Targeting Nanoparticles to Tumors using Adenoviral Vectors

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ABSTRACT

Development of novel therapies remains essential for treatment of cancer; in this regard, nanotechnology holds great promise. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs), and novel tumor treatment opportunities are exemplified by the use of gold nanoparticles (AuNPs). However, for all these applications of metal nanoparticles, selective tumor targeting is crucial for successful clinical application. Considering the progress made in targeting adenoviral (Ad) gene therapy vectors to tumors, we herein aim to couple metal nanoparticles to targeted Ad vectors to achieve selective tumor accumulation. We demonstrate that metal nanoparticles such as QDs and AuNPs can indeed be coupled to Ad vectors, without compromising viral infectivity, retargeting ability or function of the nanoparticles. This innovative combination strategy is therefore expected to lead to the development of a unique methodology for cancer detection and treatment.

Keywords: adenovirus, quantum dots, gold nanoparticles, targeting, imaging

1 INTRODUCTION

Despite advances in detection and treatment of cancer, development of novel therapies remains essential in the continuing battle against this disease. In this regard, nanotechnology holds great promise for the detection and treatment of cancer. For example, tumor imaging opportunities have expanded by the development of quantum dots (QDs) for fluorescence based detection [1], or magnetic nanoparticles for magnetic resonance imaging applications [2]. Novel tumor treatment opportunities are exemplified by the use of gold nanoparticles, which upon laser irradiation will heat up and kill neoplastic cells via hyperthermia [3,4]. However, for all these applications of metal nanoparticles, selective tumor localization is crucial for successful clinical application.

In this respect, great progress has been made in targeting gene therapy vectors to tumors. In particular, a virus that causes the common cold – adenovirus (Ad) – has been used in targeted gene therapy for cancer [5]. For example, our laboratory has developed bi-functional adapter molecules, which bind with one domain to the virus and to tumor-associated antigens (TAAs) with the other. We have previously established that these adapter molecules are able to mediate Ad vector targeting to TAAs *in vitro* and to TAAs expressed in the pulmonary vasculature after systemic administration *in vivo* [6]. Importantly, it has also recently been demonstrated that the utility of adapter molecules extends to Ad vectors targeted to TAA-expressing tumors and hepatic metastases, even when delivered systemically (Dr. H.R. Herschman, UCLA, personal communication, manuscript submitted). We therefore aim to couple metal nanoparticles to Ad vectors that are targeted to tumor cells using bi-functional adapter molecules, in order to achieve their selective tumor accumulation. This combination of novel nanotechnology developments with gene therapy targeting strategies is expected to lead to the development of a multi-pronged approach for cancer detection and treatment.

2 EXPERIMENTAL SECTION

2.1 Cell Culture

HEK-293 cells were obtained from Microbix (Toronto, Canada), MDA-MB-361 cells were obtained from ATCC (Manassas, VA, USA) and MC38 cells stably transfected with carcinoembryonic antigen (CEA), MC38-CEA-2, were kindly provided by Dr. Jeffrey Schlom, National Cancer Institute (Bethesda, MD). All cells were maintained in DMEM:Ham's F12 (1:1 v/v, Mediatech, Herndon, VA) medium, containing 10% fetal bovine serum (Hyclone, Logan, UT), 2 nM L-glutamine, 100 IU/mL penicillin and 25 μg/mL streptomycin (all Mediatech). Medium for MC38-CEA-2 cells additionally contained 500 ug/mL G418 (Mediatech). Cells were grown in a humified atmosphere with 5% CO2 at 37 °C.

