PREPARATION AND CYTOTOXICITY EFFECT OF ANTI-HEPATOCELLULAR CARCINOMA SCFV IMMUNOLIPOSOME ON HEPATOCARCINOMA CELL *IN VITRO*

G-H. ZHANG, Y-F. LIU[1] and H-Y. HU[2]

School of Nursing, the Fourth Military Medical University, Xi'an, Shaanxi; [1]Department of Patholgy, the Fourth Military Medical University, Xi'an, Shaanxi; [2]School of Pharmacy, Shenyang Pharmaceutical University, Shenyang, Liaoning, P.R. China

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The use of PE38 for cancer therapy has attracted considerable attention for a long time. However, the extensive use of PE38 is prohibited by its severe side effects. Even though immunotoxin PE38 has been researched for cancer therapy, it has displayed low antitumor activity. The aim of this study is to compare the killing efficacy on Hepatocellular carcinoma (HCC) SMMC-7721 cell of immunoliposome PE38, immunotoxin PE38 and liposome PE38. In this study, the sterically stabilized liposomal PE38 was prepared using soybean phosphatidylcholine, cholesterol, and Cholesterol-PEG-COOH. The humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) was coupled to sterically stabilized liposomes using the N-hydroxysuccinimide ester method. The immunoliposome PE38 was prepared in our lab using the above-mentioned single-chain antibody. The hdsFv25-immunoliposomes were immunoreactive as determined by ELISA assay. Immunoliposome PE38 can kill SMMC-7721 cells in vitro with higher efficiency than non-targeted liposomes. These results indicate that immunoliposome PE38 may be potential in the treatment of hepatocarcinoma.

Over the past two decades, the bacterial toxins Pseudomonas exotoxin (PE38) have attracted considerable attention for their potential use in cancer therapy (1-3), because their binding domains either mutated or removed to prevent them from binding to normal cells and are either fused or chemically conjugated to a ligand specific for cancer cells(4-6). Immunotoxin PE38 is composed of protein toxin PE38 connected to an antibody (7-8). Recently, it has become the focus of tumor therapy and been used in clinical experiment (9). To enhance the effect of immunotoxins, the liposome technology which combines to protein toxins has been introduced for cancer therapy.

HCC is a kind of insidious metastasized early malignant tumor with a high death rate. Among various therapy approaches of HCC, liposome-mediated delivery appears to be a more promising strategy, because liposomal toxins having both merits of liposome and protein toxins bind to cancer cells more easily. Liposome PE38 is one kind of those liposomal toxins. Although, liposomes are versatile carrier systems for the delivery of cytotoxic drugs in tumor therapy (10), the conventional liposomes only allow passive tumor site targeting to some degree. Liposomes directed by monoclonal antibodies or their fragments

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Mailing address: Dr Gui-hong Zhang,
School of Nursing,
the Fourth Military Medical University,
169 ChangLe Road,
Xi'an, Shaanxi, 7 10032, P.R.China
Tel: ++029 84774893 Fax: ++029 87854391
e-mail: litter8@163.com

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might be effective approaches to tumor-targeted drug delivery (11). However, immunoliposomes *in vivo* exhibit relatively low targeting efficiency (12), since they are prevented from reaching the target sites as they are easily trapped by antibody uptake in the reticuloendothelial system (RES). The coating of immunoliposomes with the poly (ethylene glycol) improves their stabilization, and turns them into potential long-circulating drug carriers, and maximizes targeting (13). It has been confirmed that antibodies can be attached to the distal end of the PEG chain, and the binding of the antibodies to the target cells can not be sterically hindered by the PEG chain, thus improving the target efficiency of immunoliposomes *in vivo* (14). Through the above-mentioned method, we prepared the immunoliposome PE38, which was named as the third generation immunoliposome, in our laboratory. In our laboratory the fluorescent density of mouse anti-human antibody MAb25 that we prepared is obviously increased in athymic mouse (15). Through the gene engineering method, the MAb25 antibody was humanized, the disulfide bond of which was stabilized and fusion proteins were formed (16-18).

