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Research Paper

Polymer-based nanoparticles loaded with a TLR7 ligand to target the lymph node for immunostimulation

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ABSTRACT

Small-molecule agonists for the Toll-like receptors (TLR) 7 and 8 are effective for the immunotherapy of skin cancer when used as topical agents. Their systemic use has however been largely unsuccessful due to dose-limiting toxicity. We propose a polymer-based nanodelivery system to target resiquimod, a TLR7 ligand, to the lymph node in order to focus the immunostimulatory activity and to prevent a generalized inflammatory response. We demonstrate successful encapsulation of resiquimod in methoxypoly(ethylene glycol)-b-poly(DL-lactic acid) (mPEG-PLA) and mixed poly(DL-lactic-*co*-glycolic acid) (PLGA)/mPEG-PLA nanoparticles. We show that these particles are taken up mainly by dendritic cells and macrophages, which are the prime initiators of anticancer immune responses. Nanoparticles loaded with resiquimod activate these cells, demonstrating the availability of the immune-stimulating cargo. The unloaded particles are non-inflammatory and do not have cytotoxic activity on immune cells. Following subcutaneous injection in mice, mPEG-PLA and PLGA/mPEG-PLA nanoparticles are detected in dendritic cells and macrophages in the draining lymph nodes, demonstrating the targeting potential of these particles. Thus, polymer-based nanoparticles represent a promising delivery system that allows lymph node targeting for small-molecule TLR7 agonists in the context of systemic cancer immunotherapy.

1. Introduction

Chemotherapy, radiotherapy and surgery have long formed the mainstay of cancer treatment, but new therapies such as immunotherapy are emerging. This strategy, which is based on the stimulation of the patient's own immune system against tumor tissues, has proven highly successful in different types of cancer. The initiation of an anticancer immune response is orchestrated by cells of the immune system, in particular macrophages and dendritic cells. These cell types are activated through triggering of pattern-recognition receptors such as the family of Toll-like receptors (TLRs), which are pivotal for the generation of many types of immune responses. TLRs play a key role in immunotherapy, as they participate in the induction of an innate immune response that controls the development of antitumoral immunity (Iwasaki and Medzhitov, 2015).

Imiquimod and resiquimod (R848), two synthetic imidazoquinolines, activate signaling of TLR7 and TLR8 (Heil et al., 2003). These receptors are mainly expressed by monocytes, macrophages and dendritic cells and are localized intracellularly in the endosomal membranes (Nishiya and DeFranco, 2004). Activation of TLR7 leads to development of NK-cell and cytotoxic T-cell responses, which are instrumental for anticancer immunity, and at the same time blocks the function of immunosuppressive cells such as regulatory T cells and myeloid-derived suppressor cells (Anz et al., 2010; Bourquin et al., 2009; Dumitru et al., 2009; Hotz et al., 2016; Spinetti et al., 2016).

Due to their proven clinical efficacy for the topical treatment of skin tumors (Papakostas and Stockfleth, 2015), imidazoquinolines have been attractive candidates as immunostimulating agents for systemic use in the treatment of non-skin cancers. Unfortunately, systemic administration has met with limited success in clinical trials so far (Iribarren et al., 2016). Part of the lack of efficacy may lie in a phenomenon of tolerance that leads to immune unresponsiveness upon repetitive applications of these TLR7 ligands (Bourquin et al., 2011). In addition, upon systemic application in clinical studies, dose-limiting toxicity restricted the therapeutic potential of these drugs (Kobold et al., 2014). The lack of solubility of these molecules in aqueous media

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may also represent a hurdle for their development as systemic treatments (Allémann et al., 1993; Fahr and Liu, 2007).

The development of polymer nanoparticles encapsulating TLR7 ligands may bypass their limitations for systemic application. The targeting of the immune activating substances by particulate delivery directly to the lymph nodes, where the anticancer immune response is initiated, could help reduce unspecific adverse effects (Kranz et al., 2016). Furthermore, encapsulation in a polymer matrix allows the formulation of poorly hydrophilic drugs as injectable suspensions and may provide a delayed release to avoid tolerance (Leroux et al., 1996; Zeisser-Labouèbe et al., 2006). To date, a limited number of studies have reported the use of nanocarriers in imiquimod or resiquimod delivery for cancer immunotherapy. Poly(DL-lactic-*co*-glycolic acid) (PLGA) particles containing a TLR7 agonist combined with a vascular disruptive agent were administered intratumorally with good results (Seth et al., 2017). In a clinical study, imiquimod and a TLR9 agonist coupled with a peptide antigen were loaded into virus-like nanoparticles and administered as intralymph node injection (Goldinger et al., 2012). The co-encapsulation of TLR3 and TLR7 ligands and antigen in PLGA nanoparticles has been reported as vaccine design and enhances the development of B cell responses (Kasturi et al., 2011).

