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High-resolution Optical Mapping of Inflammatory Macrophages Following Endovascular Arterial Injury

Amit Saxena^{1,*}, Chase W. Kessinger^{1,*}, Brian Thompson², Jason R. McCarthy³, Yoshiko Iwamoto³, Charles P. Lin^{2,3}, and Farouc A. Jaffer^{1,2,4}

¹Cardiovascular Research Center, Cardiology Division, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

²Wellman Center for Photomedicine, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

³Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

⁴Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114

Abstract

Purpose—Inflammation following arterial injury mediates vascular restenosis, a leading cause of cardiovascular morbidity. Here we utilize intravital microscopy (IVM) and a dextran-coated nanosensor to spatially map inflammatory macrophages *in vivo* following endovascular injury of murine carotid arteries.

Procedures—C57Bl/6 mice (N=23) underwent endovascular guidewire carotid arterial injury. At day 14 or day 28 post-injury, mice underwent fluorescence IVM, twenty-four hours after injection with the near-infrared fluorescent macrophage nanosensor CLIO-VT680. Adventitial collagen was concomitantly imaged using second harmonic generation (SHG) IVM. Correlative fluorescence microscopy and immunohistochemistry were performed.

Results—Two-plane IVM reconstructions detected macrophage inflammation in the arterial wall that was elevated at day 14 compared to day 28 animals ($p < 0.05$). SHG-based collagen imaging of the outer arterial wall facilitated analysis of the macrophage-rich, inflamed neointima. Histological analyses and fluorescence microscopy data demonstrated increased macrophage infiltration in day 14 compared to day 28 neointima.

Conclusions—We demonstrate that the macrophage response to arterial injury can be imaged *in vivo* using IVM-based molecular imaging, and shows a higher macrophage influx at day 14 compared to day 28 post-injury.

Keywords

Inflammation; vascular injury; molecular imaging; intravital microscopy; macrophage; nanoparticle; restenosis; percutaneous coronary intervention; fluorescence

Correspondence: Farouc Jaffer MD PhD MGH CVRC Simches Research Building, Room 3206 185 Cambridge Street Boston, MA 02114 fjaffer@mg.harvard.edu Tel: 617 724 9353 Fax 617 860 3180.

*Contributed equally

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Introduction

Percutaneous coronary intervention (PCI) treatment of obstructive coronary artery disease often utilizes angioplasty and mechanical stents to re-establish coronary blood flow. While highly effective, restenosis due to neointimal proliferation is a major cause of PCI failure, leading to recurrent angina and myocardial infarction. Significant restenosis also necessitates repeat invasive procedures, and overall is a major cause of cardiovascular morbidity following PCI.

Restenosis is now appreciated to be driven by the inflammatory response following vascular injury (1, 2). Inflammation after injury occurs as part of the normal wound healing response after vascular injury, and is characterized by the sequence of inflammation, granulation, extracellular matrix remodeling, and smooth muscle cell (SMC) proliferation and migration (3). When the inflammatory response is exaggerated, proliferative scar tissue forms in the neointima (termed restenosis), and can reduce arterial blood flow leading the sequelae above. Approaches to investigate the inflammatory response *in vivo* could therefore improve the understanding of mechanisms governing restenosis.

Macrophages are a key cellular mediator of the arterial inflammatory injury response, both in angioplasty and in stent-induced vascular injury (4–8). Recruited macrophages induce a variety of proinflammatory mediators, including adhesion molecules, chemokines, cytokines, free oxygen radicals and matrix metalloproteinases. Experimental studies employing guidewire injury-based protocols in mice have shown that inflammation is induced in the arterial wall, and is characterized by macrophage influx followed by smooth muscle cell proliferation (9), thereby recapitulating the human vascular injury response.