2.2 Construction, Production and Purification of Bi-Functional Adapter Molecules

Bi-functional fusion proteins capable of retargeting Ad vectors to either the tumor-associated antigen carcinoembryonic antigen (CEA) or c-erbB2 (HER2/neu) were constructed, consisting of the ectodomain of CAR including its own leader sequence (aa 1-236), followed by a 5-aa peptide linker (GGPGS), a 6-histidine tag (for detection/purification), followed by either the anti-CEA single chain antibody MFE-23 (a kind gift from Dr. Kerry Chester, London, UK) or the anti-c-erbB2 antibody C6.5 (provided by Dr. J.D. Marks, Department of Anesthesia and Pharmaceutical Chemistry, University of California, San Francisco, CA). To construct sCAR-MFE and sCAR-C6.5, first, cDNA encoding sCAR followed by the 6-his tag was amplified from pFBsCAR6hTf [7], introducing a HindIII (5') while maintaining the BamHI (3') restriction site. Second, the scFvs MFE-23 and C6.5 were amplified by PCR introducing a BamHI (5') and XhoI (3') restriction site. Both sCAR and scFv PCR products were simultaneously ligated into the pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA), digested with HindIII and XhoI restriction enzymes, thereby constructing pcDNA/sCAR/6h/MFE and pcDNA/sCAR/6h/C6.5. The constructed plasmids were verified by sequencing. HEK-293 cells were stably transfected with PvuI linearized plasmid using Superfect transfection reagent (Qiagen,Valencia, CA, USA), and clones were selected for high production and secretion of protein in the supernatant. After expansion of a positive clone, media was collected and protein was purified by immobilized metal-affinity chromatography (Ni-NTA Superflow, Qiagen), followed by dialysis against PBS.

2.3 Adenoviral Vectors

For labeling Ad vectors with quantum dots we utilized a virus with a biotin acceptor peptide genetically incorporated into the hexon capsid protein, generously provided by Dr. Michael A. Barry, Baylor College of Medicine [8]. This virus is metabolically biotinylated upon replication, allowing the coupling of streptavidin-labeled molecules, particles or complexes. For labeling Ad vectors with gold nanoparticles we utilized a virus with a six-histidine motif genetically incorporated into the hexon capsid protein, generously provided by Dr. Hongju Wu, University of Alabama at Birmingham [9], allowing coupling of Ni-NTA-labeled molecules, particles or complexes. To produce the viruses, HEK-293 cells were infected using medium containing 2% fetal bovine serum; following overnight incubation regular 10% medium was added to the cells and incubated until a total cytopathic effect was observed. Cells were harvested, frozen and thawed four times, and virus was purified using standard CsCl purification methods. Viral particle number was determined by measuring absorbance at 260nm using a conversion factor of 1.1×10^{12} viral particles per absorbance unit [10].

2.4 Labeling Ad Vectors with Quantum Dots

QDs labeled with streptavidin on their surface (655 nm, Invitrogen, Carlsbad, CA) were incubated with Ad vectors expressing biotin molecules on their surface in a QD:Ad ratio of 1250 (mole:particle), before being added to the c-erbB2-expressing MDA-MB-361 breast cancer cells. Cells were plated the prior day in 2-well Lab-Tek™ Chamber Slides™ (Nalge Nunc International, Rochester, NY) at a concentration of 25,000 cells per well. The Ad-QD complex (MOI 5,000 particles/cell) was targeted to c-erbB2 by adding a final concentration of 1 ug/mL of the previously described bi-functional adapter molecule sCAR-C6.5 to the reaction mixture [11]. The Ad-QD-sCAR-C6.5 complexes, or QDs by themselves, were incubated with cells for 30 min at 4 °C, after which unbound complexes were removed via washing. Cells were subsequently incubated at 37 °C for 30 minutes. Cells were then washed, fixed in neutral-buffered formalin, washed again, embedded in 90% glycerol and imaged utilizing Dual Mode Fluorescence (CytoViva Inc, Auburn, AL).

2.5 Labeling Ad Vectors with Gold Nanoparticles

Ni-NTA-labeled gold nanoparticles (AuNP; Nanoprobes, Yaphank, NY) were incubated with Ad vectors (1×10^{12} viral particles total) presenting a six-histidine motif on their surface and carrying luciferase as a transgene in a AuNP:Ad ratio of 2,000 (particle:particle). As a control, AuNP were incubated with Ad vectors lacking a six-histidine motif and Ad vectors were incubated without AuNP present. AuNP-labeled Ad vectors were separated from unreacted reagents in a CsCl density gradient. Viral particle number was again determined as described above.

