Polymeric Nanoparticle PET/MR Imaging Allows Macrophage Detection in Atherosclerotic Plaques

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Rationale: Myeloid cell content in atherosclerotic plaques associates with rupture and thrombosis. Thus, imaging of lesional monocytes and macrophages could serve as a biomarker of disease progression and therapeutic intervention.

Objective: To noninvasively assess plaque inflammation with dextran nanoparticle (DNP)-facilitated hybrid positron emission tomography/magnetic resonance imaging (PET/MRI).

Methods and Results: Using clinically approved building blocks, we systematically developed 13-nm polymeric nanoparticles consisting of cross-linked short chain dextrans, which were modified with desferoxamine for zirconium-89 radiolabeling (89Zr-DNP) and a near-infrared fluorochrome (VT680) for microscopic and cellular validation. Flow cytometry of cells isolated from excised aortas showed DNP uptake predominantly in monocytes and macrophages (76.7%) and lower signal originating from other leukocytes, such as neutrophils and lymphocytes (11.8% and 0.7%, P<0.05 versus monocytes and macrophages). DNP colocalized with the myeloid cell marker CD11b on immunohistochemistry. PET/MRI revealed high uptake of 89Zr-DNP in the aortic root of apolipoprotein E knock out (ApoE−/−) mice (standard uptake value, ApoE−/− mice versus wild-type controls, 1.9±0.28 versus 1.3±0.03; P<0.05), corroborated by ex vivo scintillation counting and autoradiography. Therapeutic silencing of the monocyte-recruiting receptor C-C chemokine receptor type 2 (CCR2) with short-interfering RNA decreased 89Zr-DNP plaque signal (P<0.05) and inflammatory gene expression (P<0.05).


Key Words: atherosclerosis • inflammation • macrophage • molecular imaging • nanoparticles • PET/MRI

During atherogenesis, monocytes and macrophages (Mo/Mϕ) invade the arterial intima and sustain and amplify local inflammation through secretion of cytokines, reactive oxygen species, and proteolytic enzymes.3,4 These innate immune cells may differentiate into foam cells and favor degradation of extracellular matrix macromolecules and thinning of the fibrous cap, features of plaques that have ruptured and triggered myocardial infarction or stroke.5 Macrophage-targeted imaging may provide the opportunity to detect upstream events that play a causative role in disease progression and thus enable preventive therapy.1,3,4 Iron oxide nanoparticle uptake assessed by magnetic resonance imaging (MRI) has begun to address this need,3,4 but this approach is mostly semiquantitative, and the detection sensitivity for these nanoparticles is about 50 times lower compared with radioisotope imaging.5

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This study aimed to develop a sensitive, clinically viable technique for detection of inflammatory leukocytes in atherosclerotic plaque with positron emission tomography (PET). The approach used core-free dextran nanoparticles (DNP) made from clinically approved components, labeled with desferoxamine to chelate the PET isotope zirconium-89 (89Zr). Dextran is biocompatible and biodegradable, making it a suitable starting material for nanoparticles intended for clinical use. We tested DNPs using hybrid PET/MRI
for detecting macrophages in atherosclerotic plaques in apolipoprotein E knock out (ApoE⁻/⁻) mice. MRI provided the anatomic reference for the source of the PET signal, which reported on nanoparticle deposition in the vessel wall. We further used the PET macrophage reporter ⁸⁹Zr-DNP to assess the efficacy of a therapeutic intervention, Mo/Mϕ-targeted RNA interference. Using lipidoid nanoparticle-enabled silencing of C-C chemokine receptor type 2 (CCR2),⁶ the chemokine receptor that dominates recruitment of inflammatory monocytes,⁷ we followed plaque inflammation with PET/MRI noninvasively and related imaging results to modified gene expression patterns in the vascular wall.

Methods

DNP Synthesis
Dextran (10 kDa; Amersham Biosciences, Sweden) was cross-linked with epichlorohydrin followed by partial amination with ethylene diamine to yield amino-DNPs.⁸ DNP-NH₂ was modified with p-isothiocyanatobenzyl desferoxamine (SCN-Bz-Df; Macrocyclics, TX) and the N-hydroxysuccinimidyl ester of the near-infrared fluorophore VivoTag680 (VT680; PerkinElmer, Waltham, MA). The remaining amines were succinylated. The quantitative radiolabeling of the nanoparticle was achieved at room temperature by complexation procedure.⁶ Lipids were dissolved in 90% ethanol and mixed with ⁸⁹Zr-DNP to give ⁸⁹Zr-DNP. The radiochemical yield and purity of ⁸⁹Zr-DNP were determined using radio-thin-layer chromatography using and instant thin-layer chromatography plates and 50 mmol/L EDTA as a mobile phase.

