行政院原子能委員會

委託研究計畫研究報告

診斷子宮頸癌 ^{111}In-Chi-Cx-99-liposome 之研製及應用研究

Production and Application of ^{111}In-Chi-Cx-99-liposome in Diagnosis of Cervical Cancer

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目　錄

中文摘要

上皮細胞衍生之癌症包括肺癌、乳癌、子宮頸癌、卵巢癌、胰臟癌等等皆有研究顯示其細胞中有角質素 19 (cytokeratin 19, CK19)之大量表現，而其表現強弱及發生率與癌症之轉移有關。我們的研究亦發現在肺癌，乳癌，卵巢癌，子宮頸癌的癌組織切片中有角質素 19 的大量表現,在上述幾種癌症的細胞株亦發現角質素 19 大量表現於細胞膜表面。據此，我們發展出一株對角質素 19 具專一性的小鼠單株抗體名為 Cx-99，再據此單株抗體經由分子生物修飾方式建立出一株人/鼠嵌合抗體，希望能用此專一性抗體發展出應用於人體之診斷試劑或是治療藥物，並減低人抗鼠抗體反應(HAMA reaction)所產生之副作用。微脂體包覆藥物的劑型用於癌症治療目前已有不錯的成效，原因在於癌症組織的血管內皮細胞間隙相對於正常組織微血管疏鬆，微脂體較易進入組織間，對正常組織影響不大，從而降低化療藥物的副作用。微脂體可包覆放射性治療藥物或是化學性細胞毒物，本計畫即是打算將放射性同位素 ^{111}In 包覆於微脂體內，再於此微脂體外接上我們發展出的嵌合抗體 MAb Chi-Cx-99，形成 ^{111}In-Chi-Cx99-liposome 複合物，並進行此複合物的特性化檢測、體外細胞毒殺試驗，以及異體腫瘤移植動物實驗，以其發展專一性放射性抗癌藥物。本年度(第二年)的工作項目及內容如下。1. 持續大量抗體製備純化；2. ^{111}In-Chi-Cx99-liposome 複合物合成、特性化檢測、以及造影應用；3. 體外細胞株毒性試驗，4. 以腫瘤異體移植之動物模型檢測藥物毒殺性。實驗初期我們採用台灣微脂體公司所提供的包覆 doxorubicin 的微脂體 doxisome^{TM} 作為結合抗體的材料。所得之免疫微脂體經過粒徑大小，ELISA 以及蛋白質檢測證明已成功將抗體結合於 doxisome^{TM}，形成 MAb Chi-Cx-99-doxisome 免疫微脂體。並於細胞毒殺以及動物腫瘤實驗中得到相對較佳之腫瘤毒殺效果。

關鍵字：子宮頸癌，單株抗體 Chi-Cx-99，微脂體，銦-111

英文摘要

Epithelial cells-derived cancers, including lung cancer, breast cancer, cervical cancer, ovarian cancer and pancreatic cancer, reveal an excess expression in cytokeratin 19 (CK-19). And the cancer metastasis is related to the incidence and expression quantity of CK19. We have found excess CK19 expression in tissue section of lung, breast, ovarian and cervical cancer. And we also found CK19 is over-expressed in the membrane surface of cell lines of lung, breast, ovarian and cervical cancer. Accordingly, we developed a mice monoclonal antibody named Cx-99 which is against CK19, and further, by molecular modification and cloning we developed another mouse/human chimeric. Using this chimeric antibody we hope to develop drugs in application for diagnosis or therapy, and to reduce the side effects of human-anti-mouse antibody (HAMA) reaction. Liposome encapsulated formulations of drugs for cancer treatment has good results at present due to vascular endothelial cell gap of cancer tissues relative to normal tissues is loose. Liposome can entry the interstitial space easier than normal tissue, thus reducing the side effects of chemotherapy drugs. Radioactive drugs or cytotoxic chemicals can be encapsulated into liposome. The present project is planning encapsulate radioisotope ^{111}In, and conjugate Cx-99 chimeric antibody to liposome to form ^{111}In-Chi-Cx99-liposome complex. We will perform the characterization of ^{111}In-Chi-Cx99-liposome complex, in vitro cytotoxicity, and tumor xenograft experiment to develop specific radioactive anti-cancer drugs. The items and contents of the 2nd year are described as follow: 1. production and purification of chimeric antibody in a mass quantity; 2. preparation and characterization of ^{111}In- Chi-Cx99- liposome; 3. in vitro cytotoxicity using cervical cancer cell line; and 4. In vivo Tumoricidal effect of MAb Chi-Cx-99-dosxisome. In production and purification of MAb Chi-Cx-99, we have obtained 16 mg

