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Intra plaque and cellular distribution of dextran coated iron oxide fluorescently labeled nanoparticles: insights into atherothrombosis and plaque rupture

Claudia Calcagno, MD, PhD[1,2] and **Zahi A. Fayad, PhD**[1,2]

[1]Translational and Molecular Imaging Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[2]Department of Radiology, Icahn School of Medicine at Mount Sinai, New York, NY, USA

Inflammation, endothelial permeability and the vulnerable plaque

The past 10–15 years have witnessed a tremendous interest in developing and validating non-invasive imaging techniques aimed at quantifying features of atherosclerotic plaque vulnerability in humans most notably plaque inflammation. Among these, Magnetic Resonance Imaging (MRI) with iron oxide nanoparticles has been widely investigated over the years as a mean to detect this feature of high-risk plaques (1).

Iron oxide nanoparticles imaging of plaque inflammation

Iron oxide nanoparticles are particles containing an iron oxide core. Based on their size, they are subdivided into several types, better suited for different applications (for example intravascular versus tissue imaging)(2). Their tissue accumulation causes local magnetic field inhomogeneities that can be measured by MRI (2). Alternatively, iron oxides (such as the dextran coated cross-linked iron oxide nanoparticles, CLIO, used in this work)(3), can be labeled with radioisotopes and/or fluorophores and functionalized as multi-modality imaging agents detectable by positron emission tomography (PET) or near infra-red fluorescence (NIRF) imaging, alone or in combination(4). Iron oxides can be used as non-targeted agents, avidly phagocytized by inflammatory cells and cells of the reticulo endothelial system (2), or can be labeled to target and image specific molecules involved in disease processes (some examples in atherosclerosis are iron oxides targeted to oxidation specific epitopes, or to adhesion molecules on endothelial cells)(5). While targeted and functionalized nanoparticles are typically used in animal models, some non-targeted iron oxides are commercially available. Ferumoxtran-10 and ferumoxytol, an agent currently approved only for the treatment of iron deficient anemia in patients with chronic kidney disease (CKD) are ultrasmall superparamagnetic iron oxides (USPIOs) that are currently or have been

CORRESPONDANCE TO: Zahi A. Fayad, PhD, Director, Translational and Molecular Imaging Institute, Professor of Radiology and Medicine (Cardiology), Icahn School of Medicine at Mount Sinai, Tel: 212-824-8452, Fax: 240-368-8096, zahi.fayad@mssm.edu, One Gustave L. Levy Place, Box 1234, New York, NY, 10029, USA.

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previously clinically approved, and whose use has been investigated to track plaque inflammation(6). The use of these and other iron oxides for pre-clinical and clinical imaging of cardiovascular disease has been described in hundreds of publications, of which we give some examples. In several studies uptake of ferumoxtran-10 was demonstrated in the symptomatic and contralateral asymptomatic carotid artery of patients with carotid (7) and coronary artery disease(8). No correlation was found between ferumoxtran-10 uptake and carotid stenosis(9). The ATHEROMA study showed a reduction in ferumoxtran-10 uptake in the human carotid arteries after 12 weeks of treatment with high dose (80 mg/day) but not low (10 mg/day) dose atorvastatin(10), suggestive of a reduction in plaque inflammation. Pre-clinical studies in animal models have offered further insights on the role of iron oxides for plaque imaging. A lower accumulation of P904, a ultrasmall superparamagnetic iron oxide (USPIO) particle with fast blood clearance, was shown in aortic plaques of atherosclerotic mice after treatment with irbesartan(11). P904 accumulation correlated with ΔT2* values and with macrophage-covered area by immunohistological analysis(11). In rabbits both ferumoxtran-10 and ferumoxytol (12) were found to accumulate in aortic plaques. In hereditary hyperlipidemic rabbits a low endothelial sheer stress (calculated using computational fluid dynamics of computed tomography (CT) images) and P904 accumulation assessed by MRI were found to be independent predictors of plaque progression (13). A different study using dextran-coated monocrystalline iron oxide nanoparticles (MIONs) demonstrated vessel wall accumulation in a similar rabbit model, which correlated with plaque macrophages content, and was reduced upon regression of plaque inflammation after treatment with rosuvastatin(14). While iron oxides have been widely used in these studies as macrophage imaging agents, as already mentioned in the article by Stein-Merlob et al. [15] in this issue of *Circulation: Cardiovascular Imaging*, some animal studies have raised questions on their pattern of deposition within the plaque, and their cellular specificity. As an additional example, a murine study (16) showed a reduction in ferumoxtran-10 uptake, rather than macrophages content, after treatment with a p38 MAPK inhibitor. Another study in rabbits (17) showed that 15nm fractionated, but not non-fractionated feridex (a superparamagnetic iron oxide, SPIO) gets deposited in atherosclerotcic plaques in this model, thereby suggesting that iron oxides particle size should be further investigated to optimize plaque macrophages targeting.

