Nanoparticle PET-CT Imaging of Macrophages in Inflammatory Atherosclerosis

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Background—Macrophages participate centrally in atherosclerosis, and macrophage markers (eg, CD68, MAC-3) correlate well with lesion severity and therapeutic modulation. On the basis of the avidity of lesional macrophages for polysaccharide-containing supramolecular structures such as nanoparticles, we have developed a new positron emission tomography (PET) agent with optimized pharmacokinetics to allow in vivo imaging at tracer concentrations.

Methods and Results—A dextranated and DTPA-modified magnetofluorescent 20-nm nanoparticle was labeled with the PET tracer $^{64}$Cu (1 mCi/0.1 mg nanoparticles) to yield a PET, magnetic resonance, and optically detectable imaging agent. Peak PET activity 24 hours after intravenous injection into mice deficient in apolipoprotein E with experimental atherosclerosis mapped to areas of high plaque load identified by computed tomography such as the aortic root and arch and correlated with magnetic resonance and optical imaging. Accumulated dose in apolipoprotein E–deficient aortas determined by gamma counting was 260% and in carotids 392% of respective wild-type organs ($P<0.05$ both). Autoradiography of aortas demonstrated uptake of the agent into macrophage-rich atheromata identified by Oil Red O staining of lipid deposits. The novel nanoagent accumulated predominantly in macrophages as determined by fluorescence microscopy and flow cytometry of cells dissociated from aortas.

Conclusions—This report establishes the capability of a novel trimodality nanoparticle to directly detect macrophages in atherosclerotic plaques. Advantages include improved sensitivity; direct correlation of PET signal with an established biomarker (CD68); ability to readily quantify the PET signal, perform whole-body vascular surveys, and spatially localize and follow the trireporter by microscopy; and clinical translatability of the agent given similarities to magnetic resonance imaging probes in clinical trials. (Circulation. 2008;117:379-387.)

Key Words: atherosclerosis ■ imaging ■ inflammation ■ nanoparticles ■ positron-emission tomography

Inflammation drives atherosclerotic plaque evolution and increases cardiovascular risk. Monocytes/macrophages enter nascent atherosclerotic lesions, ingest modified lipoprotein particles, and give rise to foam cells, a hallmark of atheromata.1 Macrophages amplify local inflammation through secretion of cytokines and reactive oxygen species and can weaken the fibrous cap of the plaque by secretion of proteases.3 Therefore, macrophages act as protagonist cells that can destabilize inflammatory atherosclerotic plaques and favor plaque disruption, a frequent cause of myocardial infarction and stroke.1

Clinical Perspective p 387

A variety of carbohydrate and polyol-coated nanoparticles have emerged as powerful affinity labels for macrophages based on their active internalization and intracellular trapping into phagocytic cells. For example, magnetic resonance imaging (MRI) studies have used nanoparticles to report areas of inflamed lesions in animals2–6 and in carotid artery plaques in patients.7,8 Although MRI provides unparalleled versatility and soft tissue contrast,9 direct visualization of abnormalities often requires relatively large amounts of nanoparticles (2 to 20 mg Fe/kg). Nuclear techniques such as positron-emission tomography (PET) potentially provide detection sensitivities an order of magnitude higher, enabling the use of nanoparticles at lower concentrations than MRI permits. Furthermore, the combination of the high sensitivity of PET with the anatomic detail provided by computed tomography (CT) in hybrid imaging has the potential to map signal to atherosclerotic vascular territories.10 However, imaging in small vessels such as coronary arteries may still...
prove challenging with today’s resolutions of PET imaging systems.