While imaging of arterial inflammation following vascular injury is feasible at lower resolution (10, 11, 12), limited information is available regarding the high-resolution topography of inflammatory cells, such as recruited macrophages, following vascular injury. The present study thus investigated a high-resolution approach to image inflammation in injured murine carotid arteries. Using fluorescence intravital microscopy (IVM) and a macrophage-targeted fluorescent molecular imaging agent, and concomitant second harmonic generation (SHG) IVM of arterial wall collagen, we test the hypothesis that macrophage influx into the neointima can be visualized *in vivo* following guidewire-induced murine carotid arterial injury. We then assess the differences at two time points following endovascular injury: day 14, to assess sub-acute inflammatory response; and day 28, to assess the later/healed vascular response.

Material and Methods

Animals

All animal experimental protocols were reviewed and approved by the MGH Subcommittee on Research Animal Care. This study used a total 23 male C57BL/6 mice, age 14–16 weeks old, for imaging (n=10) and histological studies (n=13). *In vivo* imaging was performed at either day 14 (n=5) or day 28 (n=5).

Guidewire-induced arterial injury of murine carotid arteries

Mice were placed supine and anesthetized using intraperitoneal ketamine/xylazine (80 and 12 mg/kg, respectively). The carotid artery was exposed by blunt dissection and then underwent unilateral arteriotomy (Fig 1). The right internal carotid artery was exposed and temporarily ligated, followed by ligation of proximal end of the external common carotid artery. The first 3 cm of a 0.36 mm (.014") diameter clinical coronary artery guide-wire (Stabilizer; Cordis/Johnson and Johnson) was introduced into the right common carotid and

traversed across the entire length of the carotid artery up to the carotid bifurcation. The guide-wire was gently rotated inside the vessel and then left in place for approximately 1 minute to induce endothelial denudation of the artery (wire/artery diameter ratio ≈ 1.8). Thereafter the external carotid artery was ligated permanently and blood flow was resumed via internal carotid artery. Sham surgery (incision followed by artery exposure, without guidewire injury) was performed in the contralateral artery. Approximately 10% of mice developed acute thrombosis of the injured carotid artery, and were excluded from further study.

Preparation of the macrophage nanosensor CLIO VT680

CLIO was provided by the NanoCore at the Center for Systems Biology at the MGH. To CLIO (20 mg) in PBS (2.37 mL) was added the succinimidyl ester of VivoTag 680 (ex/em 680/700 nm; 1 mg, Perkin Elmer, Waltham, MA) dissolved in 400 μ L of dimethylsulfoxide (DMSO). The reaction was allowed to proceed for 16 hours, at which time it was purified by filtration through Sephadex G-25 to yield 3 dyes per CLIO, as determined spectrophotometrically (13).

Confocal fluorescence and second harmonic generation intravital microscopy of murine carotid arteries

IVM of murine carotid arteries was performed as previously established in the lab (14–16). Briefly, a small incision was made in the neck of the mice to expose the carotid artery, which was then propped up using PE-10 tubing (Becton Dickinson, Franklin Lakes, NJ) as a scaffold to stabilize the artery and limit motion-induced blurring. Images of the wire-injured and contralateral control carotid arteries were captured over one hour, using a customized integrated confocal and SHG intravital imaging system (17). High-resolution images with cellular detail were obtained from intact carotid arteries at depths of up to 200 μ m from the carotid surface using a 30 $\times$ magnification, 0.9-NA water immersion objective lens (Lomo, Saint Petersburg, Russia), resulting in a field of view of 660 $\times$ 660 μ m.

Carotid arterial blood flow was imaged using fluorescein isothiocyanate (FITC)-dextran (0.5 mg tail vein injection, MW 2,000 kDa, Lot 120M5310V, Sigma, St. Louis, MO). FITC was excited with a 491-nm solid-state laser (Dual Calypso 20, Cobolt AB, Stockholm, Sweden) and detected with a PMT through a 528 $\pm$ 19-nm bandpass filter (FF01-528/38-25, Semrock, Rochester, New York). To image the injury-induced macrophage response, CLIO-VT680 was excited with a 635 nm helium-neon laser (Radius, Coherent Incorporated, Santa Clara, California) and detected through a 695 $\pm$ 27.5-nm band-pass filter (XF3076 695AF55, Omega Optical, Brattleboro, Vermont).

