Biomaterials 102 (2016) 175–186

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Nanoparticle tumor localization, disruption of autophagosomal trafficking, and prolonged drug delivery improve survival in peritoneal mesothelioma

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ARTICLE INFO

Article history:
Received 19 April 2016
Received in revised form
11 June 2016
Accepted 13 June 2016
Available online 23 June 2016

Keywords:
Mesothelioma
Nanoparticle
Drug delivery
Paclitaxel
Autophagosome
Tumor localization

ABSTRACT

The treatment outcomes for malignant peritoneal mesothelioma are poor and associated with high co-morbidities due to suboptimal drug delivery. Thus, there is an unmet need for new approaches that concentrate drug at the tumor for a prolonged period of time yielding enhanced antitumor efficacy and improved metrics of treatment success. A paclitaxel-loaded pH-responsive expansile nanoparticle (PTX-eNP) system is described that addresses two unique challenges to improve the outcomes for peritoneal mesothelioma. First, following intraperitoneal administration, eNPs rapidly and specifically localize to tumors. The rate of eNP uptake by tumors is an order of magnitude faster than the rate of uptake in non-malignant cells; and, subsequent accumulation in autophagosomes and disruption of autophagosomal trafficking leads to prolonged intracellular retention of eNPs. The net effect of these combined mechanisms manifests as rapid localization to intraperitoneal tumors within 4 h of injection and persistent intratumoral retention for >14 days. Second, the high tumor-specificity of PTX-eNPs leads to delivery of greater than 100 times higher concentrations of drug in tumors compared to PTX alone and this is maintained for at least seven days following administration. As a result, overall survival of animals with established mesothelioma more than doubled when animals were treated with multiple doses of PTX-eNPs compared to equivalent dosing with PTX or non-responsive PTX-loaded nanoparticles.

1. Introduction

Unlike other solid cancers where mortality commonly results from metastatic disease, patients with diffuse peritoneal malignancies often succumb to local disease progression and locoregional recurrence. The rapid local progression of mesothelioma results in a median survival of only 4–12 months following diagnosis, even with best supportive care [1–3], while systemic intravenous platinum-based chemotherapy alone offers minimal improvement [4]. Therefore, peritoneal mesothelioma, as well as other peritoneal malignancies such as ovarian, gastric, and appendiceal carcinoma, are sometimes treated with multimodality regimens consisting of cytoreductive surgery followed by intracavitary chemotherapy with the goal of ablating residual microscopic tumor [5–9]. Aggressive surgical debulking and intraoperative heated chemotherapy have been advocated for the treatment of both intraperitoneal and intrapleural malignant mesothelioma since large bulky tumors and massive pleural effusions and/or ascites severely compromise quality of life in these patients. Interestingly, due to the unique pharmacokinetics of the peritoneal-plasma barrier, clinical trials investigating

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http://dx.doi.org/10.1016/j.biomaterials.2016.06.031
0142-9612/

intraperitoneal, rather than intravenous (IV), chemotherapy demonstrate improved survival and increased intratumoral drug levels with comparatively fewer systemic side effects [10–12]. However, despite initial palliation of symptoms, the majority of patients still succumb to unrelenting locoregional tumor growth, with significant financial and emotional cost [13]. Given the high incidence of local disease failures and the rapid clearance of systemically administered paclitaxel, the treatment regimen of these patients now includes five consecutive days of intraperitoneal paclitaxel, in an attempt to keep drug levels high following surgical debulking and intra-operative chemotherapy. Cure has been reported in some patients, although the overall 5-year survival remains poor [14–18].

Given the challenge of local tumor recurrence, novel locoregional therapies are being investigated. These include mesothelin-targeted immunotherapy, which has prolonged survival in animal models of pleural mesothelioma [13], and various micro- and nanoparticle (NP) drug delivery systems designed to address many of the limitations that currently prevent optimal delivery of more traditional chemotherapeutic agents to tumors. Particle-based systems are being engineered to: increase the solubility of hydrophobic drugs; provide more consistent drug levels over prolonged periods; protect sensitive drugs from degradation or enzymatic alteration; and, in some cases, provide local or "targeted" delivery to a desired tissue [19–27]. Drug delivery systems using particles, nanorods, micelles, or hydrogels, have been developed specifically for the treatment of peritoneal carcinomatoses [28–33], with poly(lactic acid) (PLA) and poly(lactic-*co*-glycolic acid) (PLGA) being the most widely studied formulations due to availability, biocompatibility, and use in other FDA approved devices [34–36]. Unfortunately, the rapid "burst" release of >50% of encapsulated drug within the first 10–48 h prevents the clinical application of these formulations in the setting of large debulking operations as post-operative healing is critical and sustained drug release is required to kill tumor cells with extended doubling times [37,38]. To address these shortcomings, functional systems in which drug release is triggered by specific physical stimuli (e.g., pH, temperature, oxidative/reductive environments, or osmolality) are being pursued to improve the anti-tumor efficacy of NP-based therapies [39–41].