To assess Ad retargeting, CEA-expressing MC38-CEA-2 colon cancer cells cells were plated in triplicate at a density of 1×10^5 cells/well in 24-well plates. The following day, 1×10^7 viral particles (MOI 100 particles/cell) were incubated for 15 min at room temperature with 75 ng of the previously described sCAR-MFE [6], before being added to the cells in medium containing 2% fetal bovine serum. After 2 hours of incubation, medium containing the virus was removed and replaced with regular growth medium. Cells were incubated for an additional 22 hours and were subsequently washed with PBS and lysed using Reporter Lysis Buffer (Promega, Madison, WI). After one freeze-thaw cycle, luciferase activity was measured using the Luciferase Assay System (Promega), according to manufacturer's instructions.

3 RESULTS & DISCUSSION

We herein aimed to demonstrate that targeted Ad vectors can serve as a platform for tumor-selective delivery of metal nanoparticles, providing either imaging or therapeutic properties, or both. This would allow a potential combination of nanotechnology and gene therapy approaches for the imaging and treatment of cancer. We therefore analyzed whether delivery of nanoparticles inside tumor cells was feasible, and whether infection of tumor cells with nanoparticle-labeled Ad vectors would still result in transgene expression.

First, we coupled streptavidin-labeled QDs to biotinylated Ad vectors, and analyzed cellular uptake of the complexes upon targeting to the tumor associated antigen c-erbB2 using the bi-functional protein sCAR-C6.5. In contrast with untargeted QDs (Figure 1A), targeted Ad-QD complexes were taken up by c-erbB2 expressing cells and clearly visible in intracellular compartments (Figure 1B). This indicates the potential of targeted Ad vectors to carry nanoparticles inside tumor cells, where they can function as imaging or therapeutic agents.

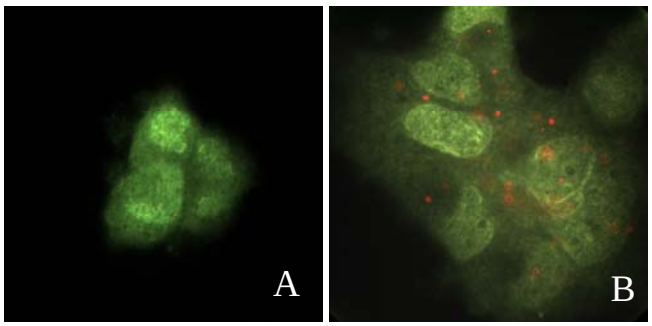

Figure 1: Dual Mode Fluorescence imaging of MDA-MB-361 cells, incubated with either (A) streptavidin-labeled QDs alone or (B) streptavidin-labeled QDs coupled to c-erbB2-targeted Ad vectors. Targeting the QDs to tumor cells utilizing the Ad platform resulted in a clear punctate pattern of red fluorescence, indicating their intracellular presence.

Next, we coupled Ni-NTA-labeled AuNPs to Ad vectors expressing a six-histidine tag in the hexon capsid protein. An increase of the density of Ad vectors in a CsCl gradient demonstrated the successful coupling of Ni-NTA-labeled AuNP to six-histidine labeled Ad vectors (Figure 2C, thick arrow), whereas the similar density of the control Ad (Figure 2A) and the Ad vector without a six-histidine tag but incubated with AuNP (Figure 2B), indicates that no unspecific interaction occurs between Ad and AuNP (thin arrow).