To better understand the spatial relationship of neointimal inflammation, the same carotid artery also underwent concomitant second harmonic generation (SHG) IVM of type I and III collagen to define the adventitial outer layer of the artery. SHG imaging was performed using 880-nm excitation (Mai Tai HP, Spectra-Physics, Irvine, California). SHG signals were acquired through a 457 $\pm$ 25-nm bandpass filter (FF01-457/50-25, Semrock). Multiple z-axis stacks with a step-size of 2 μ m were acquired to a depth of 200 micrometers deep into the carotid artery. Images were acquired at 30 frames per second using a Macintosh computer equipped with an Active Silicon snapper card (CBL-25D-SNP, Active Silicon, Chelmsford, Massachusetts). All images were captured after averaging three consecutive frames.

Fluorescence microscopy and immunohistochemistry

Excised carotid arteries were embedded in optimal cutting temperature (OCT) compound (TissueTek, Sakura Finetek, Japan) and frozen in dry-ice chilled isopentane. Six micrometer

sections were cut from the carotid artery specimens, and fluorescence microscopy (FM) was performed in multiple channels using an upright epifluorescence microscope (Eclipse 90i, Nikon, Japan) with FITC filter 460–500 ex/510–560 em; dichroic 505nm; Cy5.5 filter 628–673 ex; 685–735 em; dichroic 680nm. Exposure times ranged between 100–1000 msec. Images were analyzed using ImageJ software (NIH, ver. 1.45d).

Immunohistochemical and morphometric analysis

For morphometric analysis, carotid arteries were perfused using phosphate buffered saline and then embedded in OCT compound for sectioning. Carotid arteries were snap frozen and sectioned (6 μ m thickness) using a cryostat microtome (Leica CM3050 S, USA). Frozen sections were air dried at room temperature and incubated in -20°C acetone for 1 minute, and then stored in PBS until stained. Twenty to forty micrometer spaced adjacent sections were then immunostained to identify neointimal macrophages via a rat anti-mouse Mac-3 antibody (Clone: M3/84, BD Biosciences), and endothelial cells via a rat anti-mouse CD31/PECAM-1 antibody (BD Biosciences, Clone: MEC 13.3). Biotinylated anti-rat IgG served as a secondary antibody (Vector Laboratories, Inc.) prior to color development with AEC substrate (Dako Cytomation). Adjacent sections were also stained with hematoxylin and eosin for general morphology.

The first section was registered to the carotid artery side branch used for guidewire insertion, and subsequent sections were obtained every 20–40 μ m. Digitized images were then analyzed using ImageJ (NIH). Carotid arteries were measured for circumference of the lumen, the internal elastic membrane (IEL), the external elastic membrane (EEL), and the border of the adventitia in all 2–4 cross-sections per vessel. The following parameters were calculated: adventitial area, defined as the area between the vessel border and the EEL; medial area, comprised the area between the IEL and the EEL; and the neointimal area comprised the space between the IEL and the lumen. Both the intimal area and the intima-to-media ratio (IMR) were calculated to assess the volume of injury-induced restenotic tissue. Uninjured contralateral arteries served as controls (n=5).

Image analysis

Image analysis was performed using ImageJ software (NIH, ver. 1.45d, Bethesda, MD). IVM z-stack images were collected in the coronal plane and corrected for rotation and pitch in the x-y and y-z directions, respectively, to produce coronal datasets where the carotid was perpendicular to the axial reslice plane. Adjacent axial reslices (200 slices) were summed producing the final axial image. Coronal summation images were generated from summing 60 slices (120 μ m total depth) starting from the luminal edge of collagen SHG signal expanding into the lumen on the axial reslice datasets. Mean signal intensity (SI) measurements of the artery wall were taken from manually drawn regions-of-interest. The artery wall was defined as all areas interior to the collagen SHG signal. Target-to-background ratios (TBRs) were calculated as the mean SI of injured carotid artery datasets divided by the mean SI of control artery datasets.