To date, most clinical PET-CT studies have used 18F-fluoro-2-deoxyglucose (18FDG) to image atherosclerosis.11–15 Targeting areas of high metabolic rate with tagged glucose is an attractive concept, but the rapid radioactive decay of 18FDG has limited the careful histological and molecular studies necessary to correlate the imaging signal with plaque components. Thus, uptake of 18FDG in the region of atherosclerotic arteries may not report solely on inflammatory cells.16

Here, we report the development of a novel class of macrophage-targeted PET agents based on long-circulating, dextran-coated nanoparticles. The magnetic nanoparticle base material has been derivatized with DTPA for the nuclear tracer 64Cu and a near-infrared fluorochrome, yielding a trimodality reporter (Figure 1). We hypothesized that 64Cu-TNP accumulation correlates with macrophage burden and that the derived in vivo PET signal correlates with inflammatory plaque components identified by MRI, fluorescence imaging, and flow cytometry. Furthermore, we compare the in vivo distribution of 64Cu-TNP to 18FDG in atherosclerotic mice.

Methods

Synthesis of 64Cu-TNP

The well-characterized nanoparticle MION (monocrystalline iron oxide nanoparticle), previously used for MR imaging17, served as starting material. The dextran coating of the nanoparticle was crosslinked with epichlorin hydrin, aminated, and labeled with near-infrared fluorochrome Vivotag-680 (VT680, VisEn Medical, Woburn, Mass). The ratio of VT680 per nanoparticle was 20 nm as determined by laser light scattering. The nanoparticles were derivatized with DTPA for the nuclear tracer 64Cu and a near-infrared fluorochrome, yielding a trimodality reporter 64Cu-TNP. A schematic view of the trimodality reporter 64Cu-TNP is shown in Figure 1. The derivatized nanoparticles was used for the labeling with 64CuCl2, obtained from Isotec, Trace, Toronto, Canada, equivalent to 185 MBq 64Cu, in ammonium acetate buffer (180 μL, 0.5 mol/L, pH 5.5). After 25 minutes of incubation at 95°C, the solution was centrifuged and washed 3 times. Pure 64Cu-TNP was redissolved in 400 μL PBS. We used stable nonradioactive copper salts for labeling TNP used in MRI experiments to reduce exposure to radiation. Figure 1 shows a schematic of the agent. Aliquots were routinely analyzed by high-performance liquid chromatography (eluent A, 0.1% trifluoroacetic acid in water, eluent B, acetonitrile; gradient: 0 to 20 minutes, 95% for eluent A; 20 to 24 minutes, 95% for eluent B; 24 to 28 minutes, 95% for eluent B; 30 minutes, 95% for eluent A) using a Varian 210 high-performance liquid chromatography (Salt Lake City, Utah) with a C18 column, multiwavelength detector, and a flow-through gamma detector. The specific activity of 64Cu-TNP was 1 mCi per 0.1 mg Fe of nanoparticles (corresponding to ~300 μCi per mouse or 1.5 mg Fe/kg body weight). The average diameter of the nanoparticles was 20 nm as determined by laser light scattering. The R1 and R2 values were ~:29 and 60 mmol/L 1/sec 1 (0.47 T, 39°C).

Atherosclerotic Mice

Nine apoE−/− mice (The Jackson Laboratory, Bar Harbor, Me) were used for the PET imaging study, 5 for MRI, 3 for flow cytometry, and 4 for histology. The remaining 9 mice were used for additional biodistribution experiments (n = 4), in initial dose-finding studies, and in implementing the study design for serial PET imaging (18FDG followed by 64Cu-TNP imaging). Five wild-type (The Jackson Laboratory) mice were imaged with PET; the remaining mice were used for biodistribution studies. ApoE−/− mice had an average age of 45 weeks and consumed a high-cholesterol diet (0.2% total cholesterol; Harlan Teklad, Madison, Wis) for 20 weeks. Mice were anesthetized for all procedures and imaged under inhalation anesthesia (isoflurane 2% to 3% vol/vol + 2 L/min O2) (Forane, Baxter, Deerfield, Ill). 18FDG PET studies used ketamine/xylazine anesthesia to suppress myocardial tracer uptake (intraperitoneal injections of ketamine 100 mg/kg and xylazine 5 mg/kg). The institutional Subcommittee on Research Animal Care approved all animal studies.