The experimental result indicated that the fusion protein (hdsFv-PE38) has favorable therapy efficacy against tumor. Recently, the use of immunoliposome for cancer therapy has attracted considerable attention, and has been applied to clinic therapy. According to literature (19), the immunoliposome has therapy effectiveness for colon tumor, neuroblastoma, melanoma, etc.

In this study, PE38-loaded immunoliposome was prepared. Furthermore, there is no literature which reports making immunoliposome PE38 as we did in this paper. Our lab is the first to make immunoliposome PE38 targeting hepatocarcinoma cell, and there is no literature reporting using immunoliposome PE38 for hepatocarcinoma.The sterically stabilized immunoliposome prepared in our lab was characterized by their immunoreactivity, the binding and uptake by SMMC-7721 cells, and the results demonstrate that the immunoliposome PE38 could conspicuously increase cytotoxicity on hepatoma cells *in vitro*.

MATERIALS AND METHODS

Materials

The humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) immunotoxin PE38 and toxin PE38 were expressed and purified by our lab. RPMI 1640 nutrient medium, 3-(4, 5-dimethylthiazol-2-yl)-2 (MTT), low molecular weight standard proteinum, nitrocellulose filter (NC filter) and isopropyl thio-β-D-galactoride (IPTG) were purchased from Sigma Chemical Co. (St Louis, MO). Alpha- (3.beta) cholest-5-en-3-omega-hydoxy-Poly (oxy-1, 2-ethanediyl), (Chol-PEG-OH), Soybean phosphatidylcholine (SPC), Cholesterol, MES were gived as a present by Ph.D.Hu Haiyang of Shenyang Pharmaceutical University, the average molecular weight of PEG was 2000. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (S-NHS) were obtained from Pierce (Rockford, IL), Sephadex G-50 were obtained from Pharmacia. PE38 gene was presented by Prof. Pastan Iran in an American state-maintained health center tumor research institute.

Cell culture

The human hepatoma cell line SMMC-7721 was stored in our laboratory. The cells were cultured in RPMI 1640 culture media supplemented with 10% heat-inactivated fetal bovine serum at 37°C (5% CO^2, 95% O^2) in a humidified cell incubator, and the cells were seeded every other day and used at the logarithmic phase.

Preparation of immunoliposome PE38

Liposomes coated with PEG-Chol were prepared from SPC and Cholesterol (5:4, m/m) with Chol-PEG-COOH synthesized as described (20) by lipid film hydration-sonic method. The lipid mixture was dried to a thin film under reduced pressure. The dried lipid film was then rehydrated in MES buffer (10 mM MES/ 20 mM NaCl, pH5.5), and sonicated. Conjugation of hdscFv25 to liposomes was performed as described (21). 100 μl of 0.5 MEDC and 100 μl of 0.5 M S-NHS were added to the liposomes (5μmol lipids/ml), and the mixture reacted for 30 min at room temperature. The desired amount of FITC-labeled hdscFv25 or hdscFv25 was then added and incubated overnight at room temperature. The immunoliposome was separated on a Sepharose Cl-4B column. The coupling efficiency of hdscFv25 was estimated by the FITC fluorescence. Then we loaded PE38 to liposomes. PE38 (1mg/ml) was dissolved in Tris-HCl buffer (10 mM Tris/5 mM EDTA, 30 mM NaCl, pH 7.5), and then added to liposomes. The mixture was incubated for 2 h at room temperature. Liposomes were separated from free PE38 on a Sephadex G50 column, and the encapsulation efficiency was determined by modified Lowry assay (22).

Immunoreactivity of immunoliposome PE38

The human hepotamana cell line SMMC 7721 (1×105) was seeded in 96-well plates and cultured in RPMI 1640 media with 10% FBS at 37°C. Cells were incubated for 1 h with the free hdscFv25, hdscFv25 conjugated to

Chol-PEG-COOH. The plates were extensively washed with PBS, and coated with mouse anti-his antibody for 30 min at 37°C. After washing with PBS, HRP labeled goat anti-mouse antibody was used. The color was visible with OPD. The intensity of the color was analyzed using a BIORAD 550 ELISA reader (BIO-RAD Inc.) at a wavelength of 550 nm.

