Journal of Controlled Release 178 (2014) 79–85

Contents lists available at ScienceDirect

Journal of Controlled Release

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

Tumor priming enhances siRNA delivery and transfection in intraperitoneal tumors

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ARTICLE INFO

Article history:
Received 21 November 2013
Accepted 14 January 2014
Available online 23 January 2014

Keywords:
Intraperitoneal chemotherapy
Peritoneal carcinomatosis
siRNA
Microparticles
Paclitaxel
Pancreatic cancer

ABSTRACT

Cancers originating from the digestive system account for 290,000 or ~20% of all new cancer cases annually in the US. We previously developed paclitaxel-loaded tumor-penetrating microparticles (TPM) for intraperitoneal (IP) treatment of peritoneal tumors (Lu et al., 2008; Tsai et al., 2007; Tsai et al., 2013). TPM is undergoing NIH-supported IND-enabling studies for clinical evaluation. The present study evaluated the hypothesis that TPM, *via* inducing apoptosis and expanding the interstitial space, promotes the delivery and transfection of lipid vectors containing siRNA. The *in vivo* model was the metastatic human Hs766T pancreatic tumor that, upon IP injection, produced widely distributed solid tumors and ascites in the peritoneal cavity in 100% of animals. The target gene was survivin, an anti-apoptotic protein induced by chemotherapy and associated with metastases and poor prognosis of patients with gastric and colorectal cancers. The siRNA carrier was pegylated liposomes comprising cationic and neutral lipids plus a fusogenic lipid (PCat). PCat-loaded with survivin siRNA (PCat–siSurvivin) was active in cultured cells (decreased survivin mRNA and protein levels, reduced cell clonogenicity, enhanced paclitaxel activity), but lost its activity *in vivo*; this difference is consistent with the well-known problem of inadequate delivery and transfection of siRNA *in vivo*. In comparison, single agent TPM prolonged animal survival and, as expected, induced survivin expression in tumors. Addition of PCat–siSurvivin reversed the TPM-induced survivin expression and enhanced the antitumor activity of TPM. The finding that *in vivo* survivin knockdown by PCat–siSurvivin was successful only when it was given in combination with TPM provides the proof-of-concept that tumor priming promotes the delivery and transfection of liposomal siRNA. The data further suggest the TPM/PCat–siSurvivin combination as a potentially useful chemo-gene therapy for peritoneal cancer.

1. Introduction

Cancer originating from the digestive system, including cancers of the esophagus, gallbladder, liver, bile duct, pancreas, stomach, small intestine, colon and rectum, accounts for 290,000 new cases or about 20% of all newly diagnosed cancers annually in the United States. Peritoneal carcinomatosis due to loco-regional spread is common and presented with 10 to 30% of cases at the time of initial surgery [4,5].

The survival advantage of intraperitoneal (IP) therapy of peritoneal cancer was first demonstrated 17 years ago and has since been confirmed in multiple clinical trials [6–11]. However, several difficulties, including local toxicity and ineffectiveness against bulky tumors, have prohibited IP therapy from becoming a common standard of care. To address these problems, we developed paclitaxel-loaded tumor penetrating microparticles (TPM). TPM comprises two components, one component releases the drug rapidly to produce tumor priming (*i.e.*, induce apoptosis and thereby expand the interstitial space) to promote the transport of the remaining microparticles and the second component releases the drug slowly to provide sustained drug exposure [12,13]. Studies in mice with metastatic IP pancreatic and ovarian tumors show superior delivery and activity of TPM compared to the intravenous paclitaxel solutions used off-label in previous IP studies [1–3]. Based on its promising preclinical activity, TPM is currently undergoing NIH-supported IND-enabling studies, in preparation for clinical evaluation.

We have since made several observations that suggest it may be possible to further improve the efficacy of IP TPM. We found that tumor priming using either paclitaxel or TPM enhances the delivery and efficacy of doxorubicin-loaded liposomes (85 nm in diameter given intravenously),

Abbreviations: Blank microparticles or blank liposomes, microparticles or liposomes not loaded with drug or siRNA; DC, liposomes comprising DOTAP:cholesterol in 50:50 ratio; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-dioleoyl-3-trimethylammoniumpropane; DSPE-PEG, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]; GA, glycolide; ILS, increase in life span; IP, intraperitoneal; LA, lactide; MST, median survival time; PCat, liposomes comprising DOTAP:cholesterol:DOPE:DSPE-PEG in 50:30:19:1 ratio; PLGA, poly(D,L-lactide-co-glycolide); siNT, non-target siRNA; siSurvivin, survivin siRNA; TPM, tumor penetrating microparticles loaded with paclitaxel; CI, confidence interval; SEM, standard error of the mean.