Phantom Studies

An agar phantom was used to compare the sensitivity of nanoparticle detection with the different imaging modalities. Nanoparticles were diluted in 2% low-melting agarose to concentrations ranging from...
PET-CT Imaging
Nine apoE⁻/⁻ mice were imaged with PET-CT 1 hour after injection of 259±52Ci of ⁶⁸Ga-DOTA. On the following day, the same apoE⁻/⁻ and C57BL6 mice were injected with 307±53µCi of ⁶⁸Cu-TNP IV (1.5 mg nanoparticles per 1 kg body weight) and imaged at 24 hours after injection. This dosage provides activity within the dynamic range of the PET system 24 hours after injection. Furthermore, this concentration was ~1 order of magnitude lower than previously used for MRI (10 to 30 mg Fe/kg).² The PET-CT used a FLEX X-PET/IX-O micro PET-CT (Gamma Medica Ideas, Inc, Northbridge, Calif). To achieve appropriate intravascular contrast, iopamidol 61% (Bracco Diagnostics, Princeton, NJ) was infused continuously via the tail vein at a rate of 65 µL/min for the time of CT acquisition, which was ~2 minutes. The CT system was calibrated to acquire the 512 projectiles at 75 kVp with a 64-mm detector center of rotation. After the CT scan was complete, the mouse bed was translated axially and centered within the PET detector ring. Depending on the count rate, the PET scan was acquired for 30 to 45 minutes. Spatial resolution was 72 µm isotropic for CT and 2 mm for PET imaging. The PET and CT images were fused using a software module in Amira (version 4.1) specifically written by Mercury Computer Systems for the X-PET-PET-CT system (Mercury Computer Systems, Carlsbad, Calif). Because the mouse remains in the same position on the bed for both PET and CT acquisitions and the relative positions of the field of views of the PET and CT are known, the software module can use the positional information to fuse and coregister the PET and CT data. Fused images were converted into DICOM format and analyzed with OsiriX shareware (Geneva, Switzerland; www.osirix-viewer.com) by placing volumes of interest in the aortic root, a region of consistent location of atheromata in apoE⁻/⁻ mice, and in skeletal muscle for calculation of target-to-background ratios. Standard uptake values were calculated as follows: (tissue activity/volume of tissue)/(body activity/body weight).

MRI Studies
In vivo MRI studies were performed with a 7-T horizontal-bore scanner (Bruker Pharmascan, Billerica, Mass) as previously described.² Five apoE⁻/⁻ mice were imaged before and 48 hours after intravenous injection of 30 µg Fe/kg of a cold version of TNP. Bright-blood cine images were obtained with ECG and respiratory gating (SA Instruments, Stony Brook, NY) using a gradient-echo FLASH (fast, low-angle shot) sequence and a dedicated mouse cardiac volume coil (Rapid Biomedical, Wuerzburg, Germany). Imaging parameters were as follows: echo time, 2.7 ms; 16 frames per RR interval (repetition time, 7.0 to 8.0 ms); in-plane resolution, 200×200 µm; slice thickness, 1 mm; and number of excitations, 8. Images were analyzed with OsiriX. Signal intensities were measured by tracing a manual region of interest in the blood and adventitial wall of the aortic root. The contrast-to-noise ratio (CNR) between the blood pool and the adjacent plaque was calculated as follows: CNR=(blood signal−plaque signal)/SD of the noise).

