Molecular Imaging of Atherosclerosis using Paramagnetic Cy5.5-labeled PEG-micelles

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Introduction

Atherosclerosis is a progressive disease that can be considered a chronic inflammation of the large arteries. Several studies have shown that the vulnerability of atherosclerotic plaque is primarily determined by its cellular and molecular composition[1,2]. Imaging of these cellular and molecular targets may therefore result in improved understanding and diagnosis of the disease. For this purpose we developed a targeted paramagnetic and fluorescently labeled micellar contrast agent (CA). The targeting ligand used was the RGD-peptide, which binds to the αvβ3-integrin that is strongly up-regulated on activated endothelial cells[3]. The micelles were fluorescently labeled with the near infrared (NIR) probe Cy5.5, that is especially suitable for fluorescence imaging of histological sections and intact specimen since there is less autofluorescence and better penetration depth at higher wavelengths. The specific uptake of the RGD-PEG-micelles was evaluated using endothelial cells *in vitro*. *In vivo*, we applied this targeted contrast agent to atherosclerotic apoE-KO mice and observed enhancement of the atherosclerotic aorta wall using MRI. *Ex vivo*, the accumulation of the micelles in the plaque was confirmed using optical techniques.

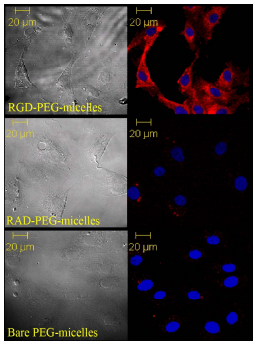


Figure 1: CLSM of HUVEC incubated with different micelles. On the left brightfield images and on the right the CY5.5 fluorescence (red) and the nuclei are shown (blue).

Materials and Methods

Pegylated micelles, composed of Gd-DTPA-BSA , PEG-DSPE, Mal-PEG-DSPE and Cy5.5-PEG-DSPE were prepared by lipid film hydration, followed by heating to 70 ºC and constant mixing for 20 minutes. The Cy5.5-PEG-DSPE lipid was obtained by covalently coupling of Cy5.5 NHS ester to NH_2-PEG-DSPE under basic conditions. The RGD-peptide was equipped with a thioacetyl group to allow conjugation to Mal-PEG-DSPE[4].

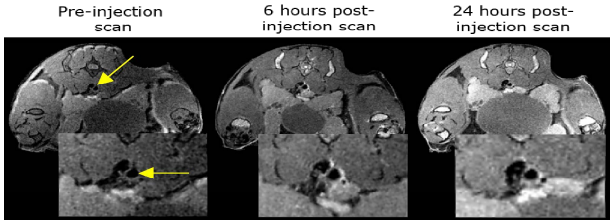
Human Umbilical Vein Endothelial Cells (HUVEC) were incubated with medium containing either RGD-PEG-micelles, RAD-PEG-micelles, bare PEG-micelles or no micelles to demonstrate the specific uptake of the RGD-PEG-micelles by these cells *in vitro*. The RAD-peptide is a non-specific control ligand that exhibits no affinity for the αvβ3-integrin.

For *in vivo* MRI, 35 to 60 weeks old apoE-KO mice (n=12) that were fed a 0.2 % cholesterol diet for at least 27 weeks were used. The mice were anesthetized and either RGD-PEG-micelles (n=9) or bare PEG-micelles (n=3) were administered intravenously at a dose of 0.075 mmol Gd/kg via the tail vein. Mice were scanned before and at 6, 24, 48, 72 and 168 hours for RGD-PEG-micelles and at 24 and 48 hours for bare PEG-micelles. MRI was done on a 9.4 T, 89 mm-bore system using a high resolution, T_1-weighted sequence (pixel size: 101x101x500 μm^3, TR: 800 ms, TE: 8.6 ms). The relative signal enhancement (RSE) of the aorta wall was determined with $RSE=[(SIW_{post}/SIM_{post})/(SIW_{pre}/SIM_{pre})-1]*100$ (SIW: Signal Intensity Wall, SIM: Signal Intensity Muscle). After the MRI, mice were sacrificed and the aortas were dissected to perform whole aorta fluorescence imaging on a Xenogen IVIS 200 system and to investigate aortic coupes with a Zeiss CLSM 510 META system in order to localize the micelles at a (sub) cellular level. CD31 staining on the aortic sections was performed to identify possible neovessels in the plaque[5].

