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Research paper

A noticeable phenomenon: Thiol terminal PEG enhances the immunogenicity of PEGylated emulsions injected intravenously or subcutaneously into rats

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ABSTRACT

Repeated intravenous injection of long-circulating methoxy-polyethylene glycol (PEG)-liposomes alters the pharmacokinetics and biodistribution of the second administration, regarded as the "accelerated blood clearance (ABC) phenomenon." Nevertheless, the effect of terminal groups of distearoylphosphatidylethanolamine-polyethylene glycol (DSPE-PEG) on the induction of the ABC phenomenon had not been reported previously. In this study, rats were injected intravenously or subcutaneously with PEG coated emulsions (DE) which were prepared using PEG terminated with either the methoxyl (OCH_3), hydroxyl (OH), amino (NH_2), carboxyl (COOH), or thiol (SH) group. DE-OCH_3 demonstrated the longest prolonged half-life in vivo after a single intravenous injection, followed by DE-SH and DE-COOH. In contrast, DE-OH was rapidly removed from the blood circulation, as was DE-NH_2. Moreover, we observed a strong positive relationship between the circulation time of initially injected PEGylated emulsions and the extent to which the ABC phenomenon was induced, but a exception of DE-SH increasing the ABC effect. Furthermore, the present study suggested that thiols might stimulate the proliferation and differentiation of B cells to induce the fastest clearance of the second intravenous administration by inducing the synthesis of the cell membrane and cytosolic proteins or reacting with follicular dendritic cells. The results strongly suggested that thiol groups played a stimulatory role in the immune response and provided a considerable implication for multiple drug therapy of thiol groups.

1. Introduction

Polyethylene glycol (PEG) is approved by the FDA and because of its non-toxicity [1] and little immunogenicity [2], the compound has been widely used as a vehicle or as a modified material in foods, cosmetics and pharmaceuticals, including injectable, topical, rectal and nasal formulations. The modification of colloidal particles with PEG, termed PEGylation [3], markedly reduces their recognition by the mononuclear phagocyte system (MPS) and produces prolonged circulation in the blood when administered intravenously [4]. Therefore, PEG is the mostly widely employed variety of nanoparticle capping and has been used on a range of systems including PLGA nanoparticles [5] and gold nanoparticles [6]. PEG has also been used in mixed monolayer capped nanoparticles with groups used as targeting peptides and drugs to produce fully functional delivery systems. Clearly, PEG has a bright future for the application of nanoparticle systems when given by intravenous or subcutaneous injections.

Nevertheless, recent reports have demonstrated that the intravenous injection of PEGylated liposomes significantly alters the pharmacokinetics and biodistribution of the second dose (after an interval of several days). For example, the prolonged circulating characteristic of the PEGylated liposomes is lost and the compounds accumulate extensively in the liver and spleen. This phenomenon is referred to as the "accelerated blood clearance (ABC) phenomenon" [7–9]. Furthermore, this phenomenon is elicited by pretreatment with not only PEGylated liposomes [7] but also PEG-containing polymeric nanoparticles [10,11] and polymeric micelles [12]. Although the mechanism of the ABC phenomenon induced by PEGylated liposomes has not been elucidated fully, studies suggest that the splenic marginal zone B plays an essential role in the induction phase [13], and the anti-PEG IgM produced following the initial injection of the PEGylated particles is thought to be responsible for this abnormal phenomenon [14]. Therefore, the ABC phenomenon can be utilized as a simple means to evaluate the immunogenicity of colloidal particles.

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A number of studies have already shown that the physicochemical properties of the first injected nanoparticles, such as lipid composition, size, surface charge, PEG-modification, greatly influence the rate of blood clearance and the tissue distribution of subsequently injected dose [15,16]. However, the effect of terminal groups of DSPE-PEG on the induction of the ABC phenomenon is unclear. In this study, we used PEGylated emulsions (DE) as the model carriers, the ABC phenomenon as the indicator, and the methoxyl (OCH_3) group of DSPE-PEG at the PEG terminal as the control to investigate the effect of the hydroxyl (OH), amino (NH_2), carboxyl (COOH), and thiol (SH) groups on the immune response to DE. The results demonstrated that regardless of the injection route (intravenous or subcutaneous), the thiol group significantly induced the accelerated removal of subsequent DE-OCH_3 doses administered by intravenous injection in all the test groups. In other words, the thiol group evoked a marked immune response. Furthermore, the study demonstrated a rapid and clear method to evaluate immunogenicity in vivo.

2. Materials and methods

2.1. Materials

The injectable soybean lecithin (S75) was obtained from LIPOID GmbH (Ludwigshafen, Germany). The distearoylphosphatidyl-N-(methoxy polyoxyethylene succinyl) ethanolamine (DSPE-PEG-OCH_3), distearoylphosphatidyl-N-(3-carboxypropionyl poly oxyethylene succinyl) ethanolamine (DSPE-PEG-COOH), distearoylphosphatidyl-N-(hydroxy poly oxyethylene succinyl) ethanolamine (DSPE-PEG-OH), distearoylphosphatidyl-N-(thiol poly oxyethylene succinyl) ethanolamine (DSPE-PEG-SH) and distearoylphosphatidyl-N-(amine poly oxyethylene succinyl) ethanolamine (DSPE-PEG-NH_2) with average molecular weights of PEG 2000, 2000, 3400, 3400 and 3400 were purchased from Genzyme Corporation (Cambridge MA, USA). The Beiya Medicated Oil Co., Ltd. (Tieling, China) provided the medium-chain triglycerides (MCT). The tocopheryl nicotinate (TN) was supplied by the Northeast Pharmaceutical Group Co., Ltd. (Shenyang, China). The fluorescent lipophilic tracers DiR (1, 1′-dioctadecyl-3,3,3′,3′-tetra-methylindotricarbocyanine Ex. 750/Em. 780) were a gift from the FanBo Biochemical Group Co., Ltd. (Peking, China). All other reagents were of chromatographic grade.

