

The Way that PEGyl-DSPC Liposomal Doxorubicin Particles Penetrate into Solid Tumor Tissue

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Abstract

Background: For enhancement of drug effectiveness and reduction of drug toxicity, liposomal drugs have been studied in laboratories and clinics for decades. Although the results obtained from in vitro are encouraging, but the results from in vivo tests were not satisfactory. The main reasons for this situation were that we do not have enough information about the way how liposomal particles penetrating into solid tumor tissue, and what happening to the liposome particles after they got into the tumor tissue. In this paper, we are going to report the results from our observations on the way folic acid targeted and non-targeted PEGyl-DSPC liposomal doxorubicin particles penetrate into solid tumor tissue.

Methods: Subcutaneous transplanted murine L1210JF solid tumors in mice were used as a model. PEGyl liposomal doxorubicins were injected through tail venue, and tumor tissue samples were collected at special time points. Cryosections were cut and dried by a flowing of air after mounted on the slides right away. Then the dried cryosections were stained in water systems; the blood vessel cells were stained with green fluorescent FITC labeled antibody against CD31 antigen; the nuclei of the living cells were stained with a blue fluorescent dye DAPI. Since the whole procedure was carried out in aquatic system, the red color fluorescent liposomal doxorubicin particles remain visible under fluorescence microscope.

Results: Both folate conjugated and non-conjugated PEGyl-DSPC liposomal doxorubicin particles were only leaking out from the broken holes of blood vessels with a special direction and spread out for a limited distance, which was similar to the results showed before, in that observation a latex microsphere sample was used as a model.

Abbreviations: DSPC:1, 2-Distearoyl-sn-Glycero-3-phosphatylcholine; PEG₂₀₀₀-DSPC:1, 2-Distearoyl-sn-Glycero-3-phosphatidylethanolamine-N-[methoxy(polyethylene glycol)-2000]; Folate-PEG3400-DSPE:1, 2-Distearoyl-sn-Glycero-3-phosphatidylethanolamine-N-[polyethylene glycol-3400]-folate.

Keywords: Solid tumor, liposome particles, blood vessel penetration.

Introduction

The toxicity of all the available anticancer drugs are too high, commonly the side effect occurs at their functional dosage. Liposomal drugs have been proven capable of changing the drug distribution in vivo, increasing the drug effectiveness, and decreasing the drug toxicity (Vail et al. 2004). After targeting liposomal drug technique was developed, we can see in culture systems the liposome particles binding onto tumor cell surface and taken up by the cells quickly. However, at the time the liposomes being used in animals, the results were showed not as good as what we expected (Jain, 2001; Nagayasu et al. 1999; Patel, 1996). People were too busy in making and testing different types of liposomes, number of papers even only reported their results without animal tests, did not pay enough attention to the study of knowing further about the real processing of the liposomal particles in the solid tumor. Actually, since we do not know the exact way from which liposomal particles getting into solid tumor tissue, and also the fortune of those liposomal particles after they got inside the solid tumor tissue. Clearly, it is not really clear what is the right direction to further improve the effectiveness of liposomal drugs in animal or clinic yet, especially in the targeted liposomes and the solid tumor cases. A number of researchers have done a lot of studies in this area with various types of equipment and experimental designs (Lichtenbeld et al. 1996; Hashizume et al. 2000; Davorak et al. 1999; Kohn et al. 1992; Uster et al. 1998). From the results of those studies we agreed that the liposomal particles may pass through

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the broken hole of the tumor blood vessels and spread out in the tumor tissue, but so far a strong or direct evidence about this hypothesis is still not available. The main obstacle in this part of study is that the liposomal particles are composed of lipid membranes, which will be destroyed immediately when the solid tumor tissue were treated in organic solvents during the procedures of histological stains. Because of this reason we have tried to use rhodamine labeled latex microsphere particles with the same size (100 nm in diameter) and negative electro charge to mimic the behaving of the liposomal particles (Pan et al. 2004). The results were very good, from the fluorescent color, we can see the red color particles leaking out from green color blood vessels through the broken areas, but not from everywhere or passing through the blood vessel wall. However, the methods of using latex microspheres has limitations, first they are rigid particles, possibly still not totally the same to the real liposomal particles, second, they will not be up taken up by the cell and kill the cancer cells as the liposomal particles probably will do. These week points become critical, when we want to compare the different situations between the non-targeted and the targeted liposomal particles. It is not impossible to label the latex microspheres, make them become targeted or carry drugs, but it is kind of difficult to control, Anyway, it will be another research, no longer the problem of liposomal drugs we want to see at present time. Nevertheless, based on the experience with the use of fluorescent latex microspheres, we realized if we omitted the acetone fixing step, instead, dried the thin cancer tissue under an air flow immediately after the cryosections have been mounted on the glass slices, then stained the tumor sections with antibody or chemical reagent in water solutions, we may be able to avoided the damage of the liposomal particles, and see the liposome particles, if the liposome particles carried a red color fluorescence. Thus, in this study, we are using the real PEGyl-DSPC liposomal doxorubicin as a tool to run the observation. The advantage of this liposome is first we can locate the liposome by the red color fluorescence of drug doxorubicine with the same method of fluorescent stains as we have used in the fluorescent latex microspheres; and the second, these folic acid targeted and non-targeted liposomes have been used in set of liposome studies in our study in the past. In this case, with the limited changes, more results can be used in comparasion,

with that we may be able to obtain some better ideas about it. Meanwhile, in another experiment, we also observed the fortune of these liposomal particles after they got into the solid tumor tissue, and compared the anticancer effectiveness of these liposomal doxorubicin in mice. The results were surprisely different to what we used to believe, the better anticancer effectiveness was not because of more folic acid targeted particles getting into tumor tissue, the better anticancer effectiveness came from the non-targeted liposomal particles kept in the tumor tissue for long time with no change, kept the drug doxorubicin inside the lipid membrane and no chance to meet the cancer cells. Those results will be reported in another paper. The abstract of these results have been present in the meeting os AACCS (Pan et al. 2005). We hope based on these new information we may be able to find out what should we do to improve the effectiveness of the liposomal drug treatment in vivo.

Materials and Methods

Reagents

Folic acid free RPMI 1640 and fetal calf serum were purchased from GIBCO (Grand island, NY, U.S.A.). DSPC and PEG₂₀₀₀-DSPC were purchased from Avanti Polar Lipids, Inc. Folate-PED3400-DSPC was obtained from the NIH. The other reagents such as cholesterol, doxorubicin and Sepharose CL-4B chromatography resin were obtained from Sigma Chemical Co (St. Louis, Missouri). Rat anti-mouse CD31 antibody was obtained from BD Pharmingen (San Diego, CA, U.S.A., Catalog number 550274). The biotinylated rabbit anti-rat, mouse absorbed antibody was purchased from Vector, Inc. (Burlingame, CA, U.S.A., catalog number BA-4001), and the FITC-streptavidin was obtained from Dako Inc. (Carpinteria, CA, U.S.A., Catalog number F0422). The nuclei stain fluorescent dye DAPI (4', 6-diamidino-2-phenylindole) containing mount medium was a gift from Dako Inc. The tissue-Tek O.C.T. compound was obtained from Sakura Finetek USA Inc. (Torrance, CA, U.S.A.).

PEGyl-DSPC liposomal doxorubicin preparation

DSPC/Cholesterol/PEG-DSPE (65:31:4, mole/mole) non-targeting liposome and DSPC/Choles-

terol/PEG2000-DSPE/Folate-PEG3400-DSPE (60:31:3.5:0.5 mole/mole) folate targeted liposomes were prepared by the procedure we used in previous studies (Pan et al. 2003; Pan et al. 2002). Briefly, mixed the lipid composition in chloroform solution in a round bottom flask, totally weight of the lipid mixture was 30 mg. then this mixture solution was dried under nitrogen. The residue was desiccated under vacuum for two hours, and re-hydrated in 5 ml pH 4.0, 400 mM sodium citrate solution, treated with ultrasonic and vortex, freezing and thawing alternately. The production was then passed through a 100-nm pore-sized polycarbonate membrane under nitrogen at 60° C in a lipid extruder (Lipex™, Northern Lipids, Inc., Vancouver, Canada). The mean size of the liposome particles was controlled at 100 nm in diameter (Ishida et al. 1999), which was measured by photon-correlation spectroscopy on a NICOMP 370 Submicron Particle Analyzer. After purified from Sepharose CL-4B column, the unilamellar liposome particles were remote-loaded with doxorubicin based on trans-membrane pH gradient of 4.0–7.4. The drug-to-lipid weight ratio was at 1:10 (wt/wt). The doxorubicin containing liposome sample was purified by passing through Sepharose CL-4B column again to separate off small amount of free doxorubicin. Then the liposomal doxorubicin preparations were diluted in pH 7.4 PBS to yield a doxorubicin concentration of 1 mg/ml suspension. The final production of liposomal doxorubicin samples were stored under 4° C, covered with aluminum foil. In our observations the liposome sample were all fresh made and used within one month, although they have been proven stable for much longer time.

