Spect imaging of cardiovascular neovascularization

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SPECT IMAGING OF CARDIOVASCULAR NEOVASCULARIZATION

Gerardus Jacobus Joseph Hendriks

“There is grandeur in this view of life, with its several powers, having been originally breathed into a few forms or into one; and that, whilst this planet has gone cycling on according to the fixed law of gravity, from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.”

Charles Darwin

On the Origin of Species, first edition.

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Promotores

Prof. dr. F.M. Mottaghy

Prof. dr. M.J. Post

Co-promotor

Dr. M. Bauwens

Beoordelingscommissie

Prof. dr. C.P.M. Reutelingsperger

Prof. dr. O. Gheysens

Dr. H. J. Verberne

Prof. dr. ir. F. Verhaegen

Dr. S.M. Schalla

Voorzitter

Katholieke Universiteit Leuven

Academisch Medisch Centrum

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CHAPTER 1

GENERAL INTRODUCTION

Imaging of neovascularization is a continuously developing area of research which is of paramount importance to cardiovascular disease and oncology. Sensitive detection of neovascularization through nuclear imaging will facilitate translation of successful therapeutic interventions in preclinical models to the bedside. However, we still lack specific tracers for neovascularization imaging. **Chapter 2** in this dissertation provides a detailed overview and discussion of pre-clinical work aimed at validating new radiotracers for neovascularization, thereby outlining the context in which my research fits.

Neovascularization is a collective term referring to three distinct processes: vasculogenesis, angiogenesis and arteriogenesis. These processes are initiated by different stimuli but all serve to restore tissue blood supply. Angiogenesis is an ischemia driven process that leads to capillary sprouting from preexisting vessels[1]. Arteriogenesis represents the shear-stress initiated outward remodeling of preexistent inter-connecting arterioles[2]. Vasculogenesis mainly occurs during embryonic development, whereas angiogenesis and arteriogenesis also occur frequently during adulthood. Therefore the latter two are major targets for therapy and diagnosis.

Pathological angiogenesis, as in cancer, is undesirable since it provides the growing tumor with its own blood supply[3]. Consequently, cancer therapy research has focused on strategies to inhibit angiogenesis. However, in response to arterial occlusion, for example after myocardial infarction (MI) or in case of peripheral vascular disease (PVD) stimulation of angiogenesis and/or arteriogenesis can be beneficial[4, 5].

To study the process of angiogenesis and/or arteriogenesis in a biological environment, several animal models are available that mimic MI or PVD. In my studies a mouse MI model was employed to monitor angiogenesis and a mouse and rat ischemic hind limb model was employed to study arteriogenesis. In the mouse MI model the left anterior descending coronary artery (LAD) is ligated just proximal to its main bifurcation. LAD ligation results in an antero-apical infarction of the left ventricular wall, involving approximately 40% of left ventricular circumference[6]. The disease progression, which is seen in this standardized MI model is well documented for several mouse phenotypes[7]. Furthermore, numerous hind limb ischemia models have been developed to study collateral artery formation in the setting of PVD[8]. We employed a model in which the femoral artery was ligated, giving rise to an ischemic area in the gastrocnemius muscle and high shear-stress area in the adductor muscle. Hence, the anatomical separation of these areas allowed us to exclusively study the arteriogenic response in the adductor muscle. Perfusion recovery measurements using laser Doppler perfusion imaging are often used to monitor the arteriogenic response.

Several preclinical studies aimed at improving angiogenesis and arteriogenesis have been performed and showed promising results[9-13]. However, while promising results were achieved in preclinical studies, clinical trials failed to reproduce these results[14-17].

Among the proposed reasons for these clinical failures are ineffective delivery, insensitive and irreproducible readout parameters, and an unresponsive patient population[18]. An insufficient duration of drug delivery is a likely cause for ineffective therapy as time is needed for pro-angiogenic growth factors (also known to play an important role in arteriogenesis[5]) to not only induce growth but also allow maturation of these vessels. Delivery of growth factors should cover a period of several weeks to months, instead of single dose administration or short term (i.e. several days) commonly studied in clinical trials[14-17]. Moreover, in a pre-clinical study it was suggested that administration of multiple factors could induce a synergistic effect and thereby enhance vessel stability and maturation[19, 20].

Regardless of the treatment setup the most important aspect when applying therapy is the ability to accurately monitor the targeted process. From clinical trials that have been executed it has become clear that clinical readout parameters such as peak walking distance in PVD trials[21] and exercise tolerance test in coronary artery disease (CAD) trials[17] are not sufficiently sensitive and reproducible to document incremental improvements in function and perfusion. Reduced angina scores and increased treadmill exercise times are often used to indicate recovery. Furthermore, perfusion imaging through angiography, MRI, SPECT (^{99m}Tc -sestamibi, ^{99m}Tc -tetrofosmin, ^{201}Tl) and PET (^{15}O , ^{13}N , ^{18}F -Flurpiridaz and ^{82}Rb) imaging are used to indicate recovery. Clearly, these methods are suitable to indicate recovery[22]. However, it often takes a few months before improvement can be detected[23]. In order to facilitate early diagnosis and early treatment for patients with ischemic cardiovascular disease we require specific and sensitive non-invasive tracers for neovascularization.

MOLECULAR IMAGING – SINGLE PHOTON EMISSION COMPUTED TOMOGRAPHY

Molecular imaging is an interdisciplinary field aimed at studying biochemical processes in living subjects. Subjected to continuous development of hardware technology, image-processing algorithms and diagnostic agents, molecular imaging is ultimately directed at improving patient health care. Several molecular imaging approaches are available for non-invasive imaging of neovascularization. Among them, the nuclear techniques positron emission tomography (PET) and single photon emission computed tomography (SPECT) are the most sensitive with detection ranges in the nano- or picomolar order.

Today, the majority of clinical imaging procedures are comprised of SPECT and PET scans, with SPECT by far making up the largest fraction. However, also in preclinical research, SPECT and PET are frequently employed tools in verification and validation steps in tracer development, drug design and therapeutic regimens[24]. The work described in this dissertation is mainly focused on pre-clinical SPECT imaging and the development of

new SPECT tracers for angiogenesis and arteriogenesis. In the pre-clinical setting, SPECT scanners outclass pre-clinical PET scanners in terms of spatial resolution through the use of collimators with dedicated pinhole geometry[24]. In the clinical setting this is reversed, although groundbreaking new developments are announced for clinical SPECT imaging in the form of G-SPECT (MILabs, The Netherlands), achieving a resolution under 3 mm.

SPECT imaging is based on the detection of gamma rays, emitted by radionuclide labeled tracers. The gamma camera used to detect these gamma rays was invented by Hal Anger in the middle of last century[25] and continues to be the main component of most commercially available SPECT systems[26]. The basic components of an Anger gamma camera are a collimator, a scintillation crystal, a photosensitive light guide layer and a set of photomultiplier tubes. A collimator is a device (most commonly made of tungsten) that only allows gamma rays traveling in parallel to a pre-defined direction to reach the detector. The detector, usually made of a sodium-iodine (NaI) crystal with thallium impurities, interacts with the gamma photons to create visible light that is emitted in every direction from the site of interaction. The origin of this interaction can be determined from the light distribution, whereas the energy deposited is proportional to the integral of the light produced. Subsequent conversion of light into a measurable signal is mostly done by a set of photomultiplier tubes (PMT) that also magnify the small signal that is produced in the photo-sensitive layer at the entrance to the PMTs. The final position and energy are then determined electronically thereby assigning each photon to an energy and detector surface position[26]. These energy-resolving gamma-cameras provide a clear advantage over PET cameras as they can distinguish between different gamma-emitting radionuclides based on their differences in emission energy[27]. This enables the possibility of imaging more than one radionuclide at the same time. Multi-tracer imaging facilitates a one-stop shop principle as it results in shorter acquisition times with perfect registration of different biological processes in space and time.

SMALL ANIMAL SPECT IMAGING

Current small animal SPECT imaging devices use a 360° covering stationary detector set-up in combination with a pinhole collimator. These set-ups provide a large number of projection angles under which the animal is observed, thereby allowing dynamic imaging since neither the detector (or collimator) nor the object has to be rotated. The SPECT system we used (VECTor, MILabs, The Netherlands) for our studies uses a similar set-up[28](Figure 1).

Figure 1. Image of the VECTOR system that was used for the studies described in this dissertation (A). Situation inside the collimator (B). Interchangeable collimator (C). Cross-sectional overviews of a mouse positioned inside the collimator with the field of view (FOV) centered around the heart. Overview of the handlings that precede, coincide and follow the imaging process. Modified from Branderhorst et al., Phys. Med. Biol., 2012[29].

This system is based on the U-SPECT-II system (without integrated CT) with a triangular stationary detector set-up, an XYZ-stage that moves the animal during scanning. In case imaging of a larger volume of interest is desired (e.g. in whole-body imaging) the animal-bed is translated into 3 dimensions using the XYZ-stage. The system can be fitted with an interchangeable cylindrical collimator (Figure 1C). With multiple dedicated collimators available (each containing 75 pinholes) a large number of options become available providing a trade-off between sensitivity and resolution. The selected collimator therefore largely depends on the tracer(s) one is using. Data is acquired in list-mode which allows flexibility in post-acquisition image reconstruction. As a result, choice of time-frame length, selection of energy windows for single- and multiple-isotope imaging, scatter correction and retrospective electrocardiogram (ECG) and respiratory gating is available[30].

Images acquired using technetium-99m (^{99m}Tc) in combination with the mouse 0.35 mm collimator can reach resolutions smaller than 0.35 mm while images acquired using ^{99m}Tc in combination with the 1.0 mm rat collimator reached resolutions smaller than 0.8 mm. Furthermore, using this system, it was found that for high resolution cardiac perfusion SPECT (using the perfusion tracer ^{99m}Tc -tetrofosmin) in mice, respiratory gating is not needed as it has no significant influence on measured left ventricular volumes and ejection

fractions determined using ECG gating. However, image filtering and animal positioning (supine or prone) do have a large influence on these parameters and should therefore be kept the same for all scans in a comparative study[31].

In SPECT imaging there are other factors besides motion that affect image quantification that are unavoidable but not insuperable. Among them are attenuation and photon scatter which can be corrected for through various methods (for detailed coverage of this topic refer to[32, 33]). Photons that originate within a study subject can interact with tissue, a process called Compton scatter. Upon this interaction, photons will lose energy which either leads to absorption (Figure 2B) or to a scatter event that causes the photon to deflect from its original course but still reach the detector (Figure 2C). It is convenient to distinguish attenuation as the reduction of the measured counts compared with what would be expected in air versus scatter as an increase in the measured counts due to the inclusion of some scattered radiation that still falls in the selected energy window (misplaced compared to the primary event)[26]. An example of attenuation and scatter can be seen in Figure 2.

Figure 2. Cross section through pre-clinical gamma camera and animal. Photon originating from within the test subject that is able to hit the detector unhindered (A). Photon that interacts with tissue and loses its energy (B). Photon that hits the detector after a scatter event (C). Photon that is unable to penetrate the collimator septum and hit the detector (D).

THESIS OUTLINE

The main goal of this dissertation was to test novel non-invasive SPECT tracers for imaging and characterizing neovascularization in murine models for MI and PVD. In chapter 2 an elaborate review, providing an overview of pre-clinically used SPECT and PET radiotracers in animal models for myocardial ischemia and PVD, is presented. This review can be regarded as an introduction to the pre-clinical work issued in the subsequent chapters. The content of these chapters is divided in three parts, each of them dealing with (targets for) cardiovascular SPECT imaging.

Part 1 describes several approaches to design a pre-clinical SPECT tracer for arteriogenesis. Despite extensive research into the pathways involved in arteriogenesis, no tracers specifically targeting the process could be developed. In chapter 3 I will discuss several important agents (i.e. intercellular adhesion molecule 1 (ICAM-1), C-X-C motif ligand 1 (CXCL1), Evasin3 and thymosin β 4) in the arteriogenic process and their ability to serve as SPECT tracer for arteriogenesis.

Part 2 focusses on perfusion and functional SPECT imaging, comparing the accuracy of SPECT to established methods like laser-Doppler perfusion imaging and 3D-echocardiography. In chapter 4 perfusion recovery through arteriogenesis in a mouse ischemic hind limb model is investigated using SPECT and laser-Doppler perfusion imaging. In chapter 5 a longitudinal follow up study investigating left ventricular cardiac function in a mouse MI model is described. In this study a comparison was made between ECG-gated μ SPECT and 3D-echocardiography.