which possess high purity above 90% and good binding affinity to CK19 after identification by SDS-PAGE and ELISA. We adopt the doxisome^{TM} provided by Taiwan Liposome Company (TLC) to produce immunodoxisome and to perform the particle size measurement, ELISA and protein quantification. These results indicated that we have successfully conjugate MAb Chi-Cx-99 to doxisome^{TM} to form MAb Chi-Cx-99-doxisome^{TM}. In the in vitro and in vivo experiments, the immunodoxisome reveal better cytotoxic effects than doxisome only.

Keywords: cervical cancer, MAb Chi-Cx-99, liposome, ^{111}Indium

壹、計畫緣起與目的

Conventional examination of cervical cancer has only limited sensitivity and specificity in diagnosis and monitoring for supravaginal lesions especially for those of recurrence. Surgical removal of tumor cells or chemotherapy cannot remove tumor cells completely. It is an immediate need to develop tools for the diagnosis and treatment of these cancers. Tumor markers used for studying cervical carcinoma include carcinoembryonic antigen (CEA) (Lam et al. 1992), epithelial membrane antigen (EMA) (Bamford et al. 1983), and cytokeratins (Ferdeghini et al. 1993). There has been limited success in the early diagnosis and monitoring of the disease. Most of these markers have considerable cross reactivity with normal human tissues which limited their roles as a marker. We have established a murine IgG1 monoclonal antibody, MAb Cx-99, that recognizes the surface antigen on epithelial cells but not on fibroblastic or hematopoietic cells. Immunohistochemical studies showed that this antigen was present in all 37 squamous cell carcinomas including 33 cervical SCC, and 30 of the 32 adenocarcinomas examined. In the normal cervix, the recognized antigen was restricted to the undifferentiated basal cells presented in 100% of cervical squamous cell carcinomas and 94% adenocarcinomas (Yuan et al., 1992). The DNA of the antigen recognized by Cx-99 was later cloned and shown to share 99% sequence identity with that of cytokeratin 19 (Yuan et al. 1997). Because of the high specificity, MAb Cx-99 has the potential to be applied in the diagnosis of cervical cancer. In the previous studies, MAb Cx-99 by conjugating ^{131}I, ^{125}I, and ^{111}In has shown its capability of localizing cervical carcinoma xenograft by either biodistribution or radioimmunoscintigraphy (Yuan et al. 1995).

However, the application of murine monoclonal antibody to human

frequently causes anti-murine immunoglobulin reaction that results in human anti-mouse antibody (HAMA) response. The HAMA reactions, cause anaphylaxis, serum sickness, urticaria, fever, and hypotension, usually occur 2 to 3 weeks after the injections and are unrelated to the dose and rate of administration. HAMA responses are likely to result in altered MAb pharmacokinetics, nonspecific targeting and therapy, and early antibody clearance (Goldenberg, 1993). Human antibodies should theoretically be more stable in serum and less immunogenic to human. However, several limitations to the traditional hybridoma-based production of human antibodies, such as instability of many human hybridoma cell lines and human MAbs are mostly of IgM isotype which is suboptimal for clinical use, hinder the development of human antibody. To minimize the undesired immune response without affecting the specificity of murine antibody in human, several types of modified antibodies have been developed such as mouse/human chimeric antibodiy and CDR-grafted humanized antibody (Colcher et al., 1998; Coney et al., 1994). The human/mouse chimeric antibody produces a monoclonal antibody that is antigenically similar to human immunoglobulin but binds the same antigen as the mouse monoclonal antibody from which the CDR sequence were derived. These recombinant monoclonal antibodies are far less immunogenic in human than the parent mouse monoclonal antibodies, and thus they can be used for the treatment of human with far less risk of analyphaxis(Janeway et al.,2001).

