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Prevention of nodal metastases in breast cancer following the lymphatic migration of paclitaxel-loaded expansile nanoparticles

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ABSTRACT

Although breast cancer patients with localized disease exhibit an excellent long-term prognosis, up to 40% of patients treated with local resection alone may harbor occult nodal metastatic disease leading to increased locoregional recurrence and decreased survival. Given the potential for targeted drug delivery to result in more efficacious locoregional control with less morbidity, the current study assessed the ability of drug-loaded polymeric expansile nanoparticles (eNP) to migrate from the site of tumor to regional lymph nodes, locally deliver a chemotherapeutic payload, and prevent primary tumor growth as well as lymph node metastases. Expansile nanoparticles entered tumor cells and paclitaxel-loaded eNP (Pax-eNP) exhibited dose-dependent cytotoxicity *in vitro* and significantly decreased tumor doubling time *in vivo* against human triple negative breast cancer in both microscopic and established murine breast cancer models. Furthermore, migration of Pax-eNP to axillary lymph nodes resulted in higher intranodal paclitaxel concentrations and a significantly lower incidence of lymph node metastases. These findings demonstrate that lymphatic migration of drug-loaded eNP provides regionally targeted delivery of chemotherapy to both decrease local tumor growth and strategically prevent the development of nodal metastases within the regional tumor-draining lymph node basin.

1. Introduction

Breast cancer is the most commonly diagnosed solid organ malignancy in women worldwide, affecting over 1.5 million women in 2010 [1,2]. Although patients with disease localized to the breast exhibit an excellent long-term prognosis, the development of metastases in the regional lymph nodes is associated with a significant decrease in survival. The NSABP B-04 clinical trial was a landmark randomized controlled study designed to assess differences in outcome for women with nonpalpable axillary nodes as a function of locoregional therapy [3]. Among women treated with radical mastectomy there was a 40% incidence of occult metastases in the axillary nodes and 18% of patients had clinical evidence of tumor growth in the axilla following total mastectomy where axillary lymph nodes are not removed. Whereas, these results established the importance of assessing regional lymph node status, subsequent studies dampened enthusiasm for aggressive lymphadenectomy by highlighting the associated complications and long-term morbidity of nerve damage, increased infectious risk, pain and lymphedema [4,5] Consequently, NSABP-32 investigated the differences in clinical outcome in over 5000 women with operable, clinically node negative breast cancer randomly assigned to regional node assessment via sentinel lymph node (SLN) biopsy with subsequent axillary node dissection vs. SLN biopsy alone [6]. Among patients with histologically negative SLN(s), nearly twice as many regional node recurrences were noted in the SLN biopsy alone cohort indicating that occult nodal disease was left behind when an axillary node dissection was not performed [4]. Although regional nodal recurrence was the first clinically evident site of treatment failure in only 21 patients, in-depth histologic analysis of all previously determined negative SLN

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samples (3887 patients) demonstrated occult metastatic disease, i.e. missed nodal disease, in 15.9% of "negative SLN". Patients with occult nodal metastases exhibited a nearly 3-fold increase in regional recurrence and statistically significantly worse outcomes in terms of overall survival, disease-free survival, and distant-disease-free interval ($p < 0.04$ for each) [7]. Importantly, these differences were noted despite complete surgical resection and adjuvant treatment with standard radiation therapy, chemotherapy, and endocrine regimens as deemed appropriate based on tumor characteristics. Given that the majority of patient received either adjuvant chemotherapy, endocrine or other systemic therapy and at least tangential external radiation therapy (XRT) to the SLN biopsy site and portions of the level I and II axillary lymph node chains, it is of interest that the number of node-negative patients that presented with locoregional failure was essentially equivalent to the number that presented with distant metastases [7]. Furthermore, although the 10-year risk of locoregional first recurrence following breast conserving therapy can be decreased from 25% to 7–8% with the addition of local radiotherapy, significant concerns of increases in 15-year mortality due to resultant cardiac disease has called for new approaches to prevent locoregional recurrence in these patients [8,9].