Statistical methods

All data are reported as mean $\pm$ SEM. Differences between two groups were assessed by the unpaired Student's *t* test. All statistics including linear regressions were performed using Prism (version 5.0, GraphPad Software, San Diego, California).

Results

The completion rate of the guidewire injury protocol (Fig. 1) was 95%, and the survival rate of animals completing the imaging protocol was approximately 85%. Extravasation of the wire or rupture of the carotid artery was not observed.

Wire injury de-endothelializes the carotid artery and induces neointimal proliferation at day 14

Neointima formation occurs as a response to vascular injury. Wire injury resulted in significant endothelial denudation as evidenced by reduced CD31 staining at day 3, which re-endothelialized by day 14 (Fig. 2). At day 14, guide-wire injury resulted in proliferation of the neointima, or restenosis, as well as a smaller lumen size, when compared to the control contralateral artery (Fig. 2c–2d).

IVM identifies recruited macrophages following vascular injury

Macrophages are a central effector cell in the inflammatory response to vascular injury. The wire-injured length of carotid arteries was imaged *in vivo* at either day 14 or day 28. The macrophage response post injury was registered using macrophage nanosensor CLIO-VT680 injected i.v. 24 hours prior to IVM. At day 14, abundant, punctate near-infrared fluorescence (NIRF) signals were evident in the injured arterial wall, indicating uptake of the macrophage reporter CLIO-VT680 (Fig. 3). In contrast, day 28 arteries showed relatively reduced CLIO-VT680 signal uptake. Coronal summations of the IVM images of 60 slices (2 μ m thickness each) showed significantly higher day 14 TBR values of 1.68 ± 0.53 compared to day 28 TBR values of 0.99 ± 0.12 at day 28 ($P < 0.023$, Fig. 3, 5a). The contralateral sham arteries also showed little NIRF signal at either day 14 or day 28, affirming that endovascular injury was required to induce macrophage infiltration.

The spatial relationship of neointimal inflammation to the arterial wall comprised of the tunica adventitia, tunica media, and neointima was studied by investigating axial reconstructions of the 3D IVM dataset (Fig. 4). FITC-dextran based angiography provided an arterial angiogram (data not shown). SHG microscopy of arterial wall collagen defined the outer boundary of the artery (Fig. 4) and facilitated axial image analyses of neointimal macrophages. Analyzing up to a depth of 120 micrometers below the arterial wall defined by the adventitial collagen boundary, we found that the day 14 macrophage influx (NIRF signal enhancement) was greater than at day 28. Adjacent axial reconstructions summation stacks obtained from 200 adjacent axial slices also demonstrated greater macrophage infiltration in the neo-intimal region of the vessel at day 14, with a TBR of 1.58 ± 0.42 , compared to day 28, with a macrophage TBR of 0.99 ± 0.24 at day 28 ($P < 0.02$, Fig. 5b).

Epifluorescence microscopy of arterial sections reveals deposition of the macrophage targeted particle CLIO-VT680 in the neointima of the injured carotid artery

Fluorescence microscopy of fresh-frozen sections demonstrated vascular neointimal formation at the two time-points investigated in this study (Fig. 6). Images acquired in the NIRF channel demonstrated that CLIO-VT680 localized at the endothelial interface of the neo-intima, and also in the peri-adventitial region of the carotid artery (Fig. 6b). The CLIO-VT680 signal present at day 14 was elevated compared to day 28, corroborating the IVM findings. Correlative immunohistochemistry confirmed that regions of Mac-3 positive immunoreactive macrophages were diffusely localized both in the neo-intima and in the adventitial wall of the wire-injured carotid artery. The intima-media ratio, a measure of restenosis, could be assessed accurately in 8 mice (5 others were limited by off-axis or sectioning artifact), and was increased at day 14 (2.62 ± 1.20), compared to day 28 ($0.59 \pm$

0.40, $P < 0.05$, Fig. 6f). The neointimal area was also significantly increased at day 14 ($67.7 \pm 33.7 \mu\text{m}^2$) compared to day 28 ($19.1 \pm 13.2 \mu\text{m}^2$, $P < 0.05$, Fig. 6f).

