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Human Ferritin Cages for Imaging Vascular Macrophages

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Abstract

Atherosclerosis is a leading cause of death worldwide. Macrophages are key components of vascular inflammation, which contributes to the development and complications of atherosclerosis. Ferritin, an iron storage and transport protein, has been found to accumulate in macrophages in human atherosclerotic plaques. We hypothesized that ferritin could serve as an intrinsic nano-platform to target delivery of imaging agents to vascular macrophages to detect high-risk atherosclerotic plaques. Here we show that engineered human ferritin protein cages, either conjugated to the fluorescent Cy5.5 molecule or encapsulating a magnetite nanoparticle, are taken up *in vivo* by macrophages in murine atherosclerotic carotid arteries and can be imaged by fluorescence and magnetic resonance imaging. These results indicate that human ferritin can serve as a nanoparticle platform to image vascular inflammation *in vivo*.

Keywords

nanoparticle; inflammation; macrophage; ferritin; atherosclerosis; molecular imaging

1. Introduction

Accumulating evidence has established that inflammation plays an important role in atherosclerosis[1,2], a leading cause of death worldwide. Inflammation is involved not only in the initiation of atherosclerosis but also its progression and complications. Thus, visualizing inflammation within the vessel wall may help to detect atherosclerosis, characterize its biological activity, and predict risk. Current clinical atherosclerosis imaging methods can visualize vessel stenosis and plaque, but offer limited information regarding the underlying biology within the vessel wall. Emerging molecular and cellular imaging

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techniques have the potential to provide functional and biological information on the pathobiology of atherosclerosis[3–10].

A variety of nanomaterials has been used for molecular and cellular imaging of cardiovascular disease[11]. We and others have previously shown that protein cage architectures, such as virus capsids[12–16], ferritins[17–19] and heat shock proteins[20,21] are useful templates for loading and/or synthesis of imaging agents within the interior cavity of the protein cages. Importantly, it has been reported that one of these protein cages - ferritin, an iron storage and transport protein - accumulates in human plaque macrophages[22–24], and is also postulated to undergo receptor-mediated uptake in inflammatory cells[25–27]. We and others have reported that engineered human ferritin is taken up by macrophages *in vitro*[19,28]. These findings imply that ferritin may serve as an intrinsic vehicle for targeting plaque macrophages. The present study tested the hypothesis that modified ferritin cages can be used as fluorescence or MR imaging agents for *in vivo* detection of vascular macrophages.

2. Materials and methods

2. 1. Preparation of Cy5.5 labeled HFn (HFn-Cy5.5) and Magnetite mineralized HFn (HFn-Fe₃O₄)

The apoferritin (iron-free ferritin) shell is assembled from 24 polypeptide chains of 2 species, the heavy (H) subunit and light (L) subunits[29,30]. Based on our prior work[19], we used a recombinant ferritin cage composed of 100% H subunit (HFn) as the platform for all experiments. Recombinant HFn was expressed and purified from *E. coli*, as previously described[19]. For fluorescence imaging, Cy5.5 mono NHS ester (GE Healthcare UK Limited, Buckinghamshire, UK) was conjugated with HFn (1mg/mL in Dulbecco's phosphate buffered saline (DPBS)), adjusted to pH8.2. The HFn solution was mixed with Cy5.5-NHS in a concentration of 7.2–480 molar equivalents per cage (0.3–20 per subunit) at room temperature for 1 h followed by overnight incubation at 4°C. The samples were purified by size exclusion chromatography in DPBS at pH7.4 to remove free Cy5.5 dye. To quantify the amount of Cy5.5 dye covalently attached to HFn, the samples were analyzed by UV-Vis spectroscopy (Agilent, Santa Clara, CA, USA). The normalized Cy5.5 absorbance spectrum was subtracted from the Cy conjugated HFn spectrum, and the protein concentration was calculated from absorbance at 280nm of the difference spectrum (HFn subunit $\epsilon = 21.09 \text{ mM}^{-1}\text{cm}^{-1}$). The concentration of Cy5.5 covalently attached to HFn was calculated from the absorbance maximum near 675nm using a value of $\epsilon = 250\text{mM}^{-1}\text{cm}^{-1}$ as per the supplier. Fluorescence emission spectra of the Cy5.5 labeled HFn samples were analyzed with a fluorescence spectrometer (TECAN). For MRI, HFn nanoparticles were mineralized with magnetite at loading factors of 3000Fe-5000Fe per cage, giving R2 values of 31–93 $\text{mM}^{-1}\text{s}^{-1}$ as described previously[19].

