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Potentiation of pH-sensitive polymer-modified liposomes with cationic lipid inclusion as antigen delivery carriers for cancer immunotherapy

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ABSTRACT

Cationic lipid-incorporated liposomes modified with pH-sensitive polymers were prepared by introducing 3, 5-didodecyloxybenzamidine as a cationic lipid to egg yolk phosphatidylcholine liposomes modified with 3-methylglutarylated hyperbranched poly(glycidol) (MGlu-HPG) as a pH-sensitive polymer. These liposomes were stable at neutral pH, but were destabilized below pH 6.0 because MGlu-HPG changed its characteristics from hydrophilic to hydrophobic in response to the pH decrease. Cationic lipid inclusion improved their pH sensitivity at weakly acidic pH and association of liposomes with murine dendritic cell (DC) lines. Cationic lipid-incorporated liposomes delivered entrapped ovalbumin (OVA) molecules not only to cytosol but also to endosome/lysosome. Treatment with cationic lipid-incorporated liposomes induced up-regulation of antigen presentation-involved molecules on DCs, the promotion of cytokine production, and antigen presentation *via* both major histocompatibility complex (MHC) class I and II molecules. Especially, antigen presentation *via* MHC class II was promoted by cationic lipid inclusion, which might correspond to efficient endosome/lysosome delivery of OVA. Subcutaneous administration of OVA-loaded cationic lipid-incorporated liposomes induced antigen-specific antibody production in serum and Th1-dominant immune responses in the spleen. Furthermore, administration of the cationic lipid-incorporated liposomes to mice bearing E.G7-OVA tumor more significantly reduced the tumor volume than liposomes without cationic lipids. Therefore, cationic lipid inclusion into pH-sensitive polymer-modified liposomes, which can achieve both efficient antigen intracellular delivery and activation of antigen presenting cell, is an effective approach to develop antigen carriers for efficient cancer immunotherapy.

1. Introduction

Cancer immunotherapy, which activates cancer-specific immune responses for the treatment, has received much attention [1–5]. Efficient delivery of cancer-specific antigens into antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages is crucial to activate such immune responses [1,4,5]. Actually, through the presentation of antigen, DCs can activate antigen-specific immunities of two types: humoral immunity and cellular immunity [6,7]. When exogenous antigenic proteins are taken up by DCs *via* endocytosis, these molecules are degraded to peptide fragments in lysosome. In addition, the antigen-derived peptides are presented on major histocompatibility complex (MHC) class II molecules to the CD4+ T cells. Then, CD4+ T cells differentiate to subsets of helper T cells such as Th1 cells and Th2 cells, which activate cytotoxic T lymphocytes (CTLs) or B cells, respectively, *via* secretion of various cytokines. In contrast, endogenous antigenic proteins existing in cytosol of DCs are degraded by proteasomes. Their derived peptide fragments are presented on MHC class I molecules to the CD8+ T cells, which then differentiate to antigen-specific CTLs. The induction of the tumor-specific CTLs is generally regarded as important to achieve efficient cancer immunotherapy because these CTLs attack the target cells directly and eliminate them effectively. Therefore, using antigen delivery systems that generate antigen-specific CTLs and Th1 cells is important for the induction of effective cellular immunity with high tumor-specificity.