Current regimens for systemic chemotherapy are hampered by systemic side effects that limit the total dose and concentration of drug that can be delivered locally to the desired target tissues, i.e. tumor bed and draining lymph node basins. Furthermore, limited vascular access to regional nodes and rapid systemic clearance results in virtually no persistent drug delivery to regional nodes of patients with breast cancer, even within 6 h of administration [10]. These limitations suggest that a local drug delivery system that can deliver chemotherapeutic drug to the primary tumor site and regional lymph nodes via lymphatic pathways may result in a significantly more efficacious means to control locoregional disease with less morbidity than axillary node dissection or radiation.

To this aim, we investigated an expansile nanoparticle (eNP) local drug delivery system previously shown to exhibit superior anti-cancer efficacy in the treatment of peritoneal carcinomatosis [11]. Paclitaxel-loaded eNP (Pax-eNP) demonstrated increased tumor affinity and resulted in both decreased local tumor burden and prolonged survival *in vivo*. Recently, we have shown that eNP are also capable of lymphatic trafficking *in vivo* suggesting that eNP may provide the means to concentrate drug delivery within the regional lymph node basins draining sites of tumor [12]. We hypothesized that locally administered nanoparticles at the site of the original tumor would be capable of migrating to regional lymph nodes, delivering the chemotherapeutic payload, and preventing primary tumor growth as well as lymph node metastases. Therefore, in the current study we investigate the utility of Pax-eNP to control both local tumor growth and occult lymphatic metastases in a sensitive bioluminescent murine model of metastatic triple negative breast cancer.

2. Materials and methods

2.1. Nanoparticle preparation

Paclitaxel-loaded expansile (Pax-eNP) and unloaded expansile (unloaded eNP) nanoparticles were prepared using a miniemulsion polymerization technique [13]. Encapsulation efficiency of 5% Pax-eNP (w/w of polymer) was assessed via HPLC and was greater than 95% for all studies. For studies involving eNP trafficking, Rhodamine-B (Sigma–Aldrich, St. Louis, MO) was covalently attached to the NP at 0.02% w/w loading and prepared using the corresponding acrylate monomer. In addition, to separately identify the location of the nanoparticle monomer and the paclitaxel payload, Rhodamine-B labeled eNP were prepared with 0.02% w/w Oregon Green 488 conjugated paclitaxel (Life Technologies Corp., Carlsbad, CA) as the payload (OGPax-Rho-eNP).

2.2. Cell culture and in vitro cell viability assay

MDA-MB-231-luc-D3H2LN (Caliper Scientific, Hopkinton, MA), was chosen for these studies as it is a firefly luciferase transfected human breast cancer cell line that is "triple negative" for estrogen, progesterone and her-2neu receptors, multi-drug resistant and highly metastatic to regional lymph nodes. It was maintained at 37 °C in 5% CO_2 using MEM media containing 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 units/mL). Anti-tumor cytotoxicity of Pax-eNP, unloaded eNP, and paclitaxel resuspended in standard cremaphor/ethanol solution (Pax-C/E) were assessed using the MTS *in vitro* cytotoxicity assay (CellTiter 96® Aqueous One, Promega, Madison, WI) as described previously [11]. Briefly, tumor cells were cultured in 96-well plates at 3.0×10^3 cells/well for 24 h. Culture media was removed and replaced with media containing Pax-eNP, unloaded eNP, or Pax-C/E (Pax control). Cell viability was measured after three days of treatment exposure with the percent viability calculated as absorbance relative to control wells (cell with culture media). A total of 4–6 wells were used per treatment per concentration for each experiment and all assays were repeated a minimum of four times.