siRNA Nanoparticle Synthesis
siRNA (short-interfering RNA) synthesis and formulation into lipid nanoparticles used previously described methods. The RNA duplex for silencing CCR2 was 5′-uGcuAAAcGucucuGcAAAdTsdT-3′ (sense), 5′-UUUG aAGAGCUGUuAGcAdTsdT-3′ (antisense), a sequence known to limit CCR2 expression.⁶ Single-strand RNAs were produced, annealed, and used as duplexes. The nanocarrier for siRNA delivery was prepared with a cationic lipid using a spontaneous vesicle formation procedure.⁶ Lipids were dissolved in 90% ethanol and mixed with siRNA solution (1:1 ratio) at fixed speed and diluted immediately with PBS to final 25% ethanol concentration. The ethanol was then removed and the external buffer was replaced with PBS by dialysis.

Animal Experiments
Female C57Bl/6 (n=10) and ApoE⁻/⁻ mice (n=22) were purchased from the Jackson Laboratory (Jackson, MI). C57Bl/6 (B6) mice were 12 to 16 weeks of age. ApoE⁻/⁻ mice had an average age of 28 weeks and consumed a high-cholesterol diet (Harlan Teklad, 0.2% total cholesterol) for at least 16 weeks, conditions that reliably produce atherosclerotic plaques in the aortic root and arch. Mice were anesthetized by digital autoradiography. Aortas were exposed to a ⁸⁹Zr-DNP in the tail vein of 5 B6 mice. After killing (48 hours), mice were perfused with 10 mL of PBS. Organs were harvested, weighed, and their activity was recorded with a γ counter (1480 Wizard 3-inch PerkinElmer, Waltham, MA). Biodistribution data were corrected for radioactive decay and residual radioactivity at the injection site. Oil-Red-O staining depicted the distribution of plaques in ApoE⁻/⁻ aortas that were subsequently photographed by a digital camera as well as analyzed by digital autoradiography. Aortas were exposed to a phosphorimager plate and read with a Typhoon FLA9000 system (GE Healthcare) 4 hours later. Visualization of scanned autoradiography images was performed using the ImageJ program (Image J 1.440, National Institute of Health, Bethesda, MD).

Quantitative Reverse-Transcriptase Polymerase Chain Reaction
Messenger RNA was extracted from the aortic root and arch using a RNasy Micro Kit (Qiagen) using manufacturer’s protocol. One microgram of mRNA was used to generate complimentary DNA (cDNA) using a high capacity RNA to cDNA kit (Applied Biosystems). cDNA was then further amplified using the QuantiTect Whole Transcriptome}
kit (Qiagen). The amplified cDNA was diluted at a ratio of 1:10 in DNase- and RNase-free H,O and 2 μL of final cDNA was used for further analysis. Levels of mRNAs encoding target genes used Taqman assays (Applied Biosystems). Variability in cDNA concentration was normalized using GAPDH, a constitutively expressed gene.

PET-CT Imaging
Imaging experiments were performed 48 hours after injection of 89Zr-DNP at 354±13 μCi/mouse. All PET-computed tomography (CT) images were acquired on a Siemens Inveon micro PET-CT. Each PET acquisition was ≈90 minutes in duration. PET was reconstructed from 600 million coincidental 511 keV photon counts. Counts were rebinned in 3-dimension by registering photons spanning ≤3 consecutive rings, then reconstructed into sinograms by using a high-resolution Fourier Rebin algorithm. Image pixel size was anisotropic, with dimensions of 0.796 mm in the z direction and 0.861 mm in the x and y directions, for a total of 512×512×768 pixels. Projections were acquired at end expiration using a BioVet gating system (M2M Imaging, Cleveland, OH), and CT acquisition time was ≈10 minutes. Reconstruction of data sets used IRW software (Siemens).