An ideal system in nanomedicine should improve the activity of drugs while decreasing adverse effects by a precise delivery to target tissues (Riehemann et al., 2009). New delivery systems with optimized properties that include biocompatibility, favorable biodistribution, prolonged circulation time, optimal pharmacokinetics, and high loading capacity are thus currently under investigation. In the present report, resiquimod was encapsulated into pegylated polymer-based nanoparticles. Poly(DL-lactic acid) (PLA) and PLGA derivative polymers have been chosen for the matrix of the particles based on their well-known biocompatibility when used in implants or microspheres for injectable slow release delivery systems. These polymers have been approved by the FDA and numerous formulations are already on the market, for example Nutropin Depot®, Decapeptyl®, Trelstar™ Depot as microparticles or Zoladex®, as implant. Methoxypoly(ethylene glycol) (mPEG) residues have been added to avoid excessive and fast opsonization of the particles upon administration leading to a rapid elimination. The therapeutic aim for the resiquimod-loaded nanoparticles is to target the drug to dendritic cells and macrophages in the lymph node, since these cells are critical for the initiation of an antitumoral immune response. This directed delivery should support the development of anticancer immunity while at the same time reducing unwanted systemic proinflammatory reactions. This proof of concept study aims at demonstrating the possibility of encapsulating R848 in nanoparticles made of two polymers without losing or shielding the pharmacological efficiency of the active compound.

2. Materials and methods

2.1. Materials for NP synthesis

PLGA (RG 504-H) and mPEG-PLA polymers were either obtained from Boehringer Ingelheim or synthesized by ring-opening polymerization, respectively. 3,3′Dioctadecyl-oxacarbocyanine perchlorate (DiO), stannous octoate, methoxypolyethylene glycol 2000 (mPEG2000), sucrose and polyvinyl alcohol (PVAL) (Mowiol 4-88, 26000 Da, 88% hydrolysis) were all purchased from Sigma-Aldrich. DL-Lactide was purchased from Polysciences. Resiquimod was purchased from Enzo Life Sciences. All materials were used as received. The water was filtered through a Millipak 40 filter by a Millipore system.

2.2. Cell line and mice

The J774A.1 macrophage cell line (ATCC) was plated in complete medium: high glucose (4.5 g/l) DMEM (Biowest), 10% FCS (Biological industries) 2 mmol/ml L-glutamine (PAA), 1 nM sodium pyruvate (PAA), 0.5% ciproxin (Bayer) in flat-bottom 96-well plates at a concentration of 5×10^5 cells/ml (100 µl/well in a 96-well plate) unless indicated otherwise, and cultured at 37 °C in 5% CO_2.

C57BL/6 mice were purchased from Janvier Labs (Le Genest Saint Isle, France) and maintained in SPF conditions. Experiments were performed at 6–12 weeks of age. All experiments were performed in accordance with Swiss regulations on animal experimentation.

2.3. Synthesis of mPEG-PLA

After three successive vacuum-argon cycles a mixture of mPEG2000 (1.0 g, 0.5 mmol), DL-lactide (4.6 g, 40 mmol), and stannous octoate (7 mg) were stirred at 115 °C for 20 h under an argon atmosphere. The resulting mixture was then diluted in dry $CHCl_3$ and precipitated in cold and dry petroleum ether. After filtration, the crude polymer was dried under vacuum to give mPEG-PLA without any further purification. The chemical structure and molecular composition of mPEG-PLA diblock copolymer was determined by ^{1}H NMR spectroscopy. ^{1}H NMR (300 MHz, $CDCl_3$) δ ppm 5.29–5.09 (m, 100 H), 3.64 (s, 146 H), 3.38 (s, 3 H), 1.63–1.44 (m, 300 H).

2.4. Nanoparticle preparation

A solution of 150 mg of polymer (PLGA/mPEG-PLA 67/33 w/w: NPa or mPEG-PLA: NPb) in 1 ml of dichloromethane was emulsified in 2 ml of a 2.5% PVAL solution by ultra-sonication with a Branson Digital Sonifier (Branson Ultrasonics, Danbury, USA) for 40 s and with amplitude of 50%. The freshly prepared emulsion was then added dropwise to 50 ml of water and stirred 1 h at 1300 rpm with an Ultra-Turrax Eurostar digital Euros ST D (IKA-Werke GmbH & Co., Staufen, Germany) in an ice bath. The stirring speed was then reduced to 600 rpm and the solution was stirred for 2 h at room temperature to remove the solvent. 3 cycles of washing and centrifugation using an Avanti 30 Centrifuge (Beckman, USA) equipped with a F1010 Fixed-Angle Rotor (Beckman, USA), each time for 15 min at 34,000 × *g*, were finally done to obtain 10 ml of a nanoparticle suspension. The latter was then divided in five fractions of 2 ml in which 1 ml of a 15% w/w sucrose aqueous solution was added. The samples were freeze dried using a Christ Alpha 2-4 LD plus freeze dryer (Christ, Osterode am Harz, Germany). In this study, one cycle of 48 h was done at a pressure of 0.6 mBar and a temperature of −85 °C, the final heating temperature was set to 22 °C. In the case of loaded nanoparticles, R848 (5 mg) or DiO (22.5 µg) were solubilized in dichloromethane and added to the polymer solution before mixing it with the PVA solution. Different types of NP were prepared, as presented in Table 1.

Table 1
Composition and characteristics of the different batches of NP.

NP	PLGA	mPEG-PLA	Loading	Size (PDI)	Drug loading µg/mg NP
R848-NPa	67%	33%	R848	278 nm (0.19)	8.36
R848-NPb	0	100%	R848	154 nm (0.14)	0.74
u-NPa	67%	33%	Ø	254 nm (0.10)	/
u-NPb	0	100%	Ø	176 nm (0.17)	/
DiO-NPa	67%	33%	DiO	230 nm (0.14)	/
DiO-NPb	0	100%	DiO	201 nm (0.16)	/