Ex Vivo Fluorescence Reflectance Imaging
After MRI, mice were killed, and the aortas were excised with fine scissors. Thereafter, the tissue was placed in a cocktail of collagenase I, collagenase XI, DNase I, and hyaluronidase (Sigma Aldrich, St Louis, Mo) and shaken at 37°C for 1 hour.¹³ The tissue was then triturated through nylon mesh and centrifuged (15 minutes at 500g and 4°C). The resulting cell suspensions were washed with Hank’s buffered salt solution supplemented with 0.2% (wt/vol) BSA and 1% (wt/vol) FCS. To visualize macrophages, neutrophils, smooth muscle cells, and endothelial cells, the suspension was incubated with a mixture of monoclonal antibodies.²⁴ The following antibodies were used: anti–CD90–PE, 53-2.1 (BD Biosciences, San Jose, Calif); anti–B220–PE, RA3-6B2 (BD Biosciences); anti–CD49d–PE, DX5 (BD Biosciences); anti–NK1.1–PE, PK136 (BD Biosciences); anti–Ly-6G–PE, IAB (BD Biosciences); anti–CD11b–APC-Cy7, M170 (BD Biosciences); anti–CD31–FITC, 390 (BD Biosciences); and anti–α-smooth muscle actin–FITC, I44 (Abcam, Cambridge, Mass). Monocytes/macrophages were identified as CD11b⁺ (CD90/BD220/CD49b/NK1.1/Ly-6G)⁺. Neutrophils were identified as CD11b⁺ (CD90/BD220/CD49b/NK1.1/Ly-6G)⁺. Endothelial cells were identified as CD3¹ CD11b⁺. Smooth muscle cells were identified as α-actin⁺CD11b⁻. For intracellular staining of α-smooth muscle actin, cells were permeabilized and fixed with a Cytofix/Cytopern Kit (BD Biosciences). Data were acquired with an LSRII (BD Biosciences) with 670/LP and 695/40 filter configuration to detect ⁶⁴Cu-TNP. Relative contribution of signal was calculated by multiplying the proportion of cells in living gate by mean gray level fluorescent intensity.

Histopathology
The aortic root was embedded in OCT (optimal cutting temperature; Sakura Finetek USA, Torrance, Calif). Serial 6-µm-thick cryostat sections were obtained starting at the aortic valve. Sections were stained with hematoxylin and eosin or for immunofluorescence with the following antibodies: primary anti-mouse Mac-3, CD 31 (macrophages and endothelial cells, BD Biosciences), and α-actin (smooth muscle cells, Laboratory Vision, Fremont, Calif). Secondary antibodies were as follows: biotinylated goat anti-rabbit, biotinylated goat anti-rat, or secondaries attached to Texas Red for immunofluorescence (Vector Laboratories, Burlingame, Calif). Finally, sections were stained with DAPI to visualize cell nuclei.

Fluorescence Microscopy
Triple fluorescently labeled cells were imaged with an upright epifluorescence microscope (Eclipse 80i, Nikon, Melville, NY) with a cooled charge-coupled device camera (Cascade, Photometrics, Tucson, Ariz) interfaced to a Macintosh computer. Fluorescent images were obtained in 3 spectrally resolved channels: ultraviolet (for DAPI nuclear staining), Texas Red (for immunofluorescent cell-specific antibodies), and far red (for ⁶⁴Cu-TNP). Exposure time was 10 ms in the ultraviolet channel and 300 ms in the other channels. Fluorescent images were acquired as digital 16-bit images.
Results

64Cu-TNP Is Detectable by MRI and PET-CT
The detection threshold of the nanoparticle in the imaging phantom was \( \approx 5 \mu g \) Fe/mL using T2-weighted MRI and 0.1 \( \mu g \) Fe/mL for PET-CT imaging (Figure 2).