PET/MR Registration and Fusion
In vivo MRI was performed for anatomic reference immediately after PET/CT imaging in ApoE−/− mice with or without siCCCR2 treatment. Cine images were obtained on a 7-Tesla Bruker Mesmac system with ECG and respiratory gating (SA instruments, Inc, Stony Brook, NY) using a fast gradient echo FLASH sequence with the following parameters: echo time 2.7 ms; 16 frames per R-R interval (repetition time 7.0–15.0 ms); resolution 200 μm ×200 μm ×1 mm; number of excitations 4; flip angle 60°. PET/CT and MR images were acquired sequentially using a custom-designed mouse bed and a PET-CT gantry adapter. Offline fusion was based on external fiducial markers. A custom-built mouse vest of several loops of PE50 tubing was filled with 15% iodine in water, visible in both CT and MRI. PET data were fused to CT as part of a standard PET-CT workflow. Registration of MRI and PET/CT data sets were then obtained by superimposing the fiducial landmarks with AMIRA software (Version 5.4, Berlin, Germany).†

Statistics
Results are expressed as mean±SEM. Differences between groups were evaluated by Student t test. We used GraphPad Prism 4.0c for statistical analysis. A probability value of <0.05 was used to indicate statistical significance.

Results
Nanoparticle Preparation, Blood Half-life, and Biodistribution
The core-free nanoparticles used in this study consisted of polymeric dextran and had a mean particle size of 13.3 nm as determined by laser light scattering. On average, 8 desferoxamine and 1 fluorochrome moieties were attached per particle. The radiolabeling yield of 89Zr-DNP (Figure 1A and 1B) was >99.5%. After centrifugation, the radiochemical purity was 100%. 89Zr-DNP was injected into 5 B6 mice to measure the nanoparticle’s kinetics in vivo. 89Zr-DNP had a blood half-life of 3.73±0.33 hours (R² of fit =0.95; Figure 1C). Biodistribution of 89Zr-DNP at 48 hours was as follows (expressed as %ID/g): liver, 11.1±1.4; spleen, 10.3±1.8; lymph node, 5.3±0.9; blood, 4.7±1.0; kidney, 3.4±0.2; heart, 1.2±0.2; aorta, 1.2±0.1; tail, 0.9±0.2; lung, 1.0±0.4; intestines, 0.9±0.1; fat, 0.6±0.1; and muscle, 0.4±0.1 (Figure 1D).

In Vivo Hybrid PET/MRI of 89Zr-DNP Detects Atherosclerotic Plaque Inflammation
89Zr-DNP uptake in atherosclerotic aortic root plaques was quantified with PET, whereas MRI provided the anatomic reference. The aortic root and arch of ApoE−/− mice had significantly higher activity compared with wild-type controls (standard uptake value 1.85±0.28 versus 1.25±0.03 and 1.46±0.09 versus 0.98±0.04; P<0.05 for both; Figure 2A and 2B). The target-to-background ratio in aortic root and arch of ApoE−/− mice also significantly exceeded that in wild-type controls (aortic root and arch, 2.56±0.19 versus 1.64±0.03, and 2.16±0.34 versus 1.29±0.07; P<0.05 for both).

Ex Vivo Scintillation Counting and Autoradiography Confirm In Vivo PET/MRI
After imaging, we measured radioactivity in excised aortas by scintillation counting and autoradiography exposure. Compared with wild type, the percent injected dose per gram tissue (%ID/g) was significantly higher in aortas harvested from ApoE−/− mice (0.69±0.09 versus 1.24±0.11; P<0.01; Figure 2C). Autoradiography showed the peak activity in the aortic root and arch. Oil-Red-O stained lipid-rich plaques correlated well with the peak isotope signal on autoradiography (Figure 2D).
DNP Accumulate in Plaque Leukocytes

Nanoparticle uptake in atherosclerotic plaques in the aortic root was assessed using fluorescence microscopy (Figure 3). Microscopic images of adjacent sections stained for the mononuclear phagocyte antigen CD11b showed colocalization of myeloid cells with DNP in aortic root plaques (Figure 3A and 3B). CD11b is expressed primarily by monocytes, macrophages, and neutrophils. Imaging of sections in the lower-wavelength fluorescein isothiocyanate channel confirmed the specificity of the observed signal for nanoparticles rather than autofluorescence (Figure 3C). Higher magnification immunofluorescent microscopy after immunoreactive staining with CD11b corroborated colocalization of the DNP signal to CD11b+ cells (Figure 3D–3F).

Cellular Sources of the Nanoparticle Signal

Quantification of DNP uptake into specific leukocyte populations (monocytes, macrophages, neutrophils, and lymphocytes) used multicolor flow cytometry detection of cell surface markers, and the fluorescence signal of DNP was compared for different cells. Flow cytometry revealed that the majority of DNP accumulated in Mo/Mφ (Figure 4); however, 23% of the cellular signal derived from other sources. This observation suggests that although the majority of the PET signal in atherosclerotic plaques resulted from uptake of nanoparticles by Mo/Mφ, DNP was not entirely specific for those cells but also taken up to some degree by neutrophils and lymphocytes. Of note, flow cytometry does not detect extracellular signal.

