Journal of Controlled Release 161 (2012) 959–966

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

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

NANOMEDICINE

Receptor-mediated transcytosis: A mechanism for active extravascular transport of nanoparticles in solid tumors

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ARTICLE INFO

Article history:
Received 6 April 2012
Accepted 5 May 2012
Available online 14 May 2012

Keywords:
Nanoparticles
Melanocortin type-1 receptor (MC1R)
Transcytosis
Agonists
Antagonists

ABSTRACT

Targeted nanoparticle-based delivery systems have been used extensively to develop effective cancer theranostics. However, how targeting ligands affect extravascular transport of nanoparticles in solid tumors remains unclear. Here, we show, using B16/F10 melanoma cells expressing melanocortin type-1 receptor (MC1R), that the nature of targeting ligands, i.e., whether they are agonists or antagonists, directs tumor uptake and intratumoral distribution after extravasation of nanoparticles from tumor vessels into the extravascular fluid space. Pegylated hollow gold nanospheres (HAuNS, diameter = 40 nm) coated with MC1R agonist are internalized upon ligand–receptor binding, whereas MC1R antagonist-conjugated HAuNS remain attached on the cell surface. Transcellular transport of agonist-conjugated HAuNS was confirmed by a multilayer tumor cell model and by transmission electron microscopy. MC1R agonist- but not MC1R antagonist-conjugated nanoparticles exhibit significantly higher tumor uptake than nontargeted HAuNS and are quickly dispersed from tumor vessels via receptor-mediated endocytosis and subsequent transcytosis. These results confirm an active transport mechanism that can be used to overcome one of the major biological barriers for efficient nanoparticle delivery to solid tumors.

1. Introduction

Many targeted nanoparticle-based delivery systems have been developed with the goals of enhancing tumor-specific uptake of nanoparticles, reducing systemic toxicity, and increasing the efficacy of anticancer therapies. However, targeting of nanoparticles to tumor cells, although extremely appealing in this era of personalized medicine, is challenging because of the presence of a number of biological barriers [1]. Furthermore, there has been a lack of comprehensive study of the various factors that contribute to the tumor uptake efficiency of "active targeting" strategies, in which tumor-specific ligands are used to direct nanoparticles to tumor cells [2]. Pirollo and Chang [2] argue that in some ligand-conjugated nanoparticle systems, tumor uptake may be due at least in part to the enhanced permeability and retention (EPR) effect of long-circulating nanoparticles, which raises the question of whether true targeted delivery has been achieved with many of the purported active targeting nanoparticles. Using 3 different targeting schemes, Huang et al. [3] showed that targeting ligands only marginally improve the total accumulation of gold nanorods in xenograft tumor models in comparison with nontargeted controls. Similar observations have been made with other nanoparticle polymers, liposomes, and gold nanoparticles [4–6]. In a study of epithelial growth factor receptor-targeting hollow gold nanospheres (HAuNS), most nanoparticles were distributed to the perivascular region [6]. These data suggest that efficient tumor delivery of targeted nanoparticles is limited by dispersion of nanoparticles in tumor interstitium.

Here, we report that an active transport mechanism, i.e., receptor-mediated transcytosis, can facilitate extravascular transport of nanoparticles and thus can effectively enhance delivery of nanoparticles within the tumor volume. Agonist and antagonist ligands of membrane receptors behave differently upon binding to their targets. An agonist fully activates the receptor upon ligand–receptor interaction and receptor internalization, while an antagonist does not provoke a biological response itself upon binding to a receptor but blocks or dampens agonist-mediated responses. We hypothesized that the nature of targeting ligands attached to the surface of nanoparticles, i.e., whether the ligands are agonists or antagonists, affects extravascular transport and thus the tumor-targeting efficiency of the nanoparticles. To test this hypothesis, we selected melanocortin type-1 receptor (MC1R), one of the five subtypes of melanocortin receptors, as a target. MC1R is overexpressed in melanoma cells [7,8]. Molecular mechanism studies on all subtypes of melanocortin receptors have shown that receptor

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doi:10.1016/j.jconrel.2012.05.014

desensitization, internalization, and downregulation are different with agonists than with antagonists [9,10]—specifically, melanocortin receptor agonists lead to receptor internalization upon binding, but melanocortin receptor antagonists do not [9,10]. Therefore, nanoparticle-based delivery systems with MC1R agonists and those with MC1R antagonists may have different effects on the transport of nanoparticles in the extravascular space.

2. Materials and methods

2.1. Conjugation of MC1R agonists and antagonists to HAuNS

HAuNS were synthesized according to our previous report [6,11]. The MC1R agonist (Ago) and antagonist (Ant) peptides were synthesized manually using Rink amide resin and N^{α}-fluorenylmethyloxycarbonyl chemistry (see Supplementary data). Both MC1R agonists and antagonists as targeting moieties were linked to HAuNS through poly(ethylene glycol) (PEG) linker (Fig. 1A, Supplementary data). For fluorescence imaging, the HAuNS were labeled with tetramethylrhodamine–PEG_{5000}–thioctic acid (TA–PEG–TMR, Fig. S1). For quantitative analysis and micro-positron emission tomography (microPET) imaging, the above-described HAuNS were labeled with the positron emitter ^{64}Cu ($t_{1/2} = 12.7$ h) according to previously reported procedure [12].