There are numerous applications of monoclonal antibodies in cancer diagnosis and treatment such as nuclear imaging with radiolabeled MAbs, intraoperative use of γ-detection probes with radiolabeled MAbs, and immiunoconjugates (toxins, drugs). RAID is a powerful method to locate the cancer lesions with high specificity which enables it to be

used in presurgical staging of extent of disease and postsurgical evaluation of residual disease (Goldenberg, 1993; Yuan et al., 1995; Ng et al., 1995). By conjugating a radioactive substance to a monoclonal antibody that recognizes tumor antigen, cancer cells can be precisely located. Usually, between 60% and 90% of known lesions have been disclosed correctly, depending on the antibody and its form, the radionuclide, or the planar and/or tomographic scanning methods (Perkins & Pimm, 1991; Golderberg & Larson, 1992). Tumors as small as 0.4 to 0.5 cm have been disclosed with Tc-99m labeled MAbs, especially with emission tomography (Goldenberg, 1993). Indium-111 satumomab pendetide was the first labeled monoclonal antibody to be approved by the FDA for tumor imaging. It mainly reacts with most colorectal and ovarian cancers (Bohdiewicz, 1998).

Due to the lack of specific markers for cervical cancers, RAID method has not been used in the detection of cervical carcinoma. The strong reactivity of MAb Cx-99 makes it a good antibody for RAID in detecting cervical carcinomas. Our interest is to apply the MAb Cx-99, especially the humanized form or chimeric antibody, in the radioimmunodetection (RAID), and radioimmunotherapy (RAIT) in the future.

For almost three decades now, liposomes have been considered promising vehicles for delivery of a wide range of encapsulated and/or membrane-incorporated pharmaceuticals, including both therapeutic and diagnostic agents, to the required areas (Torchilin, 2005). Surface modification of liposomes with flexible, hydrophilic polymers such as polyethylene glycol (PEG) (Klibanov, 1990) can effectively protect them from capture by the reticuloendothelial system (RES) and premature clearance from the blood (Klibanov, 1990; Papahadjopoulos, 1991; Allen,

1991). As a result, such long-circulating liposomes (LCL), especially those of smaller size (100–200 nm), acquire higher ability to extravasate during their passage through the highly permeable vasculature of tumours, thus enabling their efficient accumulation in the tumour interstitium, a phenomenon known as the enhanced permeability and retention (EPR) effect (Maeda, 2001). The use of liposomes as carriers for diagnostic agents has already been applied for various imaging modalities: gamma scintigraphy, magnetic resonance imaging (MRI), computed tomography (CT) and ultrasonography (Torchilin VP, 1996; Phillips, 1999). For gamma scintigraphy and MRI, appropriate chelators (such as diethylenetriamine penta-acetic acid, DTPA) loaded with atoms of heavy metals serving as the contrast moieties are incorporated into the aqueous interior of a liposome [Tilcock, 1989] or "anchored" into the liposomal membrane by preliminary chemical derivatization with a hydrophobic group [Kabalka, 1991; Schwendener, 1990]. The use of these passively targeted LCL as carriers for contrast agents now forms an important area of research, which has even found its way into clinical trials [Harrington, 2001; Harrington , 2001; Harrington , 2001]. Clinical data obtained with 111In-labelled LCL are already available on the visualization of lung cancer [Harrington , 2001; Koukourakis, 1999], head and neck cancers [Harrington , 2001; Koukourakis, 1999], Kaposi sarcoma [Stewart, 1997], skin cancer [Harrington , 2001], glioblastomas and metastatic brain tumours [Koukourakis, 2000], soft tissue sarcomas [Koukourakis, 2002], and some other malignancies [Harrington , 2001]. The therapeutic and diagnostic potential of liposomes in cancer could be still further enhanced by rendering them tumour targeted by the attachment of certain tumourspecific ligands to the liposome surface. Among various targeting ligands, monoclonal antibodies (and their fragments) against various types of cancer are frequently used[Allen, 2002].

The goal of this research proposal is to conjugate the MAb Chi-Cx-99 to liposome and the immunoliposome will be then labeled with 111In. The radiolabeled immunoliposome will be used to perform in vitro and in vivo experiments to evaluate the value in application for diagnosis or therapy of human cervical cancer.

