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Bone marrow endothelium-targeted therapeutics for metastatic breast cancer

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ABSTRACT

Effective treatment of cancer metastasis to the bone relies on bone marrow drug accumulation. The surface proteins in the bone marrow vascular endothelium provide docking sites for targeted drug delivery. We have developed a thioaptamer that specifically binds to E-selectin that is overexpressed in the vasculature of tumor and inflammatory tissues. In this study, we tested targeted delivery of therapeutic siRNA loaded in the E-selectin thioaptamer-conjugated multistage vector (ESTA-MSV) drug carrier to bone marrow for the treatment of breast cancer bone metastasis. We evaluated tumor type- and tumor growth stage-dependent targeting in mice bearing metastatic breast cancer in the bone, and carried out studies to identify factors that determine targeting efficiency. In a subsequent study, we delivered siRNA to knock down expression of the human STAT3 gene in murine xenograft models of human MDA-MB-231 breast tumor, and assessed therapeutic efficacy. Our studies revealed that the $CD31^{+}$E-$selectin^{+}$ population accounted for 20.8%, 26.4% and 29.9% of total endothelial cells respectively inside the femur of mice bearing early, middle and late stage metastatic MDA-MB-231 tumors. In comparison, the double positive cells remained at a basal level in mice with early stage MCF-7 tumors, and jumped to 23.9% and 28.2% when tumor growth progressed to middle and late stages. Accumulation of ESTA-MSV inside the bone marrow correlated with the E-selectin expression pattern. There was up to 5-fold enrichment of the targeted MSV in the bone marrow of mice bearing early or late stage MDA-MB-231 tumors and of mice with late stage, but not early stage, MCF-7 tumors. Targeted delivery of STAT3 siRNA in ESTA-MSV resulted in knockdown of STAT3 expression in 48.7% of cancer cells inside the bone marrow. Weekly systemic administration of ESTA-MSV/STAT3 siRNA significantly extended survival of mice with MDA-MB-231 bone metastasis. In conclusion, targeting the overexpressed E-selectin provides an effective approach for tissue-specific drug delivery to the bone marrow. Tumor growth in the bone can be effectively inhibited by blockage of the STAT3 signaling.

1. Introduction

Bone is not only host to hematopoietic cancers but also a major organ for metastasis of multiple solid tumors, particularly breast and prostate cancers. In the case of late-stage breast cancer, over 60% of patients carrying estrogen receptor-positive cancer and about 10% of patients with triple negative breast cancer would eventually develop bone metastasis [1,2]. Although breast cancer bone metastasis is usually not the major cause of cancer death, symptoms associated with bone metastasis such as chronic bone pain, pathological fractures, life threatening hypercalcemia, and spinal cord compression pose a severe burden on the quality of life [3–5]. Moreover, there are currently no effective treatments of cancer metastasis to the bone by targeting cancer cells, and for most patients, palliative care is usually the only option. Although several bisphosphonates [6] and a neutralizing antibody targeting RANKL [7,8] have shown promising results on management of tumor progression, benefits on improved survival was observed in only a small population of patients [9,10].

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A major challenge in the treatment of metastatic cancer is effective delivery of therapeutics to the tumor lesion. Histological analyses have revealed significantly reduced microvessel density in their bone tumor metastases in half the number of breast cancer patients [11], which poses a huge disadvantage in delivering therapeutics to bone metastases than to the primary tumors. In addition, the bone marrow perivascular region not only provides metastatic niches for the cancer cells [12], but also shields the tumor cells from therapeutic agents in the circulation. Furthermore, the perivascular stromal cells and the endothelium support the tumor cells with chemo-attractants and pro-growth factors that facilitate homing of cancer cells. One of the key molecules in enhancing tumor cell homing and promoting tumor growth inside the bone marrow is E-selectin [13], a leukocyte adhesion molecule that is expressed only by the endothelial cells in the organ [14]. It interacts with its ligands on leukocytes at the blood vessel wall to modulate the rolling and subsequent adhesion of these cells [13]. A recent study demonstrated that E-selectin is a crucial component of the vascular niche in promoting survival of hematopoietic stem cells after mice were treated with chemotherapy agents or radiation [14]. E-selectin was also demonstrated to be a promising target for tumor delivery of chemotherapy drugs [15,16].

We have previously developed a porous silicon-based multistage vector (MSV) delivery system [17]. Large payloads of therapeutic agents are packaged into liposomes or micelles and loaded into the nanopores of the porous silicon. Once delivered to the tumor site, the silicon carrier slowly degrades into a non-toxic orthosilicic acid and the drug payload gets sustainably released [18]. The system has been successfully applied to deliver siRNA oligos to primary breast cancer [18] and metastatic ovarian cancers [19,20]. We have also developed a thioaptamer (ESTA) that specifically binds to E-selectin [21]. In the current study, we tested the feasibility of delivering therapeutic siRNA by affinity targeting to E-selectin for the treatment of breast cancer bone metastasis in murine xenograft models of human cancers. We evaluated bone marrow accumulation of ESTA-conjugate MSV (ESTA-MSV) in mice bearing bone metastasis from MDA-MB-231 and MCF-7 tumors. We also explored the underlying mechanism of differential accumulation efficiency between the two tumor lines. Finally, we carried out an efficacy study to demonstrate tumor growth inhibition as a result of effective knockdown of STAT3 expression in metastatic MDA-MB-231 cells.

