**Development of a new bioactivatable fluorescent probe for
quantification of apolipoprotein A-I proteolytic degradation *in vitro*
and *in vivo***

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Abstract

Background and aims: The potential benefits of high-density lipoproteins (HDL) against atherosclerosis are attributed to its major protein component, apolipoprotein A-I (apoA-I). Most of the apoA-I in the vascular wall appears to be in its lipid-poor form. The latter, however, is subjected to degradation by proteases localized in atherosclerotic plaques, which, in turn, has been shown to negatively impact its atheroprotective functions. Here, we report the development and *in vivo* use of a bioactivatable near-infrared full-length apoA-I-Cy5.5 fluorescent probe for the assessment of apoA-I-degrading proteolytic activities.

Methods: Fluorescence quenching was obtained by saturation of Cy5.5 fluorophore molecules on apoA-I protein. ApoA-I cleavage led to near-infrared fluorescence enhancement. *In vitro* proteolysis of the apoA-I probe by a variety of proteases including serine, cysteine, and metalloproteases resulted in an up to 11-fold increase in fluorescence ($n=5$, $p \leq 0.05$).

Results: We detected activation of the probe in atherosclerotic mice aorta sections using *in situ* zymography and showed that broad-spectrum protease inhibitors protected the probe from degradation, resulting in decreased fluorescence (-54%, $n=6$ per group, $p \leq 0.0001$). *In vivo*, the injected probe showed stronger fluorescence emission in the aorta of human apoB transgenic *Ldlr*^{-/-} atherosclerotic mice (ATX) as compared to wild-type mice. *In vivo* observations were confirmed by *ex vivo* aorta imaging quantification where a 10-fold increase in fluorescent signal in ATX mice ($p \leq 0.05$ vs. control mice) was observed.

Conclusions: The use of this probe in different applications may help to assess new molecular mechanisms of atherosclerosis and may improve current HDL-based therapies by enhancing apoA-I functionality.

Keywords

Apolipoproteins ▪ Fluorescence resonance energy transfer ▪ atherosclerosis ▪ HDL ▪ bioactivatable fluorescent probe ▪ fluorescence molecular tomography ▪ MRI ▪ proteases.

1. Introduction

Atherosclerosis is a complex inflammatory process characterized by endothelial dysfunction with subsequent infiltration of the vascular wall by different cell types including lymphocytes, mast cells and macrophages leading to foam cell formation, lipoprotein deposition and extracellular matrix remodelling [1-4]. The latter is a key feature of atherosclerosis and involves collagen synthesis and elastin degradation by matrix metalloproteinases (MMPs) and cathepsins [5-8].

High-density lipoproteins (HDL)-based therapies have been shown to induce atherosclerotic plaque regression in animal models [9-11]. HDL mediate cholesterol efflux from atherosclerotic plaques but also exert anti-oxidative, anti-inflammatory and anti-apoptotic actions which may explain their atheroprotective functions [12-14]. Apolipoprotein A-I (apoA-I) is the most abundant protein component of HDL and a key factor for most of HDL's beneficial effects. However, despite strong and negative correlations between HDL-cholesterol levels and cardiovascular risk in epidemiological studies, recent pharmacological intervention trials failed to demonstrate any beneficial effects of HDL-raising therapies [15-19]. These studies have emphasized the important distinction between HDL function and circulating levels of HDL-cholesterol in the assessment of cardiovascular risk and indicate that the integrity of HDL components, such as apoA-I, is a prerequisite of HDL functionality.

Although the majority of apoA-I within the circulation resides on mature spherical HDL particles, it is known that free or poorly lipidated apoA-I plays a major role in cholesterol efflux and the macromolecular assembly of HDL particles. The clear majority of apoA-I (92%) is in the lipid-free or lipid-poor form within both normal and atherosclerotic human arterial walls [20]. Small lipid-poor particles and lipid-free forms of apoA-I accept free cholesterol from ABCA1 with the highest efficiency in comparison to more mature HDL particles, such as HDL₂ particles

[21]. Although, apoA-I content in atherosclerotic plaque is higher than that observed within normal artery wall [20, 22], apoA-I isolated from atherosclerotic arterial wall was found to be dysfunctional, with $\approx 80\%$ reduction in cholesterol efflux capacity and $\approx 90\%$ reduction in its ability to activate LCAT [20].

Thus, apoA-I and nascent HDL particles are surrounded by a hostile environment within atheromatous tissue and their protein constituents may suffer locally from several modifications, such as oxidation [23] and proteolysis [24, 25], leading to HDL dysfunction [26]. Indeed, the ability of apoA-I to mediate cholesterol efflux is abrogated by proteolytic activities of MMP-3, -7 and -12, cathepsin F and S, plasmin, chymase (rh-CMA-1) and neutrophil elastase, all of which are present in atherosclerotic plaques or plasma [25, 27, 28]. Importantly, the local impairment of apoA-I function is expected to lead to the loss of its atheroprotective benefits in HDL-based therapies.

Near-infrared fluorescence (NIRF) molecular imaging represents a useful approach for *in vivo* and *ex vivo* imaging because of its deeper tissue penetration than visible light and relative ease of NIRF dye conjugation. The broad class of imaging tools based on activatable probes and NIRF have allowed the study of diverse enzyme activities, particularly proteases such as MMPs and cathepsins in small preclinical atherosclerotic, aortic valvular stenosis and cancer models [29, 30]. Thus, our aim was to develop a bioactivatable NIRF probe for *in vivo*, *ex vivo* or *in vitro* measurements of apoA-I-degrading protease activities present in atherosclerotic plaques or samples.

