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Generation, characterization and *in vivo* biological activity of two distinct monoclonal anti-PEG IgMs

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ABSTRACT

PEGylation, the attachment of polyethylene glycol (PEG) to nanocarriers and proteins, is a widely accepted approach to improving the *in vivo* efficacy of the non-PEGylated products. However, both PEGylated liposomes and PEGylated proteins reportedly trigger the production of specific antibodies, mainly IgM, against the PEG moiety, which possibly leads to a reduction in safety and therapeutic efficacy of the PEGylated products. In the present study, two monoclonal anti-PEG IgMs — HIK-M09 *via* immunization with an intravenous injection of PEGylated liposomes (SLs) and HIK-M11 *via* immunization with a subcutaneous administration of PEGylated ovalbumin (PEG-OVA) were successfully generated. The generated IgMs showed efficient reactivity to $mPEG_{2000}$ conjugated to 1,2-distearoyl-sn-glycero-3-phospho-ethanolamine (DSPE), PEGylated liposome (SL) and PEG-OVA. It appears that HIK-M09 recognizes ethoxy (OCH_2CH_2) repeat units along with a terminal motif of PEG, while HIK-M11 recognizes only ethoxy repeat units of PEG. Such unique properties allow HIK-M09 to bind with dense PEG. In addition, their impact on the *in vivo* clearance of the PEGylated products was investigated. It was found that the generated ant-PEG IgMs induced a clearance of SL as they were intravenously administered with SL. Interestingly, the HIK-M11, generated by PEG-OVA, induced the clearance of both SL and PEG-OVA, while the HIK-M09, generated by SL, induced the clearance of SL only. We here revealed that the presence of serum anti-PEG IgM and the subsequent binding of anti-PEG IgM to the PEGylated products are not necessarily related to the enhanced clearance of the products. It appears that subsequent complement activation following anti-PEG IgM binding is the most important step in dictating the *in vivo* fate of PEGylated products. This study may have implications for the design, development and clinical application of PEGylated products and therapeutics.

Introduction

Polyethylene glycol (PEG) is widely used in the pharmaceutical industry to manipulate the pharmacokinetics of therapeutic and diagnostic agents (Allen and Cullis, 2013; Pasut and Veronese, 2012). The attachment of lipid-conjugated PEG (PEGylation) to biomedicines such as peptides and proteins increases their stability and efficacy by providing a protective and steric barrier against interactions with plasma proteins and the cells of the mononuclear phagocyte system (MPS) (Allen et al., 1991; Brinckerhoff et al., 1999; Caliceti and Veronese, 2003). In addition, PEGylation reduces the potential for the antigenicity and immunogenicity of attached products *via* prevention of the recognition by, and interaction with, host immune cells (Harris and Chess, 2003; Roberts et al., 2002).

In recent years, several PEGylated proteins have been approved for clinical use. These include Oncaspar (PEG-asparaginase) for acute lymphoblastic leukemia (Graham, 2003), Pegasys (PEG-interferon α-2a) for hepatitis C infection (Matthews and McCoy, 2004), Krystexxa (PEG-uricase) for chronic gout (Sherman et al., 2008) and Cimzia (PEG-anti-human TNF-α Fab′) for rheumatoid arthritis (Schreiber et al., 2007). These PEGylated products have shown an improved therapeutic efficacy compared with that of non-PEGylated counterparts. Accordingly, PEGylation has been considered a milestone breakthrough in the field of protein therapeutics.

PEGylation has also frequently been applied to nanoparticles in order to reduce non-selective uptake by normal tissues, to prolong blood circulation, and to increase tumor accumulation *via* enhanced permeability and retention (EPR) effect (Allen and Cullis, 2013). Doxil® is a perfect example of this phenomenon. It is a PEGylated

Abbreviations: ABC, accelerated blood clearance; BSA, bovine serum albumin; CHOL, cholesterol; CL, conventional liposome; EDTA, ethylene diaminetetra-acetic acid; ELISA, enzyme-linked immunosorbent assay; EPR, enhanced permeability and retention; FBS, fetal bovine serum; FDA, food and drug administration; HEPC, hydrogenated egg phosphatidylcholine; HRP, horseradish peroxidase; $mPEG_{2000}$-DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]; OVA, egg white albumin; PEG, polyethylene glycol; PL, phospholipid; SL, stealth liposome; ^{3}H-CHE, ^{3}H-cholesterylhexadecyl ether.

