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Preparation and targeted delivery of immunoliposomes bearing poly(ethylene glycol)-coupled humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) *in vitro*

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Peptides in bee venom (PBV) have attracted considerable attention for anti-cancer therapy. In this study, sterically stabilized liposomal PBV (PBV-SL) was prepared using Soybean phosphatidylcholine, cholesterol, and the cholesterol derivatives of PEG with terminal COOH groups. The humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) was coupled to sterically stabilized liposomes. The hdsFv25-immunoliposome has strong affinity and specificity to SMMC-7721 cells *in vitro*. PBV-loaded sterically stabilized liposomes modified with the hdsFv25 can kill SMMC-7721 cells *in vitro* with higher efficiency than non-targeted liposomes. These results demonstrate that this strategy should also be applicable to immunotherapy for other cancers.

1. Introduction

Peptides in bee venom (PBV) containing melittin as their major protein component have attracted considerable attention for potential use in cancer therapy (Kang JH et al. 1998; Ling CQ et al. 2004; Leuschner C et al. 2003; Shin SY et al. 1999; Xing Liu et al. 2002). The main factor that prohibits the extensive use of PBV in cancer therapy is its intensive hemolytic activity, wich leads to severe side effects. Liposomal drug delivery systems can reduce toxic side-effects and increase activity, which appears to be one of the more promising. However, the *in vivo* use of conventional liposomes is hampered by their rapid removal from circulation by the reticuloendothelial system (RES) (Derksen et al. 1988). Liposomes containing lipid derivatives of poly(ethylene glycol) (SL) offer increased opportunities for targeting of liposomes to specific sites. The *in vivo* properties of SL, which include low RES uptake, prolonged circulation lifetimes and also accumulation at pathological sites, have been widely reviewed (Klibanov et al. 1990). The atachment of a monoclonal antibody (mAb) to the surface of SL is one of the most effective ways to target cells (Allen et al. 1995; Hansen et al. 1995) and specific uptake by target cells is sometimes observed (Zalipsky et al. 1996). This approach is being applied principally to improve the therapeutic efficacy of anticancer drugs.

Progress in antibody engineering has allowed the derivation of specific recognition domains from Mabs (Poon et al. 1997). The recombination of the variable regions of heavy and light chains and their integration into a single polypeptide provide the possibility of using single-chain antibody variable region fragments (designated scFv) for targeting purposes (Bird et al. 1988; Worn and Pluckthun 2001). A lipid-tagged version of a scFv has been successfully incorporated into liposomes (Laukkanen et al. 1994; Ulrik et al. 2002; Tina et al. 2004) and used to target B-lymphocytes *in vito* (De Kruif et al. 1996).

In this report, we used cholesterol derivatives of PEG with terminal COOH groups for the preparation of PBV-loaded immunoliposomes. The humanized anti-hepatoma disulfide-stabilized Fv (hdsFv25) was expressed and purified in this study. These sterically stabilized immunoliposomes (SIL [hdsFv25] were characterized by fluorescence microscopy and cytotoxicity *in vitro*. The internalization of SIL [hdsFv25] into SMMC-7721 cells was examined by fluorescence intensity.

2. Investigations, results and discussions

2.1. Preparation of hdscFv25-immunoliposomes

Particle size and size distribution of liposomes were determined by dynamic laser light scattering with a Coulter COULTER^{TM} LS 230 (Beckman-Coulter Inc.). The average diameter was determined to be 74.5 nm. An overview of the coupling efficiency for different concentrations of hdscFv25 and Chol-PEG-COOH is presented in Table 1. A high mol% of Chol-PEG-COOH that was incorporated into the liposomes 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.

Table 1: Coupling efficiency and hdscFv25 density on liposomes containing Chol-PEG-COOH

Mol% Chol-PEG-COOH	Initial hdscFv25/lipid molar ratio	HdscFv25 density (μgAB/μmol lipid)	Coupling efficiency (%)
2	1 : 500	2.07 ± 0.66	3.2 ± 1.2
	1 : 1000	1.88 ± 0.21	6.7 ± 2.1
	1 : 2000	1.4 ± 0.25	10.1 ± 2.4
4	1 : 500	4.65 ± 1.4	8.3 ± 2.5
	1 : 1000	4.1 ± 0.78	14.6 ± 1.6
	1 : 2000	2.98 ± 1.3	21.3 ± 4.2
6	1 : 500	9.07 ± 1.0	16.2 ± 2.6
	1 : 1000	6.68 ± 1.2	23.6 ± 3.1
	1 : 2000	4.52 ± 0.89	32.3 ± 2.3

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

The coupling efficiency of hdscFv25 to preformed liposomes ranged from 3.2% to 32.3%. The highest efficient coupling efficiency was obtained using the MPB-PE method (Ulla et al. 1995). To use this method, the protein would have to either naturally contain free sulfhydryl groups or be thiolated by a heterobifunctional cross-linking reagent. Since the MPB-PE method would destroy the stability of the disulfide linkage in hdscFv25, we had to choose the *N*-hydroxysuccinimide ester method in this study. However, our studies demonstrated that coupling of only a few scFv molecules is sufficient to mediate strong binding and uptake into target cells.

