Effects of Photoacoustic Imaging and Photothermal Ablation Therapy Mediated by Targeted Hollow Gold Nanospheres in an Orthotopic Mouse Xenograft Model of Glioma

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Abstract
Advancements in nanotechnology have made it possible to create multifunctional nanostructures that can be used simultaneously to image and treat cancers. For example, hollow gold nanospheres (HAuNS) have been shown to generate intense photoacoustic signals and induce efficient photothermal ablation (PTA) therapy. In this study, we used photoacoustic tomography, a hybrid imaging modality, to assess the intravenous delivery of HAuNS targeted to integrins that are overexpressed in both glioma and angiogenic blood vessels. Mice were then treated with near-infrared laser, which elevated tumor temperature by 20.7°C. We found that PTA treatment significantly prolonged the survival of tumor-bearing mice. Taken together, these results show the feasibility of using a single nanostructure for image-guided local tumor PTA therapy with photoacoustic molecular imaging. Cancer Res; 71(19); 6116–21. ©2011 AACR.

Introduction
With nanotechnology, it is possible to create multifunctional nanostructures capable of mediating diagnostic imaging, treatment, and monitoring of therapeutic response. For example, pegylated gold nanorods were used for both X-ray computed tomography and photothermal ablation (PTA; ref. 1). This approach, sometimes referred to as “theranostics,” holds great promise for cancer diagnosis and therapy. Photoacoustic tomography (PAT) is a hybrid imaging modality that detects absorbed photons ultrasonically through the photoacoustic effect (2). PAT is capable of detecting optical contrast agents with high sensitivity and specificity (3–5). With the metal nanocomposites, photoacoustic imaging and temperature measurement for photothermal cancer therapy was investigated in tissue-mimicking phantoms and excised animal tissues (6).

Nanoparticle synthesis
HAuNS were synthesized as previously described (10). Cyclic RGD peptide c(KRGDf) was synthesized manually by PL-DMA resin and a–Fmoc chemistry (11). Cyclic RGD peptides have been used for imaging and treatment of glioma (4, 8, 9) because of their high binding affinity to integrin receptors such as αvβ3 receptors (IC50 = 2–40 nmol/L; refs. 12, 13). The peptide was conjugated to one terminus of a heterofunctional polyethylene glycol (PEG) precursor, N-hydroxysuccinimidy-PEG-S-acetyloxyacetate (NHS-PEG-SATA; molecular weight 5,000), through its ε-amino of lysine residue and activated ester in NHS-PEG-SATA. The sulfhydryl group on the other terminus of c(KRGDF)-PEG-SATA was released by treatment with...
mice were sacrificed by overexposure to CO\textsubscript{2}. Open-skull projection reconstruction algorithm. After data acquisition, were reconstructed by using modified delay-and-sum back-acquired 24 hours after nanoparticle injection. Imaging data per mouse). Contrast-enhanced photoacoustic images were presented as mean in injection of HAuNS. Data are (0 hour) and 24 hours after contralateral brain in mice before (bar, 5 mm). Photographs of nanoparticles 24 hours after i.v. injection of HAuNS. Data are stably transfected with luciferase gene (U87-TGL) was carried out as we previously described (3). A brief description of the PAT technique is provided in the Supplementary Data. The cell line provided by Dr. Juri G. Gelovani (University of Texas) in January 2008 was validated by short tandem repeat (STR) DNA fingerprinting by the Characterized Cell Line Core Facility at The University of Texas MD Anderson Cancer Center on April 12, 2011. The STR profiles were compared with known ATCC fingerprints, and to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1200808 (http://bioinformatics.istge.it/clima/). The STR profiles matched known DNA fingerprints of U87-MG.

On day 8 after tumor inoculation, mice were imaged by a prototype PAT scanner. Mice were then injected i.v. with c(KRGDf)-PEG-HAuNS or PEG-HAuNS (2.5 \times 10^{11} particles per mouse). Contrast-enhanced photoacoustic images were acquired 24 hours after nanoparticle injection. Imaging data were reconstructed by using modified delay-and-sum back-projection reconstruction algorithm. After data acquisition, mice were sacrificed by overexposure to CO\textsubscript{2}. Open-skull surgery was conducted to permit photography of the brain tumors.

**Micro–positron emission tomography**

To further confirm the accuracy of PAT imaging of U87 tumors, c(KRGDf)-PEG-HAuNS were labeled with the positron emitter $^{64}$Cu ($t_{1/2} = 12.7$ hours; ref. 14). Tumor-bearing mice were injected i.v. with $^{64}$Cu-labeled c(KRGDf)-PEG-HAuNS or $^{64}$Cu-labeled PEG-HAuNS at a dose of 7.5 mCi/kg ($n = 3$ per agent). Twenty-four hours later, mice were anesthetized with 2% isoflurane (Baxter). Mice were subjected to a 7-minute micro–computed tomography (\(\mu\)CT) scan followed by a 20-minute micro–positron emission tomography (\(\mu\)PET) scan with an Inveon \(\mu\)PET/CT Scanner (Siemens). The \(\mu\)PET/CT images were generated separately and then fused by Inveon Research Workplace version 3.0 (Siemens).