2. 2. Animals

The overall experimental procedure is shown in Figure 1. Macrophage-rich vascular lesions were induced in 30 FVB strain mice by the following protocol. Mice were fed a high-fat diet containing 40% kcal fat, 1.25% (by weight) cholesterol and 0.5% (by weight) sodium cholate (D12109, Research Diets, Inc. New Brunswick, NJ, USA)[31]. After 4 weeks of high-fat diet, mice were rendered diabetic by administration of 5 daily intraperitoneal injections of streptozotocin (STZ), 40 mg/kg in citrate buffers (0.05 mol/L, PH4.5, Sigma-Aldrich)[32]. At day 5 of the STZ injections, serum glucose was measured from tail vein blood using a glucometer. If the glucose level was below 200 mg/dL, animals were injected with additional STZ for 3 consecutive days. At day 14 after initiation of STZ injection, the left common carotid artery was ligated (n=21) below the bifurcation with the use of 5-0 silk

ligature (Ethicon) under 2% inhaled isoflurane as previously described[33]. In sham-operated animals (n=9), the suture was put around the exposed left carotid artery but not tightened. The wound was closed by suture and the animals were allowed to recover on a warming blanket. All procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

2. 3. Uptake and Fluorescence Imaging of HFn-Cy5.5

HFn-Cy5.5 was studied in 14 mice (11 ligated, 3 sham). Two weeks after carotid ligation, animals received intravenous HFn-Cy5.5 (8 nmol of Cy5.5/mouse) via retro-orbital injection. Under inhalational anesthesia (2% isoflurane), all mice were imaged at 48 hours including *in situ* and *ex vivo* fluorescence imaging using the Maestro™ in-vivo imaging system (CRi, Woburn, MA), which has the capability to separate fluorochromes from autofluorescence based on multispectral analysis[34]. For *in situ* fluorescence imaging, animals were euthanized and left and right carotid arteries were surgically exposed and imaged. Then, the carotid arteries and heart were carefully removed en bloc for *ex vivo* fluorescence imaging.

2. 4. Uptake and MR Imaging of HFn-Fe₃O₄

HFn-Fe₃O₄ was studied in 16 mice (10 ligated and 6 sham). Two weeks after carotid ligation, animals received intravenous injection of HFn-Fe₃O₄ (25mgFe/kg). The initial 8 mice (5 ligated and 3 sham) were analyzed by histology only (see methods below) to verify HFn-Fe₃O₄ uptake in the carotid lesion. The next 8 mice (5 ligated and 3 sham) also underwent *in vivo* MRI under inhalational anesthesia (2% isoflurane) on a warming pad to maintain temperature of 37°C. A horizontal-bore 7T scanner was used (self-shielded 30cm bore magnet, Varian Inc., Palo Alto, CA), which was equipped with an 6 cm inner-diameter radiofrequency transmit-receive coil (built in house), a 9cm bore gradient insert (770mT/m and 2500T/m/s, Resonance Research, Inc. Billerica, MA) and the GE “Micro-Signa” software environment (GE Healthcare, Waukesha, WI). To detect the T2* effects of the HFn-Fe₃O₄, bright-blood images of the neck were acquired using a gradient echo sequence (TR/TE=50/4.2, slice thickness=0.5mm, FOV=3cm, matrix=256×256, FA=50, acquisition time=9min 55sec). MRI was performed 1 hr prior to HFn-Fe₃O₄ injection and then 24 and 48hrs after HFn-Fe₃O₄ injection. The slice position was matched using the aortic arch as reference point.

2. 5. Fluorescence and MR Image Analysis

For *in situ* fluorescence imaging, ROIs were placed on the left and right carotid regions, calculating average signal intensity divided by exposure time. For *ex vivo* fluorescence imaging, surrounding tissue had been removed, so the ROIs were placed around the entire left or right carotid artery and total signal intensity divided by exposure time was used.