Part 3 concentrates on post MI angiogenesis imaging using the CD13 targeting cyclic asparagine-glycine-arginine (NGR) peptide. The synthesis and application of this newly designed SPECT tracer for angiogenesis is described in chapter 6. Chapter 7 advanced from the results described in chapter 6 with the design and application of a new tetrameric cNGR tracer with better pharmacological characteristics. Moreover, a comparison was made with the monomeric cNGR tracer which was now cyclized through native chemical ligation instead of a disulfide bridge which was used for the synthesis of the cNGR entity in chapter 6.

In Chapter 8 this thesis is concluded by a general discussion that integrates all experimental work performed in the previously described chapters.

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CHAPTER 2

SPECT AND PET IMAGING OF ANGIOGENESIS AND ARTERIOGENESIS IN PRE-CLINICAL MODELS OF MYOCARDIAL ISCHEMIA AND PERIPHERAL VASCULAR DISEASE

Geert Hendrikx
Stefan Vöö
Matthias Bauwens
Mark J. Post
Felix M. Mottaghy

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ABSTRACT

Purpose: The extent of neovascularization determines the clinical outcome of coronary artery disease and other occlusive cardiovascular disorders. Monitoring of neovascularization is therefore highly important. This review article will elaborately discuss preclinical studies aimed at validating new nuclear angiogenesis and arteriogenesis tracers. Additionally, we will briefly address possible obstacles that should be considered when designing an arteriogenesis radiotracer.

Methods: A structured medline search was the base of this review, which gives an overview on different radiopharmaceuticals that have been evaluated in preclinical models

Results: Neovascularization is a collective term used to indicate different processes such as angiogenesis and arteriogenesis. However, while it is assumed that sensitive detection through nuclear imaging will facilitate translation of successful therapeutic interventions in preclinical models to the bedside, we still lack specific tracers for neovascularization imaging. Most nuclear imaging research to date has focused on angiogenesis, leaving nuclear arteriogenesis imaging largely overlooked.

Conclusion: Although angiogenesis is the process which is best understood, there is no scarcity in theoretical targets for arteriogenesis imaging.

INTRODUCTION

Molecular imaging enables the study of molecular and cellular processes in vivo[1]. Within this field several noninvasive imaging techniques such as Magnetic Resonance imaging (MRI), Computed Tomography (CT), Optical Imaging (OI), Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) are distinguished. The latter two are the most established techniques for targeting ongoing biochemical processes and are based on the detection of injected radiolabeled probes. While the spatial resolution of MRI and CT is higher, the detection sensitivity of PET and SPECT is within the picomolar or nanomolar range and therefore significantly higher than for MRI and CT[2, 3]. Spatial resolution and detection sensitivity are two performance characteristics that play an important role in molecular imaging research using SPECT and PET tracers. Clinical gamma cameras can provide a tomographic resolution of about 10 mm while preclinical devices currently reach submillimeter resolutions using a specialized multipinhole geometry[2, 4]. The difference between clinical and preclinical PET devices is smaller. While preclinical scanners reach spatial resolutions of 1-2 mm, clinical scanners operate within the range of 4-6 mm. The application for dedicated small animal SPECT and PET imaging modalities in preclinical models is highly valuable as it has a great scope for noninvasive studying of dynamic biological processes at the molecular and cellular level[2]. Because of the high societal burden of disease, the cardiovascular system is a well-recognized target for molecular imaging. Longitudinal studies, monitoring cardiac function[5], imaging of atherosclerosis[6, 7], tissue viability and perfusion[8] and neovascularization[9, 10] are among the most studied cardiovascular areas. Molecular imaging of neovascularization has received a significant amount of attention as we still lack sensitive detection of neovascularization. It is assumed that such sensitive detection will facilitate translation of successful therapeutic interventions in preclinical models to the bedside [8, 11]. Neovascularization can be divided in three distinct processes, vasculogenesis, arteriogenesis and angiogenesis[12] and its extent determines the clinical outcome of coronary artery disease and other occlusive cardiovascular disorders. Vasculogenesis refers to the in situ formation of blood vessels from circulating endothelial progenitor cells. Despite the importance of this process during embryogenesis its further discussion is beyond the scope of this review. The term arteriogenesis describes the enlargement of pre-existing arteriolar anastomoses into large collaterals in response to enhanced fluid shear stress[13]. Angiogenesis is an ischemia driven process that represents the sprouting of new capillaries from existing microvasculature[9].

Arteriogenesis is the most important mechanism in the functional replacement of an occluded artery in peripheral vascular disease (PVD)[13, 14] but the enlargement of coronary collateral arteries in obstructive coronary artery disease is also well described[15]. Angiogenesis is associated with postinfarct remodeling and has important

implications for the prognosis following myocardial infarction (MI)[16] whereas its role in perfusion recovery in PVD is of less importance[13]. In this review we will focus on SPECT and PET based neovascularization studies in the context of MI and peripheral vascular disease (PVD). As will become apparent from this review, extensive research has been conducted concerning radiotracer imaging of angiogenesis while arteriogenesis radiotracer imaging is scarce and largely overlooked. Despite large parts of the pathways involved in arteriogenesis being unraveled, radiotracers specifically targeting this multifactorial process are yet to be developed. Alluding to the inferior amount of work being published on radiotracer imaging of arteriogenesis, we will briefly discuss the possible hurdles which have to be overcome in order to develop a nuclear arteriogenesis tracer.

PERFUSION TRACERS IN NEOVASCULARIZATION RESEARCH

Although perfusion radiotracers do not directly target angiogenesis or arteriogenesis, they are used as indicators for areas of (mainly myocardial) ischemia thereby often serving as a contrast in radiotracer guided neovascularization research. The distribution kinetics of these tracers are therefore highly important for imaging of neovascularization. Perfusion tracers are even used as surrogate markers for neovascularization in pre-clinical research (section 4). Accordingly, this section serves as a brief introduction into the uptake mechanisms, kinetics, and application of the most common SPECT and PET perfusion tracers.

Frequently employed perfusion tracers for SPECT are Thallium-201 (^{201}Tl), Technetium-99m ($^{99\text{m}}\text{Tc}$)-sestamibi, $^{99\text{m}}\text{Tc}$ -tetrofosmin and $^{99\text{m}}\text{Tc}$ -pyrophosphate while for PET, Oxygen-15 (^{15}O)-water, N-13 (^{13}N)-ammonia, Rubidium-82 (^{82}Rb) and the more recently developed Fluorine-18 (^{18}F) labeled Flurpiridaz (Lantheus Medical Imaging, Massachusetts, USA) are the most common perfusion tracers.

^{201}Tl is taken up in viable cells via the sodium-potassium pump as it has properties similar to potassium[17]. However, while ^{201}Tl has successfully been used in cardiac perfusion imaging[18] and in skeletal muscle perfusion imaging in PVD patients[19-22] $^{99\text{m}}\text{Tc}$ -labeled perfusion tracers have largely replaced the use of ^{201}Tl . Beside the considerably lower radiation exposure (6 vs. 28 millisievert) $^{99\text{m}}\text{Tc}$ -labeled tracers offer more advantages compared to ^{201}Tl . The most essential being the shorter half-life (6 hours for $^{99\text{m}}\text{Tc}$ compared to 73 hours for ^{201}Tl), allowing for injection of higher doses, in combination with the higher energy level at which $^{99\text{m}}\text{Tc}$ emits gamma rays (140 kilo electronvolt (keV) compared to 78 keV for ^{201}Tl) which results in less scatter and attenuation. Together these advantages culminate in improved imaging[23].

One $^{99\text{m}}\text{Tc}$ -labeled compound in particular, $^{99\text{m}}\text{Tc}$ -sestamibi, is omnipresent in clinical cardiology[24] and has also been incorporated in several studies examining lower-

extremity perfusion in PVD[25-27]. ^{99m}Tc -sestamibi is a lipophilic, cationic complex of six isonitriles[23]. Like ^{201}Tl , uptake of ^{99m}Tc -sestamibi after intravenous injection is proportional to blood flow[28]. Cellular uptake and retention of ^{99m}Tc -sestamibi are dependent on mitochondrial and plasma membrane potentials[29-31]. After uptake, the compound resides in myocardial cells after initial extraction and demonstrates minimal delayed redistribution[32-34]. In a case report, the merit of clinical application of ^{99m}Tc -sestamibi over Doppler ultrasound in PVD patients has already been reported on the basis of improved sensitivity in detecting differences in detecting differences of resting perfusion between the lower extremities[35]. ^{99m}Tc -tetrofosmin is an alternative lipophilic cationic complex with comparable uptake characteristics and similar widespread use in myocardial perfusion imaging[24]. However, the hepatobiliary clearance of ^{99m}Tc -tetrofosmin is reported to be slightly faster than for ^{99m}Tc -sestamibi[23]. Recently, Stacy et al. showed preliminary data and indicated that SPECT/CT using ^{99m}Tc -tetrofosmin has the potential to assess regional differences in lower-extremity perfusion in PVD patients. Furthermore, ^{99m}Tc -pyrophosphate, binding to hydroxyapatite crystals in damaged myocytes, has been frequently employed in clinical practice to identify fresh myocardial infarctions since its introduction in 1974[36, 37]. Additionally, ^{99m}Tc -pyrophosphate has been successfully used to estimate the ischemic skeletal muscle mass in a canine ischemia-reperfusion skeletal muscle model[38] and in PVD patients[39].

The most prominent PET perfusion tracers are ^{15}O -water and ^{13}N -ammonia. Both tracers have a short half-life (2.4 minutes and 9.8 minutes respectively) requiring an on-site cyclotron to enable application, thereby limiting the use of these tracers to a few centers[40]. Myocardial blood flow acquired with ^{15}O -water and ^{13}N -ammonia have been widely validated against independent microsphere blood flow measurements in animals and have yielded highly reproducible values over a range of 0.5 to 5.0 ml/g/min[40-42]. ^{15}O -water, diffusing freely into the tissue, is also frequently implemented in PVD patient studies[43-47]. The characteristics of ^{15}O -water make the tracer suitable for repeated measurements during a single visit, measurements at rest and during exercise or during vasodilator stress. An ^{15}O -water rest-stress PET study found significantly lower calf muscle flow reserve in PVD patients compared to healthy control subjects, these measurements correlated with thermodilution-derived flow reserve values[45]. Furthermore, a study by Scremin et al. showed that accurate muscle blood flow detection by ^{15}O PET in legs with severe ischemia could add valuable information about skeletal muscle viability in the residual limb when deciding the level of an amputation[46]. However, despite its frequent application, ^{15}O -water images of the myocardium are commonly of lower count density due to subtraction of the blood pool, rapid clearance of ^{15}O -water and its short half-life. Therefore, ^{15}O -water images are not suitable for the visual analysis of myocardial radiotracer uptake and, thus, are not used clinically for coronary artery disease detection[40, 48]. ^{13}N -ammonia is cleared rapidly from the circulation and is primarily

taken up by the myocardium, brain, liver, kidneys and the pituitary gland[49, 50]. In both myocardium and brain, ^{13}N -ammonia is removed from the blood by first-pass extraction (approximately 80%) and is metabolically trapped within the tissues by incorporation into the cellular pool of amino acids, mainly as glutamine[50-52]. The high first pass extraction, in combination with a sufficiently long half-life, allow high count images to be acquired. Hence, flow-limiting coronary artery disease can be visualized on stress-rest images using ^{13}N -ammonia[53, 54]. While ^{13}N -ammonia PET is frequently used to measure myocardial perfusion, its application for measuring skeletal muscle perfusion is rare, though not absent. In a patient with a right-sided static tremor, higher uptake of ^{13}N -ammonia was found in the muscles of the right leg which was related to increased perfusion produced by continuous exercise of the muscles involved in the tremor[55]. Furthermore, ^{13}N -ammonia PET was successfully used to measure local perfusion in the legs of patients with painful diabetic neuropathy[56].

^{82}Rb , a functional potassium analog, is an alternative radioactive tracer of myocardial perfusion that can be imaged with PET[24, 40]. Its diagnostic and prognostic performances appear comparable to conventional blood flow SPECT imaging[57, 58]. Although ^{82}Rb can be eluted from a commercially available Strontium-82 generator on site[40], a major limitation is its ultrashort half-life (76 seconds), which limits its use to pharmacological stress perfusion imaging[24].

