Vascular Targeting of a Gold Nanoparticle to Breast Cancer Metastasis

PUBUDU M. PEIRIS,[1,2,3] PARTHA DEB,[1,2,3] ELIZABETH DOOLITTLE,[1,2,3] GILAD DORON,[1,3] AMY GOLDBERG,[1,3] PRIYA GOVENDER,[1,3] SHRUTI SHAH,[1,3] SWETHA RAO,[1,3] SARAH CARBONE,[1,3] THOMAS COTEY,[1,3] MEILYN SYLVESTRE,[1,3] SOHAJ SINGH,[1,3] WILLIAM P. SCHIEMANN,[4] ZHENGHONG LEE,[2,3,4] EFSTATHIOS KARATHANASIS[1,2,3,4]

[1]Department of Biomedical Engineering, Case Western Reserve University, Cleveland, Ohio
[2]Department of Radiology, Case Western Reserve University, Cleveland, Ohio
[3]Case Center for Imaging Research, Case Western Reserve University, Cleveland, Ohio
[4]Case Comprehensive Cancer Center, Case Western Reserve University, Cleveland, Ohio

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ABSTRACT: The vast majority of breast cancer deaths are due to metastatic disease. Although deep tissue targeting of nanoparticles is suitable for some primary tumors, vascular targeting may be a more attractive strategy for micrometastasis. This study combined a vascular targeting strategy with the enhanced targeting capabilities of a nanoparticle to evaluate the ability of a gold nanoparticle (AuNP) to specifically target the early spread of metastatic disease. As a ligand for the vascular targeting strategy, we utilized a peptide targeting alpha(v) beta(3) integrin, which is functionally linked to the development of micrometastases at a distal site. By employing a straightforward radiolabeling method to incorporate Technetium-99m into the AuNPs, we used the high sensitivity of radionuclide imaging to monitor the longitudinal accumulation of the nanoparticles in metastatic sites. Animal and histological studies showed that vascular targeting of the nanoparticle facilitated highly accurate targeting of micrometastasis in the 4T1 mouse model of breast cancer metastasis using radionuclide imaging and a low dose of the nanoparticle. Because of the efficient targeting scheme, 14% of the injected AuNP deposited at metastatic sites in the lungs within 60 min after injection, indicating that the vascular bed of metastasis is a viable target site for nanoparticles.
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INTRODUCTION

Nanoparticles provide many potential benefits and new opportunities to address the complexity of micrometastasis. To date, though, applications of nanotechnology have mainly focused on primary tumors. While conventional agents with small molecular weight are distributed within cancer and healthy tissues in a non-specific manner, nanoparticles have been developed to exploit the leaky vasculature of primary tumors to enhance the intratumoral delivery due to the so-called "enhanced permeability and retention" (EPR) effect.[1] However, the early spread of cancer cells at metastatic sites lacks leaky vasculature for targeting by passive mechanisms. Although the EPR strategy may be effective in well-vascularized tumors of several millimeters in diameter, it is ineffective against micrometastasis, which presents small clusters of malignant cells within variable tissue types.[2] Micrometastasis is defined as cohesive deposits of tumor cells of 2 mm or less but larger than 0.2 mm. Thus, to design a nanoparticle with high sensitivity and specificity, targeting schemes must be devised according to the unique microenvironment of the target site.[3]

Targeting occult micrometastases hidden within a large population of normal cells presents a unique challenge. Micrometastases are nearly inaccessible to molecular or nanoscale agents because of several biobarriers, including tiny size and high dispersion to organs compared with primary or metastatic tumors, making them nearly inaccessible. However, micrometastatic disease upregulates specific cell-surface mol-ecules that differ from the rest of the host organ. It is well known that extravascular metastases are preceded by metastatic cancer cells residing inside the lumen of blood vessels.[4,5] Circulating tumor cells use the adhesion molecules of the leukocyte adhesion cascade to attach to the endothelium at the site of future metastasis. After the initial colonization, intravascular micrometastases continue to proliferate by producing their own microenvironment.[4–7] Specifically, integrins are functionally linked to the early development of metastatic disease at a distal site.[4,8] Following the initial adhesion onto endothelium,[9–13] the micrometastatic site transitions from P-selectin-dependent cell rolling on the endothelium to firm attachment that is mediated by $\alpha_v\beta_3$ integrin on both cancer and endothelial cells. Overall, tumor cell $\alpha_v\beta_3$ integrin promotes adhesion, migration, and invasiveness in cooperation with platelets.[14,15] Although $\alpha_v\beta_3$ integrin mediates the adhesion of cells to a large number of extracellular matrix proteins, it is minimally expressed on normal resting blood vessels.[16–18] Thus, $\alpha_v\beta_3$ integrin-mediated vascular targeting can be highly specific toward blood vessels associated with micrometastases.[19,20]

To circumvent the limitations of today's agents in targeting micrometastatic disease, we combined a vascular targeting strategy with the enhanced targeting capabilities of a gold nanoparticle (AuNP) as illustrated in Figure 1. First, vascular targeting of micrometastasis may be more effective than deep

Correspondence to: Efstathios Karathanasis (Telephone: +216-844-5281; Fax: +216-844-4987; E-mail: stathis@case.edu)