For *in vivo* MRI, the effect of HFn-Fe₃O₄ accumulation in the carotid wall was assessed by measuring the T2*-induced signal loss on the carotid lumen images. Specifically, the % reduction in carotid lumen size post-HFn-Fe₃O₄ was measured:

$$\% \text{ Lumen Loss} = 100 \times (1 - [\text{post-contrast carotid lumen area}] / [\text{pre-contrast carotid lumen area}]).$$

2. 6. Histology

Carotid arteries were cut into three 2-mm sections and frozen in OCT compound (Sakura Finetek, Torrance, CA) for histopathological analysis. Tissue samples were then cut into serial sections 5μm thick. For basic histology, sections were fixed with 10% formalin for 1

hour and then underwent hematoxylin and eosin or Perl's iron staining (for detecting HFn-Fe₃O₄). For immunohistochemistry, sections were fixed in acetone for 10 minutes at 4°C and incubated with 10% normal rabbit serum for 30 minutes at room temperature to reduce nonspecific binding. After these sections were washed in phosphate-buffered saline (PBS), they were incubated with anti-Mac3 antibody for macrophage detection (BD Biosciences, San Jose, CA) overnight at 4°C. Sections were then incubated with biotinylated secondary antibodies at room temperature for 30 minutes. Antigen-antibody conjugates were detected with avidin-biotin-horseradish peroxidase complex (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions using 3-amino-9-ethylcarbazole as chromogen. Sections were counterstained with hematoxylin. For immunofluorescence double staining, sections were stained with Alexa Fluor 488-conjugated anti-rat IgG (Molecular Probes, Eugene, OR) at room temperature for 2 hours. Sections were observed under confocal microscopy.

2. 7. Statistical Analysis

Fluorescence signal intensity and % signal loss on MRI were compared between left (ligated) and right (control) carotid arteries by Student's t-test. In addition, the % lumen loss on MRI for pre- vs. post-HFn-Fe₃O₄ time points was analyzed by one-way repeated measures ANOVA. All statistical analysis was performed by Statview (version 5, SAS Institute, Inc. Cary, NC).

3. Results

3. 1. Characterization of Cy5.5-labeled HFn (HFn-Cy5.5) and Magnetite-incorporated HFn (HFn-Fe)

Reactivity of the Cy5.5 mono NHS ester with HFn (HFn-Cy5.5) was assessed over a range of stoichiometric ratios of Cy5.5 per HFn cage. The number of Cy5.5 dye covalently attached to the HFn increased with increasing input ratios of Cy5.5 per HFn cage (Fig. 2a). The number of bound Cy5.5 was determined to be 1.2 Cy/cage, 4.0 Cy/cage, and 14.6 Cy/cage when the input molar ratio of Cy/cage was 7.2, 24 and 480, respectively. Fluorescence emission measurements of HFn-Cy5.5 with various Cy/HFn cage ratios at equivalent Cy5.5 concentration (2 µM) revealed that the emission intensity at 690nm gradually decreased with increasing the number of Cy dye attached to the cage (Fig. 2b), likely due to self-quenching of the fluorophore. When the fluorescence emission intensity was compared under the same HFn cage concentration (0.3mg/mL), the HFn-Cy5.5 with input Cy/cage = 24 exhibited the highest intensity whereas HFn-Cy5.5 with Cy/cage = 7.2 showed much lower fluorescence intensity per HFn (Fig. 2c). Therefore, the HFn-Cy5.5 with input Cy/cage = 24 was used for the *in vivo* experiments. Importantly, size exclusion chromatography of HFn-Cy5.5 showed almost identical elution volume with the unmodified HFn (Fig. 3a, b). Co-elution of the HFn protein (280nm) and Cy5.5 dye (675nm) indicates a single species consistent with the covalent attachment of Cy5.5 dye to the HFn (Fig. 3b). In addition, TEM images of the intact HFn and HFn-Cy5.5 both revealed cage-like particles of about 12nm in diameter. These results clearly suggest Cy5.5 labeling has no effect on the quaternary structure or overall cage-like morphology of the HFn.

Similarly, size exclusion chromatography of the Fe₃O₄ mineralized HFn (HFn-Fe₃O₄), which was prepared as described previously[19], showed co-elution of the protein (280nm) and the iron oxide (410nm) components (Fig. 3c), demonstrating the composite nature of the HFn and magnetite. Unstained TEM of HFn-Fe₃O₄ showed electron dense nanoparticles of about 6 nm in diameter (5000Fe/cage), which formed within the interior cavity of the cage (Fig. 3c). We have shown previously that the particles are primarily magnetite (or maghemite) and that HFn-Fe₃O₄ exhibits much higher R₁ and R₂ relaxivities compared to

endogenous ferritin and comparable to those of commercially available iron oxide contrast agents[19].