PET/MRI Detects Therapeutic Effects of In Vivo RNAi

Further experiments assessed the use of 89Zr-DNP PET/MRI for following the efficacy of a therapeutic intervention; silencing of CCR2, the monocytic chemokine receptor responsible for migration of the inflammatory monocyte subset to sites of inflammation using siRNA technology. siRNA silencing of CCR2 (siCCR2) significantly attenuates receptor protein expression in circulating and splenic monocytes. This intervention lowered the PET/MRI 89Zr-DNP signal in association with attenuated monocyte recruitment. The aortic roots of siCCR2-treated ApoE−/− mice had decreased PET signal when compared with ApoE−/− mice treated with an irrelevant siRNA. The target-to-background ratio changed from 2.25±0.11 to 1.82±0.10; P<0.03 (Figures 5A and 5B).
After imaging, quantitative reverse-transcriptase polymerase chain reaction of aortic extracts assessed gene expression. Systemic silencing of CCR2 reduced the expression of genes involved in vascular inflammation. Expression of CCR2, vascular cell adhesion molecule-1, interleukin-6, and tumor necrosis factor-α fell in siCCR2-treated mice, whereas transforming growth factor-β and arginase 1 gene expression rose (Figure 5C).

**Discussion**

Timely identification of patients with high risk for myocardial infarction or stroke remains challenging. It is even more difficult to predict the risk of rupture for a specific atherosclerotic plaque, a thrombotic trigger tightly linked to inflammation. Identification of inflamed atheromata could trigger aggressive risk factor modification, intensive pharmacological treatment,
and perhaps other preemptive interventions to enhance lesion stability and reduce the probability of plaque rupture and thrombosis. Thus, techniques for assessment of plaque inflammation might aid prevention of myocardial infarction and stroke. Inflammatory Mo/Mϕ play a central role in the pathobiology of plaque complication. Because these cells elaborate matrix-degrading proteases implicated in plaque rupture, they furnish attractive imaging biomarkers for plaque instability. The current work focused on the detection of inflammatory myeloid cells in atheromata with clinically viable, radioisotope-labeled DNPs without a metal core. Hybrid PET/MRI detection of these nanoparticles permitted tracking of RNAi-mediated silencing of CCR2, a chemokine receptor that mediates the recruitment of inflammatory monocytes in the arterial intima.

Previous studies have used PET for macrophage imaging, especially, with fluorodeoxyglucose (18F-FDG). PET has interest for detection of cellular and molecular targets because it is the most sensitive noninvasive clinical imaging technology and permits quantitation. 18F-FDG has widespread clinical availability, and studies have correlated 18F-FDG uptake to the macrophage content of atheromata. Yet, 18F-FDG uptake lacks specificity for macrophages. Many other cells, including cardiomyocytes, smooth muscle cells, and other leukocytes, all of which are present in or close to atherosclerotic plaques, accumulate high amounts of glucose, making specific imaging of plaque inflammation with 18F-FDG challenging in the coronary arteries. A recent study showed that hypoxia, but not proinflammatory cytokines, augments glucose uptake by human macrophages. Thus, there is an unmet need for more specific macrophage-targeted imaging agents. In contrast to 18F-FDG, DNP report on phagocytic rather than metabolic activity. In the current work, 13-nm DNPs were designed for clinical translatability. These nanoparticles are composed of clinically approved materials, including small cross-linked dextran strands, and do not contain a metal core; hence, they are not detectable with MRI. Modified with desferoxamine, a clinically approved metal chelator, the nanoparticles were labeled with zirconium-89 for PET imaging. Zirconium-89 has a slow radioactive decay matching the in vivo kinetics of the 13-nm DNP. Therefore, imaging can be done after the nanoparticle has cleared from the blood pool yielding a low-background signal. DNP could also be labeled with other PET isotopes. If a smaller particle size accelerated pharmacokinetics, shorter injection-imaging sequences may facilitate fluorine-18 labeling. Importantly, 89Zr-DNP are easily biodegradable, supporting translation to humans.