In this manuscript, we describe an efficacious, pH-responsive, expansile nanoparticle (eNP) drug-delivery system and investigate three unique aspects of this system including: the surprisingly discordant relationship between *in vitro* and *in vivo* efficacy, the mechanism of tumor-specific localization *in vivo*, and enhanced anti-tumor efficacy *in vivo*. First, we developed a "short duration-of-exposure" *in vitro* cytotoxicity assay model that more accurately mimics the rapid clearance of PTX *in vivo* and evaluated PTX-eNPs and PTX in both this and conventional models. Second, we investigated the kinetics and mechanisms of intratumoral eNP accumulation by characterizing the disparity in the rate of eNP internalization in native versus malignant peritoneal cells as well as the accumulation in autophagosomes and disruption of autophagosomal degradation/flux. Third, given that prolonged low drug concentrations may actually prove detrimental by facilitating the selection and growth of cancer stem cells [42,43]—we explored the ramifications of sustained high PTX concentrations over multiple cell cycles using a multiple-dose *in vivo* model of human mesothelioma. We hypothesized that PTX-eNPs would prove superior to an equivalent PTX dose due to enhanced, prolonged intratumoral delivery of PTX and, thus, significantly improve the survival of animals even with established human mesothelioma tumors.

2. Materials and methods

2.1. Nanoparticle synthesis and characterization

Expansile nanoparticles (eNPs), non-expansile nanoparticles (neNPs) and poly(lactic-*co*-glycolic) acid (MW ~50-80 k) nanoparticles (PLGA-NPs) with or without paclitaxel (PTX-eNP and PTX-PLGA-NPs) were prepared using a previously reported mini-emulsion technique with base-catalyzed polymerization of eNPs and neNPs [30,44–46]. Briefly, nanoparticle monomer and cross-linker (or polymer in the case of PLGA) with or without paclitaxel were dissolved in 500 μL of dichloromethane. This was combined with 2 mL of 10 mM pH 7.4 phosphate buffer containing 8 mg/mL sodium dodecyl sulfate and sonicated under argon for 30 min using a Sonotek probe sonicator with 20% amplitude and a 1 s on 2 s off pulse with water bath cooling. Polymerization of eNPs and neNPs was carried out by addition of 2 μL tetramethylethylenediamine and 20 μL of 200 mM ammonium persulfate. Particles were stirred under argon for 1 h and then under air overnight. Particles were dialyzed in 5 mM pH 7.4 phosphate buffer for 24 h in 10,000 MWCO snakeskin dialysis tubing and the buffer (1 L volume) was exchanged once after 4 h. Particles' PTX encapsulation efficiency was determined by quantifying PTX concentration by high performance liquid chromatography (HPLC) as previously reported [47]. PTX alone was formulated in a 50/50 mixture of Cremophor EL/ethanol (i.e., a recapitulation of Taxol®, the clinical formulation of PTX).

Fluorescently labeled eNPs were prepared by incorporating 0.02% (w/w) PolyFluor™ 407 (9-anthracenylmethyl methacrylate; PF-eNPs) or PolyFluor™ 570 (methacryloxyethyl tricarbonyl rhodamine B; Rho-eNPs) into the polymer backbone during polymerization. Fluorophores were aliquoted from a 10 mg/mL stock in dimethylsulfoxide. Rhodamine-conjugated fluorescent PLGA was purchased from PolySciTech and mixed with PLGA in a 1:5 ratio to yield the final fluorescently labeled Rho-PLGA-NPs with equivalent fluorescent intensity of Rho-eNPs and Rho-neNPs.

Nanoparticles were characterized by dynamic light scattering by diluting 10 μL of particles in 3 mL of de-ionized water and measuring the hydrodynamic radius (by number) using a Brookhaven 90Plus particle sizer. Scanning electron microscopy was performed by drying a 10 μL droplet of the same particle dilution on a silicon wafer, sputter coating with Au/Pd, and imaging on a Zeiss Supra 40 scanning electron microscope.

2.2. Cell lines

Human malignant pleural mesothelioma cells (MSTO-211H), firefly luciferase gene transfected MSTO-211H cells (MSTO-211H-Luc; a generous gift from J. Rheinwald at Harvard Medical School, Boston, MA), and healthy mesothelial cells (LP-3) were maintained at 37 °C in 5% CO_2 in complete culture media using RPMI 1640 or DMEM media, respectively, containing 10% (v/v) fetal bovine serum, streptomycin (100 mg/mL), and penicillin (100 units/mL).

2.3. In vitro cell viability

MSTO-211H tumor cells were seeded in 96-well plates at 2000 cells/well in media. After 24 h, media was replaced with media containing PTX-eNPs, unloaded-eNPs, or PTX. Treatment with equivalent PTX doses were determined based on encapsulation efficiency. Cells were incubated with treatments for 4 h and washed thrice with phosphate buffered saline (PBS) before addition of media without treatments. After further culturing for 3 days, cell viability was determined using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI).