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Epidermal growth factor receptor-targeted immunoliposomes for delivery of celecoxib to cancer cells

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ABSTRACT

Cyclooxygenase-2 (COX-2) is highly expressed in many different cancers. Therefore, the inhibition of the COX-2 pathway by a selective COX-2 inhibitor, celecoxib (CLX), may be an alternative strategy for cancer prevention and therapy. Liposomal drug delivery systems can be used to increase the therapeutic efficacy of CLX while minimizing its side effects. Previous studies have reported the encapsulation of CLX within the non-targeted long circulating liposomes and functional effect of these formulations against colorectal cancer cell lines. However, the selectivity and internalization of CLX-loaded liposomes can further be improved by grafting targeting ligands on their surface. Cetuximab (anti-epidermal growth factor receptor – EGFR – monoclonal antibody) is a promising targeting ligand since EGFR is highly expressed in a wide range of solid tumors. The aim of this study was to develop EGFR-targeted immunoliposomes for enhancing the delivery of CLX to cancer cells and to evaluate the functional effects of these liposomes in cancer cell lines. EGFR-targeted ILs, having an average size of 120 nm, could encapsulate 40% of the CLX, while providing a sustained drug release profile. Cell association studies have also shown that the immunoliposome uptake was higher in EGFR-overexpressing cells compared to the non-targeted liposomes. In addition, the CLX-loaded-anti-EGFR immunoliposomes were significantly more toxic compared to the non-targeted ones in cancer cells with EGFR-overexpression but not in the cells with low EGFR expression, regardless of their COX-2 expression status. Thus, selective targeting of CLX with anti-EGFR immunoliposomes appears to be a promising strategy for therapy of tumors that overexpress EGFR.

1. Introduction

Deregulation in the epidermal growth factor receptor (EGFR) and cyclooxygenase-2 (COX-2) signaling pathways are frequently seen in different cancer types (Bocca et al., 2014; Chong and Jänne, 2013; Hynes and Lane, 2005; Vecchione et al., 2011). Interestingly, a crosstalk between COX-2 and EGFR signaling pathways has been shown, and an increase in COX-2 expression can lead to an increase in EGFR expression and vice versa (Shin et al., 2013; Li et al., 2011). Therefore, targeting these two complementary pathways may be an alternative strategy for cancer therapy (Wang et al., 2011). Cetuximab (chimeric, anti-EGFR monoclonal antibody) is capable of binding to EGFR and EGFRvIII, preventing the ligand-dependent activation of downstream signaling pathways and promoting the internalization and degradation of the receptor (Patel et al., 2007). On the other hand, celecoxib (CLX), a selective COX-2 inhibitor, has been well exploited for its anticarcinogenic activity that may be COX-2 dependent or independent (Grösch et al., 2006; Jendrossek, 2013; Kashiwagi et al., 2014). Among several approaches of combining CLX and cetuximab for cancer therapy, we have examined the therapeutic effect of CLX specifically on cancer cells via a drug delivery system with cetuximab as the targeting ligand.

Liposomes were chosen as a suitable delivery system for CLX due to the lipophilic nature of this drug. Our group has previously shown that high CLX encapsulation can be successfully achieved in different formulations of multilamellar vesicles (MLVs) and large unilamellar vesicles (LUVs) with a sustained release profile (Deniz et al., 2010; Erdoğ et al., 2013). In addition, non-targeted long circulating liposomes containing CLX were shown to have a functional effect against colorectal cancer cell lines *in vitro* (Erdoğ et al., 2013). As a targeting ligand, cetuximab can be grafted on the

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surface of liposomes to prepare EGFR-targeted immunoliposomes (ILs). Such targeted drug delivery systems can improve the therapeutic efficacy of drugs through increased internalization and intracellular delivery of the drugs selectively to the target cells (Jain, 2010; Mamot et al., 2006; Park et al., 2002; Senzer et al., 2013).

Cetuximab represents a promising candidate as a targeting ligand for liposomes since EGFR is highly expressed in a wide range of tumors. For example, an EGFR-targeted IL for delivery of doxorubicin was shown to have clinical activity in a phase 1 dose-escalation study (Mamot et al., 2012b). These liposomes have progressed to a phase 2 clinical study.

To the best of our knowledge, there are no reported publications of CLX-loaded EGFR-targeted ILs for cancer therapy. In the present study, we aimed to develop EGFR-targeted ILs for enhancing the delivery of CLX and to evaluate its functional effects in cancer cell lines with varying expressions of EGFR and COX-2. EGFR-targeted ILs, having a size of about 120 nm, showed an encapsulation efficiency of 40% and a sustained release of CLX. Cell association studies have shown that the IL uptake was higher than that of the non-targeted LUVs in EGFR-overexpressing cells. In addition, the CLX-loaded-EGFR targeted ILs were significantly more toxic compared to the non-targeted ones in cancer cells with EGFR-overexpression but not in the cells with low EGFR expression, independent of their COX-2 expression status. Thus, selective targeted delivery of liposomal CLX with antibodies provides a promising strategy for different cancer types with targetable surface modifications.

