Folate Receptor–Targeted Single-Photon Emission Computed Tomography/Computed Tomography to Detect Activated Macrophages in Atherosclerosis: Can It Distinguish Vulnerable from Stable Atherosclerotic Plaques?

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Abstract

The need for noninvasive imaging to distinguish stable from vulnerable atherosclerotic plaques is evident. Activated macrophages play a role in atherosclerosis and express folate receptor folate receptor β (FR-β). The feasibility of folate targeting to detect atherosclerosis was demonstrated in human and mouse plaques, and it was suggested that molecular imaging of FR-β through folate conjugates might be a specific marker for plaque vulnerability. However, these studies did not allow differentiation between stable and vulnerable atherosclerotic plaques. We investigated the feasibility of a folate-based radiopharmaceutical (\(^{111}\)In-EC0800) with high-resolution animal single-photon emission computed tomography/computed tomography (SPECT/CT) to differentiate between stable and vulnerable atherosclerotic plaques in apolipoprotein E\(^{-/-}\) mice in which we can induce plaques with the characteristics of stable and vulnerable plaques by placing a flow-modifying cast around the common carotid artery. Both plaques showed \(^{111}\)In-EC0800 uptake, with higher uptake in the vulnerable plaque. However, the vulnerable plaque was larger than the stable plaque. Therefore, we determined tracer uptake per plaque volume and demonstrated higher accumulation of \(^{111}\)In-EC0800 in the stable plaque normalized to plaque volume. Our data show that \(^{111}\)In-EC0800 is not a clear-cut marker for the detection of vulnerable plaques but detects both stable and vulnerable atherosclerotic plaques in a mouse model of atherosclerosis.

Atherosclerotic plaques with a high risk of rupture are called vulnerable plaques, whereas plaques with a low risk of rupture are called stable plaques. Vulnerable plaques are generally characterized by a thin fibrous cap, a large lipid pool, and increased macrophage activity. Angiography, the “gold standard” to detect occlusive arterial stenosis, is unable to detect vulnerable plaques that are not stenotic. Therefore, the need to develop a noninvasive molecular imaging tool that can differentiate between stable and vulnerable atherosclerotic plaques is evident. To that extent, a specific target is required that is differentially expressed between stable and vulnerable plaques. Macrophages are differentially activated between stable and vulnerable plaques and may provide a specific stable or vulnerable plaque marker. Activated macrophages were shown to express folate receptor β (FR-β). Recently, FR-β-expressing macrophages were used as a target to detect atherosclerosis ex vivo in human atherosclerotic lesions and in vivo in the aortic arch of apolipoprotein E\(^{-/-}\) (ApoE\(^{-/-}\)) mice by means of folate targeting. Both groups hypothesized that molecular imaging of FR-β through folate conjugates could be a marker for plaque vulnerability, suggesting that macrophages in vulnerable plaques express FR-β, whereas macrophages in stable plaques do not. However, these studies did not consider differences between stable and vulnerable atherosclerotic plaques. We tested their hypothesis by...
targeting FR-β-expressing macrophages with a folate-based radiopharmaceutical (\(^{111}\)In-EC0800, kindly provided by Endocyte Inc., West Lafayette, IN) and high-resolution animal single-photon emission computed tomography/computed tomography (SPECT/CT) in a unique mouse model in which both atherosclerotic plaque phenotypes are present in the same vessel segment.\(^6\)-\(^{12}\)

**Materials and Methods**

Female ApoE\(^{-/-}\) mice on a C57BL/6J background (\(n = 4\)) were obtained from in-house breeding facilities (Erasmus MC, Rotterdam, The Netherlands). Normal chow diet was replaced at 13 weeks of age by an atherogenic Western diet (nr 4021.06, Arie Blok, Woerden, The Netherlands) 2 weeks prior to cast placement around the right common carotid artery (RCCA), as described previously.\(^6\) The cast with a constrictive, tapered lumen (400 \(\mu\)m to 200 \(\mu\)m over 1.5 mm) alters the arterial flow fields, which, in an atherosclerotic setting (ie, ApoE\(^{-/-}\) mice on a high-fat diet), leads to the generation of a plaque proximal to the cast with characteristics of a vulnerable plaque and a more stable plaque distal to the cast.\(^6\)-\(^{12}\) A DOTA-Bz-folate conjugate referred to as EC0800 (Endocyte Inc.) was radiolabeled with \(^{111}\)In as previously described.\(^13\) Six weeks after cast placement, mice were injected with \(^{111}\)In-EC0800 (\(\pm 50\) MBq) intravenously in the tail. Scans were obtained approximately 20 hours after injection using a four-head multipinhole SPECT/CT camera (NanoSPECT/CT, Bioscan Inc., Washington, DC). Mice were anesthetized with 1.5 to 2.5% isoflurane, and SPECT images were acquired for 60 minutes (32 projections, 210 seconds per projection, voxel size 0.3 \(\times\) 0.3 \(\times\) 0.3 mm). In addition, an anatomic CT scan was obtained. The fused images of each mouse were manually reoriented to achieve the same orientation for all scans. Quantification of the SPECT signal was performed using custom software routines (MATLAB, 7.1., The MathWorks, Natick, MA), which was validated by InVivoScope (version 1.44, Bioscan, Inc.). The volumes of interest (VOI) for quantification were based on the location of the cast in the CT scan (voxel size 0.1 \(\times\) 0.1 \(\times\) 0.1 mm), blinded to the SPECT results to avoid any bias in positioning of the VOI. Because the CT and SPECT are automatically coregistered, the VOI drawn on the CT correspond to the same spatial location of the tracer. Two VOI (20 \(\times\) 10 \(\times\) 10 mm) were defined: a VOI directly distal to the cast and a VOI directly proximal to the cast. The total signal inside the VOI was used in the analysis.

