Research Article

CT imaging of myocardial scar burden with CNA35-conjugated gold nanoparticles

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Abstract

Management of patients suffering from myocardial infarction (MI) is based on the extent of coronary artery disease and myocardial scar burden. We have developed a potentially clinically-useful X-ray molecular imaging contrast agent based on gold nanoparticle (AuNPs) functionalized with collagen-binding adhesion protein 35 (CNA35) with the capabilities of achieving prolonged blood pool enhancement for vascular imaging of the coronary arteries and specific targeting of collagen within myocardial scar. At a concentration of ~ 45 mg Au/ml, AuNPs maintained a stable blood pool enhancement at 142–160 HU within an hour of intravenous administration. At 6 hours, specific signal enhancement was detected in the myocardium scar in rats injected with CNA35-AuNPs, but not with control AuNPs or in control animals. In conclusion, CNA35-AuNPs may be considered as a CT contrast agent for both vascular imaging of coronary artery disease and molecular imaging of myocardial scar in the heart.

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Key words: Myocardial scar imaging; Computed tomography; Gold nanoparticles; Contrast agent; Collagen adhesin; CNA35

Myocardial scar burden is an important prognostic marker in patients with ischemic heart disease. Current gold standard for the detection of myocardial scar is late gadolinium enhancement imaging by contrast-enhanced cardiac magnetic resonance imaging in which the degree of late gadolinium enhancement provides incremental prognostic significance beyond coronary angiographic findings.1-3 However, the use of gadolinium-Diethylenetriamine pentaacetate (Gd-DTPA) may not be suitable for patients with renal impairment due to the risk of nephrogenic systemic fibrosis.4 Magnetic resonance imaging is also contraindicated in patients with implantable devices and some pacemaker leads. The development of another imaging modality for evaluating myocardial scar burden may provide an alternate tool for stratifying patients with ischemic heart disease.

Our previous study has demonstrated that molecular imaging of myocardial scar could be achieved with computed tomographic (CT) imaging using gold nanoparticles (AuNPs) targeting collagen in a small animal model.5 AuNPs have been developed as a new generation contrast agent for X-ray based imaging. Compared with conventional iodine-based contrast agents, gold has the advantage of superior X-ray attenuation and profile that are suitable for material differentiation in dual-energy CT.6 Thus, AuNPs may be useful as a superior blood pool agent for vascular imaging during the initial phase of imaging, which provide angiographic data on the distribution and degree of coronary lesions.7 The ability to functionalize the surface of AuNPs with a targeting ligand provides an opportunity for CT-based molecular imaging. CNA35 is a small protein expressed in E. coli that it is easy to conjugate. The collagen-binding domains of CNA35 have excellent affinity for collagen I with a Kd ranging from 20 nM to 30 μM. CNA35 is approximately five times smaller than antibodies, and this could be beneficial for tissue penetration and on-rate kinetics.8 Previous studies show binding of fluorescently labeled CNA35 to collagen in fibroblasts and in the murine arterial wall.9 When fluorescently labeled CNA35 was injected into mice, fluorescence microscopy demonstrated CNA35 binding to the collagen in the myocardium and supported the potential utility of CNA35 for in vivo targeting.

Abbreviations: AuNPs, Gold nanoparticles; CNA, Collagen adhesin; CT, Computed tomography; DLS, Dynamic light scattering; PEG, Poly (ethylene glycol); LAD, Left anterior descending; MI, Myocardial infarction.

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of cardiac fibrosis. We injected CNA35 functionalized gold nanoparticles into mice with myocardial infarction and detected low levels of signal in the vicinity of the myocardial scar. However, the size of the mouse heart and the myocardial scar are at the threshold of detection by our pre-clinical CT scanner and the localization and quantitation of myocardial scar burden and distribution are technically challenging. In this study, we used a rat model of myocardial infarction to further evaluate this imaging approach. The size of the heart in the rat is suitable for pre-clinical CT imaging and the distribution of myocardial scar burden in the rat could potentially be better defined than in the mouse as shown in our previous study.