2.2. Fluorescence microscopy

For fluorescence microscopy studies of the cells with hdscFv25-immunoliposomes, SMMC-7721 cells and Hela cells were used. FITC-hdscFv25-immunoliposomes were able to bind to the cells quickly (Fig. 1A) when incubated with SMMC-7721 cells at 37 °C. Incubation of FITC-hdscFv25-immunoliposomes with Hela cells did not result in any detectable binding (Fig. 1B). Thus, hdscFv25-immunoliposomes could enter SMMC-7721 cells more efficiently. Importantly, binding of hdscFv25-immunoliposomes results in internalization of the carrier systems. Thus, hdscFv25-immunoliposomes should be useful to deliver therapeutic drugs into tumor cells.

2.3. Internalization of hdscFv25-liposomes to SMMC-7721 cells

The ability of hdscFv25-targeted liposomes to specifically bind cancer cells was demonstrated using the SMMC-7721 cell line. As shown in Fig. 2, noticeably higher fluorescence intensity of hdscFv25-liposomes was observed compared to the nontargeted liposomes ($P < 0.05$). In this study, the total binding and uptake for SIL [hdscFv25] was 6.0-fold greater than SL. The excess hdscFv25 could block the internalization of hdscFv25-liposomes to SMMC-7721 cells. Lack of immunoliposome internalization was associated with poor cytotoxicity and lack of therapeutic benefit.

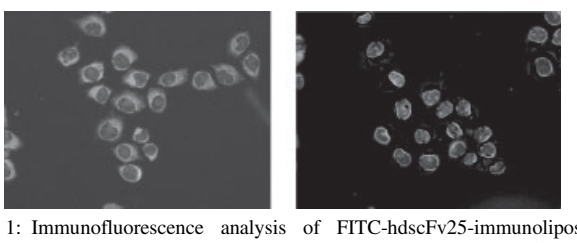

Fig. 1: Immunofluorescence analysis of FITC-hdscFv25-immunoliposomes. (A) SMMC-7721 cells incubated with FITC-hdscFv25-immunoliposomes at 37 °C for 1 h; (B) Hela cells incubated with FITC-hdscFv25-immunoliposomes at 37 °C for 1 h

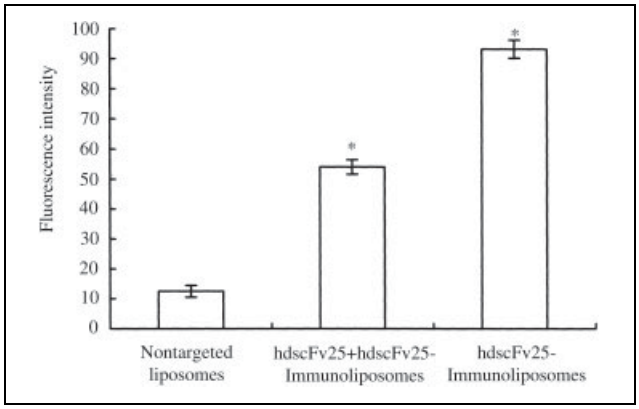

Fig. 2: Fluorescence analysis of internalization of hdscFv25-targeted and control liposomes labeled with NBD-PE with SMMC-7721 cells