貳、研究方法與過程

一、 Cell Lines

Of the two cervical carcinoma cell lines studied, Caski are obtained from the ATCC (American Tissue Culture Collection), and CC7T was obtained from Dr. T.M. Change, Veterans General Hospital (VGH), Taipei. FS-4, a foreskin fibroblast cell line, and G9T, a glioma line, are purchased from the ATCC. All cell lines are maintained in DMEM containing 10% FCS.

二、 MAb Chi-Cx-99 Preparation

The FO cells clones which stably express MAb Chi-Cx-99 are cultured in RPMI 1640(Gibco #11875) with 5% FBS until the concentration reached 0.8~1.0 x 10^6/ml.

2.1 ELISA for detection of chimeric Cx-99 Ab

Goat anti-Human kappa chain (Serotec 050802) were used as capture antibody to coated on 96-well plate with a concentration of 5μg/ml in coating buffer (Na_2CO_3 1.59g, $NaHCO_3$ 2.93 g add Q-H_2O to

1L).After incubation overnight at 4℃ and washed, the coated plates were blocked with 2% skim milk in PBS at 37℃ for 2hours.Dilted chimeric Cx-99 Ab was reacted with the capture Ab at 4℃ overnight then washed with PBST. Goat anti-Human IgG Fc- HRP detection antibody(Serotec 0102) with 10%FBS was used and the reaction was conducted at 37℃ for 1hour. After washing, ABTS (KPL50-66-01) was added and waited for 30 minutes at room temperature then OD405nm was read.

2.2 Large scale culture of transfectoma

The transfectoma clone with highest titer was first cultured in 10 cm dish until 80% confluence and expanded to two 15 cm dishes with 25ml growth medium. After confluent, cells in one 15 cm dish were collected and resuspended in 50ml RPMI1640 with 10% Low IgG FBS (Hyclone, cat: 30151.03).Despensed 10ml cell solution to 15 cm dish and supplied another 15ml serum-free medium for 1~2

days(FBS final concentration was 4%). While the color of medium getting yellow, added 25ml serum-free medium and kept culturing for another 1~2days(FBS final concentration was 2%).One hundred milliliter of 1% Low IgG medium were supplied when cell grew confluent and kept culturing for 2~4 days until the color becoming yellow. Collected the culture supernatant and centrifuged at 3000rpm for 30 minutes at 4℃, then filtered the supernatant with 0.45μm filter membrane(47mm, Supor-450, 60173, Gelmam, Michigan, USA) and stored at 4℃ with 0.02%sodium azide.

2.3 Fusion protein purification and characterization

2.3.1 Purification

Protein A-sepharose CL-4B (17-0974-04, Amersham Bioscience) was packed slowly into Eco-column (5ml, Sarstedt, D-51588, German) and connected the column to P-1 pump (18-1110-91,

Pharmacia), then washed the column with 100~200ml PBS(flow speed: 4x10; about 2ml/min). Supernatant of culture medium was applied to the column(flow speed : 2ml/min,it took about overnight) and washed with PBS with 20-fold gel volume until the O.D.280 of flowthrough lower than 0.01. The column was then washed continuously with 50~100ml sodium citrate buffer (0.15M NaCl, 100mM citrate pH5.5). We used different elution buffer (100mM glycine ,pH3.5 and pH 2.5) to elute chimeric Cx-99 antibody into collective tubes which was prepared with 200µl neutralization solution and labeled previously. Two milliliter was collected in each tube and read OD280.Selected three high-OD280 fraction and proceeded condensation procedure.

2.3.2 Condensation

Six milliliter of high concentration fraction was applied into dialysis membrane and dialysed against PEG (50% ddH2O, 50% PEG 20’000 ,Fluka) at 4℃

until the volume shrank to 2ml.The dialysis buffer was then changed to PBS, filtered with 0.22μm low protein binding filter and measured the protein concentration.