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liposomal formulation of doxorubicin that has been approved by the FDA for use in the clinical treatment of Kaposi's sarcoma and both ovarian and breast carcinomas (Chao et al., 2003; Hsiao et al., 2009). The substitution of Doxil® for the free drug results in a higher therapeutic effect because liposome encapsulation changes the pharmacokinetics and biodistribution of the drug, and leads to an enhancement of drug accumulation in solid tumors and a decreased accumulation in normal healthy tissues, particularly the heart (Uziely et al., 1995).

Despite the aforementioned merits of PEG in enhancing the therapeutic efficacy of PEGylated proteins and/or nanoparticles, PEG has been burdened by claims of immunogenicity. Several reports have emphasized the existence of naturally occurring anti-PEG antibodies in normal donors (Armstrong et al., 2007; Liu et al., 2011; Richter and Akerblom, 1983). Furthermore, Armstrong et al. (2007) have suggested that these naturally occurring anti-PEG antibodies could prime a host's immune response against treatment with PEGylated proteins, resulting in a reduced therapeutic effect. In addition to these reports that emphasize the immunogenicity of PEGylated proteins, other studies have demonstrated the immunogenicity of PEGylated nanocarriers based on the accelerated blood clearance (ABC) phenomenon that has been observed in animal models upon repeated administration of PEGylated nanoparticles and has been linked to the induction of anti-PEG antibodies (Ishida et al., 2006; Tagami et al., 2011; Wang et al., 2007). Consequently, the immunogenicity of PEG has potential implications for the development of PEGylated therapeutics and/or PEGylated drug delivery systems.

Nevertheless, although both PEGylated nanoparticles and PEGylated proteins could induce anti-PEG antibodies, it remains unclear whether they could induce the same type of anti-PEG antibodies. Roffler and colleagues (Cheng et al., 1999, 2005; Su et al., 2010) distinguished two types of anti-PEG antibodies, one recognizing the repeating (OCH_2CH_2) subunits and the other mainly recognizing other motifs of PEG such as a terminal CH_3O- motif of PEG along with some (OCH_2CH_2) units. AGP4 and 3.3 mouse monoclonal antibodies belong to the former type, and PEG-B-47 rabbit monoclonal antibody belongs to the latter type. In a similar manner, Saifer and coworkers reported that CH_3O-PEG-attached proteins induced both types of anti-PEG antibodies, while HO-PEG-attached proteins induced only the former type of anti-PEG antibodies in rabbits (Sherman et al., 2012).

Based on those results and the notion that no PEGylated nanoparticle-induced monoclonal anti-PEG antibodies have been reported, two monoclonal anti-PEG IgMs in the present study, HIK-M09 and HIK-M11, by the injection of PEGylated liposome (SL) and PEGylated ovalbumin (PEG-OVA), respectively, were generated. The issue of whether there is a significant difference in the reactivity toward the PEG moiety as well as in the PEGylated products was investigated. In addition, the *in vivo* biological activity of the generated monoclonal anti-PEG IgM on the biodistribution of PEGylated products was evaluated.

Materials and methods

Materials

Hydrogenated egg phosphatidylcholine (HEPC), methoxy (polyethylene glycol (PEG))-40000 maleimide (maleimide-activated PEG), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy (PEG)-2000] (CH_3O-PEG_{2000}-DSPE), HO-PEG_{2000}-DSPE, COOH-PEG_{2000}-DSPE, NH_2-PEG_{2000}-DSPE, and DSPE were generously donated by NOF (Tokyo, Japan). Cholesterol (CHOL) was purchased from Wako Pure Chemical (Osaka, Japan). ^{3}H-cholesterylhexadecyl ether (^{3}H-CHE) was purchased from Perkin Elmer Japan (Yokohama, Japan). All lipids were used without further purification. Egg white albumin (OVA) was purchased from SIGMA (MO, USA). All other reagents were of analytical grade.

