Systemic RALA/iNOS Nanoparticles: A Potent Gene Therapy for Metastatic Breast Cancer Coupled as a Biomarker of Treatment

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This study aimed to determine the therapeutic benefit of a nanoparticulate formulation for the delivery of inducible nitric oxide synthase (iNOS) gene therapy in a model of breast cancer metastasis. Nanoparticles comprising a cationic peptide vector, RALA, and plasmid DNA were formulated and characterized using a range of physiochemical analyses. Nanoparticles complexed using iNOS plasmids and RALA approximated 60 nm in diameter with a charge of 25 mV. A vector neutralization assay, performed to determine the immunogenicity of nanoparticle formulations in immunocompetent C57BL/6 mice, revealed that no vector neutralization was evident. Nanoparticles harboring iNOS plasmids (constitutively active cytomegalovirus [CMV]-driven or transcriptionally regulated human osteocalcin [hOC]-driven) evoked iNOS protein expression and nitrite accumulation and impaired clonogenicity in the highly aggressive MDA-MB-231 human breast cancer model. Micrometastases of MDA-MB-231-luc-D3H1 cells were established in female BALB/c SCID mice by intracardiac delivery. Nanoparticulate RALA/CMV-iNOS or RALA/hOC-iNOS increased median survival in mice bearing micrometastases by 27% compared with controls and also provoked elevated blood nitrite levels. Additionally, iNOS gene therapy sensitized MDA-MB-231-luc-D3H1 tumors to docetaxel treatment. Studies demonstrated that systemically delivered RALA-iNOS nanoparticles have therapeutic potential for the treatment of metastatic breast cancer. Furthermore, detection of nitrite levels in the blood serves as a reliable biomarker of treatment.

INTRODUCTION
An obstacle to genetic therapies is the absence of a vector with the DNA delivery ability of a virus that lacks the immunogenicity commonly associated with viral vectors. We have developed a cationic fusogenic peptide vector, RALA, that, on exposure to anionic nucleic acids, self-assembles into nanoscale particles suitable for cell membrane penetration. Endosomal escape, consequent to conformational change at low pH, ensures that the genetic cargo can reach the nucleus and achieve transgene expression. We previously demonstrated the remedial potential of RALA-delivered therapeutic cargoes.

Growth of ZR-75-1 breast cancer xenografts was abrogated by plasmid FK506-binding protein-like (FKBPL), whereas nanocomplexation of anionic bisphosphonates with RALA afforded the agents cytotoxicity against PC-3 prostate cancer cells in vitro and in xenografts following intratumoral injection. In this study, we aimed to provoke a therapeutic benefit in a model of aggressive breast cancer by nanocomplexation of plasmid inducible nitric oxide synthase (iNOS) with RALA.

The paradoxical relationship between nitric oxide (NO) and transformed tissue, whereby low concentrations of the gasotransmitter provoke an aggressive phenotype but higher concentrations are detrimental to the tumor, has led to a divergence in the discipline, with attempts being made to either promote or interfere with NO signaling. The mechanisms by which NO mediates its effects in neoplastic conditions are diverse but can be broadly characterized into promotion (low NO) or inhibition (high NO) of apoptosis, promotion (low) or inhibition (high) of proliferation, and stimulation (low) or attenuation (high) of angiogenesis. NO can react with inorganic molecules (i.e., oxygen, superoxide, or transition metals), structures in DNA, prosthetic groups, or proteins and can elicit beneficial or detrimental responses dependent on radical concentration and local environmental conditions. Host macrophages that infiltrate tumors rely partially on the cytotoxic properties of NO to evoke an anti-tumoral response. The majority of attempts to exploit the tumoricidal properties of NO involve using an NO donor molecule. Many such donors exist and are broadly represented by the organic nitrates, metal-NO complexes, S-nitrosothiols, sylonamines, diazeniumdiolates (NONOates), and NO-drug hybrids. One NO-donating prodrug that has received particular attention is JS-K. JS-K induced apoptosis in a range of

Received 30 November 2016; accepted 8 December 2016.
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breast cancer cell lines but spared normal human microvascular endothelial cells (HMECs) and MCF-10A.7 JS-K was recruited into the National Cancer Institute’s Rapid Access to Interventional Development (RAID) program, accelerating its progression as a potential therapeutic agent.8

As an alternative approach to achieving therapeutic levels of intra-tumoral NO, we,9–14 and others15–17 have demonstrated the benefit of iNOS as a therapeutic transgene. Constitutive iNOS expression abolished clonogenicity in ZR-75-1 breast cancer cells13 and sensitized to cisplatin in human cancer cell lines and murine RIF-1 xenografts9 and in A549 models of human primary and metastatic lung cancer.17 To limit NO release from an iNOS gene therapeutic to target tumors, we have deployed a transcriptional targeting approach using the human osteocalcin (hOC) promoter to drive iNOS expression. The hOC promoter is activated by transcription factors such as Runx2 and Fra-2, which are commonly overexpressed in cancers that metastasize to bone.18 hOC-iNOS-derived NO achieved almost complete elimination of colony-forming ability in PC-3 and DU145 castration-resistant prostate cancer cells and induced stasis in PC-3 xenografts.11,19

The purpose of the current study was to determine whether cationic RALA-based nanoparticles (NPs) carrying an iNOS transgene had a therapeutic effect in mice bearing MDA-MB-231 (known to be sensitive to the NO donor diethylenetriamine (DETA)/NO through generation of dinitrogen trioxide)20 micrometastases.

RESULTS

Nanoparticle Characterization

Incubation of RALA with plasmid DNA in water resulted in the formation of nanoparticles suitable for cellular internalization (Figure 1A).1,2,21 Subcellular Nanoparticle Localization

Labeling with Cy3 did not affect the physicochemical properties of nanoparticles (Figure 1B). The ability of RALA to deliver Cy3-labeled pEGFP-1 nanoparticles to the nuclei of MDA-MB-231-luc-D3H1 cells was confirmed by confocal fluorescence microscopy using orthogonal sectioning (to construct XZ and YZ images to correspond to an area of interest in an XY image following collection of a z stack of images). By 60 min following commencement of transfection, Cy3 fluorescence was evident within the confines of

Figure 1. Complexation of Plasmid DNA with RALA Produces Nanoparticles Suitable for Cellular Delivery

(A) Incubation of plasmid DNA with RALA resulted in nanoparticles that did not exceed 100 nm in diameter, with a positive charge of approximately 20–25 mV. (B) Cy3-labeled DNA forms nanoparticles with RALA that resemble those formed with unlabeled DNA. Data points represent mean ± SD. n ≥ 3. (C) Orthogonal sectioning of z stacks of MDA-MB-231-luc-D3H1 cells transfected with RALA/Cy3-pEGFP-1. RALA delivers plasmid DNA to the nuclei of MDA-MB-231-luc-D3H1 cells within 120 min. Green, actin cytoskeleton; blue, nucleus; red, Cy3.
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