2.3. Microscopic and established breast cancer model in mice

All animal experiments were approved and conducted in accordance with the guidelines for humane care and use of laboratory animals from the Institutional Animal Care and Use Committee. Orthotopic breast cancers were induced with inoculation of two million MDA-MB-231-luc-D3H2LN cells into the 4th mammary fat pad in 6–8 wks female Nude mice (Harlan, Indianapolis, IN). Local treatment with Pax-eNP, unloaded eNP or 4 mg/kg Pax-C/E were injected at the site of tumor inoculation on the same day (Day 0) for the microscopic tumor model and on Day 7 for studies utilizing the established tumor model. 100 μg Pax-eNP, the paclitaxel dose equivalent of 4 mg/kg local Pax-C/E, was used for all studies with efficacy compared against a standard dose of 12 mg/kg systemic Pax-C/E administered intraperitoneally. Tumor size was measured twice per week with a digital caliper and at the time of sacrifice, with the estimated tumor volume calculated as $\pi \times$ (length $\times$ width $\times$ height/6). Mice were euthanized 5½ weeks after treatment for both models or when tumors reached 2 cm, skin necrosis developed, body mass dropped ≥15% within one week, or animals became moribund.

2.4. Bioluminescent imaging for detection of lymph node metastases

Axillary lymph node bioluminescent imaging was performed at the designated timepoint, 5 1/2 weeks after treatment for both models, or when early termination required due to the size or necrosis of tumor. Mice received 150 mg/kg D-Luciferin (Caliper Life Sciences, Hopkinton, MA) prior euthanasia. Axillary lymph nodes (LNs) were immediately harvested and submerged in 1.5 mg/mL luciferin in PBS in black 96-well plates and imaged for 3 min with a Caliper IVIS-100 bioluminescence camera (Caliper Life Sciences, Hopkinton, MA). In initial animal studies, a threshold of positive (i.e. metastatic) nodes was set as the mean + two $\times$ standard deviations greater than signals of nodes from non-tumor control animals. Subsequent studies utilized the absolute bioluminescent signal at photons/sec for statistical analyses.

2.5. Confocal microscopy

For *in vitro* cell imaging, MDA-MB-231-luc-D3H2LN were seeded at 2.0×10^4 cells on a poly-lysine (Sigma) treated glass coverslips (No. 1.5, Fisher Scientific, Pittsburg, PA) and placed with a 24-well plate for 48 h at 37 °C in 5% CO_2. Cells were incubated with Rho-eNP loaded with or without OGPax. Coverslips were washed with HBSS three times, fixed in 2% paraformaldehyde (Fisher Scientific, Pittsburg, PA) for 10 min, and washed three times with HBSS. Cells were stained with Hoechst 33342 (Life Technologies, Carlsbad, CA) and Alexa Fluor® 488 conjugated wheat germ agglutinin (WGA 488, Life Technologies, Carlsbad, CA) for identification of the nucleus and cell membranes respectively, and mounted on slides with Antifade Prolong Gold® (Life Technologies, Carlsbad, CA) mounting media in room temperature overnight. Slides were stored at −20 °C until analysis. Images were obtained with Zeiss LSM 510 inverted confocal laser scanning microscope with Plan-Apochromat 10×/0.45 or C-Apochromat 40 × 1.2 W corr lens (Carl Zeiss Microscopy, Thornwood, NY).

Confocal imaging of labeled eNP that have migrated *in vivo* to axillary LNs was performed after the injection of 100 μL of Rho-eNP or OGPax-Rho-eNP in the 4th mammary fat pad of naïve or tumor-bearing mice. Mice were sacrificed and axillary LNs were harvested 4 or 10 days after injection, placed in Tissue-Tek O.C.T. compound (Fisher Scientific) and frozen in liquid nitrogen. Each node was cryosectioned at 5 μm intervals and sections examined for fluorescence via confocal microscopy after nuclear staining with DAPI dihydrochloride (Life Technologies, Carlsbad, CA). Adjacent tissue sections were stained with hematoxylin and eosin for general histologic evaluation. Volocity 6.0.1 software (PerkinElmer Inc., Waltham, MA) was utilized to quantify colocalization with calculation of the co–efficient parameter "MX", which indicates percentage of Rho-eNP that contains OGPax.