Nuclear Medicine and Biology 46 (2017) 25–31

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

Simvastatin augments revascularization and reperfusion in a murine model of hind limb ischemia – Multimodal imaging assessment

Julian Luke Goggi [a,b,*], Michael Ng [a], Nalini Shenoy [a], Ramasamy Boominathan [a], Peter Cheng [a], Sakthivel Sekar [a], Kishore Kumar Bhakoo [a]

[a] Singapore Bioimaging Consortium, Agency for Science, Technology and Research (A* Star), 11 Biopolis Way, #07-10, Helios, Singapore, 138667
[b] Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, 117456

ARTICLE INFO

Article history:
Received 2 August 2016
Received in revised form 27 October 2016
Accepted 12 November 2016

Keywords:
Statins
Integrin
Neovascularization
RGD
MRI
Ischemia

ABSTRACT

Introduction: Peripheral artery disease can lead to severe disability and limb loss. Therapeutic strategies focussing on macrovascular repair have shown benefit but have not significantly reduced amputation rates in progressive PAD. Proangiogenic small molecule therapies may substantially improve vascularisation in limb ischemia. The purpose of the current study was to assess the proangiogenic effects of simvastatin in a murine model of hind limb ischemia using longitudinal multimodal imaging.
Methods: Mice underwent surgical intervention to induce hind limb ischemia, and were treated with simvastatin orally for 28 days. Neovascularisation was assessed using ^{99m}Tc-RGD SPECT imaging, and macrovascular volume was assessed by quantitative time of flight MRI. At each imaging time point, VEGF expression and capillary vessel density were quantified using immunohistochemical analysis.
Results: Simvastatin significantly increased ^{99m}Tc-RGD retention in the ischemic hind limb by day 3 post-surgery, with maximal retention at day 8. Vascular volume was significantly increased in the ischemic hind limb of simvastatin treated animals, but only by day 22. Immunohistochemical analysis shows that simvastatin significantly augmented tissue VEGF expression from day 8 with increase in capillary density ($CD31^+$) from day 14.
Conclusions: Early assessment of proangiogenic therapy efficacy can be identified using ^{99m}Tc-RGD SPECT, which displays significant increases in retention before macrovascular volume changes are measureable with MRI.
Advances in knowledge and implications for patient care: Simvastatin offers an effective proangiogenic therapy as an adjunct for management of limb ischemia. Simvastatin induces integrin expression and vascular remodeling leading to neovascularisation and improved perfusion.

1. Introduction

Peripheral artery disease (PAD) is a major cause of disability in a number of pathologies, including diabetes and vascular insufficiencies. Primary therapeutic strategies include macrovascular repair or bypass of the blocked vessels [1]. Despite routine clinical use of these macrovascular approaches, amputation rates in progressive PAD have remained unchanged over the past 30 years [2]. Therapies that induce angiogenesis have been proposed for patients with peripheral ischemic disease as an adjunct to conventional therapies. Angiogenesis is the process of forming new blood vessels, sprouting from existing vessels, and is central to normal biological processes such as vascular remodeling and wound healing [3,4]. Hypoxia caused by an ischemic insult can induce limited vascular remodeling spontaneously *via* the induction of vascular endothelial growth factor (VEGF) [5]. Angiogenic therapies are designed to improve this remodeling with the aim of re-establishing tissue perfusion.

Significant work has concentrated on stimulating angiogenesis to improve perfusion and function in ischemic tissues, independent of macrovascular function, using cell based therapies or growth factors; however, efforts have been stymied by safety concerns over side effects, such as hypotension and destabilization of atherosclerotic plaques [6–11]. Small molecule pro-angiogenic alternatives based around existing cardiovascular disease therapies could prove to be an effective clinically relevant strategy. Statins, 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase inhibitors, are routinely used clinically to manage cholesterol levels. Current guidelines recommend the use of statins in all patients with PAD due to the high possibility of systemic cardiovascular events [12]. Prophylactic use of statins has also been shown to significantly reduce adverse events in PAD when used to a similar extent as with coronary heart disease [12–16]. While the mechanism of action is unclear, recent studies have shown that statins not only reduce the risk of adverse events in PAD, but can also improve limb prognosis in patients with PAD beyond lipid altering effects alone [12]. Preclinically, statins have been shown to exert proangiogenic effects, exerting protective effects on ischemic injury in the heart and promoting angiogenesis in the periphery of animals with normal cholesterol levels [17–20].