2.3.3 SDS-PAGE and Western blot of chimaeric Cx-99 Ab

Total cell lysate of cervical cancer cell line CC7T and CaSki were extracted and 20 g of crude extract were loaded on 12% SDS-PAGE. Transferred the protein on SDS-PAGE to PVDF membrane and blocked nonspecific binding with 5% skim milk. Primary antibody were used against the membrane at 4℃ overnight. After washing, secondary antibody which conjugated HRP incubated with the membrane at 37℃ for one hour. Finally HRP substrate DAB were applied to detect the result.

三、 Preparation of immunoliposome

Antibody is reacted with 5 molar excess of maleimide-PEG-DSPE in HEPES buffer (pH 7.2) at 37℃ for 5 hours. The conjugate is mixed with liposome at a ratio that should

yield about 10 antibody molecules/liposome assuming that 25% of the antibody conjugated to Mal-PEG-DSPE and 100% inserted into the liposomes. The appropriate volume of liposomes is added to the antibody-PEG-DSPE conjugate and is incubated overnight at 37℃. The next day, the unreacted maleimide is reduced by incubation with 2mM beta-mercaptoethanol for 30 min at room temperature. The antibody-conjugate liposome is purified from the unconjugated antibody by size exclusion chromatography on a 1.5 x 30 –cm ,A450m column equilibrated with 25mM HEPES/ 0.9% saline(pH 7.2). The amount of protein conjugate to the liposome is quantitated by SDS-PAGE on a 4-20% gradient gel. The protein in the gel is visualized with Sypro Orange, and the intensities of the samples are quantitated using an AlphaImager from AlphaInnotech Corporation.

四、Radiolabelling of immunoliposomes

Liposomes containing the amphiphilic chelate DTPA-PE (composition:PC:Chol:PEG2000-PE:DTPA-PE in a 3:2:0.3:0.3 molar ratio) were prepared along with the IgG- and

2C5-immuno-analogues. The loading of liposome-incorporated DTPA-PE with ^{111}In was performed via the trans-chelation mechanism. DTPA-PE-containing liposomes were supplemented with 0.1 M citrate buffer and incubated for 1 h with ^{111}In at room temperature, to allow for the trans-chelation of ^{111}In from a weak citrate complex into a firm DTPA complex, and then dialyzed overnight against HBS at 4°C to remove a free label.

五、In vitro cytotoxicity

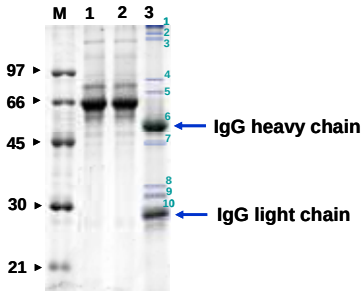
Two cell lines CC7T and CasKi will be plating onto 96-well tissue culture plate in a concentration of 5×10^3 cells/well. After 18 hrs, the 111In-labeled immunoliposome will be added and culture for 12, 24, 48, or 72h. The treated cells will be then harvest for total protein detection using SRB method.

參、主要發現與結論

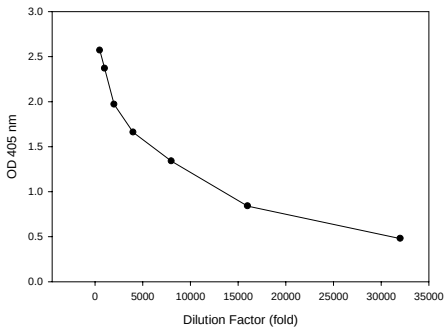
In this year we perform the production, purification and characterization of MAb Chi-Cx-99; the production and characterization of MAb Chi-Cx-99-dooxisome complex; in vitro and in vivo cytotoxicity and tumoricidal effect respectively。

一、Production, purification and characterization of MAb Chi-Cx-99

We have obtained about 17 mg MAb protein with high purity and titer (Fig. 1). In the 32000 fold dilution factor, the OD 405 nm is still higher than 0.5. It indicates a high affinity of MAb Chi-Cx-99 to antigen CK19.



number	volume	percenta
1	4011610	
2	1510620	
3	1160230	
4	352012	
5	400658	
6	1.2E+08	
7	2692872	
8	1168950	
9	1591138	
10	95943594	



A. B

Fig. 1 A. The purity of MAb Chi-Cx-99 after purification by protein G. Lane M: protein marker (Kd), lane 1:original culture supernatant, lane 2: flow through, lane 3: IgG eluent. The purity is about 94.38%. B. Serial dilution factor of culture supernatant vs OD 405 nm value in ELISA evaluation system.