Discussion

In this study we present a high-resolution optical molecular and structural imaging approach to map the *in vivo* murine macrophage response following guidewire based endovascular injury. The results may provide a new *in vivo* method to investigate inflammatory mechanisms underlying restenosis.

Experimental and clinical studies have shown that following vascular injury, monocytes infiltrate the vessel wall, differentiate into macrophages, and secrete inflammatory cytokines (4, 18, 19), and thereby modulate restenosis. Imaging of macrophage activity therefore offers a new approach to understand the *in vivo* inflammatory response to vascular injury. To image the macrophage response *in vivo*, we utilized IVM in conjunction with a magneto-fluorescent nanoparticle that offers multimodal detection of macrophages (14, 16, 20, 21, 22). The *in vivo* results from longitudinal and axial reformatted 3D IVM datasets (Fig. 3,4) demonstrated that arterial injury macrophage response is elevated at day 14 post-injury, and then returns to control artery levels at day 28. Injury-induced systemic inflammation was not found to be a significant cause of arterial wall inflammation, as the NIRF signals of the control arteries in the same mice were similar at day 14 and day 28. Findings were corroborated by histological assessment of macrophages, and by NIR fluorescence microscopy of the distribution of CLIO-VT680 (Fig. 6). The overall findings extend prior *ex vivo* histological assessment of endovascular injury in murine arteries (23–25), and provide a foundation to further understand the role of inflammation, as well as genetic/pharmacological modulators of inflammation, in restenosis following vascular injury.

Intravital microscopy offers superb spatial resolution for detecting *in vivo* aspects of vascular disease. In addition, IVM offers versatile photonic detection, which in this study enabled simultaneous detection of arterial wall macrophages via confocal fluorescence microscopy of CLIO-VT680, as well as of type I and III collagen via SHG microscopy (26). Collagen signals were instrumental in providing a registered boundary for both injured and control arteries, allowing reproducible image analyses.

Traditional vascular injury models typically utilize opaque collar or cuff approaches that constrict the artery and induce restenosis (27, 28). However such models limit the applicability of IVM approaches due to absorption of light by the cuff itself, limiting illumination of the neointima below the cuff. To overcome this limitation, we employed a guidewire-induced endovascular injury model to allow high-resolution IVM and SHG microscopy of arterial wall cellular and structural signatures. An endovascular approach to injury also more typically represents the vascular injury induced during clinical percutaneous endovascular intervention. In addition, guidewire-based injury produces broader transmural mechanical damage to the medial wall in contrast to ligation or cuff-based methods (28, 29).

Limitations of this study are present. As mouse arteries are not large enough to accommodate human coronary stents, additional studies will be required in larger animals to precisely characterize stent-induced inflammatory responses *in vivo*. In the current study, the carotid artery was stabilized with tubing that could alter normal flow characteristics; alternative strategies for IVM of large mouse arteries (30) may induce fewer disturbances in flow. While we have demonstrated an elevated murine macrophage response at day 14 compared to day 28, further time course studies will be needed to elucidate the timepoint of the peak macrophage response. Due to variable guidewire injury and thus varying neointima

across the injured length of carotid artery, it was not possible to exactly co-register of IVM and histological data sets. Nevertheless, *in vivo* coronal and axial reconstructions provided useful information on the heterogeneity and composition of the restenosis at two time points investigated. CLIO reports predominantly on macrophages, although mild uptake by endothelial cells and smooth cells may occur (19). Lastly multiphoton microscopy or lower resolution fluorescence molecular tomography (FMT) approaches could enable deeper fluorescence imaging of inflammatory macrophages, as compared to confocal IVM.