2.4. PBV loading and leakage from liposomes

As the weight ratio of PBV to phosphatidylcholine ranged from 1 : 5 ∼ 1 : 25 (w/w), the maximum encapsulation efficiency of the liposomes was reached at a weight ratio of PBV to SPC of 1 : 15. A high cholesterol/SPC molar ratio resulted in higher encapsulation efficiency of the liposomes. The encapsulation efficiency of the liposomes was highest when the cholesterol/lipid molar ratio reached 4 : 5. The type of lipid (EPC, SPC, and DPPC), the mol% of Chol-PEG-COOH, the pH value of Tris-HCI buffer, and the concentration of NaCl could all affect the encapsulation efficiency (data not shown). The highest encapsulation efficiency in this study was 80.2%. The average diameter of the liposomes ranged from 74.5 to 110 nm. The leakage experiment was done in PBS (pH 7.4) at 37 °C for 12 h. The percentage of PBV released was 23.2%. PBV is extracted from honeybee venom, and melittin is a major protein component. Owing to its amphipathic nature, melittin associated strongly with phospholipid bilayers. Furthermore, the presence of PEG-lipids in liposomes did not prevent melittin adsorption on to the interface (Sybille et al. 2002). Another experiment has demonstrated that melittin showed strong binding to the vesicles already attacked Benachir and Lafleur 1995. The leakage experiment in our study also demonstrated this. Besides leakage, the change in the average diameter of the liposomes also indicated that melittin could induce liposome aggregation and subsequent intermixing of lipids between liposome visicles.

2.5. In vitro cytotoxicity of SIL [hdscFv25]

Using the MTT assay, the IC_{50} of free PBV, various formulations of PBV, and hdscFv25 were compared, as shown in Table 2. After 1 h incubation, PBV-SIL [hdscFv25] was more cytotoxic on SMMC-7721 cells than PBV-SL ($P < 0.05$) or PBV-SIL [hdscFv25] mixed with 20-fold hdscFv25 on SMMC-7721 cells. When cytotoxicities were compared for Hela cells, no significant differences of IC50 were observed between PBV-SIL [hdscFv25] and PBV-SL ($P > 0.05$), suggesting that PBV-SIL [hdscFv25] showed relatively selective cytotoxicity on SMMC-7721 cells. As

Table 2: Cytotoxic effect of free PBV and various formulations of PBV on SMMC-7721 cells and Hela cells

Formulation	IC50 (μg/ml)	
	SMMC-7721	Hela
Free peptides	11.02 ± 1.14	7.63 ± 1.47
Peptides – SIL [hdscFv25]	14.66 ± 2.47	100.21 ± 2.12
Peptides – SIL [hdscFv25] and 20-fold hdscFv25	27.4 ± 1.31	104.57 ± 3.14
Peptides – SL	113.3 ± 4.75	103.81 ± 3.75
Peptides – SL and hdscFv25	115.21 ± 3.71	107.21 ± 3.21
Free hdscFv25	NE	NE
Empty SIL [hdscFv25]	NE	NE
Free peptides and hdscFv25	11.63 ± 0.75	7.96 ± 1.28

SMMC-7721 cells (1×10^5) and Hela cells (1×10^5) were placed in 96-wellplates and incubated with either free PBV, PBV-SL, PBV-SIL [hdscFv25] or free hdscFv25 for 1 h (37 °C, 5% CO_2). The plates were read at wavelengths of 490 nm. Cytotoxicity data were expressed as mean ± S.D. n = 6

expected, free hdscFv25 and empty SIL [hdscFv25] did not have a cytotoxic effect on SMMC-7721 cells and Hela cells.

In summary, we used an engineered recombinant human antibody fragment for the generation of immunoliposomes, which can successfully target human hepatoma cells. We have shown selective targeting and cytotoxicity of PBV-SIL [hdscFv25] against SMM-C7721 cells *in vitro*. We believe this strategy could be generally applicable in the treatment of various cancers.

3. Experimental

3.1. Materials

Soybean phosphatidylcholine (SPC) was purchased from Taiwei Pharmaceutics Corp. (Shanghai, China), cholesterol was obtained from Bodi Chemistry Inc. (Tianjin, China), alpha-(3.beta)cholest-en-3-omega-hydroxy-poly (oxy-1, 2-ethanediyl), (Chol-PEG-OH) was purchased from Nippom Oil and Fats (Tokyo, Japan), 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), Peptides in bee venom (PBV, melittin = 90%) were extracted from honey bee venom in our laboratory, Sephadex G50 and Sepharose CL-4B were obtained from Pharmacia; fluorescein isothiocyanate (FITC), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MMT) were purchased from Sigma Chemical Co. (St Louis, MO). l-Palmitoyl-2 [12-[(7-nitro-2-1, 3-benzoxadiazol-4-yl) amino] dodecanoyl]-Sn-glycero-3-phosphoethanolamine (NBD-PE) was purchased from Avanti Polar Lipids Inc. (Alabaster, AL), and chol-PEG-COOH was synthesized as described (Succinic 1981). HdscFv25 was prepared and expressed in our laboratory.