A promising ^{18}F labeled perfusion tracer was added to the available PET perfusion tracers almost a decade ago in the form of Flurpiridaz (initially evaluated as: BMS-747158-02; Lantheus Medical Imaging, Massachusetts USA). Flurpiridaz is an analog of the insecticide pyridine, which binds to the mitochondrial complex I of the electron transport chain with a very high affinity[40, 59, 60]. The radiotracer is rapidly cleared from the blood (in under 5 min) and displays stable uptake in the healthy and infarcted myocardium up to 40 min. Furthermore, ^{18}F -Flurpiridaz has a high first-pass extraction fraction above 90% (which is preserved at high flow rates) and a very slow wash out[40]. These favorable properties in combination with its half-life of 109 minutes result in high count images of high diagnostic quality for the detection of perfusion deficits underlying CAD[40, 61, 62]. ^{18}F -Flurpiridaz myocardial blood flow PET imaging was validated using radioactive microspheres in a pig model[40, 63]. Moreover, positive results from phase 2 human studies have been published[61]. The high extraction fraction of ^{18}F -Flurpiridaz may offer an advantage for evaluating lower-extremity skeletal muscle blood flow. However, so far there are no studies that assessed the potential of ^{18}F -Flurpiridaz in the setting of PVD.

Angiogenesis

The formation of new capillary arteries from pre-existing microvasculature is termed angiogenesis. Angiogenesis is a dynamic process involving endothelial proliferation and differentiation which is mainly triggered by tissue ischemia or hypoxia. During this process, new capillaries form around ischemic tissue zones, as they occur in MI, stroke, and PVD[64, 65]. Upon development of tissue ischemia, transcription factors such as hypoxia inducible factor 1 α (HIF-1 α) and inflammatory mediators are released locally resulting in vasodilation, enhanced vascular permeability, and accumulation of monocytes and macrophages which in turn secrete more growth factors and inflammatory mediators[65, 66]. These inflammatory cells facilitate degradation of the basal membrane of the parent artery and the surrounding extracellular matrix (ECM) through the release of matrix metalloproteinases (MMPs). Following ECM degradation, endothelial cells migrate and proliferate down a hypoxia sensitized chemotactic gradient of various growth factors to form a new capillary vessel with a lumen[65]. The role of integrins in this part of the angiogenic process is of paramount importance as integrins are the principle adhesion receptors used by endothelial cells to interact with their extracellular microenvironment[67]. The subsequent formation of a functioning vasculature requires the orchestrated interaction of endothelial cells, the extracellular matrix, and surrounding cells such as pericytes and smooth muscle cells[65, 68]. This sprouting process iterates until proangiogenic signals abate, and quiescence is re-established[69] (Figure 1).

Figure 1. Mechanism of angiogenesis. Capillary sprouting is guided into the ischemic area down a chemotactic gradient of growth factors. Modified from Carmeliet, 2000, *Nature Medicine*[12].

Angiogenesis is a multistep process orchestrated by a multitude of angiogenic factors and inhibitors, which offer a wide range of targets for therapeutic interventions and imaging[9, 68]. Because of its important role in the (partial) restoration of tissue perfusion in the ischemic area, angiogenesis stimulating therapy is an intensely studied subject in cardiovascular research. Nevertheless, while results from animal studies have been encouraging[70-74], the results obtained during clinical studies have not been convincing[75-78]. Plausible explanations for the latter are ineffective growth factor

delivery, irreproducible readout parameters, and an unresponsive patient population[79]. However, the most important feature in a therapeutic intervention study is its ability to accurately monitor the targeted process. Current clinical readout parameters such as peak walking distance in PVD trials[80] and the exercise tolerance test in coronary artery disease trials[78] are not sufficiently sensitive and reproducible. Surely, perfusion imaging through MRI, SPECT and PET imaging can be used to indicate enhanced perfusion of the ischemic tissue. However, it often takes several months before improvement becomes apparent[8]. In order to facilitate early diagnosis and early treatment for patients with ischemic cardiovascular disease specific and sensitive non-invasive tracers for neovascularization will be required.

Main targets for targeted radiotracer imaging of angiogenesis in myocardial ischemia

The main targets for nuclear angiogenesis imaging in animal models of MI are the $\alpha_v\beta_3$ integrin and the vascular endothelial growth factor (VEGF)-receptor, while CD105 (endoglin) and CD13 (aminopeptidase-N) were also successfully used (Table 1).

Integrins are transmembrane receptors that contribute to the angiogenic process through increased signal transduction as well as modulation of cell adhesion to the extracellular matrix[47, 67, 81]. The $\alpha_v\beta_3$ integrin (also termed vitronectin receptor) is the most abundant integrin expressed on the surface of proliferating endothelial cells and has been implicated in cell migration and cell survival signaling. Further, the $\alpha_v\beta_3$ integrin is minimally expressed on normal quiescent endothelial cells[82]. These properties have made the $\alpha_v\beta_3$ integrin a target of choice for the imaging of angiogenesis. The arginine-glycine-aspartic acid (RGD) peptide, naturally present in extracellular matrix proteins, was found to be highly selective for the $\alpha_v\beta_3$ integrin. The discovery of the RGD sequence has marked the starting point for the development of probes targeting the $\alpha_v\beta_3$ integrin[9, 82]. RGD peptides evolved from linear peptides with low selectivity and biostability to constructs with better pharmacokinetics (through attachment of carbohydrates, charged amino acids and polyethylene glycol groups) and optimized binding characteristics (through multimerization)[9, 82].

Currently, a large variety of RGD peptides have been developed that are suited for radiolabeling into SPECT or PET tracers for non-invasive imaging of angiogenesis. Pre-clinically, these tracers have been tested in animal models for MI, with and without reperfusion and in the presence of pro-angiogenic growth factors, and in hind limb ischemia. Enhanced angiogenesis post-MI has been indicated by several groups using Technetium-99m (^{99m}Tc) labeled RGD peptides. ^{99m}Tc -NC100692 (maraciclatide®) is a technetium-labelled cyclic RGD peptide that has been used in a variety of SPECT studies to noninvasively assess angiogenesis[97]. This compound has a high affinity for the $\alpha_v\beta_3$ integrin, is metabolically stable, and has a biodistribution and kinetics that are favorable for SPECT imaging. SPECT imaging of ^{99m}Tc -NC100692 was successfully used to indicate

Table 1. Radiotracers for myocardial angiogenesis imaging in pre-clinical studies.

Myocardial angiogenesis			
Biological target	Tracer	Modality	Reference(s)
$\alpha_v\beta_3$ integrin	^{99m}Tc -NC100692	SPECT	[83-85]
	^{99m}Tc -RAFT-RGD	SPECT	[86]
	^{111}In -RP748	SPECT	[16, 87, 88]
	^{123}I -gluco-RGD	SPECT	[89]
	^{18}F -galacto-RGD	PET	[90, 91]
	^{18}F -AIF-NOTA-PRGD2	PET	[92]
	^{68}Ga -NOTA-RGD	PET	[93, 94]
	^{68}Ga -NODAGA-RGD	PET	[91]
	^{68}Ga -TRAP(RGD) ₃	PET	[91]
CD13	^{111}In -DTPA-cNGR	SPECT	[8]
VEGF receptor	^{64}Cu -DOTA-VEGF ₁₂₁	PET	[95]
CD105	^{64}Cu -NOTA-TRC105	PET	[96]

post-MI angiogenesis hypoperfused (indicated by ^{201}Tl) myocardial regions of matrix-metalloproteinase-9 (MMP-9) null mice compared to wild-type mice[85]. In rats SPECT imaging of ^{99m}Tc -NC100692 was used to indicate ongoing angiogenesis in the peri-infarct region after MI[83] and in reperfused ischemic myocardium[84]. Furthermore, ^{99m}Tc -RAFT-RGD, a different RGD-based tracer for targeting $\alpha_v\beta_3$ integrin expression in vivo, was used in a rat model of reperfused ischemic myocardium. SPECT imaging was used to show enhanced ^{99m}Tc -RAFT-RGD, but not ^{99m}Tc -RAFT-RAD (negative control), uptake in the infarct and peri-infarct zone 14 days after reperfusion[86]. Additionally, specific binding of ^{99m}Tc -RAFT-RGD to the $\alpha_v\beta_3$ integrin was shown in human microvascular endothelial cells as the presence of an excess of unlabeled RGD resulted in a significant inhibition of ^{99m}Tc -RAFT-RGD binding.

The suitability of the $\alpha_v\beta_3$ integrin as a target for radiotracer imaging of angiogenesis was further established by studies that employed the indium-111 (^{111}In) labelled RP748 (also named quinolone). ^{111}In -RP748 is an $\alpha_v\beta_3$ integrin binding small molecule that specifically binds to activated endothelial cells in vitro and vivo[98]. This SPECT radiotracer has subsequently been assessed in rat[16, 87, 88] and canine[16, 88] models of MI in both early (acute) and late (3 weeks) time points after induction of MI. Binding specificity of ^{111}In -RP748 was shown by a direct comparison with the control compound ^{111}In -RP790 having similar chemical structure, although no in vitro specificity for $\alpha_v\beta_3$ -integrin. SPECT imaging revealed no uptake of ^{111}In -RP790 in hypoxic or infarct areas[16, 88].

Furthermore, in a swine model of hibernating myocardium, SPECT imaging revealed enhanced Iodine-123 (^{123}I)-gluco-RGD uptake in areas corresponding to ^{201}Tl defects in animals that received an endomyocardial injection of VEGF compared to animals that received a saline control injection. No uptake of an ^{123}I -labeled control peptide was seen in the heart of a control animal[89].

Despite the variety in SPECT radiotracers for angiogenesis, PET radiotracers for angiogenesis have actually been investigated more frequently with results that are very similar to studies that use SPECT imaging in preclinical models of cardiac angiogenesis. Tracers that have been used include ^{18}F -galacto-RGD[90, 91], ^{18}F -AIF-NOTA-PRGD2[92], ^{68}Ga -NOTA-RGD[93, 94], ^{68}Ga -NODAGA-RGD[91], and ^{68}Ga -TRAP(RGD) $_3$ [91]. Remarkably, in some studies, the uptake of RGD-based PET tracers was enhanced up to 4[92] or even 6[90] months after the angiogenesis stimulating intervention. Specificity of uptake was shown either by inhibition of binding with a specific non-radiolabeled $\alpha_v\beta_3$ integrin antagonist[90] or by co-incident and co-localized endothelial integrin markers such as CD31 or CD61 (β_3)[91, 92, 94].

Together with the $\alpha_v\beta_3$ integrin, the RGD peptide forms a reliable axis for targeted radiotracer imaging of angiogenesis. However, despite the promising results obtained in pre-clinical studies the application of RGD-based radiotracers for angiogenesis imaging in the context of myocardial ischemia or PVD in the clinic is modest to say the least. Instead radiotracer imaging of angiogenesis in patients so far has largely focused on imaging of tumor angiogenesis. Like in pre-clinical research, the main target for imaging has been the $\alpha_v\beta_3$ integrin through various radiolabeled RGD peptides[99-106], while the VEGF receptor[107], prostate-specific membrane antigen (PSMA)[108], and the extra domain B of fibronectin[109] have also received attention. To date only a few small studies in MI patients have been performed, all using the $\alpha_v\beta_3$ integrin as a target for angiogenesis imaging[110-112] (Table 2).

Makowski et al. used PET/CT imaging to target the $\alpha_v\beta_3$ integrin with ^{18}F -galacto-RGD in an MI patient and found enhanced uptake in infarcted area (defined by the extent of delayed enhancement MRI and decreased ^{13}N -ammonia myocardial blood flow) two weeks after MI. The feasibility of clinical angiogenesis imaging through targeting the $\alpha_v\beta_3$ integrin was further shown in studies by Mozid et al.[111] and Sun et al.[112]. Mozid and co-workers applied an intracoronary injection of granulocyte colony-stimulating factor mobilized bone-marrow stem cells in patients with chronic ischemic heart failure. Using $^{99\text{m}}\text{Tc}$ -NC100692 SPECT imaging they found baseline (day 0) uptake in all heart failure patients with no uptake seen in control patients. This suggests persistent angiogenesis in patients with chronic heart failure and remote MI, which is in line with the preclinical finding of enhanced uptake long after the ischemic incident. Unfortunately no proof of concept was provided that therapy-induced neovascularization can be picked up by RGD-based PET imaging in a robust manner[111]. Sun and co-workers used ^{68}Ga -PRGD2 SPECT in MI and stroke patients and found enhanced uptake in 20 out of 23 MI patients and in 8 out of 16 stroke patients. Furthermore, higher uptake of ^{68}Ga -PRGD2 was observed 1-3 weeks after the onset of MI/stroke and correlated well with the disease phase and severity[112].