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Fig. 1. Endovascular guidewire injury model in murine carotid arteries

Upper panel shows a schematic representation. **(a)** Temporary ligations are introduced to the carotid artery. **(b)** An 0.014" guidewire is next inserted via the external carotid (EC) artery and traversed along the entire length of common carotid artery up to the carotid bifurcation for 1 minute (distal to head) to induce injury to the vessel. **(c)** The EC is closed and temporary ligations are removed to allow blood flow via the internal carotid (IC) artery. The lower panel shows the procedure in detail. **(d)** The IC artery is temporary ligated and 6-0 silk suture is passed underneath the EC artery. **(e)** Distal to the head, an arterial clamp stops blood flow while passing the guidewire into the artery. **(f)** Threads are stretched and loops are created to allow the nicked area to be viewed distinctly. **(g)** The guidewire is introduced into the EC and rotated inside to induce endothelial denudation, while the arterial clamp restricts blood flow during the procedure. **(h)** Permanent ligation of the EC, followed by release of the clamp and return of blood flow through the IC artery. Arrowhead indicates point of insertion of the guidewire. **(i)** The surgical incision is sutured closed. EC = external carotid artery, IC = internal carotid artery, CC = common carotid artery.

Fig. 2. Endothelial cell layer and restenosis assessment of injured and control carotid arteries at day 3 and day 14

Endothelial cells were detected by CD31 immunohistochemistry. (a) Day 3 control carotid artery. (b) Day 3 wire-injured carotid artery (A and B - 20 x). (c) Day 14 control carotid artery. (d) Day 14 wire-injured carotid artery (C and D - 10 x). Yellow arrows show re-endothelialized regions; blue arrows show denuded artery lumen. Scale bar, 100 μ m.

Fig. 3. Representative coronal reconstruction IVM images of murine carotid arteries
 Images were generated by summing 60 coronal slices starting from the luminal edge of adventitial collagen, defined by the SHG signal. A and B show CLIO-VT680 channel images (SHG signals are not displayed). **(a)** Day 14 and day 28 injured carotid artery. **(b)** Day 14 and day 28 control carotid artery. Images were pitch- and angle-corrected. Scale bar, 100 μm .

Fig. 4. Axial reconstruction IVM and SHG images of carotid arteries

Axial reconstructions (200 slices * 2 μm thickness each) of carotid arteries were summed starting from the luminal edge of adventitial collagen defined by SHG signal **(a)** Injured arteries, day 14 and day 28. **(b)** Contralateral control arteries, day 14 and day 28. Images were pitch- and angle-corrected. Representative summation injured and matched images are shown and were equally windowed. SHG, second harmonic generation. Scale bar, 100 μm .

Coronal	D14	D28
Mean	1.682	0.9940
Stdev	0.5376	0.1284
Std.Error	0.2404	0.05741

Axial	D14	D28
Mean	1.580	0.9960
Stdev	0.4208	0.2417
Std.Error	0.1882	0.1081

Fig. 5. Macrophage infiltration in IVM coronal and axial reconstructions of carotid arteries
Quantification of CLIO-VT680 TBR values of the carotid artery at day 14 and day 28 were calculated on summation (a) coronal images and (b) axial images. Mean $\pm$ SEM of 5 mice are shown. * $P < 0.05$.

Fig. 6. Fluorescence microscopy and histological analyses at day 14 and day 28 after endovascular injury

Upper panel, day 14; Lower panel, day 28 representative images. Rows **a, c**: Mac-3 macrophage immunohistochemistry. The yellow and red dotted lines show measurements of neointimal and medial areas, respectively. Rows **b, d** – fluorescence microscopy images showing a merged FITC and CLIO-VT680 (macrophage) fluorescence, colored green and red, respectively. (**e**), Intima-to-media ratio in arteries as a measure of restenosis. (**f**) Neointimal area in carotid arteries as a measure of restenosis. Mean $\pm$ SEM of 4 mice are shown. Each data point represents the measured average of one mouse. * $P < 0.05$ and ** $P < 0.001$. Scale bars, 100 μ m.