**Photothermal therapy**

On day 8 after tumor inoculation, mice were injected i.v. with \(\nu\)-luciferin (4 mg/kg) for bioluminescence imaging. Mice were randomly allocated into 5 groups of 15 mice each. Groups I and IV were injected i.v. with c(KRGDf)-PEG-HAuNS, group II with PEG-HAuNS (both 2.5 \times 10^{11} particles per mouse), and groups III and V with saline. Groups I to III were also treated with NIR laser irradiation (16 W/cm\textsuperscript{2}, 3 minutes, 808 nm) 24 hours after nanoparticle injection. In each group, 5 mice were subjected to luciferase bioluminescence imaging at various times after nanoparticle injection to measure tumor burden (response to PTA therapy), and 10 mice were subjected to
survival monitoring. Survival data were presented by Kaplan-Meier plots and analyzed by a log-rank test. \( P < 0.05 \) was considered statistically significant.

**Results**

**PAT permits visualization of U87 gliomas in intact nude mice**

Transmission electron microscopy showed that c(KRGDf)-PEG-HAuNS were spherical, had hollow interiors, had average diameter of approximately 40 nm, and had an absorption peak tuned to approximately 800 nm (Fig. 1B). We previously showed that the molar extinction coefficient associated with the optical absorption cross-section of HauNS \( \frac{1.4 \times 10^{11}}{\text{cm}^2/\text{mol}} \) is much higher than that of hemoglobin \( \frac{\sim 1 \times 10^3}{\text{cm}^2/\text{mol}} \). Indeed, PEG-HAuNS at a concentration as low as 20 pmol/L displayed the same optical absorption as hemoglobin at its typical blood concentration of 2.3 mmol/L (3). The strong absorption of PEG-HAuNS in the NIR region permitted intense contrast in PAT.

Images of mice with U87 glioma before and after i.v. injection of c(KRGDf)-PEG-HAuNS or PEG-HAuNS are shown in Fig. 1C. Precontrast PAT only showed the normal brain vasculature. There was a low signal-to-background ratio between tumor and normal brain with no significant difference in photoacoustic signal intensities (Fig. 1D). However, 24 hours after injection of c(KRGDf)-PEG-HAuNS, PAT clearly revealed brain tumor, and the tumor location on PAT correlated with mouse brain anatomy. Quantitative analysis confirmed that the mean contrast-enhanced photoacoustic signal ratio of tumor-to-contralateral normal brain 24 hours after c(KRGDf)-PEG-HAuNS injection was approximately twice as high as that obtained from precontrast images \( (P = 0.0375) \). In contrast, 24 hours after injection of PEG-HAuNS, there was no change in tumor-to-brain photoacoustic ratio compared with that obtained from precontrast PAT images \( (P = 0.4677) \). These data supported that selective accumulation of c(KRGDf)-PEG-HAuNS enhanced PAT of U87 glioma.

**μPET/CT confirms accuracy of PAT imaging of U87 gliomas with c(KRGDf)-PEG-HAuNS**

Twenty-four hours after i.v. injection, \(^{64}\)Cu-labeled c(KRGDf)-PEG-HAuNS showed 2.9-fold higher uptake than \(^{64}\)Cu-labeled PEG-HAuNS in U87 tumors \( [1.12 \pm 0.22 \% \text{ID/g}] \) and auto-radiography showed higher radioactivity in tumor with c(KRGDf)-PEG-HAuNS than PEG-HAuNS. In addition to integrin \( \alpha_v \beta_3 \), recent study showed that c(KRGDf) may also interact with other integrins such as \( \alpha_v \beta_5 \) (14). Here, we used immunohistochemical staining of integrin \( \alpha_v \beta_3 \) receptors in tumors to confirm HAuNS localization. The intracranial distribution of radioactivity matched well with the site of tumor inoculation as well as the distribution of integrin \( \alpha_v \beta_3 \). Selective uptake of c(KRGDf)-PEG-HAuNS in U87 tumors was further supported by microscopy, which revealed greater accumulation of c(KRGDf)-PEG-HAuNS than PEG-HAuNS in tumors (Fig. 2).

**Injection of c(KRGDf)-PEG-HAuNS plus NIR laser irradiation elevates temperature of U87 gliomas**

Representative magnetic resonance temperature imaging (MRTI) maps of mouse brains at the end of laser irradiation are shown in Fig. 3A. Dynamic temperature curves based on MRTI analysis revealed that NIR laser irradiation in mice injected with c(KRGDf)-PEG-HAuNS reached a maximum temperature of 57.75 ± 0.46°C (Fig. 3B), which is more than the threshold of temperature of 54°C needed for irreversible cell damage (16, 17). In contrast, NIR laser irradiation in mice injected with nontargeted PEG-HAuNS or saline resulted in maximum temperatures of 48.14 ± 0.12°C and 41.65 ± 0.09°C, respectively, which were insufficient to induce irreversible cell damage (Fig. 3B).