Biodistribution and Half-Life of 64Cu-TNP
The mean blood half-life of 64Cu-TNP in B6 mice was 259±39 minutes assuming a 1-compartment model (constant, 0.282; fit, \( R^2 = 0.90 \)). Twenty-four hours after injection, 81.3±3.3% of decay-corrected activity remained in the animal. Biodistribution analysis of wild-type mice at this time point revealed the following profile (percent injected dose per 1 g tissue): liver, 33.6±7.3; small intestine, 15.8±2.8; kidney, 13.8±1.8; lung, 11.0±2.5; spleen, 9.4±3.6; heart, 6.0±0.9; aorta, 5.2±0.9; lymph nodes, 4.3±1.1; thymus, 2.4±1.2; and fat, 1.1±0.3. On a total-organ basis, liver (41.7±12.7%), intestine (20.0±2.1%), and carcass (28.3%) contained the majority of nanoparticles. In apoE/−/− mice, the organ distribution was similar, but the percent injected dose accumulated in aortas and carotid arteries was 260% and 392% higher, respectively, than in wild-type mice (both P<0.005).

PET-CT Shows Accumulation of 64Cu-TNP in Atherosclerotic Arteries of ApoE/−/− Mice and Is Corroborated by In Vivo MRI and Ex Vivo Fluorescence Reflectance Imaging
All imaged apoE/−/− mice showed a robust PET signal in the aortic root and arch, as identified by CT in the fused data sets (Figure 3). Intravenous application of iodinated CT contrast enhanced the anatomic detail and identification of the murine vasculature. The target-to-background ratio for 64Cu-TNP-derived PET signal in the aortic root compared with sur-
To further investigate the distribution of 64Cu-TNP within atherosclerotic lesions in apoE−/− mice, we next sought to directly compare 18FDG and 64Cu-TNP imaging in the same animal, facilitated by the short half-life of 18FDG. PET signals were detectable in areas of high plaque burden such as the aortic root for both 18FDG and 64Cu-TNP at respective peak enhancements. In a head-to-head comparison in apoE−/− mice, the PET signal emanating from atherosclerotic lesions was slightly higher for 64Cu-TNP (Figure 8) and persisted longer, consistent with cellular uptake and trapping in phagocytic cells.

**Discussion**

Traditional imaging of atherosclerosis has focused on the caliber of the arterial lumen or the structure of plaque. Advances in the basic and clinical biology of atherosclerosis have identified inflammation as a key process contributing to lesion initiation, progression, and complication. This recognition has spurred considerable effort to image inflammation in atheromata. The application of nanotechnology offers new approaches to the design of imaging agents. Optical imaging and magnetic resonance imaging have used nanoparticles, but this technology has only recently been explored for the design of new generations of PET imaging agents. Here, we report on the development and validation of a novel, flexible nanoparticle platform for PET imaging. The synergy of a high inherent phagocytic avidity of dextran-coated nanoparticles and derivatization of these nanomaterials with a radiotracer provides a highly sensitive tool to assess macrophage burden in murine atherosclerotic lesions. Furthermore, the trimodal character of 64Cu-TNP allows hybrid imaging and rigorous probe validation by fluorescence-based techniques on the cellular and molecular level.

Clinically, interest has burgeoned in developing strategies to identify inflamed, presumably rupture-prone atherosclerotic plaques. Such functional imaging might allow identification of high-risk patients and help direct therapy to prevent cardiovascular events. Goals for a suitable technology include high sensitivity, high specificity for biological processes leading to rupture of a plaque, and practicability. Phantom imaging, shown in Figure 2, established a high sensitivity for PET to detect low concentrations of 64Cu-TNP. TNP used in this study was administered at 1.5 mg Fe/kg body weight, well below the dose currently in clinical trials for oncology (2.6 mg Fe/kg) and the dose used experimentally for imaging of atherosclerosis in rabbits (14 to 56 mg
We anticipate that the detection threshold could easily be improved by several orders of magnitude with further chemical optimization aiming at higher specific activity of the nanoparticle, as well as with the higher sensitivity of the next generation of PET imaging systems.

In vivo PET-CT imaging after injection of $^{64}$Cu-TNP showed robust PET signal in regions of mouse atheromata. In vivo magnetic resonance and ex vivo fluorescence imaging established the distribution of the nanomaterials to atherosclerotic lesions. Phagocytic cells implicated in lesion growth and vulnerability ingested $^{64}$Cu-TNP as shown by immunofluorescence and flow cytometry. After in vivo distribution of $^{64}$Cu-TNP, cells that express macrophage surface markers showed the highest nanoparticle uptake as characterized by maximal mean fluorescence units per cell in flow cytometry.