Methods

Materials

Sodium citrate and gold chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Poly(ethylene glycol) methyl ether thiol (PEG-SH) and orthopyridyldisulfide-polyethylene-glycol-N-hydroxysuccinimide (OPSS-PEG-SVA) were purchased from Laysan Bio (Arab, AL, USA). PES membranes (3000 MWCO and 100,000 MWCO) were purchased from Fisher Scientific. PES membranes (10,000 MWCO) were purchased from Viva Products Inc. (Littleton, MA, USA). CNA35 was kindly donated by Magnus Hook (Texas A&M University, USA). Beuthanasia was purchased from Patterson Veterinary.

Gold-nanoparticles synthesis

To prepare AuNPs, nanopure water (500 ml) was filtered through 0.22 μm filter and boiled in a 1 L conical flask. 5 ml of gold chloride (10%) was added to the boiling water followed by 4 ml of 1% sodium citrate solution. The solution was boiled for about an hour or until 200 ml of solution was left. The solution has a burgundy color. Next, the AuNPs were pegylated with polyethylene glycol derivatives in order to avoid aggregation in vivo. The AuNPs were incubated for 1 hour with a 100:1 molar ratio of PEG-SH: AuNPs to prevent aggregation and with 50:1 molar ratio of OPSS-PEG-SVA: AuNPs for the covalent coupling of the peptide. These molar rations of PEG-SH: AuNPs and OPSS-PEG-SVA: AuNPs were determined in a previous manuscript. Briefly, AuNPs (2 × 10^13 AuNPs) were incubated with various molar ratios of PEG-SH and OPSS-PEG-SVA: AuNPs were determined in a previous manuscript. The concentration of AuNPs was calculated based on measuring the absorbance spectrum of the AuNPs solution and using the table provided by Nanopartz in which they included the molar extinction coefficients for different sizes of AuNPs depending on the maximum absorbance peak. After 1 hour incubation at room temperature, NaCl (10%) was added and the solution incubated for 30 minutes at room temperature. The absorbance spectrum (400–600 nm) and the size by DLS were then recorded for all of the above mentioned solutions. The disappearance or the shift in the signal indicates aggregation.

After pegylation, the AuNPs (50 ml) were further concentrated by centrifugation at 3270 rpm for 60 min. The pink supernatant was discarded and all the concentrated fractions were combined and filtered through 0.22 μm filters. The AuNPs collected were further concentrated using PES membrane concentrators (MWCO 10,000) to a final concentration of ~40 μg Au/ml. The AuNPs were characterized in terms of size and polydispersity by UV and dynamic light scattering after appropriate dilution.

Synthesis and characterization of CNA35-AuNPs

Recombinant CNA35 protein was a kind gift from Dr. Magnus Hook (Texas A&M University, USA). CNA35 (36.5 μg) was incubated with 500 μl AuNPs (40 mg/ml) overnight at 4 °C. Next day, the excess CNA35 was removed by centrifugation using Vivaspin filters (MWCO 100,000). The AuNPs were characterized in terms of size and polydispersity by UV Spectrophotometry and dynamic light scattering (Malvern Nano-ZS Zetasizer, Malvern Instruments Ltd., a Spectris Company; Worcestershire, UK).

Animal experiments

Female CD® rats, weighing 175–200 g, with myocardial infarction were obtained from Charles River Laboratories (Wilmington, MA). Myocardial infarction was surgically created with left anterior coronary artery (LAD) ligation and ischemic lesion comprised of 20–30% of total cardiac tissue. The animals were housed at the University of Texas Health Science Center at Houston (UTHSCH). The animal protocol was approved by the UTHSCH Center for Laboratory Animal Medicine and Care (protocol number: HSC-AWC-14-007- “CT Imaging of Myocardial Scars by Functionalized Gold Nanoparticles”); experiments were performed in accordance with institutional guidelines, and all efforts were made to minimize suffering. Animals were anesthetized with a cocktail of ketamine (43 mg/kg IP) and xylazine (8.7 mg/kg IP). The tail vein was cannulated and injected with 1.0 mL of 40–47 mg/ml of control AuNPs (control AuNPs without the CNA35 target) or CNA35-coated AuNPs. Rats without myocardial infarction were used as controls. Thirty LAD rats and 10 control rats were used for this study. Twenty LAD rats were injected with CNA35-AuNPs and 10 LAD rats were injected with control (non-targeted) AuNPs. As for the control rats, 3 rats were injected with CNA35-AuNPs and 7 rats were injected with control AuNPs. CT imaging was performed with a GE Ultra flat panel CT scanner (General Electric, Milwaukee, WI) with the following acquisition settings: 80 kVp, 22 mA with 16 s rotation/ exposure. Non-cardiac gated CT images were acquired at baseline, 1 min, 15 min, 30 min, 1 h, 2 h and 6 h after control AuNPs or CNA35-AuNPs injection. At the end of the imaging, animals were euthanized with Beuthanasia (a mixture of phenytoin and pentobarbital administered as an intravenous injection) and the carcasses with the non-beating heart and the excised hearts were imaged once more by the CT scanner. Signal enhancement is better visualized in the non-beating heart given the un gated nature of the CT acquisition sequence.