2. Materials and methods

2.1. Cell culture

The MCF-7 human breast cancer cell line was purchased from American Type Culture Collection, and the MDA-MB-231-luc-D3H2LN (MDA-MB-231) breast cancer cell line engineered with luciferase expression was from Caliper Life Sciences. MDA-MB-231 cells were cultured in high-glucose Dulbecco's modified Eagle's minimal essential medium with 10% fetal bovine serum (FBS), 100 units/mL of penicillin, 100 μg/mL of streptomycin (complete DMEM) and 0.05 mg/mL zeocin. MCF-7 cells were cultured in complete DMEM. Human microvascular endothelial cells (HMVEC) were from Life Technologies, and were maintained in Medium 131 supplied with 5% microvascular growth supplement (MVGS, Life Technologies). Cells were incubated at 37 °C with 5% CO_2.

2.2. Fabrication of porous silicon multistage vector particles

Discoidal porous silicon microparticles were fabricated by electrochemical etching of silicon wafer and surface modified with 3-aminopropyltriethoxysilane (APTES) as previously described [22]. E-selectin thioaptamer was chemically conjugated to the APTES using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as a polylinker. Morphologies of the MSV and ESTA-MSV particles were observed with scan electronic microscope (SEM). To determine grafting density of ESTA on MSV particles, 3.75 billion ESTA-MSV particles were dissolved in 3 mL 1 N NaOH solution overnight. Phosphorus concentration was detected with a Varian 720-ES inductively coupled plasma optical emission spectrometer (ICP, Varian, USA). Yttrium was used as internal control.

To evaluate ESTA stability, the aptamer (4 μg) was incubated in 400 μL murine plasma at 37 °C with moderate shaking (500 rpm). Aliquots of samples (20 μL) were collected at different time points. They were mixed with 1% sodium dodecyl sulfate (SDS), and incubated at 95 °C for 5 min to eliminate aptamer–protein interaction. The samples were separated by agarose gel (2%) electrophoresis in 1× TAE buffer containing 1× GelRed dye (Biotium, USA) with a current of 100 V. After electrophoresis, the amount of aptamer was visualized under UV light.

2.3. Preparation of siRNA polyplexes and loading of siRNA into MSV

The polyplexes were prepared with various ratios between nitrogen in cationic polymer and phosphorus in siRNA oligo (N/P ratio). siRNA oligos were first mixed with PEG(5k)2–PEI(10k) (PEG–PEI), and then incubated at 20 °C for 15 min to form the polyplexes. Hydrodynamic diameter and zeta potential of the PEG-PEI/siRNA polyplexes were characterized with a Zetasizer Nano ZS (Malvern Instruments, UK) in 20 mM phosphate buffer (pH 7.4) at 25 °C with a laser wavelength of 633 nm and a scattering angle of 90°. siRNA packaging capacity of PEG-PEI was measured by agarose gel (2%) electrophoresis in 1× TAE buffer containing 1× SYBRsafe (Invitrogen, USA).

To prepare siRNA nanoparticles, scramble (Scr) siRNA (Sigma), STAT3 siRNA (Sigma), or Alexa555-siRNA (Qiagen) was mixed with PEG–PEI polymer at N/P ratio of 15:1. The PEG–PEI/siRNA polyplexes were then loaded into the ESTA-MSV particles by sonication in a water bath for 3 min. ESTA-MSV particles were then spun down at 12,000 rpm for 5 min, and the supernatant containing excess PEG–PEI/siRNA was removed. PEG–PEI/Alexa555-siRNA loaded ESTA-MSV was prepared to calculate polyplex loading capacity, loading efficiency and release pattern. Loading capacity was determined by the difference of fluorescent intensity before and after loading. To measure siRNA release from ESTA-MSV, Alexa555-siRNA loaded ESTA-MSV was incubated in FBS at 37 °C under moderate shaking (500 rpm). Aliquots of samples were taken at different time points, silicon particles were spun down by centrifugation, and fluorescent intensity in the supernatant was measured with a Bio Tek microplate reader at excitation/emission wavelengths 550/570 nm.

2.4. Murine models of human breast cancer bone metastasis

All animal work was done in accordance with a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of The Methodist Hospital Research Institute in Houston, Texas. Female athymic nude mice (6 weeks old) were purchased from the Charles River Laboratories. They were housed in a pathogen-free facility under a 24-hour light-dark cycle, and fed with a pathogen-free diet and water *ad libitum*. To establish murine models of bone metastasis, nude mice were inoculated with 1×10^5 MDA-MB-231 or 5×10^5 MCF-7 cells by intracardiac inoculation. Tumor growth was monitored with a Xenogen IVIS 200 imaging system.

2.5. Histological analysis

Mice with tumor bone metastasis were anesthetized and euthanized at different time points. Femur and spine samples were collected, fixed in 10% formalin, decalcified with 14% ethylenediaminetetraacetic acid (EDTA), and embedded in paraffin. Four-micrometer sections were processed with hematoxylin and eosin (H&E) staining for morphology observation, or with rabbit anti-mouse E-selectin antibody (1:100 diluted, Abcam) or rat anti-mouse CD31 antibody (1:100 diluted, BD Biosciences)

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