Although thorough toxicity tests remain to be performed, we expect $^{64}$Cu-TNP to be safe at the trace amounts used. Comparable iron oxide nanoparticles have frequently been used for MRI in animal models and in patients. In vitro studies have shown that high concentrations of nanoparticles may change the cytokine profile of macrophages, yielding a dose-dependent shift toward an antiinflammatory phenotype was observed. It is unclear how the relatively high doses in this in vitro study reflect the in vivo situation. Nevertheless, it seems unlikely that iron oxide nanoparticle uptake would drive phagocytes toward a more inflammatory state. $^{64}$Cu has been used as a PET tracer in patients. A recent study by Lewis et al found no signs of acute toxicity of a $^{64}$Cu derivative in hamsters. The metal copper can be toxic in high doses; however, it was injected here in a chelated state, preventing any potential toxicity of free copper. For instance, gadolinium is highly toxic but is frequently used as a contrast agent in clinical MRI as Gd-DTPA.
Consecutive $^{18}$FDG and $^{64}$Cu-TNP PET-CT imaging established a similar but not identical macroscopic distribution for both probes, with peak vascular signal observed in the aortic root and arch. Microscopic probe distribution could be assessed for $^{64}$Cu-TNP but not for $^{18}$FDG because the radioactive signal was too low and decayed too fast to enable ex vivo autoradiography or gamma counting. Previously, $^{18}$FDG distribution was believed to correlate with plaque macrophage content in patients and in the rabbit plaques. However, unequivocal cellular distribution studies have not been published to date, given the experimental difficulty (rapid decay, unavailability of fluorescent analogs for correlative flow cytometry or fluorescence microscopy). Indeed, a recent study in apoE$^{-/-}$ mice identified brown fat, not atherosclerotic lesions, as a dominant source of $^{18}$FDG signal in vivo.

The multimodality capabilities of the nanomaterials developed here facilitated rigorous validation of the origin of the signal and the fate of the imaging probe. The addition of a fluorochrome for optical imaging proved particularly helpful to test the novel probe and to determine its fate at a cellular level in vivo. We anticipate that nanomaterials such as the one described here will advance both basic research and clinical applications for several reasons. First, the trimodality

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diagnostic capability will prove synergistic for hybrid imaging systems currently entering clinical use. Second, PET imaging has a high inherent sensitivity, allows quantification of the signal, and will facilitate whole-body screening of the entire arterial tree. Third, the described nanotechnology platform is being introduced into the clinic and is versatile. The carbohydrate coating can be amninated to attach linkers and affinity ligands to specifically target these nanoparticles to other molecular and cellular structures such as adhesion molecules, macrophage subpopulations, or cells undergoing apoptosis.

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Disclosures
Dr Weissleder is a shareholder of VisEn Medical in Woburn, Mass. The remaining authors report no conflicts.

References


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**CLINICAL PERSPECTIVE**

Phagocytic cells in inflammatory atherosclerotic lesions are a key histological component. They actively secrete enzymes involved in plaque progression and rupture and thus have been used as biomarkers for lesion severity. Here, we show that dextran-coated nanoparticles labeled with $^{64}$Cu can be used for positron emission tomography imaging of these phagocytes. Combined with computed tomography imaging for anatomic coregistration, the developed approach was highly accurate for detection of inflamed plaques in murine arteries and at tracer concentration. Compared with conventional $^{18}$F-fluoro-2-deoxyglucose positron emission tomography imaging, $^{64}$Cu-TNP yielded higher target-to-background ratio, presumably as a result of specific targeting to the phagocytic compartment. The approach may have important clinical applications in surveying lesion severity among different vascular beds and as a surrogate for therapeutic efficacy.
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