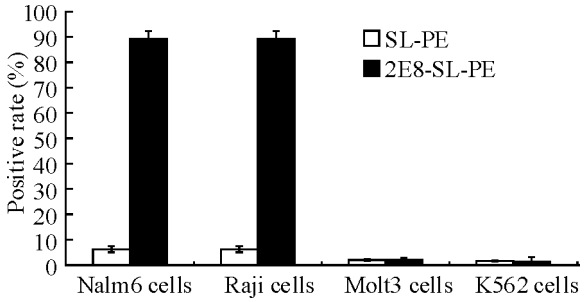

Fig. 7 Binding efficiency of SL-PE and 2E8-SL-PE to Nalm6, Raji, Molt3 and K562 cell lines. These cell lines were incubated with SL-PE and 2E8-SL-PE at 37°C for 30 min. The rate of PE-positive cells was determined by FCM

and SL-PE to Molt3 or K562 cells were much lower. To further investigate internalization of 2E8-SL-PE into Nalm6 cells, the papain was used to remove 2E8-SL-PE attached on the cell membrane. Namely, PE fluorescence on Nalm6 cells was expected to disappear following treatment with papain when those liposomes bound but not internalized into cells; PE fluorescence on Nalm6 cells would remain fluorescen when 2E8-SL-PE internalized into cells. As in fig. 8, after a 30-min incubation at 37°C following treatment with papain, approximate 50% of Nalm6 cells stained with PE previously were negative for PE fluorescence (in the positive control group, nearly 69% of Nalm6 cells incubated with 2E8-SL-PE were positive for PE fluorescence). After a 60-min incubation following treatment with papain, 73.77% of Nalm6 cells remained PE fluorescence (in the positive control group, 89% of Nalm6 cells treated with 2E8-SL-PE were positive for PE fluorescence). These results suggested that the 2E8-SL could efficiently internalized into Nalm6 cells, and it was possible that the antibody/antigen interaction was responsible for the specificity of these results. To confirm the specificity of the interaction of 2E8-SL with Nalm6 cells, a competition assay was performed using free 2E8 antibody. The binding of 2E8-SL to Nalm6 cells was inhibited by free 2E8 antibody (fig. 9).

The internalization of 2E8-SL in Nalm6 cells was further demonstrated by confocal microscopy, also. As in fig. 10, there was detectable PE fluorescence only on

cytomembrane and almost no PE fluorescence was detected in the cytoplasm after a 30-min incubation. After a 4h-incubation, PE fluorescence was detected in the cytoplasm. After 8-h incubation, PE fluorescence was full of the cytoplasm. Internalization of 2E8-SL-PE led to cytoplasmically localized red PE staining that would appear pink when merged with the intracellular DAPI generated by DNA signal.

2.3.2 Interactions of 2E8-SLwith PBMCs We used three-color immunofluorescence to determine whether 2E8-SL can specifically bind B cells in PBMCs. 2E8-SL-PE were incubated with PBMCs, and meanwhile individual populations of B and T cells were identified by staining with anti-CD20-PerCP or anti-CD4-APC and CD8-PerCP, respectively. Fig. 11 (A-F) showed a example of 2E8-SL-PE specific recognization to PBMCs. Namely, SL-PE showed lower binding to B cells or T cells, and with only 0.52% of B cells positive for SL-PE and anti-CD20-PerCP, only 0.19% of $CD8^+$ T cells labeled by SL-PE and anti-CD8-PerCP, and 1.73% of $CD4^+$ T cells labeled by SL-PE and anti-CD4-APC. In contrast, 2E8-SL-PE selectively bound B cells, and with approximate 99.24% of B cells positive for 2E8-SL-PE and anti-CD20-PerCP, and almost no 2E8-SL-PE corresponded to cells stained by anti-CD4- APC or CD8-PerCP. A summary of the results for 2E8-SL-PE and SL-PE recognizing to PBMCs from 6 donors is presented in fig. 11 (G). 2E8-SL-PE specifically targeted to a larger proportion of B cells than T cells (P<0.01). Binding ability of 2E8-SL-PE was significantly higher than that of SL to B cells (P<0.01).