3. 2. Fluorescence Imaging of HFn-Cy5.5 in Vascular Lesions

After intravenous injection of HFn-Cy5.5, both *in situ* and *ex vivo* fluorescence imaging clearly showed high signal localized to the macrophage-rich (ligated) left carotid artery and not the control (non-ligated) right carotid artery (Fig. 4a,b). Importantly, sham-operated mice did not show significant signal in either left or right carotid arteries (Fig. 4c, d). Quantitative analysis (Fig. 4e, f) showed that the fluorescent signal was significantly higher in the ligated left carotid arteries compared to contralateral right carotid artery and sham controls, both *in situ* ($p < 0.04$) and *ex vivo* ($p < 0.002$). Histology of the vessel wall demonstrated macrophage infiltration in both the neointima and adventitia of the ligated left carotid artery (Fig. 5), while no macrophage infiltration was observed in the control (non-ligated) right carotid artery. Immunohistochemistry confirmed that HFn-Cy5.5 (red signal) co-localized with macrophages (green signal) in merged images of confocal fluorescence microscopy (Fig. 5). These results demonstrate selective accumulation of HFn in macrophage-rich vascular lesions.

3. 3. *In vivo* MRI of Atherosclerosis with HFn-Fe

In vivo MRI of mouse carotids showed that the T2* signal loss effect of HFn-Fe₃O₄ caused a reduction of the carotid lumen signal post contrast (Fig. 6). Both 24 and 48 hours after intravenous injection of HFn-Fe₃O₄, the T2*-sensitive bright-blood MRI images showed concentric signal loss, which reduced the measured size of the carotid lumen cross-sectional area compared to pre-contrast images [note that the *pre-contrast* left carotid artery lumen size started off smaller than non-ligated controls, as expected, due to the ligation two weeks prior]. By quantitative analysis (Fig. 6c), the T2*-induced reduction in lumen size post-HFn-Fe₃O₄ was significant at both 24 and 48 hours ($p < 0.005$ vs. pre, $p < 0.003$ ligation vs. sham). Importantly, no T2* effect was seen in the contralateral control or sham-operated carotid arteries, showing specificity of HFn-Fe₃O₄ for the inflammatory lesion. Histological evaluation of HFn-Fe₃O₄ accumulation showed similar results to HFn-Cy5.5, as blue iron staining was observed in the neointima of the ligated left carotid arteries (Fig. 7a), but not in the contralateral control or sham arteries (Fig. 7b, c). These results further demonstrate that chemically modified HFn, now with an MR imaging agent, accumulated in plaque macrophages *in vivo* and could be detected by noninvasive imaging.

4. Discussion

We have shown that modified human ferritin nanoparticles can image vascular macrophages *in vivo* in murine carotid arteries through fluorescence or MRI. Thus, an intrinsic cage-like human protein can be used for *in vivo* macrophage and vascular imaging.

Previous animal and human studies have shown an increase in ferritin in atherosclerotic plaques, mainly in macrophages, and association with plaque rupture[22–24]. Increased ferritin in atherosclerotic lesions has also been associated with pro-inflammatory cytokines and atheroma cell apoptosis[22–24]. The heavy (H) subunit of ferritin is generally believed to play a key role in iron transport[29,30] and several investigators have also found evidence for receptor-mediated uptake of ferritin, especially the H subunit studied here, by inflammatory and other cell types[25–27]. While we and others have previously shown good uptake of modified HFn in macrophages *in vitro*[19,28], the full mechanisms for increased ferritin accumulation in macrophages *in vivo*, particularly in human atherosclerotic lesions, are not fully understood. The important finding in this study is that HFn accumulates in

vascular macrophages *in vivo* and may serve as an intrinsic macrophage imaging agent without additional macrophage targeting moieties.

A number of molecular imaging strategies have been developed for detecting macrophages in atherosclerosis, such as MRI[5,6,10], fluorescence imaging[7,35], and nuclear imaging (PET and SPECT)[9,36]. In our study, fluorescence imaging had insufficient signal penetration to allow fully noninvasive detection, requiring *in situ* carotid exposure. A more sensitive fluorescence imaging technique, such as fluorescence molecular tomography, may allow fully noninvasive imaging[37]. MRI allowed noninvasive detection, but relies on T2* signal loss, which can be challenging. The application of “positive contrast” methods to high-field small-animal MRI systems for iron detection may be advantageous[6,38].

While HFn has the advantage of being a modified human protein, the clinical translation of this approach from a short-term animal model to the chronic, complex human disease certainly requires further study. The cage-like structure of HFn also makes it a highly adaptable platform for imparting targeting and therapeutic capabilities to optimize further its “theranostic” potential[18,39].