二、Production and Characterization of Chi-Cx-99-Doxosome

As shown in Fig. 2, after separation by CL-4B sepharose chromatography, the eluent in each fraction was test by ELISA and HPLC for the binding affinity and doxorubicin concentration. A peak of OD 405 nm was shown in fraction 5, and a corresponding peak of doxorubicin was also shown in fraction 5. The compatible results of binding affinity and doxorubicin concentration indicated that the conjugation of Chi-Cx-99 to Doxisome was successful.

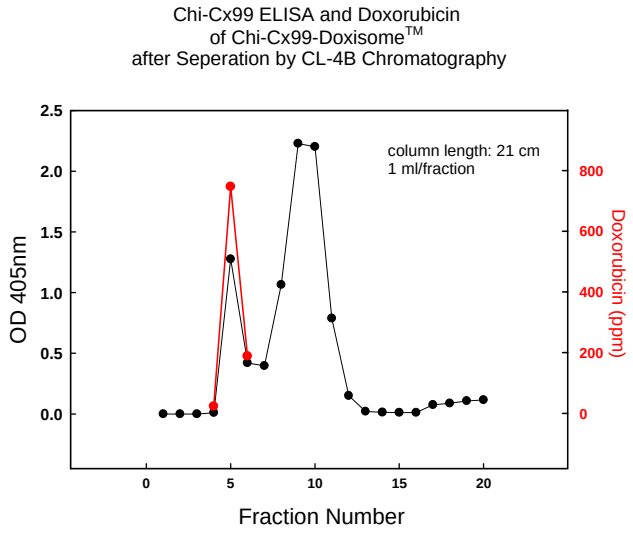


Fig. 2 Characterization of Chi-Cx-99-doxisome by ELISA and HPLC for protein binding affinity (OD 405 nm) and concentration of doxorubicin (ppm) respectively after separation via sepharose CL-4B chromatography. The eluent was collected 1 ml in each fraction.

三、*In Vitro* Cytotoxicity Test

Using the concentration of doxorubicin as a standard to test cytotoxicity of doxorubicin, doxisome and immunodoxisome on TOV-21G cells in various doses for 24, 48, and 72 h (Fig. 3). The immunodoxisome reveal a better cytotoxic effect than doxisome and doxorubicin from the concentration of 0.01 to 20 μM at 24, 48 or 72 h treatment.

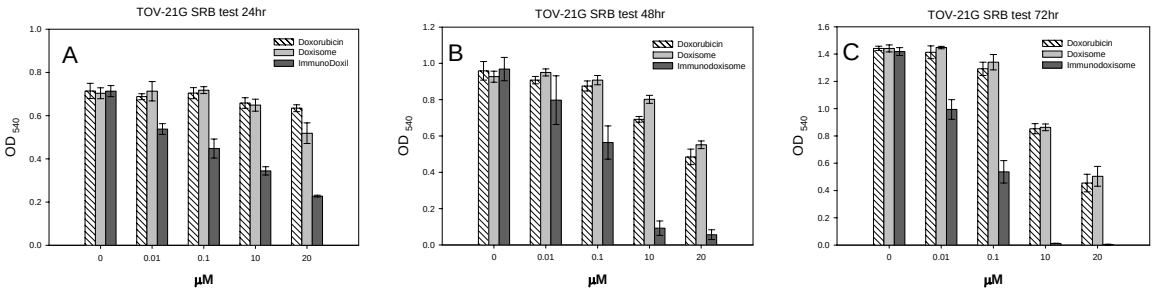


Fig. 3 Cytotoxicity of doxorubicin, doxisome and immunodoxisome on TOV-21G cells in various doses of doxorubicin. The cell viability test was performed by SRB method.

四、*In Vivo* Tumoricidal effects

The immunocompromised nude mice bearing TOV-21G were received doxorubicin, doxisome or immunodoxisome in a doxorubicin dose of 5 mg/kg body weight 5 and 12 day after the

measurement of tumor size. Figure 4 indicated that the tumors size decrease after first administration of MAb Chi-Cx-99-Doxisome at day 5, while the tumor on mice received doxisome keep growing at day 8.