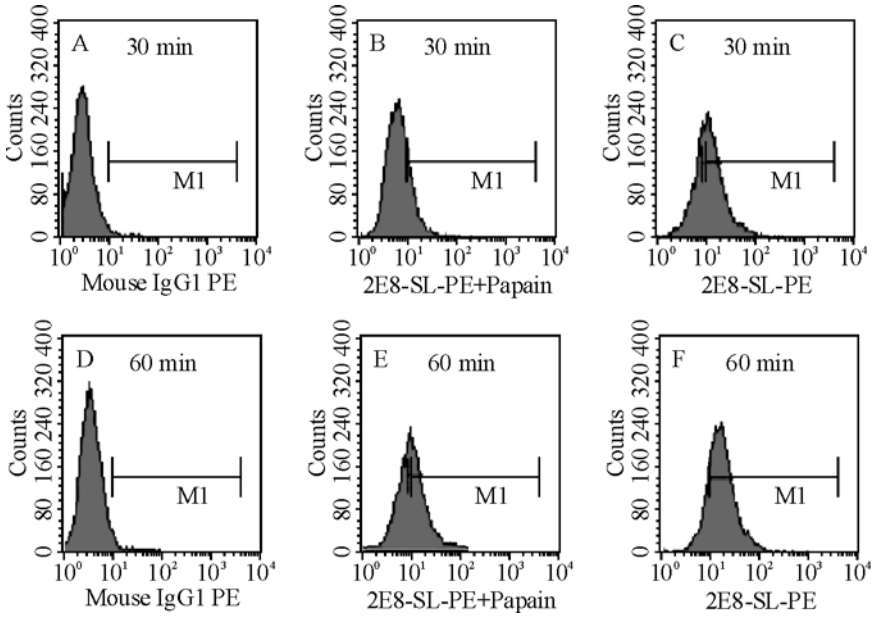

Fig. 8 Nalm6 cells treated with 2E8-SL-PE were incubated with 2 mg/ml papain for 30 min and 60 min at 37°C to remove cell surface-bound 2E8-SL-PE

(A) Nalm6 cells were incubated with mouse IgG_1-PE for 30 min; (B) Nalm6 cells were incubated with 2E8-SL-PE for 30 min, then with 2 mg/ml papain for 20 min at 37°C to remove cell surface-bound 2E8-SL-PE; (C) Nalm6 cells were incubated with 2E8-SL-PE alone for 30 min; (D) Nalm6 cells were incubated with mouse IgG_1-PE for 60 min; (E) Nalm6 cells were incubated with 2E8-SL-PE for 60 min, then with 2 mg/ml papain for 20 min at 37°C to remove cell surface-bound 2E8-SL-PE; (F) Nalm6 cells were incubated with 2E8-SL-PE alone for 60 min

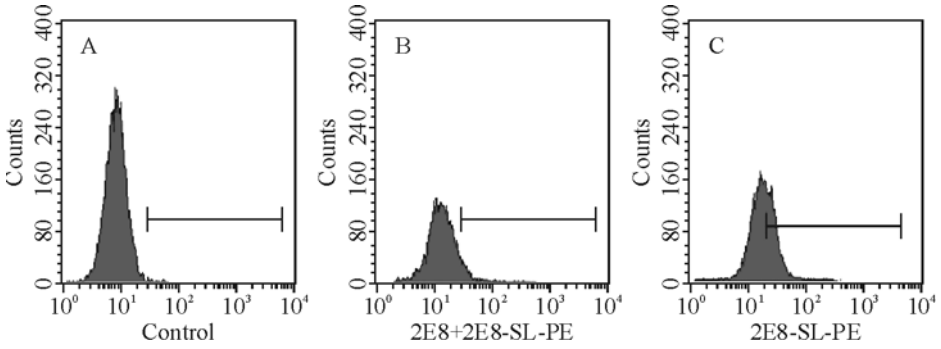

Fig. 9 Blocking of interaction of 2E8-SL with Nalm6 cells by free 2E8 antibody

(A) Nalm6 cells were incubated with mouse IgG1-PE as negative control; (B) Nalm6 cells were incubated with 100 μg/ml unlabeled 2E8 antibodies at 37°C before incubation of the 2E8-SL-PE; (C) Nalm6 cells were incubated with 2E8-SL-PE as positive control