Simple back projections were obtained from the 0.154 μm image reconstruction and exported as DICOM images. Image analysis was performed using the OsiriX software. Stacks of 3–4 consecutive sections were viewed with maximum intensity projection and window levels were adjusted to identify any signal enhancement in the myocardium.
Results

Pharmacokinetics and biodistribution of CNA35-AuNPs in vivo

The final preparation of our CNA35-AuNPs had a hydrodynamic diameter of 60–90 nm with a polydispersity index of 0.313–0.612 (based on DLS data – Figure 1, left panel). Our UV–VIS data indicate that the AuNPs have a diameter of 80 nm, exhibiting an absorption maximum at 550 nm, characteristic for a uniform preparation of AuNPs of 80 nm (Figure 1, right panel).

We envisioned the use of functionalized AuNPs as a "one-stop shop" for both vascular and molecular imaging. Gold nanoparticle is an excellent blood pool contrast agent. Rats were injected with 250 μl, 400 μl, 750 μl, or 1000 μl of AuNPs (40–47 mg Au/ml) to determine the volume of contrast agent for optimal blood pool enhancement and signal-to-noise ratio in the myocardial scar at early and late time points. A volume between 750 μl and 1000 μl (40–47 mg Au/ml) of CNA35-AuNPs or unconjugated AuNPs achieved blood pool enhancement at 142–160 HU. Peak blood pool enhancement of 90–100 HU (3–4 mg Au/ml) was observed for up to 6 hours after intravenous administration (Figure 2). In animals injected with less than 750 μl AuNPs (40–47 mg Au/ml), it was impossible to differentiate between the baseline (~79 HU) and post-contrast blood pool enhancement. The uniform and stable enhancement of the vasculature by AuNPs during the early phase of imaging may provide the blood pool contrast for visualization of the arterial systems by CT angiography. Coronary CT angiography is an accepted imaging modality for detection of the degree and distribution of coronary lesions. Revascularization of stenosed or occluded coronary arteries supplying viable myocardium may improve clinical outcomes. Gold nanoparticle may serve as a suitable alternative to conventional iodinated contrast agents as a blood pool contrast agent.

Biodistribution of AuNPs in the liver, spleen, kidneys and muscle was indirectly quantitated by CT enhancement and expressed in HU (Figure 3). A large amount of contrast was detected in the spleen which represented the major organ of elimination of AuNPs at 6 hours after intravenous administration (Figure 3). Although the increase in CT enhancement did not reach significance, the size of the liver is larger than the spleen, the total amount of hepatic retention of AuNPs was still substantial. There was no change in CT enhancement in the kidneys and muscle at 6 hours after administration of AuNPs. The metabolic fate of the AuNPs in the spleen and liver was not evaluated in the current study.

Targeted imaging of collagen deposition in myocardial scar with CNA35-AuNPs

The advantage of AuNPs over conventional iodinated contrast agents is the ability to functionalize relatively larger amount of
liposomes and extended the imaging window for blood pool contrast and others have successfully encapsulated iodixanol in pegylated contrast agent is to encapsulate the contrast agent in liposomes. We possible solution to improve the blood pool persistence of iodinated agents limit their further refinement for more sophisticated imaging applications. All iodinated contrast agents are rapidly cleared by the kidneys and do not have sufficient blood pool persistence to interrogate for surface markers or delineate microcirculation. One kidneys and do not have sufficient blood pool persistence to