Animals

Male BALB/c mice weighting 20–22 g were purchased from Japan SLC (Shizuoka, Japan). The animals had free access to water and mouse chow and were housed under controlled environmental conditions (constant temperature, humidity and a 12 h dark/light cycle). All animal experiments were evaluated and approved by the Animal and Ethics Review Committee of the University of Tokushima.

Preparation of liposomes

PEGylated liposome (SL), composed of HEPC:CHOL:CH_3O-PEG_{2000}-DSPE (1.85:1.0:0.15 molar ratio) and non-PEGylated liposome (conventional liposome, CL), composed of HEPC:CHOL:DSPE (1.85:1.0:0.15 molar ratio), were prepared as previously described (Ishida et al., 2006). The mean diameters were 112.0 ± 3.8 nm for SL and 108.7 ± 3.4 nm for CL, respectively, as determined using a NICOMP 370 HPL submicron particle analyzer (Particle Sizing System, CA, USA). The concentration of phospholipid was determined *via* a colorimetric assay (Bartlett, 1959). To follow the biodistribution of the tested SL, it was labeled with a trace amount of ^{3}H-CHE (40 μCi/μmol lipid) as a non-exchangeable lipid-phase marker.

Preparation of PEGylated protein

OVA was modified with 40 kDa maleimide-activated PEG, as reported previously (Sun et al., 2003; Veronese and Pasut, 2005). Briefly, OVA and maleimide-activated PEG were dissolved in 5 ml of 0.1 M sodium borate buffer (pH 9.2) at a molar ratio of 1:20 and stirred overnight at 4 °C. The reaction was stopped by the addition of 20 ml of 0.1 M potassium phosphate buffer (pH 5.5). To remove non-reacted maleimide-activated PEG, the reaction mixture was applied to cation exchange chromatography using a MacroCap XP (Amersham-Pharmacia Biotech, Upsala, Sweden). The fraction containing PEG-OVA was collected and concentrated using an Amicon Ultra centrifugal filter device (Millipore Corp, MA, USA). The concentration of PEG-OVA was determined by DC protein assay kit (BioRad Laboratories, CA, USA) using OVA as the standard.

Preparation of monoclonal anti-PEG IgM

Immunization with SL. Mice were intravenously injected with SL (0.005 μmol phospholipids/mouse) in HEPES buffered saline. To perform cell fusion, splenocytes were collected at day 3 after the SL injection.

Immunization with PEG-OVA. Mice were subcutaneously injected with PEG-OVA (20 μg/mouse) in complete Freund's adjuvant (Wako Pure Chemical, Osaka, Japan) and then sequentially immunized with a subcutaneous injection of PEG-OVA (20 μg/mouse) in incomplete Freund's adjuvant (Wako Pure Chemical) on days 14 and 28. On day 42, the mice were intramuscularly injected with free PEG-OVA (20 μg/mouse) in PBS. To perform cell fusion, splenocytes were collected at day 3 after the final immunization.

Development of hybridomas. The collected splenocytes were fused with the murine myeloma cell line, P3U1, using PEG-1500 (Roche, Mannheim, Germany). Hybrid cells were plated in microtiter plates and maintained in RPMI 1640 medium (Wako Pure Chemical) supplemented with 10% (v/v) heat-inactivated FBS (Japan Bioserum, Hiroshima, Japan), 100 IU/ml penicillin, 100 μg/ml streptomycin (ICN Biomedicals, CA, USA), and HAT supplements (0.1 mM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine) (Sigma-Aldrich, MO, USA) in a 5% CO_2 air incubator at 37 °C.