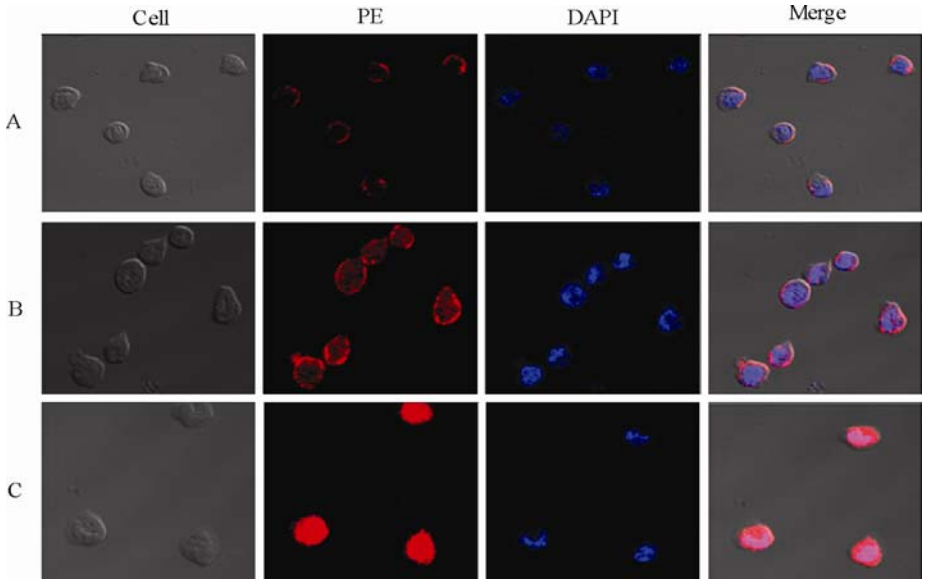

Fig. 10 Internalization analysis of 2E8-SLs by confocal laser-scanning microscopy at various positions in three-dimensional space. The images were acquired using LSM 510 META confocal and Multi-photon Imaging Systems and Laser Sharp 2000 software (×63) (A) Nalm6 cells were incubated with 2E8-SLs-PE for 30 min, then scanned at the thickness of 0.1 μm in three-dimensional space by confocal laser-scanning microscopy; (B) Nalm6 cells were incubated with 2E8-SLs-PE for 4 h, then scanned at the thickness of 0.1 μm in three-dimensional space by confocal laser-scanning microscopy; (C) Nalm6 cells were incubated with 2E8-SLs-PE for 8 h, then scanned at the thickness of 0.1 μm in three-dimensional space by confocal laser-scanning microscopy