Discussion

Iodinated contrast agent has been used as the main x-ray contrast agent for vascular imaging for many years and its popularity lies in its versatility for detecting various pathologies in different organs. However, the physical and molecular properties of iodinated contrast agents limit their further refinement for more sophisticated imaging applications. All iodinated contrast agents are rapidly cleared by the kidneys and do not have sufficient blood pool persistence to interrogate for surface markers or delineate microcirculation. One possible solution to improve the blood pool persistence of iodinated contrast agent is to encapsulate the contrast agent in liposomes. We and others have successfully encapsulated iodixanol in pegylated liposomes and extended the imaging window for blood pool contrast for up to 6 hours. Such an agent has also been used for detecting atherosclerotic plaques in a mouse model. The encapsulation of iodinated contrast agent also provides an opportunity for surface functionalization and targeted imaging. Unlike functionalization of individual iodinated molecule which links a limited number of homing ligand for each iodinated molecule, encapsulation of iodinated contrast agent within liposomes packages a large amount of contrast agents within each liposome for detection. Surface functionalization could be achieved by altering the components in the liposomal membrane. In our previous study, the “eat-me signal”, phosphatidylserine, was incorporated into the liposomal membrane to facilitate liposomal uptake by macrophages. Alternatively, homing ligands such as antibodies or peptides can be used to coat the liposomal surface for specific targeting. However, the physical properties of iodine and its interaction with x-ray remain major hurdles for further development. The distinction between calcium and iodinated contrast agent in CT imaging remains technically challenging. This is an important consideration because the coronary arteries are located within the thoracic cage and calcification is frequently encountered in atheroma. The CT values of low-attenuation bone voxels and micro calcification are similar or only slightly higher than that of contrast medium-opacified blood. Bone editing is possible in image post-processing but is time-consuming. However, the inability to differentiate between iodinated contrast uptake in the arterial wall and pre-existing calcification makes it challenging to develop iodinated contrast agent for molecular imaging.

There are only a few reports of new developments in iodinated CT contrast agents for targeted imaging. The new contrast agents reported are in most part nanoparticles. The CT contrast agent, N1177, was developed for the non-invasive detection of macrophages in atherosclerotic plaques. N1177 is an iodinated nanoparticle with a concentration of iodine of 67 mg/ml. The uptake of N1177 by macrophages was studied both in vitro and in vivo. In vitro uptake of N1177 by macrophages ~88-fold higher than that of conventional CT contrast agent. Also, in vivo work done in atherosclerotic rabbits shows strong enhancement of the aortic wall after intra-venous injection of N1177 as compared to non-atherosclerotic rabbits injected with N1177 or to atherosclerotic rabbits injected with conventional CT contrast agents. A recent review described the development of various nanoparticle CT contrast agents with an emphasis on using the nanoparticles for cell tracking applications.

We are motivated to explore other high atomic number elements for CT-based molecular imaging. There are a series of k-edge metals that had been considered for CT imaging, such as bismuth, tantalum, bromine, platinum. Bismuth is toxic, but

Figure 3. Biodistribution of AuNPs in vivo. Left panel: Substantial amount of CT enhancement was detected in the spleen 6 hours after injection of unconjugated AuNPs or CNA35-AuNPs (red asterisk). CT enhancement in the intestine was pre-existing and may represent CT attenuating materials in the diet. Right panel: The change in CT enhancement in various organs 6 hours after intravenous administration of AuNPs. Values are expressed in mean ± SEM (n = 3).
it had been stabilized with a polymer coating to improve biocompatibility. Gold is an excellent contrast agent for molecular imaging.\textsuperscript{21,22} It has a high atomic number providing a good CT contrast. AuNPs are relatively easy to synthesize and make them attractive for large-scale production. They are easy to functionalize making them not only long circulating, but also the added capability to target to specific sites.\textsuperscript{23,24} Gold is biocompatible and there is a formulation in Phase 1 clinical trials in which the AuNPs are conjugated to TNF-\(\alpha\). Different doses were tested in patients with advanced stage cancer.\textsuperscript{25} The physical characteristics of gold offer the potential for reducing the radiation dose for imaging, reducing contrast load and offering better delineation between the contrast agent and bone or micro calcifications in the arterial wall. A review of the literature reveals only a small number of studies using targeted AuNPs for molecular CT imaging.\textsuperscript{26} Most of those studies focused on the use of AuNPs in cancer molecular imaging.\textsuperscript{27-31} We are one of the few groups that employ the technique for cardiovascular molecular imaging.\textsuperscript{5}

