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Surface modification of liposomes for cardiomyocytes targeting *in vitro*

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The effect of novel 3-{4-[2-hydroxyl-(1-methyl ethylamine) propyl oxygen]phenyl}propionic acid cetylester (PAC) as a surface modification ligand on the delivery of liposomes into cultured cardiomyocytes was investigated. Small unilamellar liposomes with and without PAC (PAC-liposome and Plain-liposome) were labeled with a fluorescence marker. The cultured neonatal cardiomyocytes were incubated with liposomes under normoxia or hypoxia conditions, and then the cell-associated fluorescence was measured. A high affinity of the PAC-liposomes to cardiomyocytes was observed. The amount of cell uptake of PAC-liposomes under normoxia conditions was 4-fold higher than that of plain-liposome, and the increase was 8.5-fold when hypoxia occured. The results suggested that PAC is a potential surface modification ligand for liposome targeting the ischemic myocardium.

Liposomes have been suggested as efficient carriers for the delivery of drugs into ischemic myocardium (Caride and Zaret 1977; Phelan and Lange 1991; Smalling et al. 1995; Silva et al. 2001). This targeting phenomenon can be explained by the fact that in hypoxic areas of infarcted myocardium with increased permeability, liposomes ranging from 10 to 500 nm in size can extravasate and accumulate inside the interstitial space as a reservoir of the drug (Torchilin 2000). To enhance their activity of targeting to an ischemic myocardium or cardiomyocytes, liposomes with different characters (size, surface charge, permeability can be designed) and modified with various kinds of ligands (antibody, receptor ligand, sugar residues, etc).

It was reported that the β_1-adrenoreceptor (1-AR) predominated in mammalian ventricular cardiomyocytes (ranging from 80% in various rats, canine, and feline cardiac preparations to 60% in baboon ventricular myocytes) and its levels significantly increased when hypoxia occured (Bae et al. 2003). To improve the efficiency of liposomal drug delivery into the ischemic myocardium, 3-{4-[2-hydroxyl-(1-methyl ethylamine) propyl oxygen]phenyl}propionic acid cetylester (PAC) was synthesized, which has a homologous structure as esmolol (a selective β_1-adrenoreceptor blocker). Its effect as surface modification on the delivery of liposomes into cultured cardiomyocytes was studied.

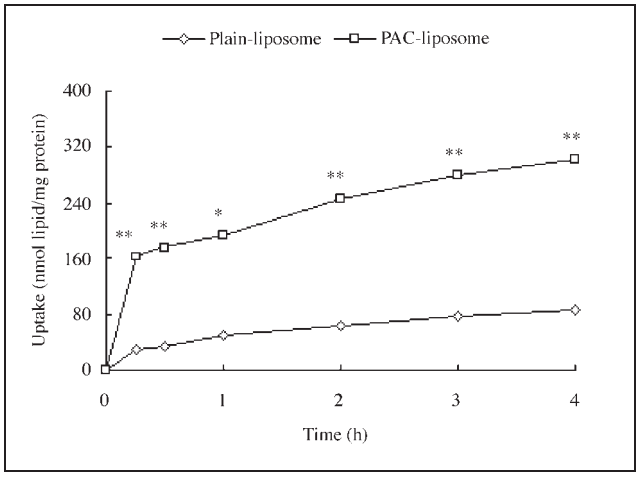

Fig. 1: Uptake of Plain-liposome and PAC-liposome by cardiomyocytes
Plain-liposomes (SPC/Chol 2 : 1 molar ratio) and PAC-liposomes (SPC/Chol/PAC 2 : 1 : 0.2 molar ratio) were labeled with NBD-PC. Cardiomyocytes were incubated at 37 °C with liposomes at a dose of 2000 nmol/dish for various lengths of time. Then the cell-associated fluorescence was measured. The amount of liposome uptake is expressed as the nmol of lipid per mg of cell protein. Each value represents the mean ± S.D. of three experiments. *$P < 0.005$, **$P < 0.001$

In our experiments, small unilamellar liposomes (SUV, mean size about 200 nm) with and without PAC (PAC-liposome and plain-liposome) were labeled with NBD-PC, which served as a bilayersituated fluorescence marker. The cultured neonatal cardiomyocytes were incubated with liposomes under experimental conditions, and then the cell-associated fluorescence was measured. When the cultured cardiomyocytes were incubated with PAC-liposomes and plain-liposomes for various time periods, a high affinity of the PAC-liposomes to the cardiomyocytes was observed (Fig. 1). The significant difference was observed from 0.25 h ($P < 0.001$), and throughout the following hours.

The levels of β_1-adrenoreceptors (1-AR) but not β_2-adrenoreceptors (2-AR) on cardiomyocyte membranes will significantly increase when hypoxia occurs (Bae et al. 2003). PAC-liposomes and plain-liposomes were added to the cultured cardiomyocytes, and incubated at 37 °C for 2 h under normoxia or hypoxia conditions. The amount of cell uptake of PAC-liposomes under normoxia conditions was 4-fold higher than that of plain-liposomes, and the increase was 8.5-fold under hypoxia conditions (Fig. 2).