Binding and uptake of immunoliposome PE38

SMMC-7721 cells (1×10^6) were incubated with NBD-labeled liposomes (1mol%of PL) for 1 h at 37°C. The PL concentration was 400nmol/ml. In some experiments, cells were preincubated for 30 min with hdscFv25 at a concentration of 100μg/ml before adding NBD-labeled immunoliposomes. After washing with cold PBS, cells were fixed using 3.7% formaldehyde and mounted with an ELITE ESP flow cytometer (BECKMAN-COULTER Inc.). Liposomes were composed of SPC and Cholesterol (5/4, m/m), and contained various mol% (2,4 and 6) Chol-PEG-COOH. Initial hdscFv25/ lipid molar ratio was prepared respectively according to Chol-PEG-COOH was 1:500, 1:1000 and 1:2000 .

Cytotoxicity assay

The concentrations prepared with immunoliposome PE38, immunotoxin PE38 and liposome PE38 for cytotoxicity assay were 0.07μg/ml, 0.148μg/ml, 0.295μg/ml, 0.59μg/ml, 1.18μg/ml, 2.36μg/ml, 4.72μg/ml, 9.44μg/ml, 18.88μg/ml and 37.76μg/ml.SMMC-7721cells (1×10^5) were plated in 96-well plates, and incubated with immunotoxin PE38 or liposome PE38 of the above-mentioned concentrations for 1 h. Some incubation with immunoliposome PE38 was preincubated with a 20-fold excess of free hdscFv25. At the end of the incubation, free hdscFv25 or liposomal PE38 were removed and the cells were further incubated for 24 h in RPMI 1640 with 10% FBS. 50 μl MTT (5 mg/ml) were added and cells were incubated for another 4 h. After incubation, 200 μl DMSO was added to dissolve the crystals. The plates were read immediately on a plate reader with a test wavelength of 490 nm.

RESULTS

Preparation of hdscFv25-immunoliposome

The Chol-PEG-COOH could be easily incorporated into liposomes and hdscFv25 could be efficiently coupled to liposomes. The average diameter was determined to be 78.9 nm. An overview of the coupling efficiency for different concentrations of hdscFv25 and Chol-PEG-COOH is presented in Table I. A high mol% of Chol-PEG-COOH resulted in a higher coupling efficiency to the liposomes. Similarly, as the molar ratio of hdscFv25/lipid increased, the total amount of bound hdscFv25 also increased substantially, and coupling efficiency of bound hdscFv25 on the liposomes decreased. The highest density was 8.97μg /μmol lipid, corresponding to approx.27 hdscFv25 per liposome estimated by the method of Enoch and Strittmatter (23).

Immunoreactivity of immunoliposomes

ELISA was utilized to determine the immunoreactivity of hdscFv25, hdscFv25 conjugated to Chol-PEG-COOH and immunoliposomes. The results showed that hdscFv25 retained its immunoreactivity to SMMC-7721 after purification, and chemical manipulation of hdscFv25 caused a slight loss in antigen binding affinity as shown in Fig. 1. The results also demonstrated an increase in ELISA signal with the increasing concentration of hdscFv25 or hdscFv25 conjugated to Chol-PEG-COOH. The degree of binding of hdscFv25-immunoliposomes to SMMC-7721 cells was dependent on both the density of hdscFv25 on liposome surface and liposome concentration as shown in Fig. 2.

Binding and uptake of immunoliposomes

Results of flow cytometry analysis demonstrated that the immunoliposomes were able to bind to SMMC-7721 cells effectively, as shown in Fig. 3. Cell binding could be partially blocked by adding additional hdscFv25. Non-targeted liposomes showed low levels of cell binding. The total binding and uptake for immunoliposomes was 5.0-fold greater than liposome PE38.

PE38 loading and leakage from liposomes

The highest encapsulation efficiency was 69.34% at a weight ratio of PE38 to SPC of 1:15 as shown in Fig. 4. The formula is: $En\%=(C_{total} - C_{free}) / C_{total}\times100\%$. The average diameters of the liposomes ranged from 74.5 to 110 nm. The leakage experiment was done in PBS (pH7.4) at 37°C for 12 h. The percentage of released PE38 was 20.1%.

