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Original article

Detection and quantification methods of monocyte homing in coronary vasculature with an imaging cryomicrotome

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ABSTRACT

Cellular imaging modalities are important for revealing the behavior and role of monocytes in response to neovascularization progression in coronary artery disease. In this study we aimed to develop methods for high-resolution three-dimensional (3D) imaging and quantification of monocytes relative to the entire coronary artery network using a novel episcopic imaging modality.

In a series of *ex vivo* experiments, human umbilical vein endothelial cells and CD14+ monocytes were labeled with fluorescent live cell tracker probes and infused into the coronary artery network of excised rat hearts by a Langendorff perfusion method. Coronary arteries were subsequently infused with fluorescent vascular cast material and processed with an imaging cryomicrotome, whereby each heart was consecutively cut (5 μm slice thickness) and block face imaged at appropriate excitation and emission wavelengths. The resulting image stacks yielded 3D reconstructions of the vascular network and the location of cells administered. Successful detection and quantification of single cells and cell clusters were achieved relative to the coronary network using customized particle detection software. These methods were then applied to an *in vivo* rabbit model of chronic myocardial ischemia in which autologous monocytes were isolated from peripheral blood, labeled with a fluorescent live cell tracker probe and re-infused into the host animal. The processed 3D image stacks revealed homing of monocytes to the ischemic myocardial tissue. Monocytes detected in the ischemic tissue were predominantly concentrated in the mid-myocardium. Vessel segmentation identified coronary collateral connections relative to monocyte localization.

This study established a novel imaging platform to efficiently determine the localization of monocytes in relation to the coronary microvascular network. These techniques are invaluable for investigating the role of monocyte populations in the progression of coronary neovascularization in animal models of chronic and sub-acute myocardial ischemia.

1. Introduction

Monocytes are an important group of cells from the innate immune system. In inflamed tissue, monocytes transform into macrophages where they clear pathogens, modulate tissue repair and healing [1], as well as support neovascularization progression [2,3].

Recent studies have shown a pivotal role for monocytes contributing to myocardial ischemic injury in myocardial infarction and atherosclerotic plaque progression [4,5]. Clinical studies have shown that high levels of pro-inflammatory monocytes in the blood of patients after acute myocardial infarction are associated with poor functional outcome [6]. Further clinical studies using post-mortem myocardial tissue of patients that died at different time points after acute myocardial infarction (AMI) have also shown distinct spatiotemporal pattern of monocyte infiltration in the infarcted myocardium [7]. Thus, monocytes play an important role in myocardial repair and healing after ischemic damage, however their infiltration can also lead to pathologic outcome. Understanding the fate of these cells in response to myocardial ischemia

Abbreviations: 3D, three-dimensional; ^{99m}Tc-HMPAO, ^{99m}Tc-hexamethylpropylene amine oxime; CMTMR, CellTracker™ Orange CMTMR; CMTPX, CellTracker™ Red CMTPX; Endo, endocardium; Epi, epicardium; LVC, left ventricular cavity; Mid, mid-myocardium; RVW, right ventricular wall.

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is thus critical in order to modulate the balance between their therapeutic and pathologic roles.

Extensive efforts have focused on advancing molecular and cellular imaging to visualize monocyte and macrophage fate [8]. Developments in numerous imaging modalities, coupled with the use of newly synthesized imaging probes, have allowed the visualization of monocyte/macrophage behavior at the molecular, cellular as well as organ levels in preclinical and clinical settings [9]. However, current techniques lack sufficient spatial resolution for detailed three-dimensional (3D) depictions of infiltrating monocytes/macrophages relative to the vasculature on an entire organ level, especially with respect to coronary neovascularization.

The objective of this study was to demonstrate the feasibility of the imaging cryomicrotome [10–13] to visualize monocytes at μm resolution on a 3D level, in conjunction with the coronary microvascular network. This imaging modality has been successfully employed in the assessment of vascular adaptation due to cryoablation [14], and in the detection of coronary collateral vessels [10–13], but not in conjunction with monocyte detection. We utilized different animal models and examined various fluorescent probes for cellular imaging, with a focus on monocytes. Particle detection software and spectral unmixing methods were used for detection and quantification of cells. Our findings ascertain a novel imaging platform for high-resolution 3D fluorescent monocyte detection and quantification in studies of coronary neovascularization. Application of these methods can yield crucial insight in the functional role of monocyte sub-populations in neovascularization progression. This knowledge would thereby help to advance the realization of appropriate therapeutic agents to modulate the beneficial and pathological effects of monocyte subsets in ischemic heart disease.

2. Materials and methods

2.1. Animal experiments

All experiments were approved by the institutional committee for animal experiments at the Academic Medical Center. Animals were purchased from Charles River Laboratories and housed in the animal care facility at the Academic Medical Center.