Table 2. Radiotracers for myocardial angiogenesis imaging in clinical studies.

Myocardial angiogenesis			
Biological target	Tracer	Modality	Reference
$\alpha_v\beta_3$ integrin	^{18}F -galacto-RGD	SPECT	[110]
	$^{99\text{m}}\text{Tc}$ -NC100692	PET	[111]
	^{68}Ga -PRGD2	PET	[112]

Large volume patient studies employing RGD-based radiotracers for imaging of post-MI angiogenesis imaging are currently lacking despite the fact that there is no scarcity in tracer constructs. This begs the question if this is because of uncertainty if targeted angiogenesis imaging has clinical benefit due to unconvincing results in the small scale clinical studies or that there is lack of scientific interest in the absence of approved therapeutic angiogenesis? The amount of pre-clinical studies suggests there is no lack of scientific interest, so it is assumed that the field is held up by a lack of therapeutic programs.

Fortuitously, angiogenesis is hallmarked by the upregulation of multiple biomarkers. Beside upregulation of the $\alpha_v\beta_3$ integrin, CD13, a membrane bound aminopeptidase, is also upregulated on angiogenically active endothelial cells[113, 114]. CD13, expressed on active endothelial cells can specifically be targeted using an asparagine-glycine-arginine (NGR) peptide motif[114]. Competition studies in tumor angiogenesis with the NGR and RGD motifs demonstrated a threefold higher target homing ratio (tumor/control organ) for NGR than for RGD[115]. In a recent study by our own group, CD13 was targeted with a cyclic asparagine-glycine-arginine (NGR) peptide, having a tenfold higher targeting efficacy than the linear entity[116], that was coupled to ^{111}In via a diethylene triamine pentaacetic acid (DTPA) chelator. Dual isotope SPECT imaging indicated significantly enhanced uptake of ^{111}In -DTPA-cNGR mainly in areas of $^{99\text{m}}\text{Tc}$ -sestamibi absence (infarct region)[8]. Given the higher target homing ratio compared to the RGD motif, it is interesting to speculate that CD13 targeting through NGR-based tracers could lead to better image quality and subsequently better possibilities for clinical translation. However, studies comparing NGR and RGD-based tracers in the same model have to be conducted before such claims can be justified. Furthermore, to gain insight into the true benefit of monitoring angiogenesis in comparison with traditional endpoints or indirect effects such as clinical state, or tissue perfusion or function, large volume patient studies have to be conducted.

Other targets for radiotracer imaging of angiogenesis in myocardial ischemia

Although various angiogenesis stimulating factors exist, VEGF is considered the most potent and predominant factor[79, 81]. VEGF ligands, of which there are four known isoforms (A-D), are released in response to ischemia and mediate their angiogenic effects by binding to specific VEGF receptors (VEGFR-1, VEGFR-2 and VEGFR-3), leading to receptor dimerization and subsequent intracellular signal transduction via tyrosine

kinases[117, 118]. The majority of VEGF-based radiotracers have been evaluated in the context of tumor angiogenesis imaging. However, peripheral angiogenesis and post MI angiogenesis have been examined as well.

In a rat MI model, increased myocardial uptake in infarcted myocardium (visualized by ^{18}F -FDG) of the PET radiotracer Copper-64 (^{64}Cu)-DOTA-VEGF₁₂₁ was shown on day 3, 7 and 17 after induction of MI. Myocardial origin of the radiotracer signal was confirmed by CT co-registration and autoradiography[95].

Among the targets that received less attention while being successfully used for nuclear imaging of angiogenesis are CD105, a 180 kDa disulfide-linked homodimeric transmembrane protein selectively expressed on the endothelial cells of newly formed vessels[119-122], and CD13, a membrane bound aminopeptidase found on activated endothelial cells[113]. PET imaging of CD105 expression in a rat MI model with ^{64}Cu -labelled TRC105, an anti-CD105 monoclonal antibody, revealed significantly enhanced uptake in infarcted myocardium (indicated by ^{18}F -FDG) 3 days after surgically induced MI compared to sham operated control animals. These findings were supported by histology, indicating increased CD105 expression following MI[96].

Radiotracer imaging of angiogenesis in peripheral vascular disease

PVD is a progressive atherosclerotic process that results in stenosis or occlusion of non-coronary blood vessels, most frequently the iliac and femoral artery [47]. Progressive ischemia, present in PVD, can lead to intermittent claudication, non-healing ulcers, limb amputation and in severe cases death[47, 123]. Despite the severity of this disease, a significant proportion of individuals with PVD remain undiagnosed in clinical practice[124].

Although angiogenesis might have less impact on perfusion recovery in PVD than arteriogenesis, targeted nuclear imaging of angiogenesis can provide valuable information of the underlying pathophysiology associated with PVD. Especially in combination with lower-extremity perfusion imaging, areas of ischemia can be identified that might have remained unnoticed on MRI images or other techniques used in present day clinical care (e.g. ankle-brachial index, duplex ultrasound or CT angiography)[47].

In pre-clinical research, numerous hind-limb ischemia models have been established in several laboratory animals to mimic the situation of PVD and to study (stimulation of) neovascularization[125]. Like in MI models, targeted imaging of angiogenesis in pre-clinical models of PVD has mainly focused on the $\alpha_v\beta_3$ integrin and VEGF receptors (Table 3).

The $\alpha_v\beta_3$ integrin

The majority of RGD-based radiotracers that were developed have been tested in cardiac angiogenesis models. However, among them, $^{99\text{m}}\text{Tc}$ -NC100692[126, 127] and ^{68}Ga -NOTA-RGD[128] have also been evaluated in preclinical PVD models. Additionally, ^{125}I -c(RGD(I) yV)[129] and a bromine-76 labeled nanoprobe (^{76}Br -Nanoprobe)[130] have only been

tested in a mouse model for PVD. Specific radiotracer uptake was concluded from the absence of uptake of a scrambled control peptide[126, 129], inhibition of binding using an excess of the non-radiolabeled tracer[128, 130] or by co-localized binding of a fluorescent tracer analogue and CD31[127]. With the majority of studies only assessing radiotracer uptake at relatively short time points after induction of ischemia (i.e. up till 14 days)[127-130] and only one study assessing uptake after 4 weeks[126], the possibility and benefit of radiotracer guided imaging of neovascularization in PVD models at later time points remains to be discovered.

Growth Factor Receptors

The involvement of numerous growth factors, such as VEGF and FGF in neovascularization has been described, also in PVD[131]. Growth factor functions are regulated in a complex fashion with multiple feedback systems influencing many cell types, hence, it is extremely difficult to elucidate unique roles of each growth factor unless it is specific to a single cell type[132]. Therefore, rather than targeting the growth factor itself for molecular imaging, their most important or abundant receptors have been used as targets as these are thought to be more specifically regulated during neovascularization than their ligands. For example, the extracellular matrix (ECM) serves as a reservoir for growth factors[133], thereby forming a storage that can be tapped on demand. Detecting presence of growth factors therefore by molecular imaging could be unrelated to active neovascularization.

As VEGF is one of the dominant growth factors inducing angiogenesis, its functional receptor, VEGFR2 has been targeted frequently for molecular imaging. Using VEGF₁₂₁, a natural splice variant of VEGF₁₆₅ that lacks an ECM reservoir binding capacity[134], the VEGFR2 receptor can be visualized in relationship to neovascularization events. In a rabbit hind limb ischemia model, 10 days after arterial ligation, uptake of ¹¹¹In-VEGF₁₂₁ was enhanced as shown by SPECT imaging and post-mortem gamma counting[135]. Corresponding immunohistological findings of increased VEGFR2 expression validated the principle. A similar finding was reported using PET and ⁶⁴Cu-labelled VEGF₁₂₁ in a mouse hindlimb ischemia study, where the level of uptake correlated well with VEGFR2 protein levels in ligated and control limbs in the presence and absence of exercise[136].

Other targets for radiotracer imaging of angiogenesis in peripheral vascular disease

Other targets that were successfully used for nuclear imaging of peripheral angiogenesis are CD105 and the natriuretic peptide clearance receptor (NPR-C). Among the 4 natriuretic peptide family members, all binding the NPR-C, atrial natriuretic peptide and C-type natriuretic peptide have been demonstrated to suppress VEGF signaling and to attenuate angiogenesis[137-140]. Liu et al. developed a C-type atrial natriuretic factor (CANF)-conjugated comblike nanoprobe that was labelled with ⁶⁴Cu. In a mouse model of hind limb ischemia PET imaging of ⁶⁴Cu-DOTA-CANF-comb showed a significantly higher uptake

in the ischemic hind limb compared to the nonischemic control limb 7 days after induction of ischemia. These results were supported by immunohistochemical findings of NPR-C upregulation with colocalization in endothelial (via PECAM-1 staining) and smooth muscle cells (via α -actin staining)[141].

Furthermore, the PET tracer ^{64}Cu -NOTA-TRC105 was used to assess the response to pravastatin treatment in a mouse ischemic hind limb model. Pravastatin is a member of the statin group of cholesterol-lowering drugs which is also known to stimulate NO-mediated angiogenesis. Significantly increased radiotracer uptake in the ischemic hind limb compared to the control hind limb was shown at day 3, 10, 17 and 24 after induction of hind limb ischemia in the pravastatin treated group with CD31/CD105 co-immunostaining validating the radiotracer uptake[142].

Table 3. Radiotracers for peripheral angiogenesis imaging in pre-clinical studies.

Peripheral angiogenesis			
Biological target	Tracer	Modality	Reference(s)
A$\nu$$\beta$₃ integrin	$^{99\text{m}}\text{Tc}$ -NC100692	SPECT	[126, 127]
	^{68}Ga -NOTA-RGD	PET	[128]
	^{125}I -c(RGD(I)yV)	SPECT	[129]
	^{76}Br -Nanoprobe	PET	[130]
VEGF receptor	^{111}In -VEGF ₁₂₁	SPECT	[135]
	^{64}Cu -VEGF ₁₂₁	PET	[136]
NPR-C	^{64}Cu -DOTA-CANF-comb	PET	[141]
CD105	^{64}Cu -NOTA-TRC105	PET	[142]

In sharp contrast to targeted neovascularization imaging in cancer patients and to a lesser extent in MI patients, radiotracer imaging in PVD patients is based entirely on the use of perfusion tracers rather than on targeted imaging of neovascularization. Although specific angiogenesis tracers are readily available, it would be more appropriate and helpful to monitor arteriogenesis as it is by far the most efficient adaptive mechanism of survival for ischemic limbs[143]. However, in the absence of specific arteriogenesis tracers, perfusion tracers are currently used for diagnosis and monitoring of perfusion recovery. In order to improve diagnosis and therapy monitoring in PVD, but also in MI patients, the development of specific arteriogenesis tracers is warranted.

RADIOTRACER IMAGING OF ARTERIOGENESIS

Arteriogenesis

Since the first observations by Fulton in 1964[144], our knowledge about arteriogenesis and its underlying cellular and molecular mechanisms has increased vastly, though the fundamental event that initiates mitogenic stimulation has not been unraveled as of today.

Figure 2. Key features in angiogenic and arteriogenic vessel growth. Both processes share their dependency on macrophage guided, controlled extracellular matrix and vessel scaffold degradation. Nevertheless, both the initial stimulus as well as the outcome differs significantly between both processes. *Established in pre-clinical models. Modified from Buschmann and Schaper, 1999, Physiology[145].