2.2. Receptor binding assay

Competitive binding experiments were carried out using murine B16/F10 melanoma cells (ATCC) as previously reported [13]. B16/F10 cells were seeded on 24-well plates 48 h before assay (20,000 cells/well). The cell culture medium was aspirated, and cells were washed twice with a freshly prepared binding buffer containing DMEM/F12 medium, 25 mM HEPES (pH 7.4), and 0.2% bovine serum albumin (BSA). For peptide competition, cells were incubated with different concentrations of unlabeled Ago or Ant and labeled [^{125}I]-NDP-α-MSH (0.1 μCi/well, Perkin-Elmer Life Science, Waltham, MA) for 40 min at 4 °C. For particle competition, different concentrations of unlabeled HAuNS conjugates were used with [^{125}I]-NDP-α-MSH. For nonspecific binding, an excessive amount of Ago, i.e., 200 μg/ml NDP-α-MSH, was used. After incubation, the assay medium was removed, and each well was washed 3 times with the binding buffer. The cells were then lysed by the addition of 250 μl of CelLytic M cell lysis reagent (Sigma). The radioactivity of the lysis solution was measured using a Packard Cobra gamma counter. IC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least squares analysis using GraphPad Prism 5 software (GraphPad).

2.3. Immunofluorescence microscopy

For visualization of intracellular translocation of MC1R, transient transfection of plasmid construct encoding GFP-tagged open reading frame clone of *Homo sapiens* MC1R (Origene) was carried out using Lipofectamine 2000 reagent (Invitrogen) as recommended by the manufacturer. Briefly, human embryonic kidney (HEK) 293 cells (ATCC) were seeded on 100-mm plates 1 day before transfection. The plasmid DNA encoding gene comprised of *green fluorescent protein* (*GFP*) and *MC1R* (5 μg) was mixed with Lipofectamine 2000 reagent in serum-free medium, incubated at room temperature for 30 min, and then added to the cells. Approximately 4 h after the addition of the plasmid DNA, the transfection mixture was replaced with DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS). The cells were then incubated for an additional 24 h. The transfection efficiency was examined under a fluorescence microscope and was found to be greater than 95%.

The MC1R-GFP-transfected HEK 293 cells were trypsinized and seeded (1×10^4) in an 8-well Lab-Tek II chambered coverglass (Thermo Fisher Scientific) 48 h before the experiment. The cells were incubated with different tetramethylrhodamine-labeled HAuNS conjugates (2×10^9 nanoparticles/ml) for 20 min at 37 °C with or without the presence of 200 μg/ml free Ant (blocking). After washing in PBS, the cells were directly visualized under an Olympus Fluoview FV1000 confocal laser scanning microscope (FV1-ASW, Olympus) equipped with a fluorescein isothiocyanate filter for MC1R-GFP and a rhodamine filter for nanoparticles.

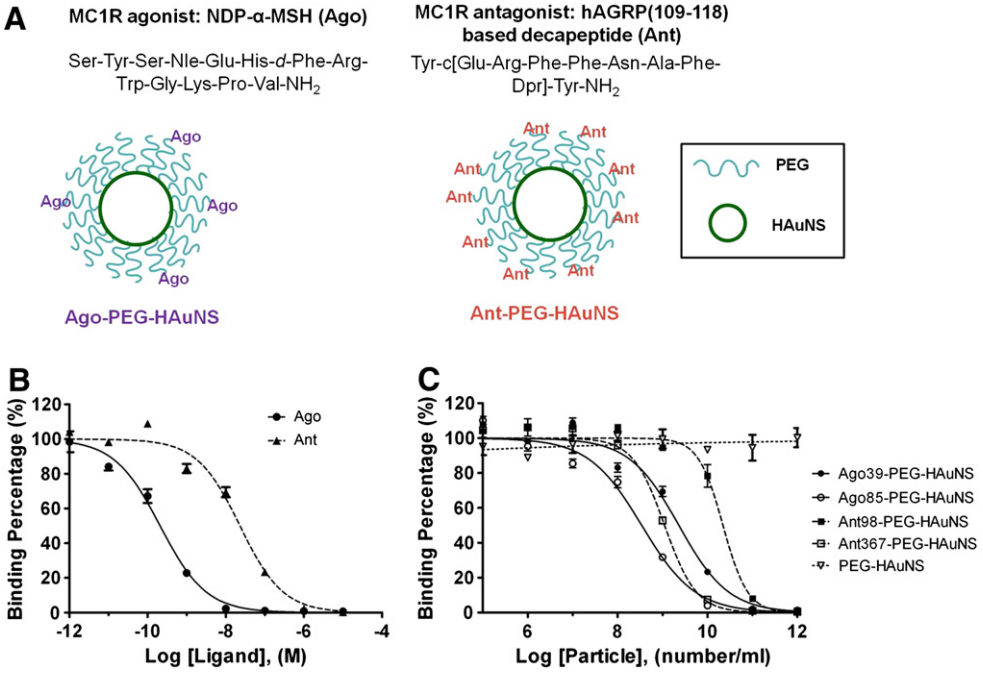

Fig. 1. (A) Chemical structure of MC1R agonist (Ago) and antagonist (Ant) and schemes of bioconjugation. (B) Competitive binding assay comparing ^{125}I-NDP-α-MSH with unlabeled Ago or Ant. (C) Competitive binding assay comparing ^{125}I-NDP-α-MSH with unlabeled Ago- or Ant-conjugated pegylated HAuNS. Ago39 and Ago85 had an average of 39 and 85 agonists, respectively, conjugated per nanoparticle. Ant98 and Ant367 had an average of 98 and 367 antagonists, respectively, conjugated per nanoparticle.