In vitro cytotoxicity of immunoliposomes

The IC50 of immunoliposome PE38, immunotoxin PE38 and liposome PE38 was 0.47μg/

Table I. *Coupling efficiency and hdscFv25 density on liposomes containing Chol-PEG-COOH.*

Mol% Chol-PEG-COOH	Initial hdscFv25/ lipid molar ratio	HdscFv25 density (μg Ab/μmol lipid)	Coupling efficiency (%)
2	1:500	2.10±0.59	3.4±1.3
	1:1000	1.90±0.23	6.6±2.4
	1:2000	1.39±0.22	99±2.3
4	1:500	4.34±1.3	8.5±2.1
	1:1000	4.09±0.67	14.54±1.4
	1:2000	2.88±1.2	21.45±4.10
6	1:500	8.75±1.0	15.35±2.4
	1:1000	6.73±1.2	23.57±3.2
	1:2000	4.49±0.76	32.4±2.2

Liposomes were composed of SPC and Cholesterol (5/4, m/m), and contained various mol% Chol-PEG-COOH. The coupling efficiency is expressed as the percent of initial hdscFv25 added to the liposomes. Data was expressed as mean ± S.D. n=3.

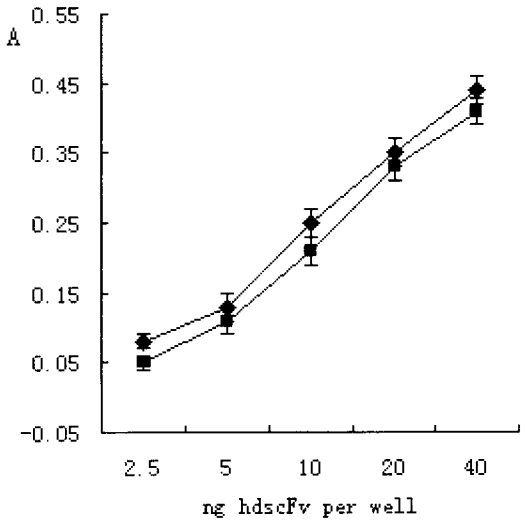

Fig. 1. *Effect of free hdscFv25 and hdscFv25 conjugated with Chol-PEG-COOH on binding to SMMC-7721 cells using ELISA assay. The cells were seeded and cultured with the free hdscFv25, hdscFv25 conjugated to Chol-PEG-COOH for 1 h. The cells were washed and coated with mouse anti-his antibody for 30 min at 37°C, and then HRP labeled goat anti-mouse antibody was used. The intensity of the visible color was analyzed using a BIORAD 550 ELISA reader (550 nm). Free hdscFv25 to SMMC-7721 cells; (■) hdscFv25 conjugated with Chol-PEG-COOH to SMMC-7721 cells. Data were expressed as mean ± S.D., n=3*

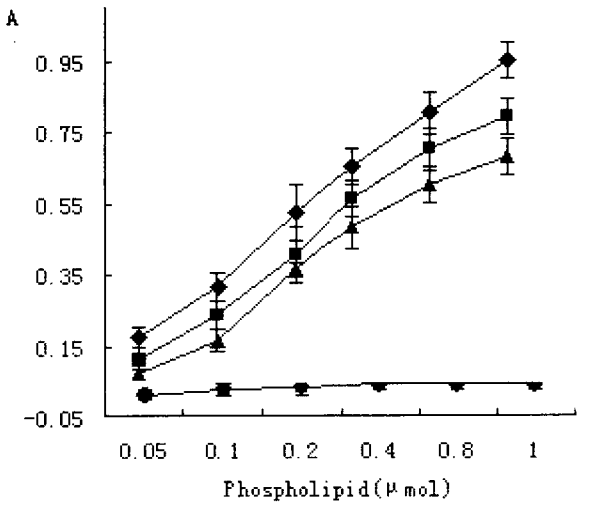

Fig. 2. *Effect of hdscFv25 density and liposome concentration on binding of immunoliposomes to SMMC-7721 using ELISA assay. The degree of binding of hdscFv25-immunoliposomes to SMMC-7721 cells was dependent on both the density of hdscFv25 on liposome surface and liposome concentration.(□) 8.75; (■) 6.73; (▲) 4.49; (●) 0 (*μg hdscFv25/μmol lipid). *Data were expressed as mean ± S.D., n=3.*