* Corresponding author. Tel.: +65 6478 8901; fax: +65 6478 8908.
E-mail address: julian_goggi@sbic.a-star.edu.sg (J.L. Goggi).

http://dx.doi.org/10.1016/j.nucmedbio.2016.11.007
0969-8051/

Indeed statins have been shown to significantly increase VEGF, eNOS, and angiogenic cytokines in preclinical ischemic models in a dose dependent manner [1,5,19,21–23]. Clinically, changes in perfusion and functional vascular remodeling after ischemic damage have been assessed using a number of different imaging methodologies; including MR perfusion imaging, angiography and laser Doppler imaging. However, these methodologies are optimized for large vessel occlusive disease and fail to differentiate between existing intra-arterial anastomoses and *de novo* blood vessel formation [24–26], and hence, are inherently less sensitive measures of angiogenesis.

New blood vessels have been shown to express high levels of integrins. The integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are expressed at low levels on epithelial cells and mature endothelial cells, but are highly expressed on activated endothelial cells during neovasculature [27]. These integrins act as receptors for proteins expressing the arginine–glycine–aspartate (RGD) tripeptide motif [28]. Integrins are attractive targets for angiogenesis imaging, and significant efforts have been made toward the development of ligands for positron emission tomography (PET) and single photon emission computed tomography (SPECT) molecular imaging incorporating the RGD tripeptide motif [29]. Such biomarkers may prove useful for the early monitoring of the efficacy of angiogenic therapies for the treatment of PAD and may help elucidate the temporal mechanisms of vascular remodeling. In the current study we use multimodal imaging to longitudinally follow angiogenesis and vascular remodeling in a mouse model of hind limb ischemia treated with statins over 28 days to determine whether integrin imaging may prove more effective than conventional single modal imaging techniques.

2. Materials and methods

Animal experiments were performed in accordance with the Institutional Animal Care and Use Committee guidelines under IACUC 120791. Male BALB/c nude mice were purchased from In Vivos (Singapore). BALB/c mice were chosen as a model animal as they have been shown to develop the most severe initial ischemia and display relatively poor endogenous recovery post arterial occlusion.

2.1. Hind limb ischemia surgery

The mice were anesthetized by intraperitoneal injection of ketamine (150 mg/kg) and xylazine (10 mg/kg). An incision of the skin from the medial thigh toward the knee was made and the membranes covering the muscle dissected away. Fat tissue was gently pulled toward the abdomen and secured. The membranous femoral sheath was pierced to expose the neurovascular bundle and the external iliac artery was gently separated from vein and nerve, ligated above the pudendoepigastric trunk proximal to the deep femoral artery twice with 7/0 polypropylene suture (Premilene, Braun, Melsungen AG), and dissected between the two ligations. The incision was closed with 5/0 polypropylene suture (Premilene, Braun, Melsungen AG). The animals received subcutaneous injection of Enrofloxacin (10 mg/kg, once daily) for 5 days and of buprenorphine (0.1 mg/kg, twice daily) for 3 days post-surgery.

2.2. Dosing

Simvastatin (S1792 Selleckchem, USA) was administered immediately after the hind limb ischemia surgery. Subsequently, simvastatin (2.0 mg/kg in saline) or vehicle (saline alone) was dosed daily by oral gavage for 28 days.

2.3. Preparation of ^{99m}Tc-Hynic-RGDfK-dimer (^{99m}Tc-RGD)

All chemicals were purchased from Sigma-Aldrich Pte. Ltd., unless otherwise specified. HYNIC-cyclo(RGDfK) dimer was purchased from ABX advanced biochemical compounds. $Na^{99m}TcO_4$ was obtained from a $^{99}Mo/^{99m}Tc$ generator (Singapore General Hospital, Department of Nuclear Medicine & PET, Radiopharmacy Lab). The radio-HPLC method used a Shimadzu Prominence HPLC system equipped with an IN/US systems β-RAM Radio-HPLC detector and Phenomenox C18 Gemini column (4.6 mm × 150 mm). The flow rate was 1.0 mL/min. The mobile phase was a gradient from 90% solvent A (Water, 0.05% TFA) and 10% solvent B (Acetonitrile, 0.05% TFA) to 38% solvent B over 20 min.