2.1.1. Infusion of cell suspensions into rat coronary arteries — ex vivo experiments

Healthy male Wistar rats (12 weeks of age, n = 2) were anesthetized by isoflurane inhalation, followed by intraperitoneal administration of heparin. Rats were euthanized by decapitation and hearts were immediately excised and perfused with heparinized buffer by cannulation of the aorta, according to Langendorff [15]. In the first rat, the coronary arteries were infused with CMTMR (25 μM) and CMTPX (25 μM) labeled human umbilical vein endothelial cells (HUVECs; 5×10^5 cells/label) through the aortic cannula. In the second rat, the coronary arteries were infused with 3.34×10^3 CD14+ monocytes isolated from peripheral blood of a healthy New Zealand White rabbit. Prior to infusion, the cells were labeled with CMTMR (72 μM). These were infused into the heart by aortic cannulation. In both experiments fluorescently labeled cells were fixed in suspension, prior to vascular infusion, with 3.7% formaldehyde followed by washing steps.

Following administration of fluorescently labeled cells, Mercox resin and respective catalyst (Ladd Research) supplemented with 425 μg/mL UV Blue (VasQtec), were infused into the coronary arteries at 100 mm Hg. After polymerization and hardening of cast material, hearts were prepared for cryomicrotome processing.

Two different cell types were employed in the *ex vivo* experiments (HUVECs and CD14+ monocytes). This was performed such that we could image at the highest practical resolution while visualizing cells of different size. HUVECs are approximately 30 μm in diameter, while rabbit monocytes are approximately 15 μm.

2.1.2. Rabbit model of chronic myocardial ischemia

Chronic myocardial ischemia was induced in New Zealand White rabbits (~3.0 kg) as described previously [16]. The animals (n = 2) were sedated with subcutaneous injections of 0.2 mg/kg dexmedetomidine (Dexdomitor; Orion Pharma) and 15 mg/kg ketamine (Nimatek, Eurovet Animal Health BV), followed by subcutaneous infusion of 0.03 mg/kg buprenorphine (Temgesic, RB Pharmaceuticals Limited). By means of a left-sided thoracotomy through the third intercostal space, an ameroid constrictor (5.5 × 1.5 mm, Research Instruments SW) was implanted on an anterolateral branch of the left circumflex artery. Progressive occlusion was induced by means of expansion of the ameroid volume which occurs in approximately 10 days, as reported earlier [16]. Immediately after ameroid implantation, the thorax was closed in layers. Fourteen days after ameroid constrictor placement, 25 mL of blood was withdrawn from the ear artery into ethylenediaminetetraacetic acid (EDTA) Becton Dickinson and Company (BD) vacutainer tubes, after which 15 mL replacement fluid was given intravenously (30 mL/h) and 15 mL NaCl subcutaneously. Monocytes were isolated from peripheral blood (7.25×10^5 cells) and fluorescently labeled as described below. Fluorescently labeled monocytes were re-administered into the host animal intravenously in PBS with 0.4% heparin (Leo) either 1 day (n = 1) or 1 h (n = 1) before euthanization. Animals were euthanized sixteen days after ameroid placement; the animals were anesthetized with dexmedetomidine and ketamine, followed by administration of 5000 IU of heparin (intravenously) and intravenous injection of 20% Euthasol (AST Farma). The heart was immediately excised and the aortic root cannulated and perfused retrograde with saline buffer supplemented with 5 IU/mL of heparin at 100 mm Hg. Batson no. 17 plastic replica material (Polysciences Inc.) supplemented with 425 μg/mL of UV Blue at 100 mm Hg was then infused through the same aortic cannula. We report imaging cryomicrotome and two photon laser scanning microscopy (TPLSM) assessment of one rabbit heart, along with histological assessment by immunofluorescence and confocal microscopy for the second rabbit heart.

2.2. Endothelial cell culture

HUVECs were obtained from multiple donors (Lonza) and were cultured in fibronectin coated 6-well plates (Corning Inc.) until 70–80% confluency. Cells were cultured in EGM-2 MV culture medium, consisting of EGM-2 SingleQuot Kit supplements and growth factors along with EBM-2 basal medium (Lonza). All experiments were conducted on cells at passages 4–7.

2.3. Monocyte isolation and culture

Peripheral blood from New Zealand White rabbits was diluted 1:1 with phosphate buffered saline (PBS), overlayed onto Ficoll Paque PLUS (GE Healthcare) and centrifuged at room temperature at 1000 *g* for 30 min with no break. The peripheral blood mononuclear cell (PBMC) enriched layer was extracted and washed with buffer consisting of PBS supplemented with 0.5% bovine serum albumin (BSA; Sigma-Aldrich) and 2 mM EDTA (Sigma) at 600 *g* for 5 min. Platelets were then washed away with PBS/0.5%BSA/2 mM EDTA at 200 *g* for 10 min. PBMCs were finally washed with cold buffer at 600 *g* for 5 min and then resuspended in cold PBS/0.5%BSA/2 mM EDTA buffer.

Monocytes in the PBMC mix were probed with a monoclonal mouse anti-human CD14-FITC antibody (1:20 dilution; T K4 clone; AbD serotec) for 15 min in the dark at 4 °C. Cells were washed with PBS/0.5%BSA/2 mM EDTA at 350 *g* for 5 min at 4 °C and finally resuspended in cold PBS/0.5%BSA/2 mM EDTA. Non-viable cells were probed with 7aad (7-amino-actinomycin D, 1:100, eBiosciences). CD14+ monocytes were selectively isolated by cell sorting using a BD FACSAria Standard, whereby dead cells were excluded by selection of 7aad negative cells.