![Figure 2. Targeted delivery of \(^{64}\)Cu-labeled c(KRGDf)-PEG-HAuNS to U87 human glioma in mouse brains. Shown are μPET/CT images (obtained 24 hours after i.v. injection of nanoparticles); photographs of mouse brains stained with hematoxylin and eosin (H&E) and \( \alpha_v \beta_3 \) and autoradiographs of mouse brains; and immunofluorescence micrographs of tumor tissue at high magnification. Red, \( \alpha_v \beta_3 \); green, scattering signal of HAuNS under dark field; blue, 4′,6-diamidino-2-phenylindole (DAPI)-stained cell nuclei; arrows, tumors; bar, 10 μm.](cancerres.aacrjournals.org)
that of the other groups (PEG-HAuNS plus laser (28 days) was significantly more than treatment. alone, or saline, luminescence intensity increased throughout day 9. In mice treated with laser alone, c(KRGDf)-PEG-HAuNS plus laser, luminescence intensity in tumors decreased only on day 9. In mice treated with laser alone, c(KRGDf)-PEG-HAuNS alone, or saline, luminescence intensity increased throughout treatment.

The median survival time of mice treated with c(KRGDf)-PEG-HAuNS plus laser (28 days) was significantly more than that of the other groups (P < 0.001): PEG-HAuNS plus laser, 19.5 days; laser alone, 17.5 days; c(KRGDf)-PEG-HAuNS alone, 17.5 days; and saline, 16.5 days (Fig. 4C). Histologic examination confirmed that c(KRGDf)-PEG-HAuNS plus laser was the treatment causing the most extensive necrotic response (Fig. 4D). Extensive pyknosis, cytoplasmic acidophilia (10, 17), and corruption of the extracellular matrix of the tumor appeared throughout tumors of mice treated with c(KRGDf)-PEG-HAuNS plus laser. The tumor cells were completely ablated; there were no discernible residue viable tumor cells in the tumor periphery (Fig. 4D, arrow). In mice treated with PEG-HAuNS plus laser or laser alone, approximately 45% and 30% of tumor tissues were necrotized, characterized as pyknosis and medium cytoplasmic acidophilia, and there were large numbers of viable tumor cells in the periphery (Fig. 4D, asterisks). In tumors of mice treated with c(KRGDf)-PEG-HAuNS alone or saline alone, most tumor cells were intact.

Discussion

We have shown effective PAT imaging and PTA therapy of glioma mediated by a single preparation of targeted HAuNS on the basis of their intrinsically high optical absorption cross-section. The findings from this study suggest potential applications of HAuNS as a novel theranostic platform. First, c(KRGDf)-PEG-HAuNS can serve as efficient optical contrast agents for photoacoustic imaging, which may provide cancer diagnosis with high resolution and high sensitivity in addition to conventional imaging modalities. PAT may also be useful for pretreatment diagnosis and real-time monitoring of treatment, as well as assessment of treatment outcome. Second, under the guidance of PAT imaging, accurate and efficient PTA of tumor cells would be instituted simply by switching the laser power from diagnostic level (pulsed laser, 50 mW/cm²) to therapeutic level (diode laser, 16 W/cm²) on the basis of the high photothermal coupling efficiency of HAuNS. Third, given that μPET/CT using ⁶⁴Cu-labeled c(KRGDf)-PEG-HAuNS confirmed selective uptake of the nanoparticles in U87 tumors, PET/CT with ⁶⁴Cu-labeled targeted HAuNS should provide an alternative imaging method for HAuNS-mediated PTA therapy. With these advantages, we envision that for treating an entire tumor volume, delivery of laser energy to the region of interest would be achieved in a way similar to that of interstitial high-dose-rate brachytherapy in that multiple applicators are used to cover the area of interest (18). For individuals with small recurrent tumors, who represent a large and important patient population, this would provide a minimally invasive technique to both diagnose and target the recurrence, using a single fiber. Remaining PAT signal from the tumor bed or beyond after surgery could guide further resection and/or provide opportunity for selective ablation mediated by the targeted HAuNS. If successfully implemented in the clinical setting, our approach described here offers an alternative option over current clinical investigations of glioma theranostics involving CT-guided thermotherapy with superparamagnetic iron oxide nanoparticles in an alternating magnetic field (19, 20). In this case, the nanoparticles are administered intratumorally under neuronavigational control and pretreatment MRI.
the presence of residual tumor cells after treatment. Combining PTA therapy and other treatment modalities, such as photothermal chemotherapy made possible with drug-loaded HAuNS or photothermal transfection made possible with HAuNS coated with therapeutic siRNA (14), should lead to further enhanced therapeutic outcome and even cure. Studies toward this goal are currently underway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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