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In vivo antitumor effect of cromolyn in PEGylated liposomes for pancreatic cancer

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ABSTRACT

A PEGylated liposomal formulation of cromolyn, composed of dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC) and 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine-N-[methoxy(polyethyleneglycol)-2000] (DSPE-mPEG2000), has been developed with the purpose of improving the antitumor activity of cromolyn for human pancreatic adenocarcinoma. In stability study, the amount of proteins adsorbed onto the PEGylated liposomes encapsulating cromolyn was 4.5-fold lower than the non-PEGylated liposome. *In vitro* study showed that the cromolyn in PEGylated liposome exhibited better anti-proliferative effect in BxPC-3 cells than in Panc-1 cells, which indicates higher level of endogenous S100P protein in BxPC-3 cells than in Panc-1 cells as a target protein for this drug. Moreover, the combination of cromolyn with gemcitabine in PEGylated liposomes demonstrated the strongest cytotoxicity to BxPC-3 pancreatic cancer cells *in vitro* and the highest anti-tumor activity against the BxPC-3 tumor bearing nude mice *in vivo*. Thus, this PEGylated liposomal formulation of cromolyn is expected to provide a novel approach to the treatment of pancreatic cancer in the future.

1. Introduction

Pancreatic cancer is the fourth most common cause of adult cancer death, accounting for an estimated 42,470 new cases and 35,240 deaths in USA in 2009. The high mortality rate is due to the high incidence of metastatic disease at initial diagnosis, the aggressive clinical course and the lack of adequate systemic therapies. Therefore, the overall 5-year survival rate is no more than 5% [1–3]. Radical surgery is still the only procedure that can completely eradicate this disease, and gemcitabine has been a global reference regimen for pancreatic cancer because of its favorable toxicity profile and modest ability to palliate typical pancreatic cancer symptoms. However, surgery and/or gemcitabine do not prolong the survival rate remarkably, so other drugs have been given in combination with gemcitabine such as 5-fluorouracil, oxaplatin, capecitabine and erlotinib [4,5]. Since the therapeutic efficacy of these gemcitabine combinations used in clinics is not enough to dramatically increase the therapeutic efficacy of pancreatic cancer, new targeting strategies and therapeutic approaches are needed to improve the survival rate and quality of life for pancreatic cancer patients.

S100P is a member of the S100 family of EF hand Ca^{2+} binding proteins and have been implicated in the regulation of a variety of intracellular and extracellular processes, including cell cycle, cell growth, differentiation and metabolism [6]. Overexpression of S100P has been observed in various cancers such as breast cancer, lung cancer, colon cancer and pancreatic cancer, with the extent of the overexpression being positively correlated with the degree of malignancy. Furthermore, it is recently reported that S100P is able to bind to the receptor for advanced glycation end-products (RAGE) which is associated with many inflammation-related pathological states such as vascular disease, cancer, neurodegeneration and diabetes [7]. Activation of RAGE is known to stimulate extracellular signal-regulated kinase (ErK) and nuclear factor-κB (NF-κB) activity and to increase the cell invasion and growth in pancreatic cancer cells [8].

Originally, cromolyn was developed as an anti-inflammatory drug used for prophylactic treatment of bronchial asthma and allergic rhinitis and it is currently administered as an intranasal solution, powder or inhalation [9,10]. Regarding the mechanism and involvement of S100 proteins in pancreatic cancer, cromolyn has previously been shown to bind specifically to other members of the S100 protein family (S100A1, S100A12, S100A13) [11,12]. In addition, cromolyn has recently been found that it binds S100P, blocks the interaction with RAGE, and inhibits tumor growth [13], which provides useful information on the use of cromolyn for cancer therapy. However, the way to deliver cromolyn into the body is very limited due to its physico-chemical properties.