Although PET and MRI were done sequentially and data were fused offline in our study, newer scanners allow for synchronous data acquisition in mice and in patients. Our data highlight the advantages of combining both modalities. MRI is a leading modality for imaging the cardiovascular system, and may thus prove to be particularly useful in conjunction with PET. Sparse molecular or cellular targets can be quantitated by PET, whereas MRI delivers high-contrast, high-resolution, and time-resolved cardiovascular imaging without additional radiation exposure. This approach permits analysis of molecular targets in anatomic and physiological context because MRI may deliver information on tissue motion and strain, blood flow, oxygenation, diffusion, and fiber orientation. PET/MRI could also enable dual target molecular imaging by simultaneous use of MRI agents. For example, one could use 89Zr-DNP for imaging of inflammation in conjunction with MR agents that detect cell death, collagen, or fibrin deposition.

Preclinical and clinical studies have highlighted the role of chronic persistent inflammation in the pathophysiology of atherosclerosis; hence specific anti-inflammatory therapy might produce clinical benefit. Here, we encapsulated siRNA in lipidoid nanoparticles for delivery to phagocytic cells. The target was CCR2, a receptor for the chemokine monocyte chemotactic protein-1 that specifically recruits the inflammatory monocyte subset to sites of inflammation in mice and in humans. As shown in landmark studies on the role of this chemokine/chemokine receptor axis, deficiency of either CCR2 or monocyte chemotactic protein-1 prevents recruitment of monocytes, thereby reducing macrophage burden and inflammation in plaques. We previously validated the delivery of siRNA to circulating and splenic monocytes by lipidoid nanoparticles and the efficiency of CCR2 silencing for the RNA target sequence. The splenic monocyte reservoir is particularly a suitable target because these cells rapidly ingest lipidoid nanoparticles used for siRNA delivery. In atherosclerotic mice, the spleen contributes monocytes to atherosclerotic plaque. Our current study followed monocyte-targeted siRNA therapy by plaque macrophage PET/MRI. We found significantly reduced PET signal in the aortic root and arch of ApoE−/− mice treated with siCCR2. The decreased macrophage PET signal was accompanied by a significantly reduced inflammatory gene expression pattern in the imaged arterial bed. The expression of CCR2 and several other proinflammatory genes fell, whereas the mRNAs that encode transforming growth factor-β and arginase 1, genes associated with resolution of inflammation, increased. Future studies will clarify whether 89Zr-DNP PET/MRI will be useful for imaging of therapeutic efficiency.

In conclusion, this study demonstrates the feasibility of nanoparticle-facilitated hybrid PET/MRI of inflammatory leukocytes in murine atherosclerotic plaques. The majority of cellular 89Zr-DNP signal (77% by flow cytometry) was derived from Mo/Mϕ; hence, the cellular specificity was limited. Specific targeting of monocyte recruitment with RNAi reduced the macrophage PET signal and dampened inflammation in plaques. Clinical translation of this cellular imaging strategy could identify macrophage-rich plaques at risk for complications and evaluate the effectiveness of macrophage-targeted therapies in atheromata.

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Disclosures

Anna Borodovsky and Kevin Fitzgerald are full-time employees at Alnylam. Daniel Anderson holds Alnylam shares.

References


Novelty and Significance

What Is Known?

- Macrophages are effector cells of innate immunity that can destabilize atherosclerotic plaques by secreting proinflammatory cytokines and proteases.
- Macrophages have been linked to plaque rupture, which leads to myocardial infarction and stroke in patients with atherosclerosis.
- Nanoparticles are avidly taken up by macrophages and may report on plaque stability.

What New Information Does This Article Contribute?

- Nanoparticles made from dextran building blocks accumulate in macrophages that reside in atherosclerotic plaques.
- Derivatization of these particles with the radioactive isotope zirconium-89 enables imaging of aortic plaques of apoE−/− mice by positron emission tomography.
- In vivo silencing of monocyte C-C chemokine receptor type 2 receptor reduces the nanoparticle imaging signal and attenuates inflammatory gene expression.

Many ischemic complications arise from atherosclerotic plaques that do not significantly obstruct blood flow. Conventional anatomic imaging therefore cannot predict which lesions are at high risk of rupture. However, this information could guide individual risk assessment and direct preventive therapy. In this study, we describe the use of 13-nm dextran nanoparticles for imaging plaque inflammation in mice with atherosclerosis. Nanoparticles were derivatized with a radioactive isotope and detected by positron emission tomography, joined with magnetic resonance imaging for anatomic reference. Mice with silenced monocyte chemokine receptor C-C chemokine receptor type 2 accumulated fewer inflammatory cells in atherosclerotic lesions, in parallel with reduced expression of inflammatory genes. Clinical translation of this technology may be helpful in identifying patients at risk for plaque rupture and for developing preventive measures to avoid the outcomes of thrombotic events, such as stroke and acute myocardial infarction.
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