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To date, many antigen delivery systems have been produced using polymer-based or membrane-based nanoparticles for induction of cellular immunity [8–15]. Some membrane-based nanoparticles such as liposomes with membrane-active properties are regarded as especially good candidates for this purpose because such systems can introduce antigenic proteins into cytosol of DCs by destabilizing or fusing with cellular or endosomal membranes and thereby inducing the antigen-derived peptide presentation on MHC class I molecules. To date, liposomes modified with fusion-active or membrane-lytic molecules such as viral proteins and their derived peptides and pH-sensitive polymers have been studied for antigen delivery into DCs for induction of cellular immunity [11–15]. From a safety perspective, to avoid unexpected biological effects including immune responses derived from viral molecules, synthetic molecules might be more desirable than virus-derived molecules for the construction of liposomes as an antigen delivery vehicle. Although synthetic molecules of various types have been used to provide membrane disruptive or fusion abilities to liposomes [16–22], synthetic polymers might be chosen for this purpose because of the high efficiency attributable to their large molecular size and the wide freedom they provide for the design of molecular architectures. Especially, pH-sensitive fusogenic polymers, which generate a strong fusion capability under weakly acidic conditions, are beneficial because these polymers generate fusion activity only under weakly acidic environments, such as that inside an endosome. Therefore, when taken up by APCs *via* endocytosis, liposomes modified by these polymers can introduce entrapped antigens into their cytosol by fusion with endosomal membrane.

In a previous study, we used egg yolk phosphatidylcholine (EYPC) liposomes modified with 3-methylglutarylated hyperbranched poly(glycidol) (MGlu-HPG), which exhibit highly fusogenic and membrane disruptive abilities under weakly acidic conditions, for the delivery of antigenic protein ovalbumin (OVA) into cytosol of DCs. Results showed that the MGlu-HPG-modified liposomes delivered OVA efficiently into cytosol of DCs [15,21,22] and that they induced antigen presentation *via* MHC class I molecules, which causes induction of antigen-specific cellular immunity. In addition, subcutaneous administration of the MGlu-HPG-modified, OVA-loaded liposomes into mice bearing OVA-expressing tumors induced the shrinkage of tumors through the OVA-specific immunity.

For T cell activation, DCs must express co-stimulatory molecules such as CD80 and CD86 molecules on their surface, which stimulates T cells *via* interaction with CD28, together with the antigen peptide presentation on MHC molecules [23–25]. The expression of these co-stimulatory molecules on DCs is enhanced when DCs are activated [26,27]. Therefore, antigen delivery systems should have capabilities not only to deliver antigen into DCs but also to activate DCs, which is known as an adjuvant function, for the induction of effective antigen-specific immunity. Molecules and materials of many types have adjuvant functions: monophosphoryl lipid A (MPLA), CpG-DNA, β-glucan, and so on [27–32]. Among them, cationic lipids might be potent adjuvants because liposomes containing cationic lipids, such as 1,2-dioleoyl-3-(trimethyl ammonium) propane (DOTAP), have been shown to activate APCs and to induce antigen-specific immune responses effectively [32–34].

Toward development of potent antigen delivery systems that achieve induction of antigen-specific immunity, we designed MGlu-HPG-modified liposomes containing cationic lipids as a membrane component, which introduce antigen molecules into cytosol of DCs and activate them simultaneously (Fig. 1). For this study, we used 3,5-didodecyloxybenzamidine (TRX) as a cationic lipid because TRX is stably incorporated in phospholipid-based membranes and because its pDNA complexes induced up-regulation of MHC class I molecules when taken up by DCs [35]. Here, we examine the capabilities of MGlu-HPG-modified, TRX-containing liposomes to deliver antigenic proteins into DCs, to activate DCs, and to induce antigen-specific immune response. In addition, we investigate the importance of synergistic effect of MGlu-HPG-induced antigen delivery into DCs and TRX-induced DC activation for the induction of antigen-specific immunity.