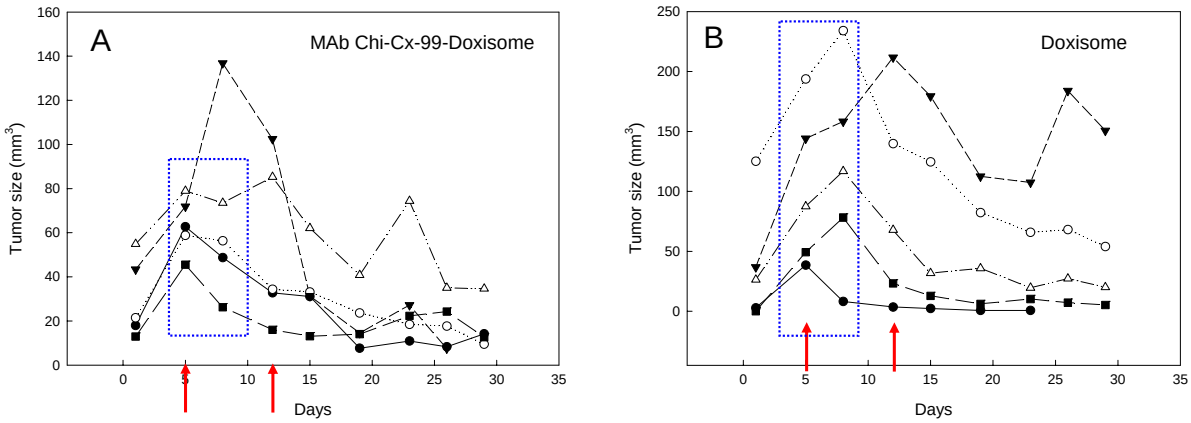


Fig. 4 The tumor growth curve in nude mice after administration of MAb Chi-Cx-99-doxisome (A) or doxisome (B). Each line indicates an individual mouse. Red arrows indicate the administration day in a dose of 5 mg/kg body weight of doxorubicin.

五、The Biodistribution and Radioactive Molecular Imaging

We use three gynecological cell lines that express the CK19 on the outer surface of cell membrane to choose the optimal one for tumor xenograft animal model. The results are shown as Fig. 5. The CC7T and SKOV3 cells formed tumors from relative higher radioactivity than most organs except liver and spleen. Since the SKOV3 cell is ovarian cancer cell line, then we choose the CC7T as our imaging model. However, either the

biodistribution(Fig 6) or imaging experiment (Fig. 7) did not show that the Chi-Cx-99-NanoX is better than IgG-NanoX or NanoX only.