The difference between the current and previous study\textsuperscript{5} was the use of a slightly larger animal model. Our pre-clinical CT scanner has a two-point resolution of \(\sim 0.3\) mm. Normal

Figure 4. In vivo and ex vivo CT imaging of rats with myocardial infarction in the left anterior descending artery territory (A-F) and control rats without myocardial infarction (G-H). Injection of CNA35-conjugated AuNPs in rats with myocardial infarction resulted in enhanced signal detection in the infarcted myocardium at 6 hours (A-E) while control AuNPs injected in rats with myocardial infarction did not produce any CT enhancement at 6 hours (F). In control rats without myocardial infarction, CNA35-conjugated AuNPs (G) and control AuNPs (H) did not generate any non-specific CT enhancement. The scale shows the intensity of the signal in Hounsfield Units (HU).
myocardial wall thickness in the mouse heart is ~0.5 mm and the infarcted zone is even thinner. Clearly, the size of the mouse heart approaches the detection limit of our CT scanner. Thus, we employed the rat in the current study to further evaluate this molecular imaging approach. The thickness of the rat myocardium is ~1–2 mm and can be represented by 4–6 voxels. At 6 hours, CNA35-AuNPs enhances the infarcted territory in the myocardium. As predicted, the area of CT enhancement and intensity were better seen in the rats (Figure 5, right panel) than in our previous study with the mice (Figure 5, left panel).

The binding of CNA35-AuNPs appears specific for myocardial scar in vivo. No detectable enhancement was noted when non-functionalized AuNPs was injected into rats with or without LAD infarction or CNA35-AuNPs in control rats without myocardial infarction. Quantitatively, the degree of CT enhancement as determined by the percentage of enhanced area seems to underestimate the percentage of infarcted area as determined by histological staining. This suggests that not all the myocardial scar is bound by CNA35-AuNPs in vivo and this may be explained by a number of reasons. First, partial volume effect of CT imaging may lead to underestimation. Second, the contrast agent requires access to the myocardial scar and the availability of vascular access may be a limiting factor. With the LAD being occluded, the entry of CNA35-AuNPs into the myocardial scar relies on the microcirculation which is rather inefficient for myocardial perfusion. Furthermore, the myocardial scar is avascular and only the periphery of the myocardial scar can directly interact with circulating CNA35-AuNPs. Third, the size of AuNPs is larger than Magnevist, the contrast agent used in late gadolinium enhancement with MRI. The 80 nm gold nanoparticle has a molecular mass of $2.1 \times 10^9$ g mol$^{-1}$, which is also larger than that of Magnevist (545.56 g mol$^{-1}$) and this may account for the poorer penetration of AuNPs into the myocardial scar. Future studies will look into the myocardial infarction scar penetration by using smaller CNA35-AuNPs. Finally, the myocardial wall is very thin in the infarcted areas and it is uncertain if CNA35-AuNPs with CT imaging has the capability to determine the transmural extent of the myocardial scar.

Other observations were made during our studies and those need to be considered when developing AuNPs as a contrast agent. As a blood pool contrast agent, the amount of gold required to generate comparable CT contrast is substantially lower than that of iodine. However, as a molecular imaging agent, a relative large amount of functionalized AuNPs was needed to generate sufficient contrast signals in the myocardium. This is related to the low concentration of the bound AuNPs at the target sites and the low sensitivity of x-ray imaging. The large amount of AuNPs also translated into a number of unanticipated effects in our animals. After intravenously administration via the tail vein, the color of the eyes and lower limbs turned dark blue. For some uncertain reasons, the animals also displayed lower limb disorder upon awakening. Since all studies were terminal, we were not able to determine if the lower limb disorder was temporary or permanent.

Our results clearly demonstrated that gold nanoparticle is an excellent blood pool contrast agent and targeted imaging of myocardial scar in a rat model of myocardial infarction with CNA35-AuNPs was possible. We confirmed that the myocardial wall thickness was an important determinant for visualizing myocardial scar burden using CT-based molecular imaging, which could be applicable in larger animal models and human subjects. However, the amount of AuNPs necessary for sufficient
contrast for molecular imaging was relatively high and this may dampen the enthusiasm of using CT imaging for molecular imaging in clinical studies.

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