It is well known that liposomes made from naturally occurring phospholipids are biocompatible carriers, and their application to drug delivery systems is known to reduce the drug toxicity and increase the therapeutic efficacy [14]. Although rapid clearance of the conventional liposomes by the reticular endothelial system

Abbreviations: cro, cromolyn; gem, gemcitabine; lipo, liposome; PEG-lipo-cro, PEGylated liposomal cromolyn; PBS, phosphate buffered saline; MTT, 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotide transferase (TdT)-mediated dUTP nick-end labeling.

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(RES) is recognized as one of the major drawbacks in anticancer drug delivery, this can be overcome by modifying the liposomes with flexible hydrophilic polymers such as polyethylene glycol (PEG) [15–17]. Therefore, PEGylated liposome as a drug delivery carrier for cromolyn is expected to enhance the anticancer effect and *in vivo* stability of cromolyn after systemic administration into the body.

In this study, we report on the development of a novel formulation of cromolyn using sterically stabilized PEGylated liposomes. The PEGylated liposomal formulation is evaluated for its particle size, zeta potential, encapsulation efficiency, SEM and biological stability. We also examined the effect of PEGylated liposomal cromolyn in combination with gemcitabine on the cell proliferation, long-term cell viability, NF-κB activity in pancreatic cancer cell and the *in vivo* antitumor activity in xenograft nude mice.

2. Materials and Methods

2.1. Materials

Dipalmitoylphosphatidylcholine (DPPC), dimyristoylphosphatidylcholine (DMPC), distearoylphosphatidylcholine (DSPC), cromolyn sodium salt (disodium 1,3-bis [(2'-carboxylatochromon-5'-yl)oxy]-2-hydroxy-propane), 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), chloroform ($CHCl_3$), trypan blue, crystal violet and Sephadex® G-75 were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-$mPEG_{2000}$) was purchased from the Avanti Polar Lipids (Alabaster, AL). Gemcitabine HCl, commercially known as Gemzar®, was purchased from Shinwon Pharmacy Co. (Seoul, Korea). All reagents and solvents were of analytical grade or better.

2.2. Preparation of PEGylated liposomes

PEGylated liposomes, composed of DPPC : DMPC : DSPC : DSPE-$mPEG_{2000}$ (4:1:1:0.1 ~ 1.0 molar ratio), were prepared using a reverse-phase evaporation vesicle (REV) method. Briefly, DPPC, DMPC, DSPC and DSPE-$mPEG_{2000}$ were dissolved in 1 mL of chloroform. After evaporation of solvent at room temperature, the formed lipid film was suspended in 1 mL of freshly hydrated diethyl ether, to which cromolyn in 1 mL of phosphate buffered saline (PBS, pH 7.4) was added. The mixture was sonicated using a bath type sonicator (Laboratory Supplies Co., Inc., NY) for 3 min until the mixture became a homogeneous dispersion and the organic solvent was then removed by a rotary evaporator (Laborota 4000, Heidolph, Italy) at 41 °C. Liposomes were downsized by extrusion through 0.45 μm and 0.2 μm polycarbonate membrane filters 10 to 20 times using a Lipex™ extrusion device (Avestin Inc. Toronto, Canada). The unencapsulated drug was removed from the liposome suspension by gel chromatography on a 1 × 12 cm Sephadex® G-75 column eluted with PBS.

2.3. Characterization of liposomes

The encapsulation efficiency of cromolyn in liposomes was evaluated by the Bligh and Dyer extraction method. Briefly, 100 μL of liposomes was mixed with 150 μL of PBS, 250 μL of methanol and 1 mL of chloroform in a glass tube to form a homogenous solution. After centrifugation, the mixture was separated into two clean layers. The bottom layer contained phospholipids and other hydrophobic materials, and the top layer contained cromolyn and other hydrophilic materials. The organic phase (bottom layer) was removed and chloroform was added again. The mixture was centrifuged, and these procedures were repeated three times. The amount of cromolyn in the supernatant was determined spectrophotometrically at 326 nm using an UltraSpec 2000 UV–VIS spectrophotometer (Pharmacia Biotech, Cambridge, UK).