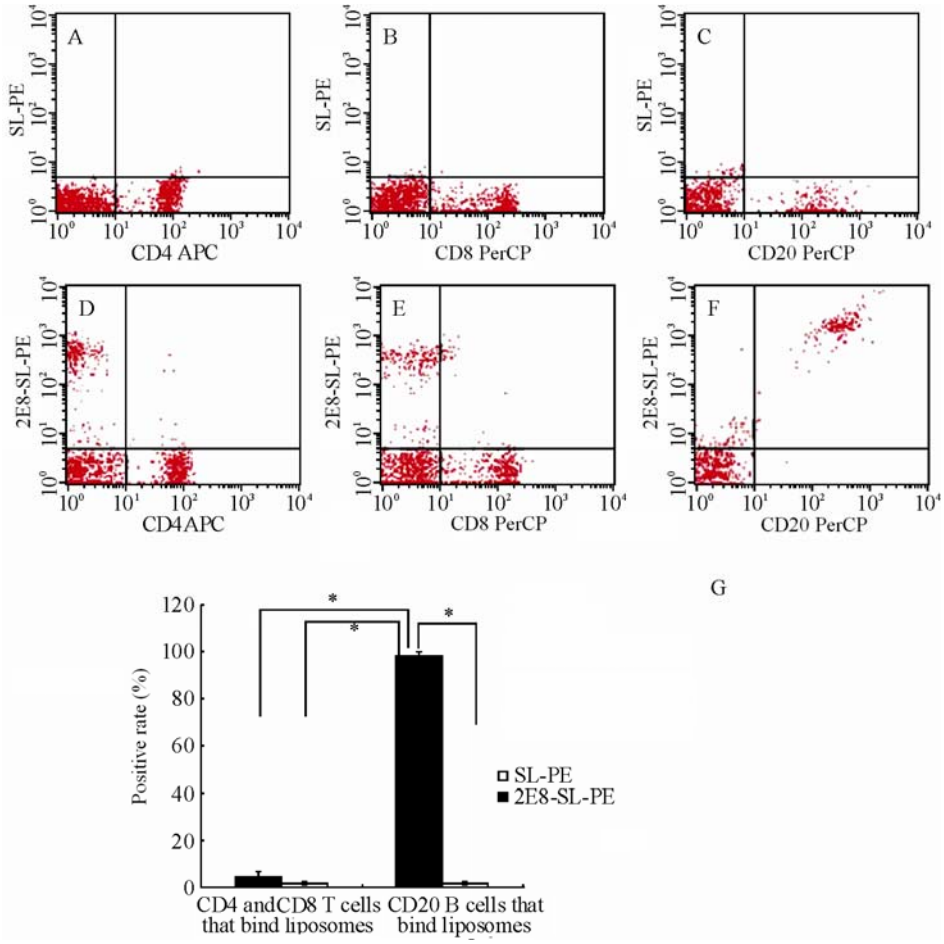

Fig. 11 Three-color flow cytometry of the binding of 2E8-SL to PBMCs. PBMCs were purified using Ficoll gradients and were incubated with either 2E8-SL-PE or SL-PE, then stained with either anti-CD20-PerCP for B cells or anti-CD4-APC, CD8-PerCP for T cells. Dot plots illustrate the intensity of the two stains on cells treated with: (A) SL-PE and anti-CD4-APC; (B) SL-PE and anti-CD8-PerCP; (C) SL-PE and anti-CD20-PerCP; (D) 2E8-SL-PE and anti-CD4-APC; (E) 2E8-SL-PE and anti-CD8-PerCP; (F) 2E8-SL-PE and anti-CD20-PerCP. (G) A summary of the results for 2E8-SL-PE and SL-PE recognizing to PBMCs from 6 donors . $^{*}P<0.05$, $n=6$

3 DISCUSSION

With development of liposomal technique, the liposome is increasingly applied in cancer therapy. Especially, SL emerged by grafting of polyethylene glycol (PEG) to conventional liposomes. On the hand, SL can escape from the capture of reticuloendothelial system and circulate longer than conventional liposomes; on the other hand, SL promotes the progress of sterically stabilized immunoliposome (SIL). So-called SIL, namely modification of SL with mAbs or antibody fragments, or small molecular ligands, can actively and specifically target to tumor cells and is a promising strategy for tumor therapy. It was reported that such SIL has better anti-cancer efficacy as deliver system of anticancer drugs than nontarge SL [19-21].

To select appropriate receptors or antigens is important for the optimal efficacy of SIL. The best targets should have stable and homogeneous expression in tumor tissues, have negligible or low expression in healthy tissues. Moreover, the targets have little or no soluble form of antigen (to avoid rapid antibody clearance) and are expressed on the cell surface where they are easily accessible to SIL [22]. CD19 is expressed on malignant and normal B-cell lymphomas with high levels, while slightly expressed on normal HSCs. Normal B may be killed during therapy, but bone marrow stem cells are expected to reconstitute the normal lymphocyte population. So, CD19 still provides a unique target for targeting therapy of SIL against malignancy with CD19 high express. And a recent research demonstrated CD19 is expressed on B-cell lymphoblast LSCs; so CD19-targeted liposomes possibly selectively recognize B-cell lymphoblast LSCs, which provide a novel effective drug carrier for anti-cancer drugs specific killing LSCs.