The initiation of arteriogenesis is, in sharp contrast to angiogenesis, independent of ischemia and instead relies on physical factors (Figure 2 and 3). Following the occlusion of a conductance artery it is generally accepted that the arteriogenic process is initiated in the pre-existing collaterals that circumvent the obstruction by deformation of the endothelial cells as a consequence of increased pulsatile fluid shear stress. Initially, these pre-existing collateral arteries are incapable of conducting the mandatory blood supply to the tissue that is situated distally of the occlusion. In order to cope with the increased perfusion pressure diametrical growth and artery maturation of the pre-existing collateral artery are required. Successful maturation into a conductance artery relies on the creation of a transient inflammatory environment. Increased expression of adhesion molecules, cytokines and growth factors by the endothelium in response to increased shear stress hallmark the inaugural events in a complex cascade[146]. Subsequently, circulating monocytes attach to the endothelium, migrate to the peri-collateral space, and differentiate into macrophages[147, 148]. The inflammatory reaction that ensues is vital to arteriogenesis and driven by the secretion of growth factors and cytokines from endothelial cells, smooth muscle cells, monocytes, and macrophages. Among these secreted factors are monocyte chemotactic protein 1 (MCP1) which induces the attraction of more monocytes, tumor necrosis factor α (TNF α) which provides the inflammatory environment in which collateral

vessels develop, and MMPs that control the digestion of the internal elastic lamina and the surrounding extracellular matrix[147]. Simultaneous to the controlled digestion of the extra-cellular scaffolding, a burst of mitotic activity of smooth muscle cells and endothelial cells is initiated which results in an outward remodeling and a subsequent larger cross sectional area of the collateral artery. The enhanced cross sectional diameter causes the blood flow velocity and shear stress to normalize. Hence, the arteriogenic process is self-limiting after collateral arteries reach a sufficiently large diameter. Further maturation of the vessel occurs through the orderly arrangement of smooth muscle cells in circular layers, establishment of cell-cell contacts and the synthesis of elastin and collagen[149].

Figure 3. Mechanism of arteriogenesis. Increased shear stress over pre-existing collateral arterioles triggers a macrophage guided outward remodeling that results in the (partial) restoration of perfusion. Modified from Schirmer et al., 2009, Heart[150].

However, despite the extensive pre-existing collateral network (and thereby possible opportunities for arteriogenic repair) in the human body, most molecular neovascularization imaging research to date has focused on angiogenesis, leaving molecular arteriogenesis imaging largely overlooked. This is not particularly useful as arteriogenesis is more capable of restoring tissue blood supply than angiogenesis[15, 151] as collateral vessels have the capacity to carry a larger volume of blood than sprouting capillary networks[152]. At present, determining precise morphology is still dependent on post mortem angiography as some collateral arteries (i.e. collateral arteries in the subendocardial plexus of the left ventricle) are rather poorly represented in clinical angiography[15]. Molecular tracers for arteriogenesis imaging are also lacking despite the identification of a fairly extensive set of molecular circuits that are perturbed[149].

Radiotracer imaging of arteriogenesis in models for myocardial ischemia

Despite the lack of specific radiotracers for arteriogenesis, there are studies in which histological findings to indicate ongoing arteriogenesis were supported by nuclear imaging (and vice versa), both in the context of MI as well as PVD. As already alluded to in section 2 in this review, perfusion tracers for PET and SPECT can be used for this purpose.

Li et al. employed gene therapy using a plasmid encoding human platelet derived endothelial cell growth factor (PD-ECGF) cDNA in a dog chronic myocardial ischemia model. A double immunohistochemical staining for von Willebrand factor and α smooth-muscle actin (α SMA) demonstrated that angiogenesis and arteriogenesis occurred. These findings were accompanied by enhanced myocardial blood flow 2 weeks after induction of gene therapy as indicated by N^{13} -ammonia PET imaging[153]. In a different study, Zuo et al. subjected pigs to coronary artery ligation and subsequent intramyocardial administration of a recombinant adeno-associated virus construct coding CD151 (a tetraspanin superfamily protein). Using N^{13} -ammonia PET they found significantly enhanced regional myocardial perfusion 8 weeks after viral transduction, which was accompanied by a marked increase in capillary (indicated by von Willebrand staining) and arteriolar density (indicated by α SMA staining)[154]. Furthermore, in rats subjected to coronary artery ligation, Kainuma et al. showed that combined treatment of the ischemic myocardium with skeletal myoblast cell-sheet plus an omentum-flap resulted in a greater amount of functionally (CD31+/Lectin+) and structurally (CD31+/ α SMA+) mature blood vessels 4 weeks after treatment. Additionally, N^{13} -ammonia PET showed better global coronary flow reserve in the group receiving this combined treatment[155].

While the majority of studies linking enhanced myocardial blood flow to enhanced arteriogenesis (in combination with angiogenesis) employed N^{13} -ammonia (getting metabolically trapped in viable tissue) PET perfusion imaging, there are also studies that used SPECT imaging to link augmented myocardial perfusion to enhanced arteriogenesis.

Crottogini et al. investigated the effect of intramyocardial plasmid-mediated human VEGF₁₆₅ gene transfer on the proliferation of vessels with smooth muscle in a pig model of myocardial ischemia. Using 99m Tc-sestamibi SPECT imaging they reported enhanced myocardial perfusion accompanied by a significant increase in small sized collateral vessels compared to placebo treated pigs. However, angiographic quantification of collateral development using the Rentrop score failed to indicate a significant difference between the groups[156]. In a different study by the same group, Janavel et al. investigated the effect of VEGF gene transfer on the evolution of experimental myocardial infarction in adult sheep. They found an increase in angiogenesis (7 days after coronary artery ligation) and arteriogenesis (10 and 15 days after coronary artery ligation). Additionally, using 99m Tc-sestamibi SPECT they found increased resting myocardial perfusion in VEGF-treated sheep 15 days after coronary artery ligation[157].

Although several studies relate enhanced perfusion and smooth muscle positivity to increased arteriogenesis caution has to be taken when using this ambiguous term. Both the maturation of angiogenic vessels as well as collateral formation are coined arteriogenesis despite the difference in impact they have on perfusion recovery. While both cause enhanced smooth muscle detectability, collateral formation is more potent to drive perfusion. Hence, ascribing perfusion recovery to collateral formation requires more than showing an increase in smooth muscle positivity. Validation of nuclear perfusion imaging with angiography (using the Rentrop scoring index) will likely provide a better insight into whether perfusion recovery is caused by maturation of angiogenic vessel or collateral formation. For example, in a Yorkshire swine ameroid constrictor model Mack et al. linked significant ^{99m}Tc -sestamibi SPECT perfusion recovery in VEGF₁₂₁ adenovector injected animals to significantly enhanced Rentrop scores (ex vivo coronary angiography) compared to control animals. However, no αSMA staining was performed[158]. In the same pig myocardial ischemia model, several other studies reported enhanced collateralization and perfusion after therapeutic stimulation, although no nuclear perfusion imaging was performed. For example, in a pig ameroid constrictor model Tio et al. showed enhanced myocardial perfusion (indicated by colored microspheres), in animals intramyocardially injected with a VEGF₁₆₅ encoding DNA containing plasmid. Enhanced perfusion at maximal vasodilation (adenosine) was accompanied by an increased Rentrop score compared to control animals[159]. In a similar model Sato et al. investigated the effect of intracoronary administration of FGF2. Using angiography they found significant improvement in collateralization (assessed by Rentrop scoring) which was supported by enhanced perfusion (microspheres and MRI) and function (MRI)[160]. More evidence on the collateralization enhancing effects of FGF2 was gathered by Laham et al., also in a pig model of myocardial ischemia. Using angiography they found enhanced collateralization (Rentrop scoring). Additionally, improved myocardial perfusion (microspheres and MRI) and function (MRI) in the ischemic territory were found. Moreover, histological evidence of increased myocardial vascularity was reported[161].

Radiotracer imaging of arteriogenesis in models of peripheral vascular disease

Stacy et al. investigated serial changes in lower extremity arteriogenesis and muscle perfusion in a pig model for PVD[162]. Significant increases in collateral artery formation in the biceps femoris and semimembranosus muscle area was shown using CT angiography 4 weeks after ligation. They concluded that arteriogenesis within the semimembranosus and biceps femoris presumably resulted in improved downstream perfusion, which was quantified using Ti^{201} SPECT and validated by postmortem gamma-counting 4 weeks after induction of hindlimb ischemia.

More recently, our own group used ^{99m}Tc -sestamibi in a mouse model for PVD to show that perfusion recovery through arteriogenesis after femoral artery ligation appears

to happen much faster than suggested by standard laser-Doppler perfusion imaging. Perfusion in the ligated hind limb restored to levels comparable to the control limb on day 7 after ligation surgery and was accompanied by a significant increase in collateral artery diameter (α SMA). Additionally, ^{99m}Tc -pyrophosphate was used to indicate muscular damage. Peak uptake of ^{99m}Tc -pyrophosphate was found 3 days after femoral artery ligation which recovered to baseline levels 14 days after surgery. The ^{99m}Tc -pyrophosphate data was further invigorated by histological findings showing peak monocyte/macrophage infiltration (CD68 staining) and DNA fragmentation (TUNEL staining) on day 3 post femoral artery ligation [163].

DEVELOPING CANDIDATE TRACERS FOR ARTERIOGENESIS IMAGING

Molecular radiotracers for imaging the arteriogenic process are lacking despite the fact that there is no scarcity in theoretic targets. Due to the larger diameters of collaterals classic, non-nuclear imaging (i.e. microspheres and angiography) has been used for detection and quantification. However, these methods are restricted to late stage arteriogenesis and lack sensitivity or the quantitative capacity compared to nuclear imaging techniques such as SPECT or PET. Early detection of arteriogenesis through radiotracer imaging might be a valuable diagnostic with therapeutic or prognostic implications. As growing collateral arteries are hallmarked by the upregulation of adhesion molecules and subsequent invasion of monocytes (followed by T-lymphocytes) which in turn are a rich source of different cytokines[164], there is a variety of mechanisms that can be targeted through radiotracer imaging. Although there is definitely an inflammatory component involved in the arteriogenic process[165, 166], for example the MCP-1 pathway that recruits monocytes to areas of collateral artery development[167], applying ligands that bind to inflammatory targets might not be suitable for operating in a transient inflammatory environment.

Designing tracers for arteriogenesis imaging requires suitable targets that have the potential to be labelled with a radiotracer (e.g. through attachment of a chelating agent). Furthermore, a meaningful animal model both for cardiac arteriogenesis as well as arteriogenesis in PVD, and a sensitive and specific imaging readout are needed. To study arteriogenesis in the setting of PVD we have been using a mouse[163] and rat[148] hindlimb model which are well established models of collateral formation. For the cardiac arteriogenesis, we have been using a mouse infarct model[168, 169] although the distinction between angiogenesis and arteriogenesis cannot be clearly made. Furthermore, a porcine model of chronic myocardial ischemia by an ameroid constrictor has been used in combination with whole mount cryomicrotome imaging to unambiguously show collateral development[165].

Targets we have been chasing include an ICAM-1 antibody, chemokines such as CXCL1, and other ligands that bind to chemokines (e.g. Evasin3, binding to CXCL1). When designing tracers we focused on a double-labelling approach (i.e. fluorescent tag as well as the ability to attach a radioisotope through a chelator). We opted for this approach to enable correct spatiotemporal presentation of target binding of our tracers. While the fluorescent tag facilitates the option to perform fluorescence microscopy or high resolution two-photon laser scanning microscopy (TPLSM), the chelator provides the option for radiotracer imaging. Subsequent chasing experiments with non-radiolabeled agents could serve as indication for target binding specificity.

So far, these novel tracers, however, proved to be ineffective as imaging agents. Reasons for this failure might be that most targets reside at endothelial cells, are expressed at low intensity and exposed to a high flow circulatory environment. Nevertheless, while the target should be abundantly expressed in the area of collateralization, it should not (or only marginally) be expressed in non-specific areas. The monoclonal anti ICAM antibody we tested in vivo appeared to bind to constitutively expressed ICAM-1 on the endothelial lining in every vessel, thereby making it impossible to distinguish specific binding patterns. Moreover, it is conceivable that an arteriogenesis specific tracer should engage in polyvalent binding before being able to overcome the high shear stress in the area of collateralization.

CONCLUSION

Advances in radiotracer imaging are made through continuously improving camera hard- and software as well as through the development of new radiotracers with favorable imaging characteristics. In this review we discussed the role of perfusion tracers and provided an extensive overview of pre-clinical research into radiotracer imaging of angiogenesis and arteriogenesis in the context of MI and PVD.