Materials and methods

Detailed Material and methods are available as Supplementary data.

2.1 Animal model

We used a hypercholesterolemic/atherosclerotic mouse model (ATX, $n=12$; 12 to 13 months old) which is deficient for the LDL receptor (*Ldlr*) gene and express the human apolipoprotein B (*Ldlr*^{-/-}; transgenic (for human apoB)). The mice were kindly given by Dr Éric Thorin. Wild-type mice (C57BL/6J, $n = 4$; 10 months old) (Jackson Laboratory) were used as control mice. Animal care and procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the animal research ethics committee of the Montreal Heart Institute.

2.2 Generation of apoA-I-Cy5.5 activatable probe

2 mg/ml of full-length purified (95% pure on SDS-PAGE) human apoA-I solution in 0.1 M sodium phosphate (pH 8.5) were added to 3.53 mM (final concentration) of mono-reactive N-hydroxysuccinimide ester cyanine 5.5 (Cy5.5) fluorescent dye solution. The final molar dye/protein ratio was 50:1. The mixture was incubated for 2h at room temperature in the dark with gentle mixing. Unbound Cy5.5 dye was removed by spin-separation cycles at 14,000 g using a 10-kDa Amicon ultra centrifugal filter followed with additional purification by size-exclusion using a Sephadex G25 filtration column.

2.3 *In vitro* activation and characterization of apoA-I-Cy5.5 probe

2.3.1 *Protease preparations*

Recombinant human chymase (rh-CMA), trypsin from porcine pancreas, recombinant human cathepsin S (rh-Cath S) and recombinant mouse metalloproteinase 12 (rm-MMP12) were used for the *in vitro* activation of the apoA-I-Cy5.5 probe. Prior to experiment, 100 µg/ml of rm-MMP12, rh-Cath-S were first activated in the appropriate activation buffer, then diluted to 10 µg/ml into reaction buffers. The proteases were >90-95 % pure.

2.3.2 *In vitro* protease-induced activation of apoA-I-cy5.5 probe and SDS-gel electrophoresis

Purified native apoA-I and apoA-I-Cy5.5 probe (500 µg/ml) were incubated with rh-CMA, (1, 5, 10 µg/ml) or rm-MMP12 and rh-Cathepsin S (1, 5 µg/ml) for 90 minutes at 37 °C. All incubations occurred in the appropriate reaction buffer for each protease tested. Digested samples were then denatured for 5 minutes at 90 °C in Laemmli sample buffer and loaded for migration in a 15% polyacrylamide-SDS gel. The fluorescence released from proteolytic activated apoA-I-Cy5.5 probe was visualized using a Caliper IVIS Lumina II multispectral imaging system using 680/720 nm Ex/Em filters. Immunoblotting with anti-human apoA-I polyclonal antibody was also performed for rh-CMA (0.5, 1, 5, 10 µg/ml) and rm-MMP12 (0.5, 1, 5, 10 µg/ml) incubated for 90 min at 37 °C with purified native apoA-I (500 µg/ml) or apoA-I-Cy5.5 (500 µg/ml) probe to detect and compare the generated fragments. The amount of protein loaded on gel was 2 µg for both native apoA-I or apoA-I-Cy5.5 probe. All experiments were conducted in parallel using the same conditions (incubation time, temperature, protease concentration, gel migration and transfer).

2.3.3 *In vitro kinetics of protease-induced activation of apoA-I-Cy5.5*

40 µg/ml of apoA-I-Cy5.5 probe substrate were added to rh-CMA (0.05, 0.1, 0.25, 0.5, 1, 2 µg/ml), trypsin (0.1, 0.25, 0.5, 1, 2, 4 µg/ml), rh-Cath-S (0.25, 0.5, 1, 2, 3, 4, 6 µg/ml), and rm-MMP12 (0.01, 0.025, 0.05, 0.1, 0.25 µg/ml). Fluorescence was measured at 37 °C every 20 seconds using a Multi-Mode Microplate fluorescence reader (Ex/Em; 674/700 nm). We used the slope equation from the free Cy5.5 standard curve to extrapolate the increase of fluorescence in terms of equivalent amount of Cy5.5 released from the early phase of protease activated apoA-I-Cy5.5 kinetic curves. The early phase of the reaction was defined as the linear portion of the fluorescence emitted early after initiation of apoA-I probe degradation by the tested protease.

2.4 *In situ zymography*

To assess apoA-I proteolysis *in situ*, 7-µm thoracic aorta cryosections from six ATX mice (13 months old) were incubated during 3 hours at 37 °C with premixed 40 µg/ml apoA-I-Cy5.5 probe in a 0.1% agarose solution prepared in protease reaction buffer, in presence or absence of a broad-spectrum protease inhibitor mix (see Suppl. data file). DAPI was added to the mixture for nuclear staining. The images were analyzed using a Zeiss LSM-710 confocal microscope (Ex/Em; 633/650-750 nm). Total fluorescence (intensity/µm²) in the atherosclerotic plaque or in the atherosclerotic plaque and the external elastic lamina was quantified using ZEN lite 2008 software from Zeiss.

2.5 *Hybrid FMT-MRI for in vivo imaging of apoA-I-Cy5.5 activation*

A combined Fluorescence Molecular Tomography (FMT)-Magnetic Resonance Imaging system (MRI) system was used for *in vivo* molecular imaging [31]. Imaging was performed to

evaluate the *in vivo* activation of our apoA-I-Cy5.5 probe following its exposure to the protease activities degrading apoA-I present in the aorta of atherosclerotic mice model.

Five 12-month-old ATX mice and three 10-month-old wild-type mice were tail-injected with 100 μ l of 1 mg/ml apoA-I-Cy5.5 probe diluted in saline. Imaging under isoflurane anaesthesia was conducted before and 24 hours after probe administration. One additional ATX and one C57BL/6J mice were only injected with saline and imaged to estimate the background signal. For FMT, the field of view (FOV) of the optical recording was designed to approximately cover the heart area. For MRI, a 2D CINE bright blood sequence (in rapid single phase mode) was used with cardiac, but no respiratory gating (see Suppl. data file for detailed parameters). After the imaging sessions, the mice were sacrificed by cardiac puncture under anaesthesia followed by intensive saline perfusion of the heart and aorta. The harvested aortas were imaged *ex vivo* to confirm the *in vivo* observation.

2.6 *Ex vivo imaging using apoA-I-Cy5.5 probe*

Dissected aortas were pinned in a glass petri dish filled with saline and then imaged at 37 °C using a epi-illumination fluorescence imaging system (IVIS Lumina II, EX/EM; 675 nm (± 20)/720 (± 20)). The total fluorescence density/cm² values were analyzed using a computer-based digitizing image system software (Image Pro Plus, version 7.0).

2.7 *Validation of ex vivo imaging results by confocal microscopy*

7 μ m cryosections from thoracic aorta region of the ATX and C57BL/6J apoA-I-Cy5.5 probe-injected mice were mounted into an anti-fading medium; 1,4-diazabicyclo[2.2.2]octane in glycerol and imaged using confocal microscopy (Ex/Em; 633/650-750 nm).

2.8 *ApoA-I-Cy5.5 biological activity*

2.8.1 *DMPC clearance assay*

A 25 mg/ml solution of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine in chloroform (DMPC), was dried under nitrogen to form a thin film on the wall of a 13 x 100 mm glass tube. Dried DMPC was resuspended in 0.1 M guanidine-HCl buffer by sonication and vortexed for 1 min. Dilution to 0.5 mg/ml was performed in clearance buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 8.5 % KBr, 0.5 mM EDTA, room temperature) and stabilized at room temperature for 4 hours. The solution was vortexed and resulted in a turbid suspension of multilamellar vesicles (mLV) suspension with an absorbance of 0.8 to 0.9 at 480 nm. Native apoA-I (0.1 and 0.2 mg/ml) and apoA-I-Cy5.5 (0.1 and 0.2 mg/ml) were added to the 0.5 mg/ml DMPC solution in a 96-well UV microplate (final volume 300 μ l/well). Time-course kinetic measurements were obtained by monitoring the change in absorbance at 480 nm during 12h at 26.0 ± 0.2 °C using a Biotek Synergy H1 Hybrid Multi-Mode Microplate reader (temperature was set on the plate reader).

2.8.2 *Intracellular localization of apoA-I-Cy5.5*

Prior to experiment, confluent J774 murine macrophages were incubated for 18 hours with 0.3 mM of cAMP in DMEM supplemented with 1% FBS and 0.2% BSA to induce ABCA1 expression. The cells were then incubated for 4 hours at 37 °C in serum-free DMEM medium premixed with apoA-I-Cy5.5 probe (20 μ g/ml). After incubation, the cells were washed twice with saline buffer and incubated at 37 °C for 30 min with 0.2 μ M of LysoTracker Green (lysosome selective stain). Paraformaldehyde solution (4% in PBS) was used to fix the cells. Nuclear staining was performed using DAPI. The cells were then imaged using confocal

microscopy for apoA-I-Cy5.5 and LysoTracker Green intracellular detection (Ex/Em; 633/650-750 and 504/520 nm, respectively).

2.9 *Statistical analysis*

Analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). A significance level of 0.05 was used for all analyses. Data are presented as the mean $\pm$ standard error of the mean (SEM). Paired Student t-test, ANOVA or Wilcoxon-Mann-Whitney tests were performed depending on the type of data analyzed. Details are reported in the Supplementary Data.

2. Results

3.1 Generation of an apoA-I-Cy5.5 activatable probe

We conjugated the apoA-I protein using different excess ratios of Cy5.5 fluorescent dye. The conjugation of apoA-I with 100:1 dye/protein molar ratio of Cy5.5 yielded the highest degree of fluorescence quenching, after removal of unbound Cy5.5 dye, in comparison to 80:1 and 50:1 dye/protein molar ratios (Suppl. Fig. S1A). However, the 50:1 dye/protein molar ratio resulted in the highest fold increases in fluorescence emission after apoA-I-Cy5.5 probe unquenching by proteolytic activation with either rh-chymase, rh-cathepsin-S or rm-MMP-12 (Suppl. Figs S1, S2 and S3, respectively). Therefore, we performed all further experiments with the apoA-I-Cy5.5 probe generated using a 50:1 dye/protein molar ratio.

3.2 In vitro characterization and activation of the apoA-I-Cy5.5 probe by chymase

Our native apoA-I has an apparent molecular mass of 27.1 +/- 0.4 kDa (Suppl Fig. S4A). Our conjugated purified apoA-I-Cy5.5 probe appeared at a position slightly higher than 37 kDa, estimated as 39.7 +/- 0.8 kDa after migration through SDS polyacrylamide gel. This suggests that approximately 12-15 Cy5.5 molecules, which have a molecular weight of 931.12 Da, were bound to the apoA-I protein to generate the probe (Fig. 1A and C, lane with no added proteases; Suppl. Fig. S4B). After proteolysis of the apoA-I-Cy5.5 probe with different concentrations of rh-CMA, we noted that the cleavage products showed increased fluorescence compared to undigested apoA-I-Cy5.5. This was expected as a consequence of the decreased quenching of fluorescence of the protein fragments relative to that of the intact protein (Fig. 1A). The pattern of cleaved fluorescent fragments around the 15-kDa molecular weight marker (Fig. 1A) is similar to that of protein fragments detected using Coomassie staining on the same gel (Fig. 1C). We

quantified the signals from the different fragments, which were dependent on the concentration of protease used in the proteolysis assay. Integration of fluorescence quantification of the bands showed an increase by 7 to 11 times of the total fluorescence intensity compared to the fluorescence of the undigested probe (Fig. 1B, Suppl. Fig. S2B, S3B and S5I and S5J). We observed similar results when the apoA-I-Cy5.5 probe was cleaved using different concentrations of cathepsin-S or MMP-12, which also generated small fluorescent fragments, although the profile and the number of fluorescent fragments were dependent on the protease used (Suppl. Fig. S2A and S3A).

Next, we addressed the potential effect of excessive conjugation of apoA-I with Cy5.5 dye on the susceptibility to proteolysis of the apoA-I-Cy5.5 probe. We performed a head-to-head comparison between native apoA-I (500 $\mu\text{g/ml}$) and apoA-I-Cy5.5 probe (500 $\mu\text{g/ml}$) incubated in the same conditions with rh-CMA or rm-MMP12 (0.5, 1, 5, 10 $\mu\text{g/ml}$). Upon reaction with rh-CMA (Suppl. Fig. S5A-B) and rmMMP-12 (Suppl. Fig. S5D-E), apoA-I-Cy5.5 generates a pattern of protein fragments with variable lengths and different intensities from 30 kDa down to 10 kDa. In contrast, protease cleavage of native apoA-I produced a progressive disappearance of the main protein band around the full-length native apoA-I with little detection of the intermediate fragment generated (Suppl. Fig. S5C, in presence of rh-CMA, and S5F, in presence of rmMMP-12). Quantitative analysis of the most intense high molecular weight band remaining following digestion of the apoA-I-Cy5.5 probe (with apparent weight around 41 kDa) showed that 97 and 98% of the protein was cleaved after incubation in the presence of 10 $\mu\text{g/mL}$ rh-CMA 10 $\mu\text{g/mL}$ rmMMP12, respectively (Suppl. Fig. S5G and S5H, dark gray columns). For native apoA-I (with apparent weight around 28 kDa), the protein was 61% digested in presence of 10 $\mu\text{g/mL}$ CMA and 74 % with 10 $\mu\text{g/mL}$ of rm-MMP12 (Suppl. Fig. S5G and S5H, light gray columns). These results suggest an overall lower susceptibility of native apoA-I to proteolysis as

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4 compared to the apoA-I-Cy5.5 probe. However, due to resolution limitations of the gel, a direct
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6 mass spectrometry analysis would be necessary to confirm an intact structure/nature of the
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8 analyzed main band.
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10 11 12 13 14 3.3 *In vitro assessment of proteolytic activities using the apoA-I-Cy5.5 probe*

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16 *In vitro* kinetic experiments were carried out in which selected proteases were added to the
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18 probe and fluorescence intensity was measured over time on a microplate fluorometer (Fig. 2A
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20 and Suppl. Fig. S6A, S6B and S6C). The rh-CMA protease (0.05, 0.1, 0.25, 0.5, 1 and 2 µg/ml)
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22 promoted an increase in fluorescence of apoA-I-Cy5.5 probe by 350, 580, 755, 900, 1040 and
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24 1200% respectively, over a period of 2.5 hours (Fig. 2A). A similar time-dependent increase of
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26 fluorescence was observed over time when we added different concentrations of the apoAI-Cy5.5
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28 probe to a fixed concentration of rh-CMA (Fig. 2B).
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34 The highest protease concentration used in the assays for trypsin, MMP-12 and cathepsin-
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36 S with the apoA-I-Cy5.5 probe promoted increases in fluorescence of 470%, 550% and 960%
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38 over time, respectively (Suppl Fig. S6A, S6B and S6C).
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42 43 44 3.4 *In vitro measurement of proteolytic activities degrading the apoA-I-Cy5.5 probe*

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46 The increases in fluorescence after proteolysis of the bioactivatable fluorescent probe with
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48 increasing protease concentrations or when substrate concentration is increased in the presence of
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50 fixed protease concentration can be fitted using third-order polynomial equation with an r^2
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52 typically > 0.99. We built standard curves of Cy5.5 free dye in each specific protease activity
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54 buffers used in our tests and observed straight lines after log-log graphing, indicating power-law
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56 relationships between the free Cy5.5 concentration in solutions and fluorescence intensity
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58 (Fig. 2E, Suppl. Fig. S7). This enabled us to convert fluorescence readings to Cy5.5
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concentrations and use the slope of the free Cy5.5 dye standard curve equation to calculate protease activities from the linear phase of the kinetic assays of fluorescence measurements with each protease tested.

To determine the usefulness and reliability of the apoA-I-Cy5.5 probe, we assayed *in vitro* Cy5.5 fluorescence unquenching over a wide range of rh-CMA, trypsin, cathepsin-S and MMP-12 protease concentrations or initial protease velocity over a wide range of apoA-I-Cy5.5 probe concentrations (showed here only for rh-CMA). We observed a good fit between the proteolytic activity (Cy5.5 unquenching in equivalence of Cy5.5/ (time x amount of probe)) and rh-CMA concentrations (1.7×10^{-9} to 6.7×10^{-8} M) using a third-order polynomial relationship (Fig. 2C), and also between rh-CMA initial velocity and the concentration of apoA-I-Cy5.5 probe (3.4×10^{-8} to 1×10^{-6} M) (Fig. 2D). Similar results were obtained with trypsin, cathepsin-S and MMP-12 as we also observed polynomial relationships between proteolytic activities (Cy5.5 release) and protease concentrations: between 5.4×10^{-9} to 1.7×10^{-7} M for trypsin, 6.4×10^{-9} to 1.5×10^{-7} M for cathepsin-S and from 1.9×10^{-9} to 1.9×10^{-8} M for MMP-12 (Suppl Fig. S6D, S6E, S6F, respectively).

3.5 *In situ* detection of proteolytic activities degrading the apoA-I-Cy5.5 probe

We wanted to characterize the usability of the apoA-I-Cy5.5 probe for the detection of proteolytic activities degrading the apoA-I protein in atherosclerotic tissue. *In situ* zymography using our quenched apoA-I-Cy5.5 probe shows increased fluorescence in atherosclerotic lesions of thoracic aorta sections from 13-month *Ldlr*^{-/-};Tg (human apoB) mice, probably explained by the activation of the apoA-I-Cy5.5 probe due to the protease activities present in the plaques, resulting in a strong fluorescent signal emission (Fig. 3A, upper right panel). The addition of broad-spectrum protease inhibitors, which significantly reduced apoA-I-Cy5.5 activation *in vitro*

(Suppl. Fig. S8), resulted in a significant decrease of the fluorescent signal in the atherosclerotic plaque area, indicating the contribution of proteolytic activities to the observed increase in fluorescence (lower and upper panels of Fig. 3A). After quantification, the fluorescence was 53.7% lower in the atherosclerotic plaque in presence of protease inhibitors ($p \leq 0.0001$; Fig. 3B) and 54.4% lower in the region including both plaque area and the external elastic layer compared to the values obtained in the absence of protease inhibitors ($p \leq 0.01$; Fig. 3C).

3.6 *In vivo and ex vivo detection of proteolytic activities degrading the apoA-I-Cy5.5 probe*

We next tested the ability of the quenched apoA-I-Cy5.5 probe to detect the proteolytic activities degrading apoA-I protein *in vivo* and *ex vivo*. Five 12-month-old *Ldlr*^{-/-};Tg (human apoB) mice (ATX) and three 10-month-old C57BL/6J mice (CTL) were tail-injected with our apoA-I-Cy5.5 quenched-probe, and FMT-MRI imaging was conducted 24 hours after the injection. As detailed previously [31], the co-registered MR images were used to provide the anatomy of the targeted tissue, as well as numerically guide the functional fluorescence reconstruction. The reconstructed fluorescence image was then used as an index of apoA-I-Cy5.5 accumulated and activated in the diseased tissue. Subsequently, the MR and fluorescence images were co-registered for visualization to facilitate the biological interpretation (Fig. 4A, left panel). A Monte Carlo-based approach was used to model photon propagation in biologically realistic MR-derived anatomy for forward modeling. Fig. 4A (right panel) depicts fluorescence intensities measured in ATX and control mice in the aortic region. To facilitate interpretation of the data, the video of axial view sequence from ATX-1 mice (Suppl. Sequence-1) and MR axial slices (YZ) are provided for anatomical structure visualisation (Fig. 4B, left column panels) and fluorescence

signal localisation (Fig. 4B, middle and right column panels). The latter support localisation of the fluorescence signal from the heart and aortic regions.

The differences in fluorescence intensity between the two treatment groups, however, were not statistically significant (Fig. 4C, $p = 0.089$). The unknown Cy5.5 dye concentration accumulated in the lung, the small number of mice used in the study and the nature of the diffuse fluorescence technique where the resolution is typically 1-3 mm and for which literature values for tissues optical properties are used may explain these results.

Therefore, to further validate these results, *in vivo* FMT-MRI observations were compared with an *ex vivo* quantification of fluorescence of the aortas (Fig. 5A) showing that the fluorescence signal from the apoA-I-Cy5.5 probe injected *in vivo* and detected in the atherosclerotic region of the aorta of the ATX mice was 10-fold higher than that observed from CTL mice aortas (Fig. 5B, $p \leq 0.05$). Auto-fluorescence controls showed negligible signal (Fig. 5A, top and bottom left panels). *In vivo* accumulation and activation of the probe were furthermore characterized by confocal microscopy of cryosections from thoracic aorta (Suppl. Fig. S9A-C). The fluorescence signal was stronger in the atherosclerotic plaque area of ATX mice aorta in comparison to the CTL mice.

3.7 ApoA-I-Cy5.5 biological activity: DMPC assay and intracellular localization

Lipid-binding activity is one of the main biological property of native apoA-I lipoprotein. To assess the apoA-I-Cy5.5 lipid binding capability, we carried out a DMPC clearance assay of both native apoA-I and conjugated apoA-I-Cy5.5 probe. The DMPC clearance assay is widely used to characterize lipid-binding activity of apolipoproteins [32, 33]. Fig. 6A shows the results of DMPC clearance kinetic assay for different concentration (mg/ml) ratios between native apoA-I: DMPC and apoA-I-Cy5.5: DMPC (1:5 and 1: 2.5) compared to control DMPC solution alone.

Our results demonstrate over 90 % (at 60 min) to 94 % (at 180 min) and 55 % (at 60 min) to 65 % (at 180 min) of DMPC mLV clearance, respectively, for the 0.2 mg/ml apoA-I and apoA-I-Cy5.5 concentration used in the assay (Fig. 6A). Moreover, we obtained 80 % of clearance with the apoA-I-Cy5.5 probe, however it was observed after a longer period of incubation (12 h 30 min of reading, data not shown). This finding suggest that our probe has preserved a significant fraction of its lipid binding ability although it does have a slower and lower clearance capacity of the DMPC mLV compared to native apoA-I.

We also validated the endocytosis route of our probe in ABCA1-expressing macrophages, a property that has been previously reported [34]. In the latter study, Dr Denis and his colleagues characterized the specific route of apoA-I endocytosis by tracking minimally conjugated fluorescently-labeled (Cy3.5) apoA-I and tested whether their Cy3.5-apoA-I probe behave in the same manner as native apoA-I. Their study showed that similarly to native apoA-I, Cy3.5-apoA-I was rapidly endocytosed and targeted to the endosomal system. However, at later time points (2 h post incubation), the majority of the Cy3.5-apoA-I had reached the lysosomal compartment. We conducted a similar experience to assess if excess (Cy5.5) labelling of the apoA-I would impair the intracellular localization of the apoA-I. We incubated ABCA1-expressing J774 macrophages cells with our apoA-I-Cy5.5 probe for 4h and then stained the lysosomes using LysoTracker-Green. Concomitant with the previously reported study [34], we found a strong colocalization of our apoA-I-Cy5.5 probe with LysoTracker suggesting that conjugated apoA-I-Cy5.5 had reached the lysosomal compartment (Fig. 6B) and behave much similarly to native apoA-I.

3. Discussion

This is the first study demonstrating the applicability of a protease-activatable fluorescent probe composed of a full-length apoA-I protein and the Cy5.5 near-infrared fluorescent dye. This new probe offers several advantages including a low background noise due to efficient fluorophore quenching as well as activation through cleavage at multiple sites and by different proteases that target apoA-I. Indeed, some full-length conjugated proteins exhibit a high fluorophore conjugation and quenching efficiency due to their globular structure [35-37]. In addition, while short peptide probes offer a high sensitivity for the detection of specific protease activity [38, 39], use of these probes would not allow conclusions regarding the overall apoA-I-degrading activity. The development of a gallium-68-labeled 24-amino acid apoA-I mimetic peptide probe (known as (68)Ga-DOTA-FAMP) was reported [40]. This probe removes cholesterol via ABCA1, and was used as a diagnostic tracer for imaging the atherosclerotic lipid burden in rabbits by positron emission tomography imaging. Again, it would be difficult to interpret apoA-I proteolysis in atherosclerotic plaque from the use of such (68)Ga-DOTA-FAMP probe. We reasoned that a probe based on full-length apoA-I could be more useful to detect conditions leading to the loss of its integrity resulting from proteolysis.

Structural studies of apoA-I have demonstrated that the apoA-I molecule exhibits a loosely folded, molten globular-like structure [41]. The observed quenching property of the apoA-I-Cy5.5 probe may therefore be related to the presence of Cy5.5 residues being brought in close proximity to the three-dimensional apoA-I structure. Within the apoA-I protein, 21 lysines and 16 arginines residues are the putative targets for the Cy5.5 dye conjugation (Suppl. Fig. S10). In fact, our results indicate that the Cy5.5 dye had access to about 12 to 15 of these 37 side-chains as indicated by the change in molecular weight of the apoA-I following conjugation.

The resulting fluorescence energy transfer (FRET) of the excited-state energy between neighboring Cy5.5 molecules covalently attached to apoA-I leads to the so-called autoquenching phenomenon. Importantly, heat or SDS denaturation did not significantly impair the quenching state of the probe as demonstrated by very low fluorescence observed in the absence of proteases on denaturing SDS-gel. In contrast, proteolytic activities of chymase, trypsin, cathepsin S or MMP-12 resulted in a strong and consistent increase in fluorescence (≈ 11 -fold) following unquenching due to protein fragmentation.

Trypsin and cathepsin S exhibit similar specificities in their proteolytic activity, cleaving proteins at basic amino acid (Lys/Arg) residues [42-44]. Chymase specifically hydrolyzes at the carboxyl side of aromatic amino acids (Trp/Tyr, Phe residues) on the apoA-I protein, in particular at Tyr¹⁸ and Phe²²⁵ and possibly also at Phe²²⁹ [20, 21], which is compatible with the appearance of two smaller fluorescent fragments of approximately 15 and 14 kDa in our analysis. Proteolysis by MMP-12 resulted in multiple fragments of the apoA-I probe, which confirms previous data from Kovanen et al. who reported at least 5 MMP-12 cleavage sites in apoA-I, mostly present in the C-terminal region [45]. Of note, however, since a direct mass spectrometry analysis was not performed in our study, we are currently unable to determine the exact cleavage sites for the probe and the nature of the resulting fragments.

Although kinetically different, at least 50 % of the ability of native apoA-I to bind lipids was preserved in the apoA-I-Cy5.5 probe, as demonstrated by the probe's DMPC mLV clearance capability. Given that the flexibility of the lipoprotein is the limiting factor in the DPMC clearance reaction [33], it seems likely that reduction of the conjugated apoA-I's DPMC clearance ability occurs due to a structural and flexibility change of apoA-I following excessive Cy5.5 conjugation.

Despite these structural changes, however, our data indicate that the apoA-I probe had maintained the same intracellular endocytosis route as the native apoA-I [34], as indicated by its lysosomal compartment localization. Further, the presence of multiple Cy5.5 molecules on apoA-I may have accounted for the modification of the probe's proteolytic susceptibility. In fact, although the probe's cleavage by proteases was not significantly hampered, as indicated by detection of multiple apoA-I fragments following protease exposure, we observed that the pattern and intensities of apoA-I fragments following probe cleavage differed from those seen with native apoA-I protein. These different cleavage pattern allude to a possible alteration of apoA-I protein structure by Cy5.5 conjugation. Indeed, it is likely that the lipophilic and negatively charged Cy5.5 dye is prone to induce structural and functional changes of apoA-I. Thus, use of a neutral fluorescent dye such as Alexa 680 [46] may help to overcome these limitations in future studies.

Although alteration of Cy5.5-conjugated apoA-I's structure and function may raise concerns regarding the probe's applicability to detect tissue-specific proteolytic activity against apoA-I, it is notable that our apoA-I probe showed an increase in fluorescence that was equivalent to previously reported activatable fluorescent probes including BSA-BODIPY, elastin-FITC, casein-FITC or a Cy5.5-R4-Cy7-CLIO peptide probe [35, 37, 47, 48]. We further confirmed our *in vitro* data by *in situ* zymography analysis, where we could demonstrate a significant fluorescence-increase induced by *in situ* proteolysis of the apoA-I-Cy5.5 probe. In contrast, a >50% reduction of the fluorescence signal was measured following tissue incubation with a broad-spectrum proteases inhibitor mix. These data indicate that the apoA-I probe fulfills essential requirements of a quantitative protease assay and may allow a sensitive and reliable quantification of apoA-I-degrading activities, albeit with limitations regarding extrapolation to native apoA-I, as discussed above.

Although a variety of *in vivo* imaging systems have evolved during the last decade, very few are able to detect biological activity in mouse aorta based on fluorescence during atherosclerotic plaque progression. Thus, we aimed to test the applicability of the apoA-I-Cy5.5 probe for *in vivo* imaging of apoA-I-degrading proteolytic activities in diseased aortic vessel walls of atherosclerotic mice. To achieve this, we applied a combination of fluorescence molecular tomography (FMT) and MRI [31,49] which allows the construction of a heterogeneous model by improving the simulations of photon propagation and attenuation of background signal, particularly from lung tissues, where air-gaps need to be taken into account. The results obtained when comparing *in vivo* FMT-MRI and *ex vivo* fluorescence reflectance imaging confirmed that heterogeneous modelling is beneficial to reconstructions and improved localization and quantification of the reporter [31, 50, 51]. In addition, we observed an increase in fluorescence signal in the aorta of atherosclerotic mice. However, quantification of the fluorescence signal resulted in a non-significant difference between atherosclerotic and control mice. Despite a trend that was confirmed *ex vivo*, failure to detect a significant *in vivo* fluorescence augmentation in diseased aorta might be attributed to Cy5.5 dye accumulation in the lung, the inherent limitation of diffuse optical imaging and the small number of mice used in our study. Thus, further characterization of *in vivo* kinetics and proteolytic activities of the apoA-I probe will be needed to refine our observation.

In summary, we developed a new bioactivatable full-length apoA-I-Cy5.5 conjugated near-infrared probe which may be applicable for *in vitro*, *in vivo* and *ex vivo* detection and quantification of apoA-I-degrading protease activities. In fact, this probe might be useful to analyze apoA-I-degrading activities in tissue homogenates or plasma from atherosclerotic patients by applying different techniques such as *in situ* zymography or *in vitro* assays. Use of different protease inhibitors will further allow to identify proteases that exert the most deleterious

effects on apoA-I integrity and may help to design HDL surrogates that are resistant to adverse modification by these proteases. Of note, however, our data indicate that excessive use of Cy5.5 has the potential to induce structural and functional alterations of the conjugated apoA-I protein and may limit the interpretation of data with respect to a native apoA-I. Therefore, future studies will have to assess the probe's susceptibility to Cy5.5-induced modifications or investigate alternative fluorophores in order to optimize its diagnostic value and to allow a broader applicability.

Conflict of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Author contributions

Conceived and designed the experiments: FM BL FL DR ER JCT. Performed the experiments: FM BL CG MB LV. Analyzed the data: FM BL CG WN ER JCT. Contributed reagents/materials/analysis tools: JCT. Wrote the paper: FM ER JCT.

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Fig. Legends

Fig. 1. *In vitro* characterization of the bioactivatable apoA-I-Cy5.5 fluorescent probe.

(A) Electrophoretic gel showing proteolytic degradation and fluorescence emission of the purified bioactivatable apoA-I-Cy5.5 fluorescent probe (500 $\mu\text{g/ml}$) incubated with recombinant human chymase (rh-CMA) protease (1, 5, 10 $\mu\text{g/ml}$); the arrow indicates the position of the undigested apoA-I-Cy5.5 fluorescent probe. (B) Gel quantification of the total fluorescence emission (density/ cm^2) per lane. (C) Coomassie staining of proteins. Data are presented as the mean $\pm$ standard error of the mean (SEM) (n=5, A and C). (*: $p = 0.0129$; $P=0.015$; $p = 0.0288$, respectively, for CTL vs. CMA 1 $\mu\text{g/ml}$, CTL vs. CMA 5 $\mu\text{g/ml}$ and CMA 1 $\mu\text{g/ml}$ vs. CMA 5 $\mu\text{g/ml}$, **: $p = 0.0073$ and $p = 0.005$, respectively, for CTL vs. CMA 10 $\mu\text{g/ml}$ and CMA 1 $\mu\text{g/ml}$ vs. CMA 10 $\mu\text{g/ml}$; ns: $p = 0.102$ for CMA 10 $\mu\text{g/ml}$ vs. CMA 5 $\mu\text{g/ml}$).

Fig. 2. *In vitro* measurement of chymase (rh-CMA) proteolytic activity using apoA-I-Cy5.5 probe.

Increase in fluorescence (n=3) over time mediated by proteolysis of apoA-I-Cy5.5 probe (40 $\mu\text{g/ml}$) using rh-CMA (0.05 $\mu\text{g/ml}$ to 2 $\mu\text{g/ml}$) (A), or different concentrations of apoA-I-Cy5.5 probe incubated with 1 $\mu\text{g/ml}$ of rh-CMA (B). The fluorescence emitted was measured for the indicated time using 20-second intervals on a fluorescent microplate reader. (C) Relationship between the proteolytic activity of the rh-CMA protease (1.7×10^{-9} to 6.7×10^{-8} M) and apoA-I-Cy5.5 probe (1×10^{-3} M), rh-CMA activity was analysed between 20 sec and 320 sec of reaction time (n=3). (D) Relationship between the concentration of the ApoA-I-Cy5.5 probe (3.4×10^{-8} to 1×10^{-6} M) and the rh-CMA initial velocity (n=3). (E) Log-log standard curve showing the relation between free Cy5.5 dye concentration (2.5×10^{-6} to 3×10^{-8} M) and

fluorescence measured in the rh-CMA-specific activity buffer (n=5). Third-order polynomial trend curves were used for fitting curves in panels (C) and (D). Data are presented as the mean $\pm$ standard error of the mean (SEM).

Fig. 3. *In situ* zymography for apoA-I-targeting proteolytic activity in mouse thoracic aorta.

(A) Representative sections of thoracic aorta of an atherosclerotic mouse (LDLr^{-/-};Tg(human apoB)), incubated for 3 h with the apoA-I-Cy5.5 fluorescent probe (40 μ g/ml) in the presence or absence of a broad-spectrum protease inhibitors cocktail. (TPMT: Transmitted light photomultiplier tube). (B and C) Quantification of the relative total fluorescence (intensity/ μ m²) in the plaque or in the plaque + elastic lamina (B) and (C). Data are presented as the mean $\pm$ standard error of the mean (SEM) (n=6 mice) (***: $p \leq 0.0001$; **: $p = 0.0023$).

Fig. 4. *In vivo* activation of the apoA-I-Cy5.5 probe assessed by hybrid FMT-MRI imaging system.

(A) The images in the left column are the magnetic resonance (MR) slices for each mouse. The heart and part of the aorta of ATX #1 are indicated with red arrows. In the right column, the reconstructed FMT/MRI images were overlaid with transparency (alpha=0.5) on the MR slices. Three orthogonal MR slices were chosen for each mouse: axial slice (YZ), coronal slice (X-Y) and sagittal slice (X-Z). (B) Three distinct ATX-1 mouse MR axial slices (YZ) were chosen for anatomical visualisation (yellow arrows, left column), fluorescence signal localisation (middle column) and fluorescence signal/MR co-localisation (right column). Anatomical structure abbreviations stand as follows: RL: right lung; LL: left lung; Svc: superior vena cava; Rpa: right pulmonary artery; Mpa: main pulmonary artery; RV: right ventricle; LV: left ventricle; Ao: aorta

(C) FMT quantification of the fluorescent intensity signal ratio. Data are presented as the mean $\pm$ standard error of the mean (SEM) (relative fluorescence ratio ATX/CTL, n=3 mice; $p=0.089$).

Fig. 5. Characterization of the *in vivo* activation of the apoA-I-Cy5.5 probe by *ex vivo* imaging.

(A) Near infrared fluorescence signal in aorta of the atherosclerotic mice (ATX) compared to normal mice (C57BL/6J). The images are presented as overlays of fluorescent image with white light image. (B) Lumina II Living Image software quantification of the total fluorescence (density/cm²). Data are presented as the mean $\pm$ standard error of the mean (SEM) (ATX n=5; CTL n = 3; *: $p=0.0253$).

Fig. 6. Biological activity of apoA-I-Cy5.5 probe.

(A) Dimyristoyl-phosphatidylcholine (DMPC) clearance kinetics. Time-course experiment for clearance (at 26.0 ± 0.2 °C) of DMPC mLV (● CTL DMPC mLV : 0.5 mg/ml) by native apoA-I (■: 0.1 mg/ml, ▲: 0.2 mg/ml) and apoA-I-Cy5.5 probe (▼: 0.1 mg/ml, ◆: 0.2 mg/ml). Time-course data were obtained by monitoring the change in absorbance at 480 nm. The DMPC concentration was 0.5 mg/ml in all cases. (B) Z-stack images (20X) by confocal microscopy for intracellular localization after 4h incubation of apoA-I-Cy5.5 probe with ABCA1-expressing J774 murine macrophages. (Red: apoA-I-Cy5.5 staining, Green: LysoTracker Green DND-26 for lysosomal staining, Blue: DAPI for nuclear staining).

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Figure 1

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