3.2. Preparation of hdscFv25-immunoliposomes

Liposomes coated with PEG-Chol were prepared from SPC and cholesterol (5 : 4, m/m) with various mol% of Chol-PEG-COOH by a lipid film hydration-sonic method. The lipid mixture was dissolved in chloroform and dried to a thin film under reduced pressure for 2 h. The dried lipid film was then rehydrated in MES buffer (10 mM MES/20 nM NaCl, pH 5.5). The liposomes were sonicated, and extruded three times through a 220 nm pore size polycarbonate membrane. Conjugation of hdscFv25 to the liposomes was performed as previously described (Maruyama and Takizawa 1995). 100 μl of 0.5 M EDC in water and 100 μl of 0.5 M S-NHS in water were added to the liposomes (5 μmol lipids/ml), and the mixture reacted for 30 min at room temperature. The excess EDC and S-NHS were separated using a dialysis assay. The desired amount of FITC labeled hdscFv25 or hdscFv25 was then added and incubated overnight at room temperature. The hdsFv25-targeted liposomes (SIL [hdscFv25]) were separated from the unbound hdscFv25 on a Sepharose Cl-4B column. The coupling efficiency of hdscFv25 was estimated by FITC fluorescence.

3.3. Fluorescence microscopy

SMMC-7721 cells were grown in 12-well plates to subconfluency and fixed with ethanol/acetone (1 : 1, v/v). FITC-hdscFv25-immunoliposomes (1 μmol/ml) were incubated with SMMC-7721 cells and Hela cells for 1 h at 37 °C. The cells were then washed three times with PBS and examined through a fluorescence microscope (Olympus, Inc., Tokyo, Japan).

3.4. Internalization of hdscFv25-liposomes to SMMC-7721 cells

SMMC-7721 cells (1×10^4) in RPMI 1640 medium with 10% fetal bovine serum were seeded in dishes and incubated for 24 h (37 °C, 5% CO_2) prior to binding assay. NBD-labeled liposomes (1 mol% of PL) were then added at 400 nmol/ml lipid concentration and the mixture was incubated at 37 °C for 60 min. To examine the specificity of the antibody mediated binding, cells were incubated with excess hdscFv25 (100 μg/ml) at 37 °C for 30 min before adding NBD-labeled hdscFv25 liposomes. After washing the cells with PBS (4 °C), the suspension was centrifuged three times (800 rpm, 3 min), and the supernatant was removed. After lysis with 200 μl of 5% Triton X-100, fluorescence was determined on a Aloka LSC-3000 counter (Aloka Co., Tokyo, Japan).

3.5. Loading PBV to liposomes

PBV (1 mg/ml) was dissolved in Tris-HCl buffer (10 mM Tris/5mM EDTA, 30 mM NaCl, pH 7.5), and then added to liposomes at a weight ratio of PBV to SPC of 1 : 15. The mixture was incubated for 2 h at room temperature. Liposomes were separated from free PBV on a Sephadex G50 column, and the encapsulation efficiency of the liposomes was calculated. Liposomes were stored at room temperature under nitrogen and used within one week after preparation.

3.6. Cytotoxicity assay

The cytotoxicity of various formulations of PBV was determined using the MTT assay. SMMC-7721 cells (1×10^5) and Hela cells (1×10^5) were plated in 96-well plates in a RPMI 1640 medium with 10% fetal bovine serum, and were incubated with free PBV, PBV-SL, PBV-SIL [hdscFv25] or free hdscFv25 for 1 h (37 °C, 5% CO_2). Some incubated with SIL [hdscFv25] were preicubated with a 20-fold excess of free hdscFv25 in cell wells, to block special sites of attachment of hdscFv25. At the end of the incubation, free or liposomal PBV were removed by gentle washing with PBS (ph 7.4), and the cells were further incubated for 24 h. 50 μl MTT (5 mg/ml) was added to each well, and the mixture was incubated for 4 h at 37 °C, 5% CO_2. After incubation, the supernatant was carefully aspirated from the plate, and 200 μl DMSO were added to each well and mixed thoroughly until all crystals were dissolved. The plates were read immediately on a plate reader with a test wavelenght of 490 nm.

3.7. Statistical analysis

The 50% inhibitory concentration (IC_{50}) of various formulations of PBV was obtained using the nonlinear Hill equation with the Kaleida Graph computer program (Synergy Software, Reading, PA). Comparison of cytotoxicity was done using a one-way analysis of variance (ANOVA) with SPSS 12.0. Data were presented as mean values with the standard deviation (mean ± S.D.), and P-values less than 0.05 were considered significant.

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