Abundant pre-clinical research has resulted in the identification and development of angiogenesis tracers while the development of specific arteriogenesis tracers remains largely overlooked. So far, perfusion tracers have been used to indicate enhanced perfusion through arteriogenesis (in combination with angiogenesis). Currently, only a few angiogenesis (targeting the $\alpha_v\beta_3$ integrin) tracers have found its way to the clinic and broad scale implementation of specific angiogenesis and arteriogenesis imaging in MI and PVD patients is still lacking. Future research should therefore focus on improving the translation of neovascularization tracers into the clinic, especially in the case of arteriogenesis. However, as illustrated in the last section, designing a tracer, specific for arteriogenesis imaging is not easy. When designing an arteriogenesis tracer it is important to keep in mind the high shear stress environment that has to be overcome (potentially

requiring polyvalent binding) as well as the notion that inflammatory factors might not operate well within a transient inflammatory setting. Clinical implementation of specific angiogenesis and arteriogenesis imaging can aid tailored therapy and would be a huge asset to MI and PVD patient risk stratification.

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CHAPTER 3

PRE-CLINICAL APPROACHES TO DESIGN AN ARTERIOGENESIS SPECT TRACER

INTRODUCTION

The growth of pre-existent collateral arterioles into functional collateral arteries is termed arteriogenesis[1]. Following an arterial occlusion, arteriogenesis is initiated by deformation of the endothelial cells through increased fluid shear stress resulting from the steep pressure gradient between the high pre-occlusive and very low post-occlusive pressure regions that are interconnected by collateral vessels[2].

The increase in fluid shear stress over the endothelium causes the activation of endothelial cells which in turn starts the inflammatory component of the arteriogenic process, which is needed to continue the process. Activated endothelial cells transform and assume a proliferative phenotype which involves the upregulation of adhesion molecules, in particular intercellular adhesion molecule 1 (ICAM-1). Beside the upregulation of adhesion molecules, activated endothelium increases the production of cytokines such as monocyte chemotactic protein 1 (MCP-1), granulocyte macrophage colony stimulating factor (GM-CSF) and tumor necrosis factor α (TNF α)[3-7]. Upregulation of these inflammatory factors results in the recruitment and transmigration of monocytes. Furthermore, the chemokine C-X-C motif ligand 1 (CXCL1) and its receptor chemokine C-X-C motif receptor 2 (CXCR2) were shown to play a role in monocyte adhering to shear stressed endothelium[8]. More importantly, in a rat model of peripheral artery disease, CXCL1 was shown to promote arteriogenesis by enhancing monocyte adherence to arteriogenic collaterals[9]. Ultimately, triggering these inflammatory pathways results in enlargement of the vascular diameter thereby partially restoring tissue blood supply.

The process of naturally occurring arteriogenesis is self-limiting and ends at 30% of maximal conductance (coronary) and 40% in the vascular periphery[2]. Therefore, the stimulation of arteriogenesis could be of potential benefit to patients with obstructive arterial disease. However, in order for such therapies to be monitored accurately (i.e. before macrostructural changes become apparent), we need non-invasive imaging tracers that are highly sensitive and specifically targeting the arteriogenic process. Despite the potential of arteriogenesis to (partially) restore tissue blood supply we currently lack a specific imaging tracer for arteriogenesis.

In the following subchapters I will describe own, non-published data from pre-clinical studies, all aimed at establishing a new non-invasive radiotracer for arteriogenesis imaging. Early markers for arteriogenesis such as ICAM-1, CXCL1, and the chemokine binding evasin3 will be discussed. These tracers were chased and developed as part of a center for translation molecular medicine (CTMM) project (EMINENCE) to improve monitoring of neovascularization (therapy). When designing these tracers we focused on a double labeling approach (i.e. fluorescent tag as well as the ability to attach a radioisotope through a chelator). We opted for this approach to enable correct spatiotemporal presentation of target binding of our tracers. While the fluorescent tag facilitates the

option to perform fluorescence microscopy or high resolution two-photon laser scanning microscopy (TPLSM), the chelator provides the option for radiotracer imaging. Subsequent chasing experiments with non-radiolabeled agents could serve as indication for target binding specificity.

Furthermore thymosin β 4, a G-actin monomer binding protein implicated in reorganization of the actin cytoskeleton known to play a role in angiogenesis and arteriogenesis[10], will be discussed.

INTERCELLULAR ADHESION MOLECULE 1 (ICAM-1)

Considering the important role of ICAM-1 in the initiating phase of arteriogenesis, we explored a commercially available phyco-erythrin (PE) coupled monoclonal anti (α)CD54 antibody against ICAM-1 as a probe for early onset arteriogenesis in a mouse (C57Bl/6) ischemic hind limb model (model as described in[11]). We expected the PE labeled antibody to reveal a specific binding pattern in the area of collateral formation in ligated hind limbs. Furthermore, to enable radiolabeling and subsequent SPECT imaging the antibody was successfully coupled to a DTPA chelator at our institution.

During a pilot study using the non-DTPA labeled α CD54-PE antibody in the mouse hind limb ischemia model we performed in vivo fluorescence microscopy. Thereto, the skin on the inner side of the ligated and the contralateral control limb was taken off to expose the area of collateral artery formation. We found that intravenous (i.v.) administration of the α CD54-PE antibody, 2 and 24 hours post ligation did not result in a distinct binding pattern in the area of collateralization in the ischemic hind limb. We observed the PE labeled antibody in the ligated as well as the contra lateral control limb without notable differences in signal intensity over time. Furthermore, we were able to detect the PE labeled antibody signal in the vasculature of the hind limbs of control animals (Figure 1).

After fluorescence microscopy experiments, animals were sacrificed, the vasculature of the mice was flushed and muscular tissue was harvested from the ischemic and contralateral control hind limb for TPLSM. TPLSM revealed fluorescent antibody signal in the vascular wall, but not in the lumen, of both ligated and contra-lateral control limbs of animals injected 2 and 24 hours after ligation surgery (only images of the right hind limb of a mouse subjected to ligation surgery and a non-operated control mouse are shown in Figure 1). Moreover, in harvested tissue from sham operated animals we were also able to detect the fluorescence signal. Subsequently, muscular tissue was harvested from hind limbs of control animals that were injected with the fluorescent antibody but not subjected to ligation or sham surgery. Remarkably, using TPLSM we were still able to detect the fluorescent signal of the antibody on the vascular wall throughout the entire tissue specimen.

Figure 1. Representative fluorescence (A,B) and TPLSM (C,D) images of the right hind limb of mice subjected to ligation surgery and non-operated control mice. Panel A and B represent fluorescent images of the vasculature of the upper part of the right hind limb with the skin reflected and the anatomical outlines contoured in red. Images were acquired 2 hours post ligation and 15 minutes post injection of the PE-labeled α CAM-1 antibody. Panel C and D represent TPLSM images of the musculature in the area of collateral artery formation. PE signal is displayed in red while the green signal originates from the muscular auto fluorescence.

CHEMOKINE C-X-C MOTIF LIGAND 1 (CXCL1)

Recently, the importance of the chemokine C-X-C-motif ligand 1 (formerly termed growth related oncogene alpha (GRO α) and the human homologue of murine keratinocyte-derived chemokine (KC)) in early onset arteriogenesis was shown by Vries et al[9]. In a rat (Sprague Dawley) hind limb ischemia model they showed early upregulation of CXCL1 (mRNA elevated already 1 hour after ligation) which reached baseline levels again at day 3 after ligation. Moreover, osmopump CXCL1 infusion directly into the collateral circulation enhanced arteriogenesis on day 3 and 7 after ligation as measured by laser Doppler perfusion imaging. Furthermore, in a mouse ischemic hind limb model continuous CXCL1 infusion and CXCL1 releasing microsphere injection enhanced perfusion recovery 7 days after femoral artery ligation compared to saline and empty microsphere conditions. Additionally, CXCL1 releasing microspheres show enhanced perfusion recovery 21 days after femoral artery ligation compared to continuous CXCL1 infusion, saline and empty microsphere conditions, thereby indicating that local injection of CXCL1 is a possible new tool to stimulate arteriogenesis in vivo[12]. Clearly CXCL1 appears to act as an important player in the process of arteriogenesis. However, the mechanism that underlies the effects

of CXCL1 makes this protein an interesting basis for an arteriogenesis specific imaging tracer. Recently it was shown that shear stressed endothelium binds and takes up CXCL1[9], most likely via the upregulated number of binding sites present in the glycocalyx[13, 14]. Targeting these increased binding sites through injection of exogenous CXCL1 coupled to a radioactive and fluorescent label is an attractive strategy for imaging of arteriogenesis.

Using native chemical ligation and oxidative protein folding CXCL1 was synthesized in our institution. Additionally, CXCL1 was coupled to a DTPA chelator as well as an Oregon Green 488 (OG488) fluorescent tag to enable radiolabeling and fluorescence microscopy. However, already before radiolabeling we noticed that DTPA-CXCL1-OG488 failed to run through a 10 kDa molecular weight cut-off filter despite a molecular mass of 9612.12 Da. Although this experiment took place at the edge of the capacity of the filter, it seems to indicate multimer formation or, less likely, precipitation of the compound. Nevertheless, we pursued to radiolabeling CXCL1 with the SPECT radionuclide ^{111}In . Upon radiolabeling DTPA-CXCL1-OG488 with ^{111}In multiple radioactive peaks kept appearing on the HPLC chromatogram. Most of these peaks were interwoven which hampered purification on HPLC, and thus, in vivo application.

EVASIN3

Isolated from tick saliva, evasins were shown to bind and neutralize chemokines in vitro and in vivo[15, 16]. Evasin3 has been shown to specifically bind and neutralize the bioactivity of CXC chemokines such as CXCL1 and CXCL2. Given the enhanced production of CXCL1 in combination with the increased opportunities for CXCL1 to bind to shear-stressed endothelium, evasin3 might be a suitable basis for an arteriogenesis specific imaging tracer.

Like CXCL1, evasin3 was synthesized at our institution and coupled to a DTPA chelator and an OG488 fluorescent tag. After successfully radiolabeling DTPA-Evasin3-OG488 with ^{111}In , the radioactive tracer was injected in two rats subjected to a hind limb ischemia model (model as described in Vries et al.[9]). The first rat was injected i.v. with ^{111}In -DTPA-Evasin3-OG488 3 hours post induction of ischemia, the other rat was injected i.v. with ^{111}In -DTPA-Evasin3-OG488 24 hours post induction of ischemia. Unfortunately in both rats we were unable to detect a specific signal from the ^{111}In -DTPA-Evasin3-OG488 tracer in the area of collateralization using μSPECT imaging. All activity was concentrated in the kidneys and bladder, suggesting rapid clearance of the tracer.

THYMOSIN β 4

T β 4 is a 43 amino acid counting peptide involved in cardiac survival and repair[17]. T β 4 is known to induce angiogenesis[18-20] and T β 4 serum levels were shown to be related to the development of coronary collateral vessels in patients with CAD[21]. T β 4 is currently being produced by RegeneRx Biopharmaceuticals, a publicly held biopharmaceutical company. The food and drug administration (FDA) cleared the way for RegeneRx Biopharmaceuticals to initiate a phase I clinical trial in patients who present to the clinic with an acute myocardial infarction[22].

We set out to explore the possibilities of T β 4 as an imaging tracer for angiogenesis or arteriogenesis. T β 4, kindly provided by RegeneRx Biopharmaceuticals, was coupled to the radioactive PET isotope ^{68}Ga using a NOTA-benzyl-NCS chelator (average coupling ratio of 1:1). As T β 4 is linked to both angiogenesis and arteriogenesis[22] we studied this newly designed tracer in two different mouse models. The Matrigel plug assay[23] was used to study ^{68}Ga -NOTA-T β 4 in a model for angiogenesis and the ischemic hind limb model was used to study ^{68}Ga -NOTA-T β 4 in a model for arteriogenesis.

Unfortunately, in Matrigels implanted subcutaneously in the flanks, we were unable to observe specific uptake of our tracer on reconstructed PET (Focus 120 microPET, Siemens Medical Solutions USA, Inc.) images. Moreover, gamma-counting of explanted Matrigels showed that FGF2 supplemented Matrigels did not display higher uptake of ^{68}Ga -NOTA-T β 4 than non-FGF2 supplemented Matrigels, thereby confirming the lack of specific uptake of our tracer.

Additionally, in the hind limb ischemia model, animals were injected i.v. with ^{68}Ga -NOTA-T β 4 at 5 different time points (i.e. baseline, post-ligation, day 3, 7, and 14) to study binding of ^{68}Ga -NOTA-T β 4 over time. However, on PET images we were unable to detect specific uptake of ^{68}Ga -NOTA-T β 4 in the area of collateralization because of high non-specific uptake in the rest of the body.