2E8 antibody was produced from the lab of our own and had been identified to be anti-CD19 mAb (IgM). In this study, 2E8 was conjugated with SL in intact form using post-insertion technique. The post-insertion technique is a more reproducible and flexible coupling procedure, and offering a chance to optimize two separate processes. Namely, the thiolated 2E8 is easily conjugated with Mal-PEG_{2000}-DSPE by forming the thiol–ether bond between the thiol and maleimide groups. As thiol–ether bond dose not easily hydrolyze, it maintains the stability of 2E8-PEG_{2000}-DSPE[18]. Moreover, the hydrophobic DSPE domain may incorporate itself into lipid bilayers of liposomes[23]. So large IgM molecules with pentamer conformation were conjugated to the terminus of PE-Glipid derivatives in a micellar phase via a simple incubation step (1 h at 60°C), thereafter, are transferred to the bilayers of preformed liposomes.

In this research, we also demonstrated that 2E8-SL was stable by observation of the shape and assay of the diameter. We infer the uptake of 2E8-SL would possibly be decreased by the RES *in vivo*.

Here，when we kept initial IgM (2E8) relative to a fixed amount of linker lipid at a reasonable molar ratio (1:50), coupling efficiency of IgM reached 58.70% ± 3.32, similar to coupling efficiency of ScFv[24]. However, the amounts of 2E8 with IgM conformation incorporated into each liposome were less than that of CD19 with ScFv conformation[25]. It is interesting that 2E8-SL remained effectively targeting to cancer cells with CD19 high expression (fig. 6-7). The binding ability of 2E8-SL to tumor cells with CD19 high expression was much higher (more than 15 times of nontarget SL) than that of other reported CD19- targeted liposomes (approximate 2 times of nontarget SL)[26]. A possible reason is that each IgM has five antigen binding sites and higher inherent affinity than ScFv to antigens. Therefore, even if one liposome was conjugated to only 9 IgM molecules, it remained enough available binding sites which allow 2E8-SL to effectively bind cancer cells with CD19 high express. Besides, that is possible associated with using post-insertion technique to prepare pendant-type 2E8-SL. And the pendant-type immunoliposome recognizes targeted cells more efficiently than the kind of liposome of which antibodies are directly incorporated into bilayer[27].

Further, the internalization of immunoliposome is necessary for good therapeutic responses. It was reported that CD19 antigen underwent rapid internalization on anti-CD19 antibody binding[28]. In this study, we demonstrated 2E8-SL may initiate endocytosis triggered by the internalizing CD19 receptor, and free 2E8 inhibited the interaction of the 2E8-SL with CD19 receptors, suggesting that 2E8-SL may act as passing membrane drug transporter and allow delivery of high levels of anti-cancer drugs to resistant malignant cells with CD19 high expression.

In PBMCs, 2E8-SL selectively targeted to CD19 high-expressed B cells, while not binding T cells (fig. 11F). The selective recognized of 2E8-SL to circulating normal B cells *in vitro* may have been used in delivering anti-dugs to malignance with CD19 high expression while making least anti-cancer dugs to T cells, thereby preserving the immune responses mediated by T cells.

To sum up, in this study, the 2E8-SL was successfully prepared by post-insertion technique. The 2E8-SL is very stable in various buffers, and can selectively recognize and efficiently internalize into tumor cells with CD19 high expression by antibody/antigen specific interaction. 2E8-SL may serve as a useful delivery carrier of anti-cancer drugs targeting to hematological malignance with CD19 high expression.

Acknowledgments

We thank Zhejiang University School of Pharmacy for providing technical support. We also appreciate the hospital staff of the Children' Hospital, Zhejiang School of Medicine for their support in sample collection from patients.

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(Received Jan. 7, 2009)