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http://dx.doi.org/10.1016/j.jconrel.2014.01.012

the penetration and dispersion of fluorescent latex beads (2 μm in diameter given IP) in peritoneal tumors, and the penetration and dispersion of siRNA-loaded cationic liposomes in 3-dimensional tumor cell spheroids and tumor histocultures [1,13,14]. Based on these observations, we hypothesized that TPM can be used to promote the delivery of siRNA vectors.

siRNA produces post-transcriptional gene silencing and represents a promising approach to reverse the chemotherapy-induced upregulation of chemoresistance genes. Its therapeutic potential is indicated by the demonstration of gene silencing in patients with melanoma [15]. siRNA, typically comprising 20–27 nucleotides, has high negative surface charge that is unfavorable for cellular uptake. A popular siRNA carrier is cationic liposome that forms multilamellar structures with the anionic siRNA upon mixing; the resulting lipoplex protects siRNA from degradation and facilitates cellular uptake. Lipoplexes, however, are relatively large in size (*e.g.*, >100 nm in diameter) which may impede effective delivery *in vivo* [16–18].

The present study evaluated whether TPM promotes the delivery of lipid vectors containing siRNA and whether the efficacy of TPM is enhanced by combining it with siRNA targeting the anti-apoptotic survivin. Survivin was selected because (a) it is highly and selectively expressed in a majority of human cancers including gastric and colorectal cancers and peritoneal metastases, compared with most differentiated adult normal tissues including the liver [19–25], (b) high survivin expression is correlated with more extensive peritoneal metastases (depth of invasion, lymph node metastasis) and shorter overall survival of patients with gastric and colorectal cancers [26–29], (c) high level survivin expression correlates with chemo/radio-resistance in multiple tumor types and its inhibition enhances cell death induced by chemo/radio-therapy [30,31], and (d) paclitaxel induces survivin expression whereas survivin siRNA (siSurvivin) significantly increases paclitaxel-induced cell death [32].

The present study used pegylated cationic liposomes (PCat) as siRNA carrier and measured the effectiveness of *in vivo* delivery and survivin knockdown in a metastatic IP pancreatic human H766T xenograft tumor model. This model was selected due to its relative ease; IP injection of Hs766T cells produced carcinomatosis and ascites in the peritoneal cavity, and deaths in 100% of animals with median survival time (MST) of 24 days post-implantation [3].

2. Materials and methods

2.1. Chemicals and reagents

Poly-lactide-co-glycolide (PLGA) polymers were purchased from Boehringer Ingelheim (Ingelheim, Germany), paclitaxel from Hande Tech (Houston, TX), lipids (1,2-dioleoyl-3-trimethylammoniumpropane or DOTAP, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine or DOPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] or DSPE-PEG, cholesterol) from Avanti Polar Lipids, Inc. (Alabaster, AL, USA), cefotaxime sodium from Hoechst-Roussel (Somerville, NJ), poly(vinyl alcohol) or PVA from Sigma Chemical (St. Louis, MO), chromatography solvents from Fisher Scientific (Fair Lawn, NJ), gentamicin from Solo Pak Laboratories (Franklin Park, IL), and all other cell culture supplies from Life Technologies (Grand Island, NY). Survivin siRNA (siSurvivin, human specific, #6351), survivin monoclonal antibody (71G4B7E), and caspase-3 polyclonal antibody (#9661) were purchased from Cell Signaling Technology (Danvers, MA), survivin polyclonal antibody (C-19, sc-8807) from Santa Cruz Biotechnology (Santa Cruz, CA), and Ki67 (MM1 clone) antigen kit from Novocastra Lab (Newcastle, UK). All chemicals and reagents were used as received.