Mice model of L1210JF solid tumor

Murine leukemia L1210JF cells were cultured in a folic acid free RPMI 1640 medium, supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 mg/ml streptomycin, in a humidified incubator, atmosphere containing 5% CO₂ and the temperature was 37° C. Before injection, the cells were spun down and washed with pH 7.4 PBS solution three times.

DBA2 mice weighting 18–22 g were purchased from Charles River Laboratories (Wilmington, Massachusetts, U.S.A.). The mice were fed a folate free special rodent diet (Catalogue No. 117772, Dyets Inc.) for one week before use (Pan et al. 2003). They were then inoculated subcutaneously

with 0.1 ml suspension of 1×10^6 L1210JF cells in PBS at the left flank. The tumor was allowed to grow until it reached a size of about 0.5 cm³. In our animal experiments, all the procedures strictly followed the regulations of the Ohio State University Institutional Animal Care and Use Committee.

Cryosections of solid tumor tissue sample preparation.

Each mouse was injected through the tail vein with PEGyl-DSPC-liposome sample containing 0.2 mg doxorubicin. At each of the selected time points, three mice were sacrificed with CO₂ gas. Then the tumor tissues were collected immediately and embedded into Tissue-Tek O.C.T. under dry ice right away. All the frozen samples kept under minus 80°C before use.

Six μm thick Cryosections were cut and mounted on positive-charged glass slides, and dried right away under an electric fan. The dried sections were kept in a minus 20° C freezer before staining. The staining procedure was processed on a Dako autostainer. Briefly, first the sections were re-hydrated in pH 7.4 PBS, and blocked with 10 % normal rabbit serum. A 1:50 dilution of rat anti-mouse CD31 primary antibody was applied and then the slides were incubated for 30 minute at room temperature (Rubin et al. 1999; Rogatsch et al. 1997; Pan et al. 2004). After wash, a 1:200 dilution of biotinylated rabbit anti-rat, mouse absorbed antibody was used as the second antibody. After 30 minutes incubation, the sections were washed and then a 1:40 dilution of FITC-Streptavidin solution was applied. Lastly, the slides were counterstained with fluorescent dye DAPI contained mounting medium to stain the alive cell nuclei and covered with a regular cover glass.

Observation on the penetration and distribution of the liposomal doxorubicin

Stained cryosections were viewed immediately under a Zeiss fluorescence microscope. A 480 nm ultraviolet exciting, 535 nm emission system was used for green fluorescent labeled blood vessel observation, and 360 nm/420 nm system was applied for observation of color fluorescent labeled cell nuclei and a 545 nm/610 nm system was used for red fluorescent doxorubicin containing liposome particle observation.

Results and Discussion

The results of the observation showed that the methods used in cryosection cutting, the tissue stainings and the selection of the liposomes were suitable. The omission of the acetone fixing step did not interfere the staining of the blood vessel cells and the nuclei of the tumor cells. Clearly, the red color doxorubicin containing liposomal particles were not penetrating out from the blood vessel wall, since the red color liposomal particles did not distributed along the blood vessels like a sleeve or as a cycle surround the cross section of the blood vessel. The liposomal particles were only passing through the holes of the broken blood vessels with a direction and spreading into the cancer tissue in the space between the cells in the tumor tissue. Compare to the total number of blood vessels in the sections, the number of leaking blood vessels were limited in our L1210JF experimental cancer tissues. Those broken blood vessels commonly located near the necrosis areas, or those areas where the necrosis is starting, since in those necrosis areas, the number of the cells along the blood vessel were high, but in a short distance showed no many alive cells there. It could be nicer if we could have a chance to quantitatively determine the ratio or the number of leaking holes in different tumor tissue samples, However, from our observations in different experiments, the frequency of the necrosis happening in the tumor tissues might different from different species of the tumors, and determined by the location on back or at the flank of the mouse where the tumor was growing. The level of necrosis also related to the size of the tumor and time how long the solid cancer had been growing.