5. Conclusions

We have shown that human ferritin, an iron storage and transport protein found in inflamed human atherosclerotic plaques, can be engineered as a vascular macrophage imaging contrast agent. Human ferritin protein cages, either conjugated to the fluorescent Cy5.5 molecule or encapsulating a magnetite nanoparticle, were taken up *in vivo* by macrophages in murine atherosclerotic carotid arteries and imaged using fluorescence and MRI. These results indicate that human ferritin can serve as a nanoparticle platform to image vascular inflammation *in vivo*.

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Figure 1. Overall experimental procedure

A macrophage-rich carotid lesion was formed by inducing hyperlipidemia and diabetes, followed by ligation of the left common carotid artery. *In vivo* imaging was performed at 14 days post-operation, followed by histology.

Figure 2. Fluorescence characterization of HFn-Cy5.5

a, Extent of HFn labeling with Cy5.5 mono NHS ester under various molar equivalent input ratios of Cy5.5/HFn subunit. **b**, Fluorescence emission of the Cy5.5 labeled HFn under same Cy5.5 concentration of 2 μ M. Cy/cage listed are input molar equivalents of Cy5.5 per HFn cage. **c**, Fluorescence emission of the Cy5.5 labeled HFn with various Cy5.5/HFn cage input ratios under same HFn concentration of 0.3 mg/mL.

Figure 3. Size characterization of HFn-Cy5.5 and HFn-Fe₃O₄

Size exclusion chromatography elution profiles (top) and TEM images (bottom) of intact HFn, Cy5.5 labeled HFn (HFn-Cy5.5) and magnetite mineralized HFn (HFn-Fe₃O₄). The intact HFn and Cy5.5 labeled protein cages were negatively stained with uranyl acetate for TEM observation while the mineralized HFn was unstained. Elution volumes of the three samples are almost identical, indicating that the overall morphology of the HFn cage is retained regardless of Cy-5.5 labeling or magnetite incorporation. Co-elution of the protein (280nm) and the Cy5.5 (675 nm) or the magnetite (410 nm) indicates composite nature of HFn cage and Cy5.5 dye or magnetite particle. Unstained image of HFn-Fe₃O₄ revealed that size of the magnetite particle is about 6 nm in diameter.

Figure 4. *In situ* and *ex vivo* fluorescence images of HFn-Cy5.5 nanoparticles in mouse carotid arteries

a, The ligated left carotid artery (LCA) showed enhanced fluorescence signal, but not the contralateral control right carotid artery (RCA). **b**, *Ex vivo* imaging further confirmed the enhanced signal was localized to the ligated LCA. **c**, **d**, With sham operation, no significant signal was seen in either LCA or RCA. **e**, **f**, Quantitative analysis of both *in situ* (**e**) and *ex vivo* (**f**) imaging showed the fluorescence signal from the ligated LCA was significantly greater than from non-ligated RCA and sham-operated LCA.

Figure 5. Colocalization of HFn-Cy5.5 with macrophages by fluorescence microscopy
a, Immunohistochemistry depicts the macrophage-rich (red) vascular lesion involving the neointima and adventitia of the ligated left carotid artery. **b–d**, Fluorescence microscopy shows that macrophages labeled with FITC-Mac-3 (**b**, green) colocalize with HFn-Cy5.5 (**c**, red) in the merged image (**d**, yellow).

Figure 6. *In vivo* MRI of carotid arteries in ligated and sham-operated mice with HFn-Fe₃O₄
a, The ligated left carotid artery (LCA) is smaller, as expected, than the non-ligated right carotid artery (RCA) prior to contrast injection. Post-contrast, there was concentric signal loss of the LCA lumen at 24 and 48 hours, but not for the RCA. **b**, No change was seen in the sham-operated LCA. **c**, Quantitative analysis of *in vivo* MRI T2* signal loss from HFn-Fe₃O₄ showed significant reduction of lumen area in the ligated LCA at 24 and 48 hours after injection, but no significant change in sham or non-ligated carotid arteries.

Figure 7. HFn-Fe₃O₄ localization in the neointima of ligated carotid arteries

a, b, HFn-Fe₃O₄ was detected by Perl's iron staining (blue) in the ligated left carotid artery, not in the control right carotid artery. **c,** No iron was detected in the left carotid artery of sham-operated mice.