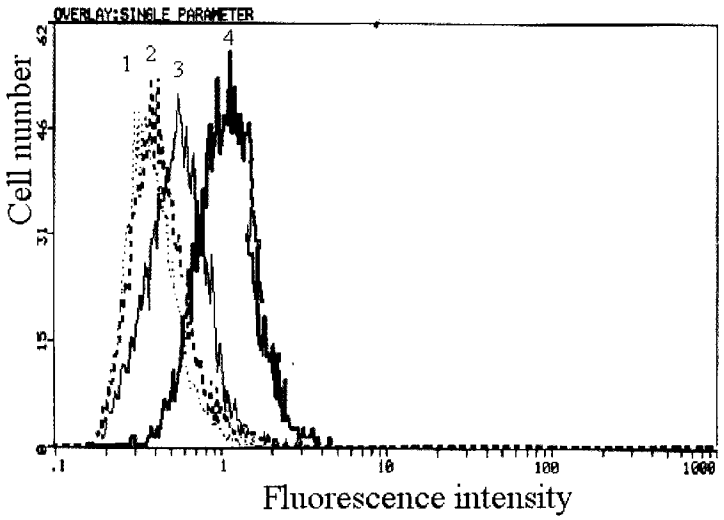

Fig. 3. *Flow cytometry analysis of cellular binding and uptake of liposomes, immunotoxin and immunoliposomes by SMMC-7721 cells. The SMMC-7721 cells were cultivated with NBD-labeled liposomes for 1 h at 37°C. The PL concentration was 400nmol/ml. After washing with cold PBS, cells were fixed using 3.7% formaldehyde and mounted with an ELITE ESP flow cytometer (BECKMAN-COULTER Inc.).*
1 control; 2 NBD-labeled liposome PE38; 3 immunotoxin PE38; 4 NBD-labeled immunoliposomes PE38.

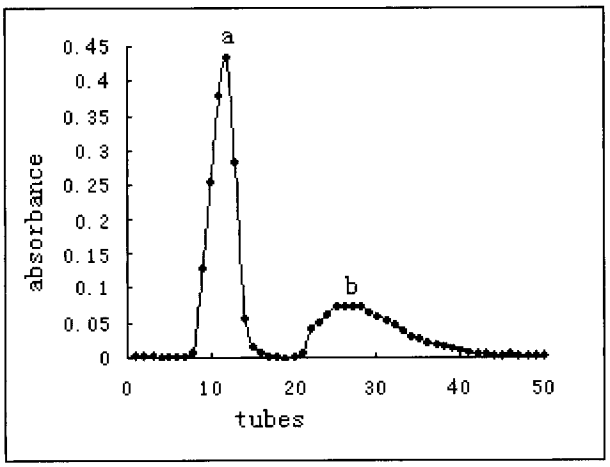

Fig. 4. *The separation curve of liposome PE38 and the free protein toxin PE38. The liposome PE38 we prepared 1 ml was eluted in Sephade G-50 (1.6cm×30cm) by the buffer (pH7.8 10mmol/L Tris-HCl). The flow rate was 0.5mL/min,2ml/tube. Absorbance was measured in ultra-violet (280 nm). a: liposome PE38; b: the free toxin protein PE38*

mL, 4.81μg/mL and 73.32μg/mL, respectively. Their cytotoxicities were compared, as shown in Fig. 5. The formula is: death rate (%) = (control A_{495nm}-experiment A_{495nm}) / control A_{495nm}×100%. After 1 h incubation, immunoliposome PE38 was more cytotoxic on SMMC-7721cells than immunotoxin PE38 and liposome PE38 ($P<0.05$). Namely, the result demonstrated the killing effect on HCC cells of immunoliposome PE38 is best, but that of liposome PE38 is worst.