2.2. Preparation of TPM

TPM comprised two paclitaxel-loaded PLGA microparticles, *i.e.*, Priming and Sustaining TPM. The Priming TPM, which releases paclitaxel rapidly (70% of dose in one day under sink condition [1]), was prepared using 50:50 LA:GA with an inherent viscosity of 0.12 dl/g in chloroform. The Sustaining TPM, which releases paclitaxel slowly (1% of dose in one day under sink condition [1]), was prepared using 75:25 LA:GA with an inherent viscosity of 0.39 dl/g in chloroform. Preparation of paclitaxel-loaded PLGA microparticles was as previously described [1,2]. Briefly, PLGA and paclitaxel were co-dissolved in methylene chloride and emulsified in PVA. After evaporation of methylene chloride, microparticles were collected, washed and lyophilized. Particle size was determined using LS230 laser diffraction particle size analyzer (Beckman Coulter, Brea, CA); the average volume-based diameter was 4.8 μm. Blank PLGA microparticles were prepared similarly, except without paclitaxel.

2.3. Preparation of liposomes and lipoplexes

PCat liposome comprised two neutral lipids (DOPE, cholesterol), one cationic lipid (DOTAP), and one pegylated lipid (DSPE-PEG2000) at ratio of 50:30:19:1 of DOTAP:cholesterol:DOPE:DSPE-PEG. DOTAP provides the positive charge. Cholesterol was used to increase the liposome stability. DOPE was used to increase the elasticity of the liposome bilayer and to promote the fusion of liposome with cell membrane and with endosomal membrane [33]. DSPE-PEG was used to provide stealth property and to improve the *in vivo* stability of liposomes. Briefly, lipids were combined and dissolved in 9:1 v/v mixture of chloroform and methanol (*e.g.*, 10 mg lipids in 5 ml chloroform/methanol). The organic phase was evaporated under nitrogen to yield a thin lipid film, which was dried under vacuum in a desiccator for approximately 12 h. The lipids were then hydrated with RNase-free buffer (1 ml per 10 mg of lipids) at 60 °C for 2 h, with gentle vortexing every 20 min. The resulting liposomal suspension was passed through a liposome extruder with a 100 nm membrane to obtain liposomes of about 100 nm diameter.

siRNA lipoplex was formed by gently mixing liposomes with siRNA solution (between 2 μM and 10 μM) at room temperature, in a 1:4 siRNA-to-DOTAP charge ratio. Particle size distribution and zeta-potential were measured using Zetasizer Nano ZS90 (Malvern, Westborough, MA). Pilot studies showed that more than 90% of siRNA remained in the PCat lipoplex after 24 h.

The *in vitro* and *in vivo* toxicities of blank PCat carrier (no siRNA) or PCat–siRNA lipoplex were compared to another cationic liposome that had been given to humans, *i.e.*, 50:50 DOTAP:cholesterol (referred to as DC liposomes [34,35]). DC liposomes were prepared using the procedures described above.

2.4. Effect of paclitaxel on siRNA transfection in vitro

As TPM contains paclitaxel, we evaluated whether paclitaxel affected the siRNA transfection in human pancreatic Hs766T cells (gift from Dr. Byoungwoo Ryu, John Hopkins Medical Institute, Baltimore, MD). Cells were maintained in DMEM medium containing 10% fetal bovine serum, 2 mM L-glutamine, antibiotics (90 μg/ml gentamicin and 90 μg/ml cefotaxime sodium, or 100 units/ml penicillin and 100 μg/ml streptomycin), at 37 °C in a humidified atmosphere with 5% CO_2. All transfections were carried out in nearly confluent cultures (over 80% confluence); this was to align with the cell density found *in vivo*.

Cells were treated with vehicle (0.1% ethanol), paclitaxel, and PCat loaded with survivin siRNA (PCat–siSurvivin), paclitaxel plus PCat loaded with non-target siRNA (PCat–siNT), or paclitaxel plus PCat–siSurvivin. The study used 10 nM paclitaxel, which was near its IC_{50} in the clonogenicity assay (see Results). Antitumor activity was measured using the clonogenic assay (cells were seeded 24 h post-siRNA treatment; colonies were counted 10 days later). Survivin mRNA and protein levels were measured using RT-PCR and Western blot, respectively. Briefly, total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), and reversed transcribed to cDNA using qScript™ cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD). Real-Time RT-PCR (triplicate samples, 5 μl cDNA per reaction) was performed with