DISCUSSION

In this chapter we discussed 4 possible targets that, based on literature, seemed to be a suitable basis for an arteriogenesis tracer. However, while in literature a key role in arteriogenesis was ascribed to these 4 agents, their application as a radiotracer for arteriogenesis imaging was not straightforward. Besides having to bind in a high shear stress environment, tracers have to bind to targets that are sufficiently abundant to generate contrast.

When selecting 3 of these agents we opted for a double labeling approach involving a fluorescent marker and a chelator enabling radiolabeling. This approach would

allow perfect spatiotemporal validation of our tracers as the fluorescent tag facilitates fluorescence microscopy and TPLSM on in vivo exposed vascular beds, while radiolabeling would allow nuclear imaging. Moreover, radiolabeling would also allow testing of binding specificity through blocking experiments.

Remarkably, in case of the PE labeled anti ICAM-1 antibody, we were able to detect the fluorescent signal in the entire vasculature of the ligated limb using TPLSM. Even more noticeable was the fact that, after injection of the antibody, we were able to detect the fluorescent signal in control limbs of animals with hind limb surgery and in both limbs of sham operated animals. Clearly ICAM-1 is expressed on the vessel walls in the area of collateralization. However, this expression is not restricted to the collateral arteries solely. Our findings might be explained by the constitutive expression of ICAM-1[5].

CXCL1 and evasin3 were the other double labeled agents we pursued. However, experiments with these tracers came to a premature halt. In case of CXCL1, multiple interwoven peaks appeared on HPLC after radiolabeling with ¹¹¹In which hampered product purification. An explanation might be found in the tendency of chemokines, such as CXCL1, to form dimers at micromolar concentrations[24]. Dimerization of CXCL1 is caused by the 4 disulfide-bridge forming cysteine residues in the protein[25]. Notwithstanding the potency of the dimeric CXCL1 form, which is comparable with the potency of the monomeric form[24], protein dimerization is likely to complicate radiolabeling through sterically hindering the chelator binding site thereby preventing effective radiolabeling. Unfortunately these problems appeared intractable and experiments were abrogated.

Unlike with CXCL1, we succeeded to radiolabel evasin3 with the SPECT radionuclide ¹¹¹In. However, shortly after injection, SPECT images only showed uptake in the kidneys and bladder, suggesting rapid clearance of the tracer. Since the tracer was rapidly cleared both 3 and 24 hours post ligation surgery we decided to stop the experiments. Despite unfavorable initial results we think that by prolonging the circulation time this tracer still has the potential to be a successful arteriogenesis tracer. A possible strategy to accomplish this is to increase the molecular size by adding a poly-ethylene glycol (PEG) chain between the chelator and the evasin3 molecule. PEG conjugation increases the retention of a molecule in the circulation by protecting against enzymatic digestion, slowing filtration by the kidneys and reducing the generation of neutralizing antibodies[26, 27]. However, given the one time administration, antibody presence is a less likely contributor to short half-life of evasin3

⁶⁸Ga was successfully coupled to Tβ4 through a NOTA chelator. Since Tβ4 is linked to both angiogenesis and arteriogenesis we tested Tβ4 as a nuclear tracer in two dedicated and established animal models.

With regard to the angiogenesis inducing model we expected to see higher uptake of our Tβ4-based tracer in FGF2 supplemented Matrigels compared to vehicle supplemented controls. Unfortunately, we were unable to observe specific uptake of ⁶⁸Ga-NOTA-Tβ4

in FGF2 supplemented Matrigels. This is an interesting finding since uptake of T β 4 was suggested to occur through internalization or binding to an unknown cell surface receptor during vessel formation[18], the latter being enhanced in hFGF2 supplemented Matrigels compared to control Matrigels. However, further confirmation of the lack of specific uptake followed from the fact that gamma-counting did not show a difference in uptake between explanted FGF2 supplemented Matrigels and non-supplemented control Matrigels.

In the arteriogenesis inducing hind limb ischemia model, PET imaging of ^{68}Ga -NOTA-T β 4 was hampered by high non-specific uptake at any time point. This high uptake outside the area of collateral artery formation might be related to the fact that T β 4 is involved in a substantial amount of processes in the body through its function in actin polymerization[10, 28].

The development of an arteriogenesis specific radiotracer appears to be a difficult task. Apparently this is much more difficult than for angiogenesis given the substantial amount of (pre-clinically) available tracers for angiogenesis and the lack of arteriogenesis tracers. There are at least three obvious reasons for this difference. First, molecular addresses in collateral arteries are subjected to high and increasing shear stress due to high flow and initially small diameters. Increased shear stress might interfere with the molecular interactions required for labeling studies[29]. Binding of an arteriogenesis radiotracer possibly requires a binding of polyvalent nature in order to overcome the high shear stress in the area of collateral artery formation. Lack of a polyvalent binding capacity might be a reason as to why the ^{111}In -labeled evasin3 SPECT tracer was cleared rapidly via the urinary tract and did not show specific binding. A different potential reason for the rapid clearance of the ^{111}In -labeled evasin3 SPECT tracer could be the small molecular size. In this case PEGylation is a possible strategy to prevent rapid clearance by increasing molecular size. Second, the arteries and their surrounding tissue are in a transient inflammatory status during collateralization. Therefore, the expression of molecular markers that are shared with arteriogenesis may generate non-specific signals when targeted by a (radio)tracer. For instance, applying the chemokine binding evasin3 as a tracer in a transient inflammatory environment requires testing different time points after ligation in the ischemic hind limb model as the point of optimal binding is currently not exactly known. Third, the underlying mechanisms that drive angiogenesis are known in finer detail compared to the underlying mechanisms of arteriogenesis. This makes testing of angiogenesis tracers for example easier in dedicated animal models. Better understanding of arteriogenesis and detailed characterization in animal models is essential for the design and application of arteriogenesis specific radiotracers.

CONCLUSION

Developing a successful nuclear tracer for arteriogenesis imaging is a delicate task that requires exact knowledge of the spatiotemporal behavior of the target. The most important hallmark of a successful (nuclear) tracer is its ability to establish a specific binding to a biomarker. In case of the fluorescently-labeled anti-ICAM-1 antibody we found high levels of non-specific binding using fluorescence microscopy and TPLSM, while PET scanning indicated high levels of non-specific binding of ^{68}Ga -labeled T β 4. We therefore had to conclude that these tracers were not suitable as nuclear tracers for non-invasive imaging of arteriogenesis.

Binding of an arteriogenesis radiotracer possibly also requires a binding of polyvalent nature in order to overcome the high shear stress in the area of collateral artery formation. Lack of a polyvalent binding capacity is a possible explanation for the rapid clearance of ^{111}In -labeled evasin3 via the urinary tract. However, a different potential reason for the rapid clearance of this tracer might be the small molecular size. In this case increasing the molecular size through PEGylation is an option to increase the retention time of the tracer.

Moreover, as unsolvable multimerization hampered testing our ^{111}In -labeled CXCL1 tracer in vivo we are unable to draw conclusions about its suitability as an arteriogenesis imaging tracer.

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CHAPTER 4

COMPARISON OF LDPI TO SPECT PERFUSION IMAGING USING $^{99\text{M}}\text{Tc}$ - SESTAMIBI AND $^{99\text{M}}\text{Tc}$ -PYROPHOSPHATE IN A MURINE ISCHEMIC HIND LIMB MODEL OF NEOVASCULARIZATION

Geert Hendrikx

Mark H. Vries

Matthias Bauwens

Marijke De Saint-Hubert

Allard Wagenaar

Joël Guillaume

Levinia Boonen

Mark J. Post

Felix M. Mottaghy

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ABSTRACT

Background: We aimed to determine the accuracy of laser-Doppler perfusion imaging (LDPI) in an animal model for hind limb ischemia.

Methods: We used a murine (C57Bl/6 mice) ischemic hind limb model in which we compared LDPI with the clinically used ^{99m}Tc -sestamibi SPECT perfusion imaging (n = 7). In addition, we used the SPECT tracer ^{99m}Tc -pyrophosphate (^{99m}Tc -PyP) to image muscular damage (n = 6).

Results: LDPI indicated a quick and prominent decrease in perfusion immediately after ligation, subsequently recovering to 21,9% and 25,2% 14 days later in the ^{99m}Tc -sestamibi and ^{99m}Tc -PyP group respectively. ^{99m}Tc -sestamibi SPECT scans also showed a quick decrease in perfusion. However, nearly full recovery was reached 7 days post ligation. Muscular damage, indicated by the uptake of ^{99m}Tc -PyP was highest at day 3 and recovered to baseline levels at day 14 post ligation. Postmortem histology supported these findings, as a significantly increased collateral diameter was found 7 and 14 days after ligation and peak macrophage infiltration and TUNEL positivity was found on day 3 after ligation.

Conclusions: Here we indicate that LDPI strongly underestimates perfusion recovery in a hind limb model for profound ischemia.

INTRODUCTION

Numerous ischemic hind limb models have been developed in mice^[1-10], rats^[11,12] and rabbits^[1] in order to study post-ligation perfusion recovery through neovascularization. Most hind limb ischemia studies have been conducted in mice and rats and regardless of the studied mode of neovascularization (i.e. arteriogenesis or angiogenesis), follow up of post ligation perfusion recovery is often performed by laser Doppler perfusion imaging (LDPI)^[2-6,9,10,13,14]. Other methods frequently used in pre-clinical research for the assessment of post ligation perfusion recovery are angiography^[3,4,9-11,14,15], microsphere injections^[2,4,11,12,15], contrast enhanced ultrasound^[16], micro CT^[10], and magnetic resonance imaging (MRI)^[14]. However, despite the variety in methods to monitor perfusion recovery in the ischemic hind limb, LDPI remains the most frequently used. The main reasons are that LDPI is a fast, efficient and non-invasive technique to document extremity blood-flow^[17]. However, since LDPI is limited by the penetration depth of the laser light^[8,18], allowing measurement of superficial skin perfusion only, the question arises whether LDPI scanning is accurately reflecting the incremental perfusion improvement of skeletal muscles through arteriogenesis in the upper hind limb or angiogenesis in the lower hind limb of the animal.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are noninvasive, radioisotope-based nuclear imaging techniques that can be used to image changes in tissue blood flow, which can provide early, sensitive and specific detection of ischemic diseases at the molecular level^[19,20]. With increasing availability of better and dedicated small animal cameras as well as the development of new radiotracers these nuclear imaging techniques provide excellent tools for perfusion imaging.

Here we used the SPECT perfusion tracer technetium-99m sestamibi (^{99m}Tc-sestamibi) to study whether LDPI accurately reflects perfusion recovery in a murine ischemic hind limb model for arteriogenesis. Additionally, we used technetium-99m pyrophosphate (^{99m}Tc-PyP) as a molecular tracer for muscular damage as it binds to hydroxyapatite crystals in damaged myocytes^[21,22]. Acquired 3-dimensional (3D) information on perfusion and myocyte damage provided by SPECT scans may prove to be a useful addition to standardized pre-clinical perfusion recovery analysis by LDPI.

MATERIALS AND METHODS

Animal model

C57Bl/6 mice were anaesthetized with isoflurane (1.5-2%) throughout the whole surgical procedure to induce hind limb ischemia. Analgesia was applied 30 minutes before and 6 hours after the procedure with temgesic (0.5 mg/BW). In short, a longitudinal incision was made in the skin overlying the middle portion of the right hind limb of the mice.

The femoral artery was dissected for several millimeters in length from the femoral nerve and femoral vein. The artery was ligated proximal to the superficial epigastric artery by electrocoagulation. Subsequently, the skin was closed with a continued suture. Next, a longitudinal incision was made in the skin just above the knee of the right hind limb. The femoral artery was dissected for several millimeters in length from the femoral nerve and femoral vein. The artery was ligated distal from the bifurcation of the saphenous artery and the popliteal artery by electrocoagulation. 7 C57Bl/6 mice were used in the ^{99m}Tc -sestamibi group and 6 C57Bl/6 mice were used in the ^{99m}Tc -PyP group.

Animals were held under the guidelines of the animal care facility (Maastricht University) with unlimited access to food and drinking water. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University conform the Directive 2010/63/EU of the European Parliament.