2. Materials and methods

2.1. Materials

Celecoxib was purchased from Santa Cruz Biotechnology (Texas, USA). Phosphatidylcholine (1,2-distearoyl-sn-glycero-3-phosphocholine, 18:0, DSPC), cholesterol (ovine wool, >98%), 18:0 mPEG (2000)-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-(methoxy(polyethylene glycol)-2000) and DSPE–PEG(2000) maleimide, mini-extruder set, filter supports and nucleopore track-etch polycarbonate (PC) membranes (100 nm) were purchased from Avanti Polar Lipids (Alabaster, AL; USA). SP-DiOC18(3) was obtained from Invitrogen (Carlsbaad, CA). Lissamine™ Rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (Rhodamine DHPE or Rh-PE) was purchased from Invitrogen (Carlsbaad, CA, USA). SlowFade® gold antifade reagent was purchased from Invitrogen (Oregon, USA). Cysteamine hydrochloride (2-MEA) was purchased from Applichem (Darmstadt, Germany). Mouse IgG (free of azide and BSA, lyophilized) was obtained from Sigma–Aldrich (St. Louis, MO, USA). Anti-EGFR monoclonal antibody (chimeric mouse–human IgG1 clone C225; free of azide and BSA) was either purchased from Thermo Scientific, Pierce Protein Research Products (Rockford, IL, USA) or LifeSpan BioSciences, Inc. (Seattle, WA, USA). MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Invitrogen (Carlsbaad, CA). Zeba™ spin desalting columns were obtained from Thermo Scientific, Pierce Protein Research Products (Rockford, IL, USA). Ultra filtration device (VivaSpin2) with MWCO 300 kDa membrane was purchased from Sartorius (Goettingen, Germany). Puradisc™ sterile 0.2 μm polyethersulfone (PES) filters were purchased from Whatman Inc. (NJ, USA). Chloroform and methanol were obtained from Merck (Munich, Germany). Human colon cancer cell line HCT-116 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ, Germany), SW620 was purchased from American Type Culture Collection (ATCC, USA) and HT-29 was purchased from ŞAP Enstitüsü (Ankara, Turkey). The human breast cancer cell line MDA-MB-468 was kindly provided by Dr. Elif Erson-Bensan from METU, Department of Biological Sciences. Cell culture media and supplements were obtained from Biochrom (Berlin, Germany). Cell culture grade plastic-ware was obtained from Greiner Bio-One GmbH (Goettingen, Germany).

2.2. Preparation of liposomes

2.2.1. Preparation of LUVs

Liposomes were prepared according to thin lipid film hydration method (Bangham et al., 1965). The liposomes were composed of DSPC, cholesterol, mPEG(2000)–DSPE (molar ratio 100:10:4) and 7 mol% of celecoxib (CLX) relative to the total mole number of liposome components. All of the components were dissolved in chloroform to prepare stock solutions and mixed according to the molar ratio stated above. Empty liposomes were prepared without adding CLX. A stream of argon was used to evaporate chloroform to form a thin lipid film and the residual chloroform was removed by vacuum in a HETO spin-vac system (HETO, Allerod, Denmark). Lipid films were hydrated with 1 mL PBS (0.1 M, pH 7.4) while heating to a temperature above the melting temperature of DSPC (approximately 56 °C) and vortexing to form MLVs. The MLVs were then extruded 15 times through 100 nm PC membranes (Avanti Polar Lipids) at 75 °C using a mini-extruder set (Avanti Polar Lipids, USA) to form LUVs. When necessary, the resulting LUVs were purified from un-encapsulated CLX using an ultra-filtration device (VivaSpin2) with MWCO (molecular weight cut off) 300 kDa membrane (Sartorius, Germany) following the manufacturer's instructions. For cell culture studies, liposomes were diluted with cell culture medium and filter sterilized through sterile 0.2 μm polyethersulfone (PES) filters (Whatman Puradisc). The final liposome suspensions were stored at 4 °C for further experiments.

For confocal microscopy and flow cytometry, 0.5 mol% of the rhodamine (Rh) labeled lipid Rh-PE and 0.3 mol% of SP-DiOC18(3) relative to the total phospholipids were incorporated into the liposome formulation, respectively. All procedures involving fluorescence-labeled dyes were carried out in the dark.

2.2.2. Immunoliposome preparation

An isoform-specific mouse IgG was used to optimize the preparation of ILs, their characterization and release of CLX. The monoclonal anti-EGFR mouse IgG (clone C225) was used for confocal microscopy, flow cytometry, and cell cultures studies. For the preparation of ILs, mPEG(2000)–DSPE and DSPE–PEG(2000) maleimide were included in the lipid film as 3 mol% and 1 mol% of total phospholipids, respectively, in order to form maleimide functionalized LUVs. The lipid film was hydrated with HBS (HEPES buffered saline: 20 mM HEPES, 140 mM NaCl, pH 7.4) and the subsequent steps to prepare the maleimide functionalized LUVs were as described above.

ILs were prepared by conjugating a half-IgG to the preformed maleimide functionalized LUVs. Mouse IgG or monoclonal anti-EGFR mouse IgG (clone C225) was reduced with the mild reducing agent 2-MEA (75 mM) for 90 min at 37 °C in the presence of HBS/EDTA (10 mM EDTA) in order to form half IgG with a free sulfhydryl group. The reducing agents from the reduced IgG mixture were removed with a Zeba Desalting column (Thermo Scientific) with MWCO of 7 kDa following the manufacturer's instructions. The reduction of the antibody to half IgG was confirmed by SDS–PAGE.

The conjugation of the antibody fragments to the liposomes was carried out by overnight incubation of the purified reduced IgG fragments (200 μg/mL) with 10 mM pre-formed maleimide functionalized LUVs at 4 °C. At the end of the conjugation reaction, the excess maleimide was quenched with 2 mM β-mercaptoethanol for 30 min. The un-conjugated antibodies, un-encapsulated CLX and other un-incorporated components of