2.2. Animals

The male Wistar rats weighing 180–200 g were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The animal care and experiments were performed in accordance with the guidelines of the local Animal Welfare Committee.

2.3. Preparation of emulsions

The PEGylated emulsions were prepared in accordance with the following procedure: briefly, the mixtures containing the TN, MCT, S75 (7.2/30/7, wt/wt/wt) and various groups of DSPE-PEG at the PEG terminal (S75 and DSPE-PEG, 9/1, molar ratio) were heated at 55 °C until fused. The sterile water for injection was heated to 55 °C and was added quickly, with stirring, to the oil phase. The mixture was agitated using a magnetic stirrer and was incubated at 55 °C for 10 min to produce the prime emulsions. Alternately, the entire product was sonicated (in an ice bath) using a laboratory ultrasonic cell pulverizer (JY92-II, Ningbo Scientz Biotechnology Co., Ltd. Zhejiang, China), at 200 W for 2 min and at 400 W for 6 min. The emulsions were sized by extrusion through polycarbonate membranes with a pore size measuring 0.22 μm and were adjusted to an isotonic level with the injection of 50% glucose. The Submicron Particle Sizer (Nicomp 380™; Particle Sizing Systems, Inc., Santa Barbara CA, USA) was used to estimate the particle size distribution using the dynamic light scattering method. The DiR-loaded PEGylated emulsions (DiR-DE) were prepared using a similar procedure except that the TN was substituted with DiR at the beginning of the process. The procedures were performed in the dark.

2.4. Biodistribution and pharmacokinetics of the PEGylated emulsions

For the first injection, the rats were administered DE containing a combination of different groups by the intravenous (via the tail vein) or subcutaneous injection of 5 μmol phospholipids/kg, and the control animals were administered intravenous injections of 5% glucose solution instead of the DE. After 7 days, the DE containing the methoxyl group was injected intravenously at the same dose. At 1, 5, 15, 30, 60, 240, 480, 720 min after the injection, the blood samples were collected in a volume of 0.5 mL and were centrifuged at 4000 rpm for 10 min to isolate the plasma. After withdrawing the last blood sample at 720 min, the rats were sacrificed and livers, spleens and the sites of subcutaneous injection were excised, rinsed in ice-cold normal saline. The blood samples and tissue samples were maintained at −20 °C until used.

The concentration of TN in plasma and tissue samples was analyzed by high performance liquid chromatography (HPLC) using a P230 pump and a UV230 UV/Vis Detector (Da Lian Elite Analytical Instruments Co., Ltd., Liaoning, China) and separated using a Hypersil BDS C18 column (200 mm × 4.6 mm) containing particles measuring 5 μm in diameter at 30 °C. The ultraviolet wavelength was 264 nm. The mobile phase was methanol/isopropanol (80/20, *v/v*) at a flow rate of 1 mL/min. Before the analysis, 100 μL of the plasma samples and homogenates (equivalent to 0.1 g tissue) were mixed with methanol (100 μL), internal standard (100 μL) (tocopheryl acetate, 100 μg/mL) and *n*-hexane (600 μL). The entire mixture was vortexed for 5 min and centrifuged at 10,000 rpm for 10 min. The supernatant (500 μL) was dried using a CentriVap Centrifugal Vacuum Concentrator (Labconco Corporation, USA) and dissolved in the mobile phase (100 μL). The resulting mixture was vortexed for 1 min and centrifuged at 10,000 rpm for 10 min. The supernatant (20 μL) was collected and used for the HPLC analysis.

2.5. In vivo fluorescent imaging

The in vivo fluorescent imaging was performed using Carestream Molecular Imaging in vivo (FX Pro, Kodak, USA) as described previously [17,18]. The DiR-DE was injected subcutaneously into the right hind footpad. At predetermined times post-injection, the rats were anesthetized using isoflurane and placed on an animal plate. The imaging parameters were as follows: excitation/emission = 750 nm/790 nm, exposure time = 3 s, f/stop = 2.5, and field of view = 160 mm. The scans were performed at 0.5 h, 1 h, 2 h, 4 h, 8 h and 12 h after administration. The rats were sacrificed, and the heart, liver, spleen, kidneys, lung, brain and popliteal lymph nodes were harvested for isolated organ imaging to evaluate the distribution of the DiR-DE. The fluorescence images and X-ray images were fused using the Carestream Molecular Imaging Systems software.

2.6. Detection of anti-PEG IgM antibodies [19]

DSPE-PEG (50 μL), at a concentration of 10 nmol, containing different terminal groups in 100% ethanol was added to the wells of a 96-well plate (Corning Incorporated, New York, USA). The coated