After imaging, mice were euthanized and flushed with phosphate-buffered saline followed by excision of the RCCA, which was embedded in Tissue-Tec (OCT compound) and frozen on isopropanol for further histologic analysis. Serial sections (5 \(\mu\)m thick) were stained for lipids (oil red O, Sigma-Aldrich, Zwijndrecht, The Netherlands), general lesion morphology (hematoxylin [Klinipath, Duiven, The Netherlands] and eosiin [hematoxylin-eosiin, Sigma-Aldrich]), macrophages (CD68, 1:200, AbD Serotec, Düsseldorf, Germany), and endothelium (CD31, 1:100, BioLegend, Uithoorn, The Netherlands). Quantification of histologic data was performed on 5 \(\mu\)m cryosections at 100 \(\mu\)m intervals of the complete plaques. The intensity of each staining method was quantified using BioPix iQ 2.0 imaging software (BioPix, Göteborg, Sweden).

Data are presented as mean ± SD, with statistical significance evaluated using a paired, two-tailed, Student \(t\)-test (SPSS release 17.0, SPSS Inc., Chicago, IL). A value of \(p \leq .05\) was considered significant. All experiments were performed under the regulation and permission of the Animal Care Committee of Erasmus Medical Center, Rotterdam, The Netherlands.

**Results**

Following 6 weeks of cast placement, comparing the radioactivity in the proximal and distal VOI, representing a vulnerable and a stable plaque, respectively, the total radioactivity was significantly 1.5 times higher (\(p = .015\)) in the region proximal to the cast compared to the region distal to the cast (Figure 1). However, a clear difference in plaque volume, defined as the area between the internal elastic lamina and the lumen, between the proximal and the distal plaque, was observed (Figure 2, A and B). Therefore, we determined tracer uptake per plaque volume. Subsequently, we found that more radioactivity was present in the distal plaque (240 kBq/mm\(^3\) ± 109 kBq/mm\(^3\)) compared to the proximal plaque (39 kBq/mm\(^3\) ± 20 kBq/mm\(^3\)) (\(p = .05\); Figure 3A) normalized to plaque volume. Similarly, expressing tracer uptake per volume of macrophages, more FR-β-expressing macrophages were present in the distal plaque (663 kBq/mm\(^3\) ± 318 kBq/mm\(^3\)) compared to the proximal plaque (86 kBq/mm\(^3\) ± 30 kBq/mm\(^3\)) (\(p = .04\); Figure 3B) normalized to macrophage volume. The proximal plaque contained a higher percentage of lipids than the distal plaque (Figure 2, C and E; 29 ± 9% vs 18 ± 14%, \(p = .14\)) but a comparable percentage of macrophages (CD68-positive area, Figure 2, D and F; 44 ± 11% vs 37 ± 2%, \(p = .24\)), which is consistent with previous findings.\(^6\)
Discussion

We investigated the use of noninvasive imaging of FR-β-expressing macrophages with $^{111}$In-EC0800 to distinguish between vulnerable and stable plaques in an atherosclerotic mouse model in vivo. We made use of an established model with a plaque with characteristics of vulnerability and a more stable plaque. Previously, we and others successfully used folate radiopharmaceuticals. In this study, we found folate uptake in both types of plaques, suggesting the presence of FR-β-expressing macrophages in both types of plaques. Some tracer uptake was observed outside the vessel at the level of the cast (see Figure 1) due to the presence of macrophages in the foreign body response. Therefore, we did not analyze tracer uptake in the cast area itself and made use of a VOI proximally and distally to the cast (see Figure 1C). Tracer uptake proximal to the cast was much higher compared to the uptake distal to the cast (see Figure 1). However, histologic analysis revealed a considerable difference in plaque size between the two plaques (see Figure 2), the plaque with the highest tracer uptake being the largest. As such, the difference in tracer uptake could simply be due to a difference in plaque size or macrophage content and not due to a difference in plaque phenotype. To test this, we expressed tracer uptake per plaque volume (see Figure 3A) or amount (area) of macrophages (see Figure 3B). Surprisingly, this revealed a higher tracer uptake in the smaller, stable plaque normalized to plaque volume, indicating that there are either more FR-β-expressing macrophages in the stable plaque compared to the vulnerable plaque or that the macrophages in the stable plaque express more FR-β individually.

Conclusion

We demonstrated that $^{111}$In-EC0800 imaging detects both stable and vulnerable atherosclerotic plaques, suggesting that $^{111}$In-EC0800 is not a clear-cut marker for the detection of vulnerable plaques, in a mouse model for atherosclerosis. However, combining folate-based imaging with imaging of plaque size might still be an interesting target for differentiating between stable and vulnerable plaques. This will have to be confirmed in a larger study setting. Initially, this technique can be used for animal studies following the development of atherosclerotic plaques over time. Further investigations should determine whether the technique can be used to differentiate between stable and vulnerable plaques in humans, aiming at the identification of patients who are at risk for plaque rupture.
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