DISCUSSION

Immunotoxin has become the focus in tumor

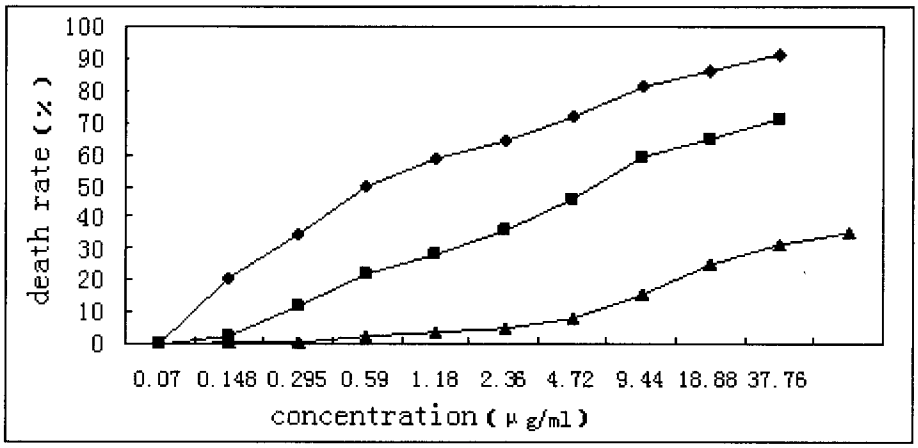

Fig. 5. *Concentration-dependent effect of various formulations of PE38 on SMMC-7721 cell mortality. Cytotoxicity effect of toxin PE38, immunotoxin PE38 and immunoliposome PE38 on hepatocellular carcinoma cells was determined via MTT assay. Data were expressed as mean ±S.D, n=6. *P<0.05, compared with the control group. (◆) immunoliposome PE38; (■) immunotoxin PE38; (▲) liposome PE38*

treatment. Some immunotoxins, such as immunotoxin PE38, have been introduced in clinical experiment (24-29), but immunotoxins cannot kill tumor cells effectively, the problem being that they are only extracellular not intra-cellular. In order to solve this problem, James D. Marks (University of California San Francisco) introduced the immunoliposome technology. The immunoliposome composed of liposome and antibody is a new technology to internalize anticancer drugs.

To date, the immunoliposome has developed for three generations. The first generation immunoliposome is single-chain antibody linked directly to liposome, but cannot kill tumor cells effectively because they are easily quickly removed from the blood by mononuclear macrophage system. The second generation immunoliposome can obviously reduce the cleaning rate owing to the long chain of hosphatidyl-ethanolamine forming a hydrophilia overlayer on the surface of adipose membrane. Nevertheless, the chain of PEG is too long to significantly decrease the conjugations of immunoliposomes to target cells 30-31. Adhibiting carboxy at the end of PEG is able to solve the above problems. Single-chain antibody linked directly to carboxy is the key characteristic of the third generation immunoliposome.

In our laboratory, the mouse anti-human single-chain antibody on hepatoma was reconstituted using a genetic engineering method to obtain stable humanized single-chain antibody hdscFv. The hdscFv has several merits, such as high specificity and affinity, small molecule and low immunogenicity. We first prepared hdsFv25-lip.PE38, named as the third generation immunoliposome by the method described previously (32-33), and then observed the killing effect of hdsFv25-lip.PE38, hdsFv-PE38 and lip.PE38 on Human Hepatoman Cell line SMMC-7721 in *vitro* using MTT assay. According to the results, we can discern that the killing effect of liposome PE38 on hepatoma carcinoma cells was worst, because the toxin was enclosed in the adipose membrane and not targeted by single-chain antibody to hinder killing tumor cells. However, the immunoliposome PE38 has distinct cytotoxicity effects. The aim of this study is to look for a better new type of drug to treat hepatoma. We have shown selective targeting and cytotoxicity of immunoliposome PE38 against SMM-C7721 cells in *vitro*. We believe this strategy could be potentially applicable in the treatment of hepatoma.

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