The size distribution and zeta potential of liposomes were determined by a dynamic laser-light scattering system (NICOMP 380ZLS, Inc., Santa Barbara, CA) using He-Ne laser light source. Measurements were carried out at room temperature with a 90 degree detection angle.

2.4. Stability assay

The *in vitro* stability of liposome in the presence of serum proteins was determined by measuring the amount of proteins adsorbed onto the liposomes. One percent (w/v) of bovine serum albumin was dissolved in PBS (pH 7.4) and mixed with liposomal solution of each formulation at 37 °C. After incubation for 0.5, 3, 12, 24 and 48 h, samples were centrifuged at 13000 × *g* for 15 min. The free serum albumin in the supernatant was removed and PBS was added, and these procedures were repeated three times. The amount of proteins adsorbed onto the liposomes was measured using a BCA Protein Assay Kit (Pierce, Rockford, IL) with bovine serum albumin as a standard. Twenty-five μL of samples were put into 96 well plates and mixed with 200 μL of BCA working reagent. The absorbance was measured spectrophotometrically at 570 nm using 1420 Multilabel Counter (Victor3, PerkinElmer Life and Analytic Sciences, USA).

2.5. MTT assay

The viability of Panc-1 and BxPC-3 cells treated with cromolyn was assessed by MTT assay. Briefly, Panc-1 (5×10^3/well) and BxPC-3 cells (6×10^3/well) were seeded in 96-well plates and incubated overnight at 37 °C. Culture medium was removed and replaced with fresh medium containing PBS, empty liposome, 300 μM free cromolyn (cro), 300 μM PEGylated liposomal cromolyn (PEG-lipo-cro), 30 μM PEGylated liposomal gemcitabine (PEG-lipo-gem), or a combination of 300 μM PEG-lipo-cro plus 30 μM PEG-lipo-gem. After 48 h incubation at 37 °C, 10 μL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 4 h. The supernatant was removed, and then the formed formazan salts were dissolved by 100 μL of DMSO in shaking plates for 30 min. Absorbance was measured at 570 nm using an ELISA reader (EL 800, BIO-TEK, USA). The percent growth (% Growth) was calculated according to the following equation:

$$\%\text{Growth} = OD_{570}(\text{sample})/OD_{570}(\text{control}) \times 100$$

2.6. In vivo animal study

Five-week-old female BALB/c-nu nude mice (from Japan SLC, Inc., Japan) were maintained in appropriately isolated cages with free access to drinking tap water and food on a daily 12-hour light/dark cycle. The BxPC-3 cells, which had been grown in RPMI 1640 with 10% FBS, were collected and resuspended in PBS. A total of 1.5×10^7 cells in 70 μL of PBS mixed with 50 μL of Matrigel Matrix (BD Bioscience, Bedford, USA) were inoculated subcutaneously on the right flank of a nude mouse and the tumors were allowed to grow (Day 0).

One week after cell injection when the tumor volume has reached about 140 mm^3 (Day 7), the mice were randomly divided into 6 groups, each group having 6 mice (n = 6) except for the control group (n = 4). Each mouse was treated with PBS (control), empty liposome, cromolyn (10 mg/kg), non-PEGylated liposomal cromolyn (10 mg/kg), PEG-lipo-cro (10 mg/kg), PEG-lipo-gem (80 mg/kg) or the combination of PEG-lipo-cro (10 mg/kg) and PEG-lipo-gem (80 mg/kg). Six different formulations were injected into the mice intravenously twice a week for 4 weeks, a total of 8 injections. The tumor size was measured twice a week with Vernier caliper (Mitutoyo Co., Japan) across its two perpendicular diameters, and the volume was estimated using the following equation:

$$\text{Tumor Volume} = 0.5 \times \left(W^2 \times L\right)$$