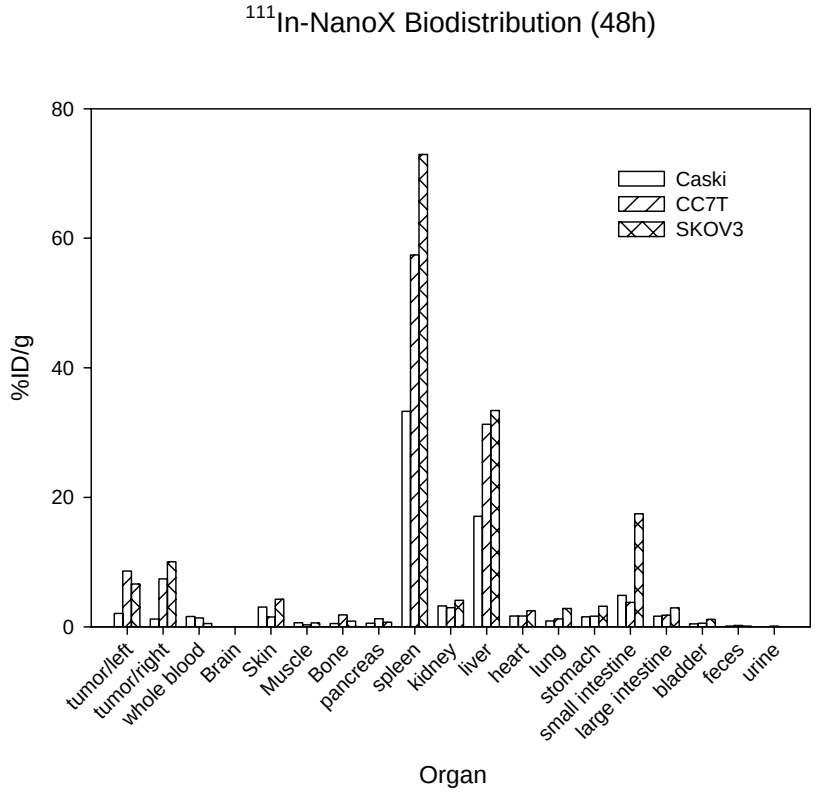


Fig. 5 The biodistribution of ^{111}In-NanoX liposome in Caski, CC7T, or SKOV3 tumor bearing NOD-SCID mice. The data was shown as percentage injection dose per gram organ/tissue weight.

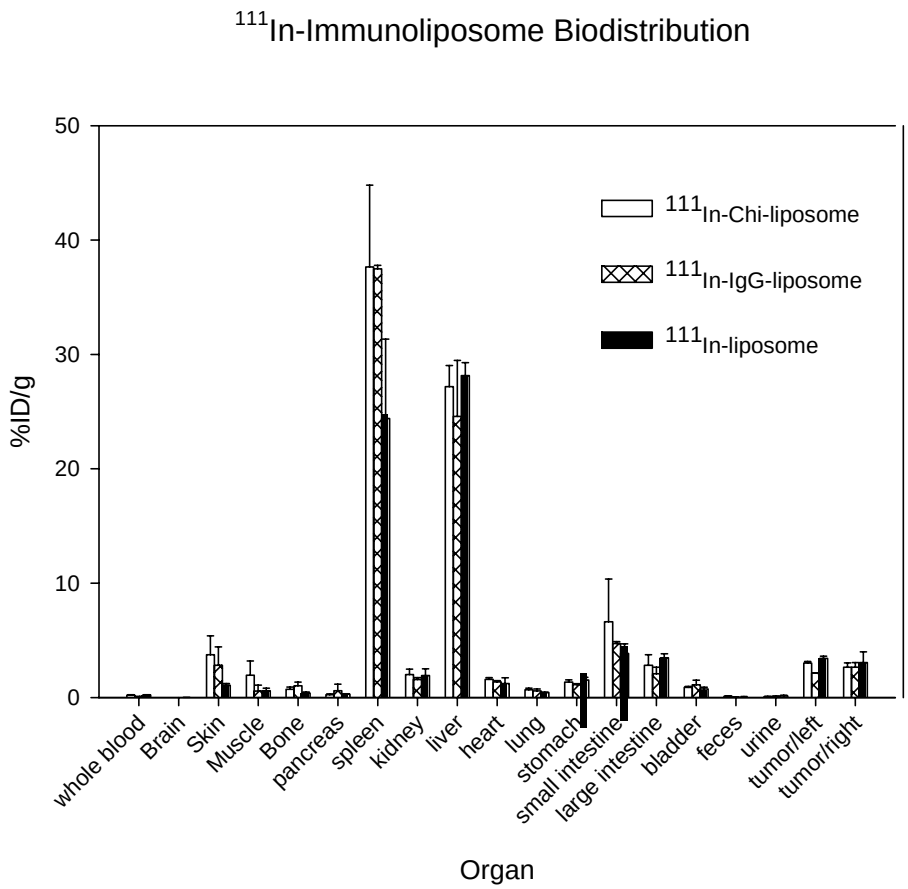


Fig. 6 The biodistribution of ^{111}In-Chi-NanoX liposome in CC7T tumor bearing NOD-SCID mice. The data was shown as percentage injection dose per gram organ/tissue weight.

Conclusions

According to the results, the immunoliposome can be applied as a good strategy to treat the cancer cells that express unique surface marker. However, since the characteristics of liposome, the liposome only and immunoliposome have the same expression in imaging system. Hence, we conclude that the immunoliposome is not better than liposome in diagnostic application, especially in radioactive imaging system.

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