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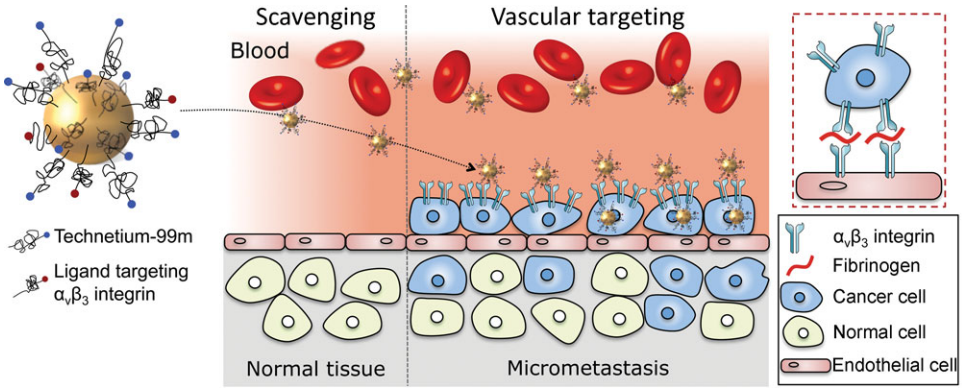

Figure 1. Illustration of the vascular targeting strategy of a AuNP radiotracer to metastatic sites. Inset: Interactions of circulating tumor cells and vascular bed.

tissue targeting, which requires the EPR effect as a prerequisite. Most notably, the size of nanoparticles makes them ideal for vascular targeting because of their significantly increased targeting avidity (i.e., latching on vascular target) resulting in geometrically enhanced multivalent attachment on the vascular bed.[19] This work shows that AuNP can effectively gain access to and be deposited at micrometastatic sites. The target site of the selected vascular targeting strategy involved $\alpha_v\beta_3$ integrin on metastatic foci resident in blood vessels as well as $\alpha_v\beta_3$ integrin on the remodeled endothelium of micrometastasis (inset in Fig. 1). Second, we employed an efficient radiolabeling method to incorporate Technetium-99m into the AuNPs for single-photon emission computed tomography (SPECT). As accurate detection requires sufficient signal difference between the lumen of the blood vessels and the blood vessel walls (i.e., target site), imaging must be delayed for days after injection of a long-circulating nanoparticle to allow the agent to clear from the bloodstream.[19,21] To decrease "interfering signals," we identified a low dose of the agent that generated significantly detectable signal on the tumor-associated vascular bed and insignificant background "noise" due to the blood circulation of the AuNP radiotracer or its non-specific uptake in various organs (e.g., liver and spleen). In this work, the combination of these complementary features facilitated accurate targeting of micrometastasis in the 4T1 mouse model of breast cancer metastasis using a low dose of the nanoparticles.

EXPERIMENTAL

Materials

The 4T1-GFP-luc cell line was received as a gift from Dr. Ruth Keri (Case Western Reserve University, Cleveland, Ohio). Female Balb/c mice were purchased from Charles River (Wilmington, Massachusetts). The primary antibody for the specific endothelial antigen CD31 was purchased from BD Biosciences Pharmingen (San Diego, California). Secondary antibodies and cell culture media were obtained from Invitrogen (Carlsbad, California). The cyclo (Arg-Gly-Asp-D-Phe-Cys) or c(RGDfC) peptide was purchased from Peptides International Inc. (Louisville, Kentucky). The bifunctional chelator 2-(4-isothiocyanatobenzyl) -diethylenetriaminepentaacetic acid (*p*-SCN-Bn-DTPA) was obtained from Macrocyclics (Dallas, Texas). The cross-linker sulfosuccinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) was obtained from Thermo Fisher Scientific (Cleveland, Ohio). Polyethylene glycol (PEG) conjugates were purchased from Laysan Bio (Arab, Alabama). General solvents and chemicals were obtained from Thermo Fisher Scientific.

Synthesis and Characterization of AuNPs

Gold nanoparticles were synthesized using the Brust-Schiffrin method with some modification.[22] Briefly, $HAuCl_4$ solution (367 μL) and tetraoctylammonium bromide (0.1367 g) were dissolved in toluene at room temperature. Dodecylamine (0.112 g) was added and stirred at room temperature for 10 min. A reducing agent, $NaBH_4$ solution in ice-cold water was added slowly in to the reaction mixture. Following the addition of $NaBH_4$, the solution turned from orange to wine red solution. The resulting AuNPs were precipitated with ethanol, centrifuged at 4,700 g for 15 min, and dried under nitrogen for 30 min. Upon dissolving the nanoparticles in chloroform, SH-PEG-NH_2 was added in large excess and allowed to react for 2 days at room temperature. Chloroform was removed by air-drying. The AuNPs were suspended in water and the excess PEG was removed by a 2-day dialysis against water.

The AuNPs were characterized in terms of their size and structure using dynamic light scattering (DLS; Brookhaven Instruments, Holtsville, NY, USA) and transmission electron microscopy (TEM; Tecnai F30; FEI, Hillsboro, OR, USA). The concentration of gold was determined *via* inductively coupled plasma optical emission spectrometry (ICP-OES, Optima 7000 DV; PerkinElmer, Waltham, MA, USA) and UV spectroscopy based on AuNP's absorption at 520 nm (Synergy HT; BioTek Instruments, Winooski, VT, USA).

Functionalization of Nanoparticles

The amines on the distal end of the PEG on the nanoparticle's surface were used to functionalize AuNP with the c(RGDfC) peptide and the DTPA chelating agent. As shown in Figure 2, step 1 consisted of the conjugation of about 50 DTPA molecules per AuNP particle. Briefly, the bifunctional chelator, *p*-SCN-Bn-DTPA agent (25 μg), was conjugated to a fraction of the surface amines on AuNP (80 pmol) in saline solution for 6 h. Excess DTPA was removed by a 1-day dialysis against saline solution. In step 2, the thiol of the cysteine residue on the c(RGDfC) peptide (15 μg) was used to conjugate the peptide to the remaining