As early as six hours after liposome injection, the red color liposome particles already showing in the tumor tissue. At 24 hours after liposome injection the liposomal particles spread out for a longer distances and then the liposomal particles stopped leaking out from the blood vessel at about 48 hours after liposome injection. In this paper, for showing the clearer picture of the way by which non-targeted and folic acid targeted liposomal particles penetrating out from the blood vessel, we showed two photos for each liposome and all of tumor tissue samples were taken at the time 12 hours after liposome injection (see Fig. 1). In Figure 1, A and B showing the non-targeting liposomal doxorubicin particles penetrating in L1210JF solid tumor tissue; C and D are showing the folic

acid targeted liposomal doxorubicin particles penetrating in L1210JF solid tumors. No obvious differences could be pointed out in Figure between the non-targeted liposome and folic acid targeted liposome, only the diffusion of the folic acid targeted liposomal particles looked faster.

We have been wary about that the low temperature frozen cryosection cutting step might break the liposomal particles or changed particle size. But, from the results we obtained, we believed that was not happened, or only happened at very limited level. Anyway, even it happened, at the time the cryosections were cutting, the liposome particles already leaked out from the blood vessel, the size change at that time will not interfere our observation. Also under the experimental condition the limited amount of free doxorubicin which leaked out from the broken liposomal particles would be wash off from the thin section tissue during the staining procedure, it would not be seeable. As showed quite often, that there are not many living cells near the leaking blood vessels, we believe those broken blood vessels are located closely to the necrotic areas or those areas where the solid tumor tissue was starting to form necrotic area. Lately, Kirpotin et al. observed the distribution and up taken of anti-HER2 antibody targeted long circulating liposomal doxorubicin particles and its correlated non-targeted liposome in HER2 expressed BT-474 cell solid tumor and non-HER2 antigen expressed MCF-7 cell solid tumor in nude mice (Kirpotin et al. 2006). In their report, the amount of liposomal particles distributed into the solid tumor tissues were the same, no difference in both antigen expressed BT-474 solid tumor or HER2 antigen not expressed MCF-7 solid tumor. And no difference between the antibody targeted or non-targeted liposome. It is agreeable, the amount of liposomal particles can penetrate into solid tumor tissue is determined by the hole size and number of the blood vessels, but not determined by the targeting group which located on the liposomal particle surface. However, after got into the tumor tissue the functional folic acid ligands bound on to folic acid receptors. Besides, as it was already reported that a number activated macrophages were presenting in the solid tumor tissue (Murdoch et al. 2004) they also actively expressing folic acid receptors on their surface. So, in our folic acid targeted liposomal particle case, the macrophage cells also up taken the folic acid targeted liposomal particles, then free drug doxorubicin