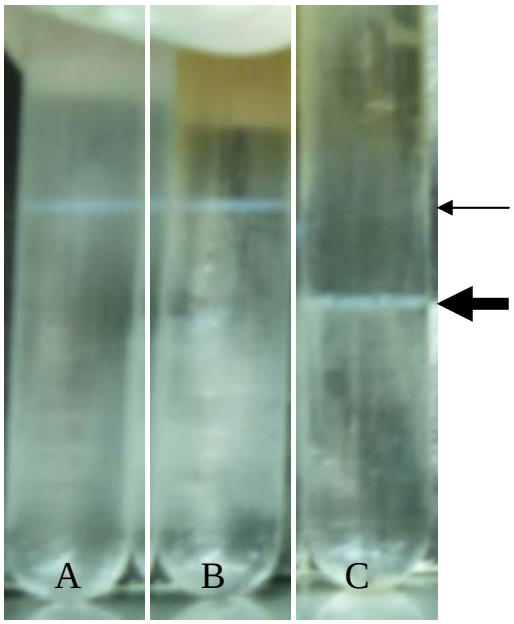

Figure 2: Photographs of CsCl density gradient centrifugation of (A) Ad vectors alone, (B), Ad vectors without a 6-His tag but with Ni-NTA AuNP and (C) Ad vectors labeled with 6-His in hexon, coupled to Ni-NTA-AuNP.

After successful coupling of AuNP to Ad vectors was demonstrated by the increase in density in a CsCl gradient, we analyzed the ability of the Ad vector to target the tumor-associated antigen carcinoembryonic antigen (CEA) and express the transgene it encodes.

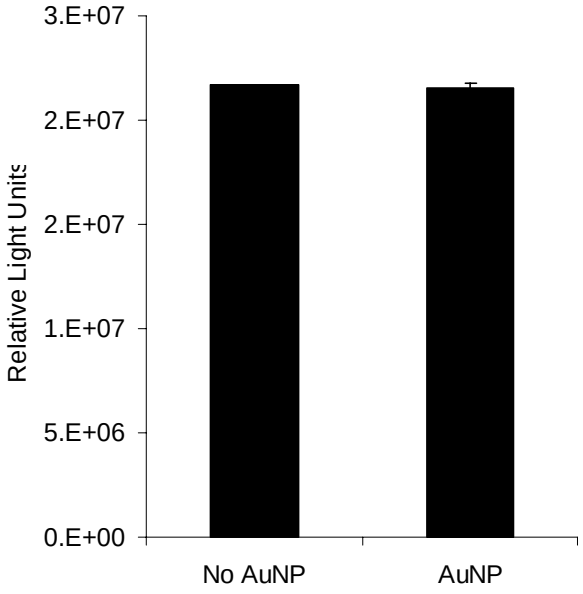

Figure 3: Luciferase expression in MC38-CEA-2 cells, 24 hours after infection by Ad vectors incorporating a 6-his tag in hexon, either without (left) or with (right) Ni-NTA AuNP coupled to their surface. Bars represent mean ± sd.

This is particularly important if gene therapy and nanotechnology will be used as synergistic therapeutic approaches within one multifunctional nanoscale system. Since the AuNPs were selectively coupled to the hexon capsid protein of the virus, which is not important for the viral retargeting and infection pathway, it was anticipated that transgene expression would not be reduced upon nanoparticle coupling. As expected, luciferase analysis indeed demonstrated that AuNP coupling to Ad did not negatively affect virus infectivity and retargeting ability to CEA-expressing MC38-CEA-2 cells (Figure 3). This is a significant improvement on coupling methods employed thus far, where AuNP were non-specifically coupled to lysine residues present in all capsid proteins, resulting in reduced infection and retargeting abilities of Ad at high ratios of AuNP:Ad [12].

4 CONCLUSION

The presented data demonstrates the feasibility of coupling metal nanoparticles to targeted Ad vectors. Importantly, Ad vector infectivity and retargeting ability were retained upon nanoparticle coupling. Therefore, Ad can provide a versatile platform for selective binding of nanoparticles, resulting in a multifunctional agent capable of simultaneous targeting and treatment of cancer by utilizing gene therapy and nanotechnology approaches. This will provide new opportunities for the diagnosis and treatment of tumors that are refractory to currently available classical therapeutic interventions.

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