2.4. Synthesis of ^{99m}Tc-Hynic-RGDfK-dimer (^{99m}Tc-RGD)

To a clean vial were added 50 μL of HYNIC-cyclized RGDfK dimer (1 mg/mL in water), 100 μL of tricine (100 mg/mL in 0.025 M sodium succinate buffer), 20 μL of tin (II) chloride (3.0 mg/mL in 0.1 M HCl solution) and 1.85–2.22 GBq (50–60 mCi) of $Na^{99m}TcO_4$ solution. The reaction solution was incubated at room temperature for 10 min, after which was added 3 mg of triphenylphosphine-3,3′,3″-trisulfonic acid trisodium salt (dissolved in 100 μL of 0.25 M sodium succinate buffer, pH 5.0) and the mixture was heated at 99 °C for 30 mins. After the radiolabelling reaction, an aliquot was analyzed by radio-HPLC. In our radiotrace the ^{99m}Tc-Hynic-RGDfK-dimer appeared as a double peak (tR = 16 mins). Previous studies have shown that the ^{99m}Tc-Hynic-RGDfK dimer displays better binding than the monomeric version and that the observed double peak is due to the presence of two diastereomers [30] and that the binding affinity of these two diastereomers is equivalent [31].

2.5. Doses preparation for animal studies

The radiolabelled compound was purified using C18-Sep.-Pak (Sep-Pak C18 light, 100 mg cartridge) before animal studies. The Sep.-Pak C18 cartridge was conditioned with 10 mL of acetonitrile followed by washing with 10 mL of water. The radiotracer was loaded onto the Sep.-Pak cartridge. The Sep.-Pak cartridge was then washed with 0.9% NaCl solution (10 mL) to elute unbound ^{99m}Tc. ^{99m}Tc-RGD was eluted with 1.4 mL of 80% ethanol and collected in 3 fractions of 0.4, 0.6 and 0.4 mL each. The fractions were analyzed by radio-HPLC and fractions with ^{99m}Tc-RGD were combined. Ethanol was removed under a stream of nitrogen gas. Doses for animal studies were prepared by dissolving the purified radiotracer in 1× phosphate buffered saline solution to obtain a concentration of 150 MBq/mL. The overall radiochemical yield was 49–65%, non-decay corrected over 112–143 min (n = 5). The specific activity of ^{99m}Tc-RGD was 31.2–44.3 GBq/μmol (n = 5). The radiochemical purity of the formulated product was above 95%.

2.6. SPECT imaging with ^{99m}Tc-RGD

BALB/c nude mice (n = 6 per imaging group) were injected with a solution of ^{99m}Tc-RGD (~30 MBq in 0.2 ml) *via* the lateral tail vein, and the animals imaged under isoflurane anesthesia (2% alveolar concentration). Biological monitoring for respiration and temperature was performed using a BioVet system (m2 m imaging, Cleveland, OH). Small-animal SPECT imaging was performed using a nanoSPECT (MEDISO, Hungary). Static images were acquired at 70–90 min post injection (based on prior dynamic imaging studies to optimize SNR; data not shown). Low dose CT images (40 kV, 500 μA; 4 × 4 binning, 200 μm resolution) were acquired for anatomical information. Images were reconstructed using in-built image reconstruction, visualization and analysis software supplied by the manufacturer, whilst SPECT and CT data were analyzed by using Amide software (Sourceforge 10.3, http://amide.sourceforge.net). The SPECT and CT images were co-registered to confirm anatomical location of the ^{99m}Tc-RGD uptake. Uptake of radioactivity was determined by placement of a region of interest (ROI) over the muscle of interest delineated using the CT images. The tissue concentrations were measured using ROI analysis and are presented as percent injected dose/g (%ID/g).