Results and discussion

CLSM on the HUVEC (Figure 1) revealed that the RGD-PEG-micelles were specifically taken up by proliferating HUVEC *in vitro*, while very limited association of RAD- or bare PEG-micelles with the HUVEC was observed, demonstrating the specificity of the multivalent RGD-conjugated micelles.

In Figure 2A, typical MR images of the abdominal region of an apoE-KO mouse are shown. Pronounced signal enhancement of the aorta wall was observed at 6 hours post injection. In Figure 2B, a graph of the mean RSE post injection is given. The mean RSE of the aortic wall at 24 hours post injection of bare micelles was determined to be 12±5%, not significantly different from control, compared to a significant enhancement of 42±15% ($p<0.001$) for RGD-PEG-micelle injected mice.



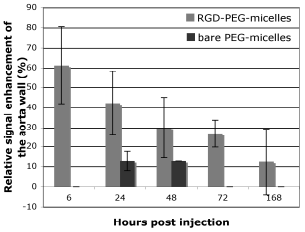


Figure 2: Typical images of the abdominal region pre and post injection of RGD-PEG-micelles are shown on the left. The arrows indicate the aorta and the insets show the aorta magnified. On the right a graph of the post-injection RSEs is shown.

Ex vivo whole aorta fluorescence imaging demonstrated the absence of autofluorescence at the emission wavelengths of Cy5.5. It also revealed that the RGD-PEG-micelles specifically accumulated in atherosclerotic regions of the aorta wall, most pronouncedly present around the renal and iliac bifurcation, and in the aortic arch (Figure 3D). CLSM on aortic sections confirmed the uptake of the RGD-conjugated micelles into the plaque (Figure 3). The indicated dark regions at 6 hours post injection (arrows Figure 3A) and the accumulations at 24 hours post injection indicated cellular uptake of the RGD-PEG-micelles in the plaque. CD31 staining revealed the absence of neovessels in the plaque, therefore we attribute the uptake to either the activated luminal endothelium or activated macrophages. In order to elucidate the exact mechanism of uptake, additional cellular and integrin stainings will be performed.

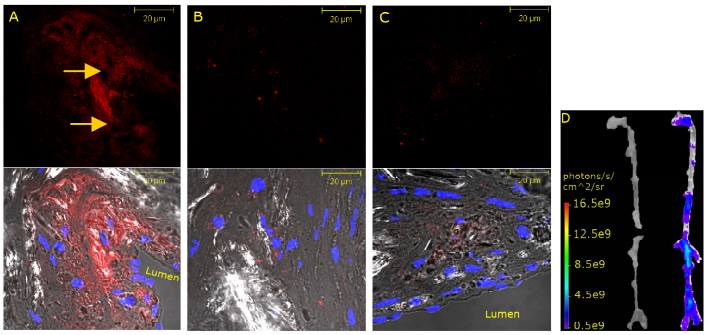


Figure 3: Figure A,B,C are CLSM images of aortic sections at 6, 24 and 48 hours post injection of RGD-PEG-micelles, respectively. The images in the bottom row are overlays of brightfield, Cy5.5 fluorescence (red) and nuclei (blue). Figure D shows two aortas imaged with the IVIS 200 system. The aortas were dissected 24 hours post injection of saline (left) and RGD-PEG-micelles (right).

Conclusions

In vivo MRI and *ex vivo* fluorescence techniques demonstrated active uptake of RGD-PEG-micelles into atherosclerotic plaque. The limited non-specific uptake of bare PEG-micelles in mouse plaques makes this CA platform very attractive for molecular imaging of atherosclerosis, also when directed to other targets. The NIR Cy5.5 fluorophore proofed to be very valuable for the application of *ex vivo* and *in vitro* fluorescence techniques.

1. Virmani, R. et al. *Arterioscler. Thromb. Vasc. Biol.* 20, 1262–1275 (2000); 2. Davies, M. J, et al. *Br. Heart J.* 69, 377–381 (1993); 3. Albert J.M. et al. *Int. J. Radiation Oncology Biol. Phys*, 65, 1536–1543 (2006); 4. Mulder et al. *FASEB J*, 19 2008-10 (2005); 5. Moulton, K.S. et al. *Circulation*, 99, 1726-1732 (1999)