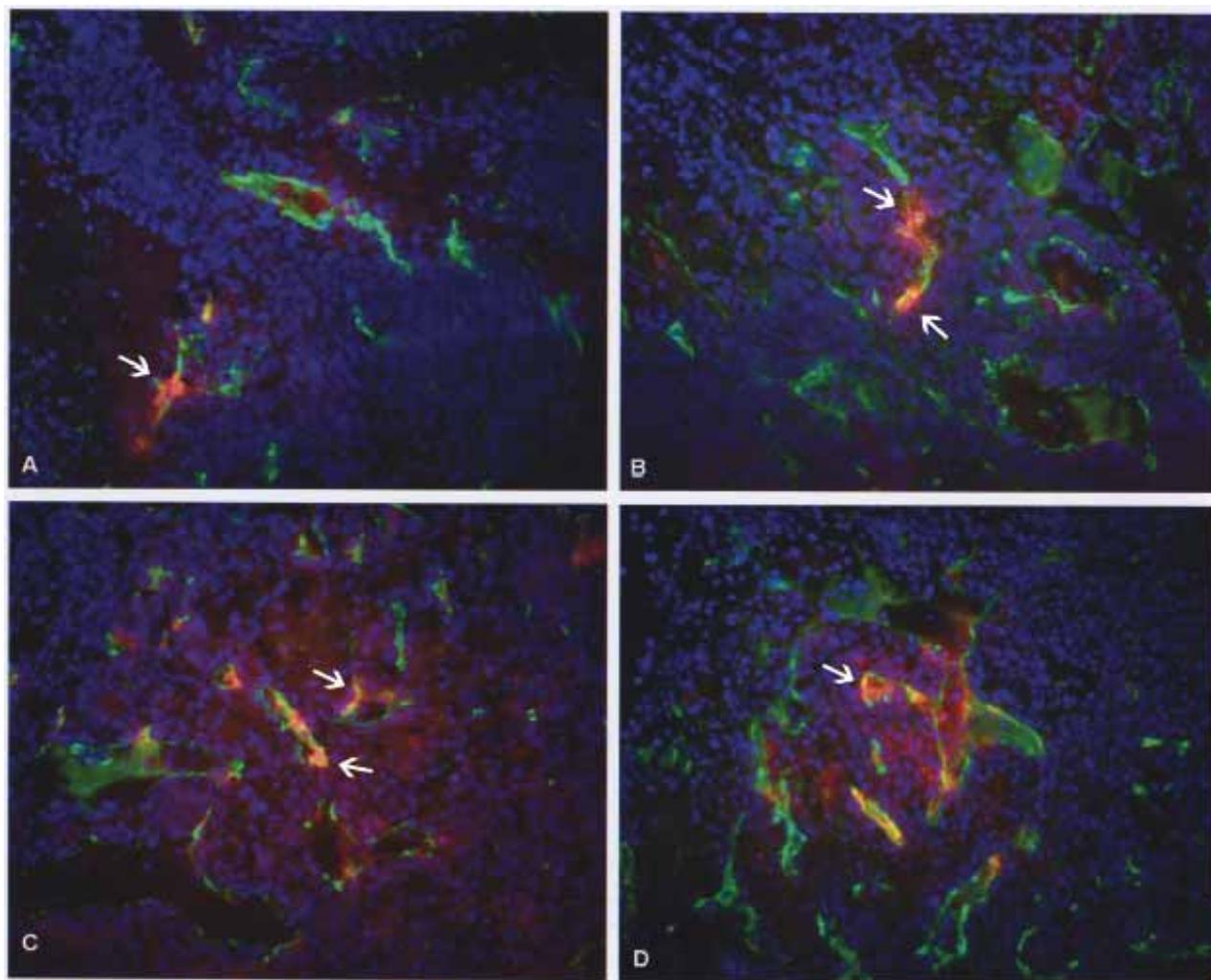


Figure 1. The PEGyl-DSPC liposomal doxorubicin particles are leaking out from broken blood vessels in L1210JF solid tumor tissue growing subcutaneously in DBA2 mice. Photo **A & B** samples were collected at 12 hours after non-targeting liposomal doxorubicin was injected from the tail vein; **C & D** samples were taken at 12 hours after folic acid targeting liposomal doxorubicin was injected from the tail vein.

released out after the lipid membrane broken down, which killed the tumor cells in neighborhood as well. After the cancer cells were killed, the blood vessel further damaged, we saw quite often the red blood cells presented in the damage tumor tissue. In this situation, more space and bigger holes allowed the targeted liposomal particles leaked into cancer tissue. No doubt the folic acid targeted liposomal doxorubicin showed better anticancer effectiveness compared to the non-targeted same liposomal doxorubicin. We will discuss about this in another paper.

Here, we need to mention that since the liposomal particles penetrated through directly into solid tumor the blood-brain barrier will no longer show (Vail et al. 2004), which will be good for the treatment of brain tumors. But the problem is since

the number of liposomal particles, in another words the amount of liposomal drug which can leak into the solid tumor is determined by the number of the leaking blood vessels, or say the necrosis level of the solid cancer tissue. So, in the case even the drug sensitivity of the cancer cells are the same, the efficiency of the cancer treatment will be still different at least at the beginning will be determined by situation of the blood vessels say by the necrotic level of the solid tumor tissue. The same problem will be more important for the study on early diagnosis of cancer, People nowadays are trying hard to use liposomal particles or other nanoparticles to carry special reagents or radio active labeled materials to find the location of the earliest stage tumor. Here we can see that we need to re-consider that strategy, since with that idea we

may not be able to show the tumor location before the tumor grow into special size, and the break blood vessels showed up. Furthermore, some cancer even naturally do not have blood vessels, such as some of the non-small-cell lung carcinoma (Pezzella et al. 1997).

At the end of this paper, we would like to make a suggestion that it might be interesting, if we mix the rhodamine labeled latex microspheres with an anticancer drug sample, or a liposomal form drug suspension, we may be able to trace the drug penetration process in a solid tumor tissue, not like doxorubicin its fluorescence will be diminished after doxorubicin reacted to the cells, the fluorescence of rhodamine can last much longer.

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