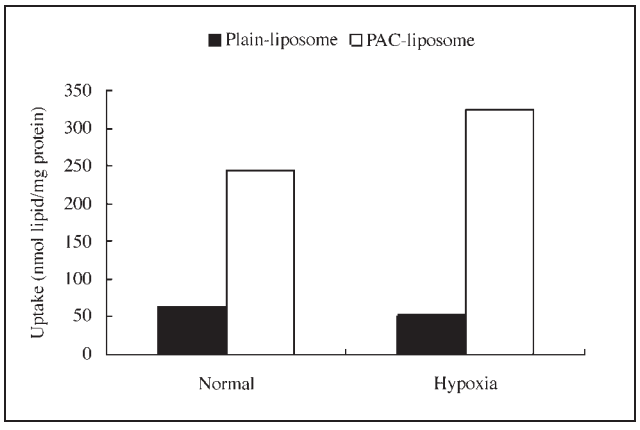

Fig. 2: Uptake of plain-liposomes and PAC-liposomes by normoxia or hypoxia cardiomyocytes
Plain-liposomes (SPC/Chol 2 : 1 molar ratio) and PAC-liposomes (SPC/Chol/PAC 2 : 1 : 0.2 molar ratio) were labeled with NBD-PC. In one group cardiomyocytes were incubated under normoxia conditions with liposomes at a dose of 2000 nmol/dish for 2 h, while cardiomyocytes were incubated under hypoxia conditions in the other group. Then the cell-associated fluorescence was measured. The amount of liposome uptake is expressed as the nmol of lipid per mg of cell protein. Each value represents the mean ± S.D. of three experiments

In conclusion, surface modification with PAC could significantly increase the delivery of liposomes into cardiomyocytes especially when hypoxia occurs. We could presume that PAC had the abilities of recognizing and blinding the β_1-adrenoreceptor on the cardiomyocyte membrane. The result indicate that PAC is a potential surface modification ligand for liposome targeting to an ischemic myocardium.

Experimental

1. Preparation of liposomes

Plain-liposome composed of SPC/Chol (2 : 1 molar ratio) and PAC-liposome composed of SPC/Chol/PAC (2 : 1 : 0.2 molar ratio) were prepared by the reverse-phase evaporation method as previously described (Szoka and Papahadjopoulos 1981). NBD-PC was incorporated into the lipids with 1 mol%, which served as a bilayersituated fluorescence marker. In brief, lipids and NBD-PC were dissolved in ether/chloroform (1 : 1 v/v) and then 150 mmol/L NaCL solution was added into the lipid solution. A water in oil (w/o) emulsion was obtained with 5 min sonication, using the bath-type sonicator. The solvent was dried in vacuum in a round-bottomed flask, and then the emulsion changed into the gel form. Phase inversion occurred after the organic solvents were completely removed from the gel, liposomes were then obtained. To homogenize size distribution, the liposomes were extruded under high pressure a nitrogen through a sterile polycarbonate membrane (Nucleopore) with a final pore size of 0.2 μm subsequently. All of the NBD-PC added was entrapped in liposomes as determined by the method of gel filtration.

2. Preparation of cardiomyocyte cultures

Cultured rat neonatal cardiomyocytes were prepared as described previously (Tanaka et al. 1994; Patten et al. 1996; Yonemochi et al. 1998). Briefly, ventricles were obtained from 1–2 day old Wister rats, and cardiomyocytes were isolated by digestion with 0.12% collagenase II. Cardiomyocytes were separated from nonmyocytes by 90 min differential plating. The estimated purity of cardiomyocytes after differential plating was 90% to 95%. The viability of the cardiomyocytes was tested using the method of Trypan Blue exclusion, and more than 95% of the isolated cardiomyocytes were viable. IMDM medium (pH 7.2) containing 10% fetal bovine serum (FBS) was supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin. The purified cardiomyocyte suspension in the above mentioned medium was prepared at a concentration of 5×10^5 cells/mL and plated in laminin-coated 35-mm plastic culture dish at 2 mL per dish. The cardiomyocytes were incubated under the conditions of 37 °C, humidified atmosphere of 95% air-5% CO_2. The 72–84 h cultured cardiomyocytes were used in the following experiments. At the time of use, the cells were single cells, small groups, or interconnected net works and strands. Cells selected for the study had spontaneous contraction frequencies of approximately 80–120 beats/min. When hypoxia was needed, cardiomyocytes were incubated at 37 °C, and under a humidified atmosphere of 95% N_2-5% CO_2.

3. Determination of liposome uptake by cardiomyocytes

For liposome uptake determination, liposomes were diluted with IMDM (without FBS) to 1000 nmol lipid/mL and added into the cultured cardiomyocytes 2 mL/dish. Unless otherwise indicated the cardiomyocytes were incubated with liposomes at 37 °C, and under a humidified atmosphere of 95% air-5% CO_2. The non-associated liposomes in solution were washed away at the end of incubation by rinsing three times with 2 mL ice-cold PBS. The washed cells in a dish were solubilized with 2 mL PBS containing 1% triton X-100. The fluorescence intensity of the cellular lysate at 460 nm excitation and 534 nm emission was measured using a fluorescence spectrophotometer (Hitachi, 650–60). The protein concentration of the cells was determined by BCA protein assay. The association of liposomes by cells was expressed as nmol of liposomal lipid per mg of cell protein. Liposomal lipid was represented by the concentration of SPC.

4. Statistics

Our experiments were done in triplicate, and the maximum deviation from the average value was 15% within each experiment. Results were expressed as mean ± S.D., the significance of the difference obtained was evaluated by Student's-*t* test.

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