Investigating the intraplaque and cellular distribution of iron oxides: CLIO-CyAm7 nanoparticles

In this paper Stein-Merlob et al. [15] use a well-validated rabbit model of atherosclerosis to evaluate the intra-plaque and cellular uptake of a NIR fluorophore-labeled (CyAm7) dextran coated nanoparticle (CLIO-CyAm7). A total of 31 rabbits were included in the study. 9 animals were injected with either CLIO-CyAm7 (n=6) or saline (n=3) and euthanized 24 hours after injection. In these animals, *ex vivo* macroscopic NIRF demonstrated increased CLIO-CyAm7 deposition in rabbit aortic plaques compared to control areas, such as the uninjured renal arteries (signal-to–noise ratio, SNR, 18.1±11.3 versus 10.64±1.2, p=0.03). *Ex vivo* fluorescence microscopy of the same samples demonstrated a non-homogeneous circumferential nanoparticle distribution in atheromas. Analysis of the nanoparticles cellular distribution revealed accumulation in plaque macrophages, but also smooth muscle cells,

and endothelial cells. Preferential accumulation was observed in the superficial tunica intima in 67% of the animals, despite the presence of these cell types deeper into the plaque. This characteristic distribution of nanoparticles within the plaque suggests that restricted diffusion may also play a role in their pattern of deposition. In the remaining 33% of animals, the authors observed nanoparticles accumulation deeper within the plaque. Histological examinations demonstrated evidence of neovascularization within these deeper regions, thereby pointing to a possible role of the disrupted endothelial barrier in facilitating nanoparticles extravasation.

Endothelial permeability, CLIO-CyAm7 accumulation and arterial thrombosis

To test this hypothesis, a group of 7 rabbits was co-injected with CLIO-CyAm7 and Evans Blue (EB), a fluorescent albumin-binding dye that is known to accumulate in areas of increased permeability. EB and CLIO-CyAm7 plaque accumulation were found to be highly correlated ($r=0.64$, $p=0.003$). Based on these findings the authors proceeded to further investigate the possible relationship between increased plaque permeability and atheroma CLIO-CyAm7 deposition as predictive traits of plaques prone to thrombosis. An additional 15 rabbits were injected with CLIO-CyAm7 and imaged 24 hours afterwards with intravascular in vivo NIRF, which confirmed CLIO-CyAm7 deposition in atheromas identified by intra-vascular ultrasound (IVUS). After imaging, rabbits underwent a well-validated triggering procedure with intra peritoneal injection of viper venum and histamine to induce arterial thrombosis. Follow-up IVUS after triggering revealed the formation of thrombotic areas in 67% of the animals, confirmed by histology. Quantitative analysis of pre-triggering intra-vascular in vivo NIRF revealed higher CLIO-CyAm7 accumulation in thrombotic versus non-thrombotic plaques at the post-trigger IVUS. Receiver Operating Characteristic (ROC) curves analysis revealed that the positive predictive value of pre-triggering CLIO-CyAm7 aortic deposition for plaque thrombosis was 59.4% (CI 40.6% to 76.3%), while the negative predictive value was 84.0% (CI 63.9% to 95.4%).

Imaging inflammation and endothelial permeability as predictors of atherothrombosis: current status and future directions

This important study by Stein-Merlob et al. [15] sheds some light into the intra-plaque distribution and cellular specificity of multi-modality functionalized CLIOs. CLIOs plaque distribution is found to be inhomogeneous along the plaque luminal circumference, and preferentially around the superficial intima. Accumulation within deeper plaque regions is co-localized with plaque neovessels in these same areas. Further *ex vivo* investigation demonstrates a relationship between plaque endothelial permeability and nanoparticles accumulations, and a predictive value of CLIO-CyAm7 deposition for arterial thrombosis in the rabbit model. Importantly, the authors demonstrated feasibility of using intravascular NIRF to quantify CLIO-CyAm7 accumulation *in vivo*, in a vessel of comparable size to the human coronary arteries(18). These findings indicate that in the near future, in vivo NIRF, alone or in combination with other intravascular imaging modality, may further enhance our ability to characterize atheromas in this challenging vascular territory.

The mechanisms of uptake and intra-plaque distribution of nanoparticles used for diagnostic or theranostic purposes, including but not limited to iron oxides, is the subject of active investigation. A similar dependency of nanoparticles accumulation to plaque permeability has been previously demonstrated for another NIR fluorophore Cy7-labeled long circulating liposomal nanoparticles also in the rabbit model(19). In this case nanoparticles vessel wall uptake was found to correlate with both *ex vivo* NIRF imaging with Evans Blue, and the *in vivo* quantification of endothelial permeability with dynamic contrast enhanced (DCE) MRI. Interestingly, in this specific case the correlation was stronger with nanoparticles accumulation half an hour after injection, and was found to weaken and then disappear at 6 and 24 hours after injection, reflecting the particles gradual diffusion within the plaque from the luminal side.

The predictive value of endothelial permeability for plaque thrombosis has also been recently investigated in rabbits using in vivo T1 mapping with MRI after injection of a gadolinium based, albumin binding contrast agent, performed pre and post triggering to induce atherothrombosis(20). Similarly to what is presented here, the authors found that rupture-prone plaques are characterized by increased endothelial permeability before triggering. ROC analysis revealed that this parameter was highly predictive of plaque thrombosis in the post-trigger scans.

Here, CLIOs plaque uptake is shown to depend on both endothelial permeability, and internalization by plaque macrophages and other cells type, making its predictive value for arterial thrombosis potentially more complex to interpret. New discoveries on the relative contributions of endothelial dysfunction, plaque neovascularization, permeability, and inflammation to atherogenesis, will allow us to better understand the predictive value of iron oxides plaque accumulation for vulnerability, thrombosis, rupture and cardiovascular events. In the pre-clinical arena, the flexibility to functionalize these particles into multi-modality agents (as shown here) will allow gathering tremendous mechanistic insights on their plaque-and cell –targeting properties in the context of atheroma progression, and/or regression upon treatment. This information will be crucial to understand the added value and complementary information of plaque iron oxides imaging in humans in comparison with the other clinical imaging techniques available to characterize plaque vulnerability(1, 19, 20).

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