2. Materials and methods

2.1. Materials

EYPC was kindly donated by NOF Co. (Tokyo, Japan). 3,5-Didodecyloxybenzamidine hydrochloride (TRX) were kindly donated by Terumo Corp., Ltd. (Kanagawa, Japan). Lissamine rhodamine B-sulfonyl phosphatidylethanolamine (Rh–PE) was purchased from Avanti Polar Lipids (Birmingham, AL, USA). OVA, bovine serum albumin (BSA), Lipid A, monophosphoryl from *Salmonella enterica* serotype minnesota Re 595 (Re mutant) (MPLA), and fluorescein isothiocyanate (FITC) were purchased from Sigma (St. Louis, MO.). Pyranine, sodium azide and Triton X-100 were obtained from Tokyo Chemical Industries Ltd. (Tokyo, Japan). *p*-Xylene-bis-pyridinium bromide (DPX) was from Molecular Probes (Oregon, USA). Tween 20 was obtained from Nacalai Tesque, Inc., (Kyoto, Japan). FITC–OVA was prepared by reacting OVA (10 mg) with FITC (11.8 mg) in 0.5 M $NaHCO_3$ (4 mL, pH 9.0) at 4 °C for three days and subsequent dialysis [21]. 3-Methylglutarylated hyperbranched poly(glycidol) with polymerization degree of 60 (MGlu-HPG) was prepared as previously reported [21]. The ratios of hydroxyl units, MGlu units and decyl amide units for MGlu-HPG was 9/80/11, as estimated using ^{1}H NMR [21].

2.2. Cell culture

DC2.4 cells, which were an immature murine DC line, were provided from Dr. K. L. Rock (University of Massachusetts Medical School, USA) and were grown in RPMI-1640 (Nacalai Tesque) supplemented with 10% FBS (MP Biomedical, Inc.), 2 mM L-glutamine (Wako), 100 mM MEM nonessential amino acid (Nacalai Tesque), 50 μM 2-mercaptoethanol (2-ME, Gibco), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C [36]. EL4, a C57BL/6 mice-derived T lymphoma, was obtained from Tohoku University (Sendai, Japan). E.G7-OVA, which is a chicken egg OVA gene-transfected clone of EL4 and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) [37]. CD8–OVA1.3 cells, a T–T hybridoma against $OVA_{257-264}$/H–$2K^b$ complex, were kindly provided by Dr. C.V. Harding [38], and were grown in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, 50 μM 2-ME, 100 U/mL penicillin and 100 μg/mL streptomycin. OT4H.1D5 cells, a T–T hybridoma against $OVA_{265-277}$/I–A^b complex, were kindly provided by Dr. J.A. Kapp [39], and were cultured in RPMI-1640 medium supplemented with 10% FBS, 50 μM 2-ME, 100 U/mL penicillin and 100 μg/mL streptomycin.

2.3. Animals

Female C57BL/6 mice (H–2^b, 7 weeks old) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The experiments were carried out in accordance with the guidelines for animal experimentation in Osaka Prefecture University.

2.4. Preparation of liposomes

To a dry, thin membrane of EYPC and various mol% of TRX (total lipids; 1.25×10^{-5} mol) was added 500 μL of 35 mM pyranine, 50 mM DPX, and 25 mM phosphate solution (pH 7.4) and the mixture was sonicated for 2 min using a bath–type sonicator. The liposome suspension was further hydrated by freezing and thawing, and was extruded through a polycarbonate membrane with a pore size of 100 nm. The liposome suspension was centrifuged with the speed of 55,000 rpm for 2 h at 4 °C twice to remove free pyranine from the pyranine-loaded liposomes. MGlu-HPG-modified liposomes were also prepared according to the above procedure using dry membrane of a lipid mixture with MGlu-HPG (lipids/polymer = 7/3, w/w).

2.5. Dynamic light scattering and zeta potential

OVA-loaded liposomes were prepared as described above except that mixtures of polymers and lipids were dispersed in OVA-containing phosphate buffered saline (PBS) solution (pH 7.4, 4 mg/mL). Diameters and zeta potentials of the liposomes (0.1 mM lipids) in 0.1 mM phosphate aqueous solution were measured using a Zetasizer Nano ZS ZEN3600 (Malvern Instruments Ltd, Worcestershire, UK). Data present the number average diameters obtained as an average of more than three measurements on different samples.

2.6. Release of pyranine from liposome

Release of pyranine from liposome was measured as previously reported [20,40]. Liposomes encapsulating pyranine (lipid concentration: 2.0×10^{-5} M) were added to PBS of varying pHs at 37 °C and fluorescence intensity at 512 nm of the mixed