Laser Doppler Perfusion Imaging

Under standardized conditions, blood flow in the hind limbs of all animals was measured using LDPI (Moor Instruments, United Kingdom). Measurements were performed 4 days prior to the surgical procedure (baseline scan), directly after the surgical procedure and at post-operative days 3, 7 and 14. During the measurements mice were under anesthesia (isoflurane: induction 2,5%; maintenance 1,5%) and placed on a heating pad in a climate controlled chamber (37°C) to ensure minimal variation between flow measurements. The perfusion ratio per time point was calculated by dividing the perfusion of the ligated (ischemic) by the non-ligated (non-ischemic) hind limb. Regions of interest (ROI) were drawn manually around the foot of the ischemic hind limb and the contralateral non-ischemic foot (Figure 1 baseline panel).

Figure 1. A typical representation of perfusion recovery in the mouse hind limb over time as can be seen by LDPI. A representative ROI placement for LDPI is shown in the baseline panel.

SPECT tracers

^{99m}Tc -sestamibi

^{99m}Tc -sestamibi was prepared from a lyophilized sestamibi kit according to manufacturer guidelines (Mallinckrodt Medical, Petten, The Netherlands). Quality controls were

satisfactory for every synthesis (radiochemical purity > 98 %). The activity was $59,8 \pm 21,0$ MBq (mean $\pm$ SD).

^{99m}Tc-pyrophosphate

6 ml of 0.9% NaCl solution was added to the lyophilized PyP-kit (Technescan PYP, Mallinckrodt Medical, Petten, The Netherlands). 150 μ l of the stannous ion containing PyP kit (10 times diluted) was injected through a custom made tail vein catheter in order to reduce red blood cell bound proteins. Fifteen minutes post stannous PyP injection, freshly eluted pertechnetate (activity: $55,5 \pm 19,4$ MBq, mean $\pm$ SD) was injected through the same tail vein catheter. In vivo formed ^{99m}Tc-stannous PyP binds to hydroxyapatite crystals in damaged cells, thereby acting as a molecular marker for cellular damage.

SPECT imaging

Imaging protocols

Mice were anesthetized with isoflurane (induction 2.5 %; maintenance; 1.5 %), a catheter was placed in the tail vein and the animals were positioned in the micro-SPECT camera (MiLabs, Utrecht, the Netherlands). Imaging of the mice in the ^{99m}Tc-sestamibi group and the ^{99m}Tc-pyrophosphate group was performed in 1 time frame of 30 min.

SPECT reconstruction

U-SPECT-II reconstruction software version 2.38 (MiLabs, Utrecht, the Netherlands) was used to reconstruct images^[23]. ^{99m}Tc images were reconstructed by selecting the photopeak (PP) and background (BG) windows. We used a PP window of 126 – 154 KeV and BG windows of 115 – 120 and 190 – 200 KeV.

SPECT quantification

To allow quantification of radiotracer uptake in vivo, a conversion factor (CF) was determined for the 1.0 mm collimator system in a representative phantom (20 ml) for ^{99m}Tc. During the SPECT reconstruction the total amount of counts was distributed over the different voxels, resulting in a counts/voxel expression for the average concentration of counts. Taking into account the voxel volume (Vv), the CF was calculated using equation (a):

$$\text{Conversion Factor (MBq)} = \frac{\text{Activity concentration } \left(\frac{\text{MBq}}{\text{ml}} \right)}{\frac{\text{SPECT counts per voxel}}{\text{Vv (ml)}}} \quad (a)$$

Using this formula we were able to determine the following CF: CF_{Tc} = 640 MBq.

Using the PMOD 2.95 view tool (PMOD Technologies, Zürich, Switzerland) volumes of interest (VOI) were drawn in the lower, upper part of the hind limb (i.e. the area of

collateralization). Each VOI had a volume of 27 mm³. For both the ^{99m}Tc-sestamibi and ^{99m}Tc-PyP images the uptake was measured for each VOI and expressed as mean standardized uptake value (SUV_{mean}) using equation (b).

$$SUV = [SPECT \text{ counts per ml} \times CF](MBq/ml) \times \left[\frac{Body \text{ weight (g)}}{Injected \text{ dose (MBq)}} \right] \quad (b)$$

For body weight we assumed that 1 gram of body weight equalled 1 ml.

Histology

A group of 11 C57Bl/6 mice was used solely for histological purposes at follow up of 0, 3, 7 or 14 days with n=2, 3, 3 and 3 respectively. After euthanasia, tissues were harvested and prepared for histological examination of paraffin sections.

α-Smooth Muscle Actin (αSMA)

Tissue sections (5 μm) were routinely stained for α-Smooth Muscle Actin (αSMA) and CD68. In short, tissue sections for the αSMA staining were deparaffinized in xylene, rehydrated in ethanol and rinsed in distilled water. Endogenous peroxidases were blocked in 0.3% hydrogen peroxide in PBS. Subsequently, slides were incubated overnight with the 1A4 anti-smooth muscle actin antibody (Sigma-Aldrich, Zwijndrecht, Netherlands) in a dilution of 1:3000. On the following day the slides were incubated with the peroxidase conjugated rat anti-mouse secondary antibody (dilution 1:250) and sites of SMA expression were visualized by chromogenic detection via 3,3' diaminobenzidine (DAB) (DAKO, Heverlee, Belgium) in a dilution of 1:50. Finally, after dehydration, slides were mounted using Entellan mounting medium (Merck, Darmstadt, Germany). Photomicrographs were acquired using StereoInvestigator software (MBF Bioscience, Williston, USA) on a BX51WI spinning disk confocal fluorescence microscope (Olympus, Tokyo, Japan) with a QImaging color camera (QImaging, Surrey, Canada). In every tissue specimen, the diameter of 5 collateral arteries was determined using ImageJ (NIH).

CD68

After rehydration, transversal tissue sections were blocked in 0.3% hydrogen peroxide in PBS and 2% bovine serum albumin in PBS/ 0,05% Tween. Nonspecific binding of the avidin/biotin system was prevented using the avidin/biotin blocking kit (Vector Laboratories, Burlingame, USA). Subsequently, slides were incubated overnight with the anti-CD68 antibody (Bio-Connect, Huissen, Netherlands) in a 1:400 dilution. The following day, in successive order, slides were incubated with secondary antibody (1:200 dilution), the avidin/biotin complex (Vectastain ABC kit, Vector Laboratories, Burlingame, USA) (dilution 1:100) and DAB (dilution 1:50) for visualization. Slides were mounted using Entellan mounting medium (Merck, Darmstadt, Germany). Photomicrographs were acquired using

Stereoinvestigator software (MBF Bioscience, Williston, USA) on a BX51WI spinning disk confocal fluorescence microscope (Olympus, Tokyo, Japan) with a Qlcam color camera (QImaging, Surrey, Canada). Counting CD68 positive cells in the peri-muscular fascia was performed using ImageJ (NIH).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

After rehydration, transverse tissue sections were incubated with proteinase K (20 µg/ml in PBS). After washing in PBS, tissue sections were blocked in 20% fetal bovine serum / 3% bovine serum albumin in PBS. Double- and single-stranded DNA breaks were subsequently enzymatically labelled with fluorescein-dUTP using the in situ cell death detection kit (Roche, Mannheim, Germany) according to manufacturer guidelines. After washing in PBS the slides were mounted using glycerol mounting medium with 4',6-diamidino-2-phenylindole (DAPI) and 1,4-diazabicyclo 2,2,2 octane (DABCO) (Abcam, Cambridge, United Kingdom). Photomicrographs were acquired using Leica application suite X software (Leica microsystems, Eindhoven, Netherlands) on a microscope coupled to a computerized morphometry system (Quantimed 570, Leica, Eindhoven, Netherlands). Counting TUNEL positive cells in the peri-muscular fascia was performed using ImageJ (NIH).

Statistics

Data were expressed as averages for each group $\pm$ standard error of the mean (SEM). A student's T-test was performed to test for significance between ischemic and non-ischemic control data. A two way analysis of variance (ANOVA) with a Bonferroni post-test was used to test for significant differences in collateral artery size, CD68 positivity and TUNEL positivity in adductor muscles between ischemic and non-ischemic limbs. $p < 0.05$ was considered statistically significant. Data were analysed in GraphPad (GraphPad software, La Jolla, USA).

RESULTS

LDPI post femoral artery ligation

Figure 1 illustrates the typical representation of perfusion recovery as seen on LDPI. Following femoral artery ligation, average perfusion ratios immediately dropped to ~2% of average baseline ratios. Subsequent perfusion recovery as measured over time by LDPI did not differ between the two groups (Figure 3A and Table 1).

SPECT imaging post femoral artery ligation

To study the perfusion recovery and (clearance of) muscular damage in the total volume of the ischemic versus the contralateral non-ischemic hind limb we performed

Table 1. Overview of the average LDPI ratios ischemic/non-ischemic limb in the ^{99m}Tc-sestamibi and ^{99m}Tc-PyP scanned group per time point. Represented p-values indicate the difference compared to the non-ischemic limb. N represents the number of animals available for analysis.

LDPI			
^{99m} Tc-sestamibi group			
Time point	Ratio ± S.E.M.	P-value	N
Baseline	0.98 ± 0.039	NS	7
Post-operatively	0.02 ± 0.004	p < 0.001	7
Day 3	0.10 ± 0.023	p < 0.001	7
Day 7	0.19 ± 0.035	p < 0.001	7
Day 14	0.22 ± 0.016	p < 0.001	7
^{99m} Tc-PyP group			
Time point	Ratio ± S.E.M.	P-value	N
Baseline	1.00 ± 0.015	NS	6
Post-operatively	0.02 ± 0.002	p < 0.001	6
Day 3	0.10 ± 0.006	p < 0.001	6
Day 7	0.19 ± 0.008	p < 0.001	5
Day 14	0.25 ± 0.022	p < 0.001	5

quantitative SPECT scans on both groups of mice. In Figure 2 the perfusion recovery and muscular damage as seen on SPECT images is illustrated. In the ^{99m}Tc-sestamibi scanned group, average SUVs in the ischemic hind limb dropped significantly compared to the contralateral non-ischemic hind limb directly after ligation (ratio 0.54 with p = 0.007) and remained decreased 3 days post ligation (ratio 0.50 with p = 0.006) (Table 2 and Figure

Table 2. Overview of the average SUVs (± SEM) in the non-ischemic and ischemic hind limb and the average ratio (± SEM) calculated from SPECT scans in the ^{99m}Tc-sestamibi and ^{99m}Tc-PyP group per time point. Represented p-values indicate the difference compared to the non-ischemic limb (NS=non-significant). N represents the number of animals available for analysis.

SPECT					
^{99m} Tc-sestamibi group					
Time point	SUV non-ischemic	SUV ischemic ± S.E.M.	Ratio ± S.E.M.	P-value	N
Baseline	0.30 ± 0.04	0.29 ± 0.04	0.97 ± 0.05	NS	7
Post-operatively	0.27 ± 0.04	0.16 ± 0.04	0.54 ± 0.10	p = 0.007	7
Day 3	0.24 ± 0.04	0.13 ± 0.04	0.50 ± 0.11	p = 0.006	6
Day 7	0.30 ± 0.04	0.28 ± 0.04	0.93 ± 0.10	NS	7
Day 14	0.26 ± 0.04	0.21 ± 0.04	0.81 ± 0.05	p = 0.022	7
^{99m} Tc-PyP group					
Time point	SUV non-ischemic	SUV ischemic	Ratio	P-value	N
Baseline	0.17 ± 0.03	0.15 ± 0.03	0.92 ± 0.04	NS	6
Post-operatively	0.15 ± 0.04	0.35 ± 0.17	3.08 ± 1.50	NS	6
Day 3	0.16 ± 0.04	0.57 ± 0.08	4.93 ± 1.25	p = 0.004	5
Day 7	0.13 ± 0.04	0.42 ± 0.10	3.27 ± 0.19	p = 0.013	5
Day 14	0.16 ± 0.07	0.18 ± 0.06	1.21 ± 0.21	NS	3

Figure 2. A typical representation of perfusion recovery as seen by ^{99m}Tc -sestamibi SPECT and muscular damage as seen by ^{99m}Tc -PyP SPECT. In all images the lower part of the body is displayed in supine position. Image signal intensity is color coded and relative to the injected dose per animal. * Marks the ligated hind limb.

3B). Remarkably, 7 and 14 days post ligation surgery average SUVs indicated nearly full perfusion recovery (Table 2 and Figure 3B).

Average SUVs obtained from ^{99m}Tc -PyP SPECT scans showed a different course, probably reflecting uptake of the tracer by damaged skeletal muscle cells. The uptake of ^{99m}Tc -PyP in the ischemic limb increased drastically upon ligation and peaked 3 days post ligation surgery (ratio 4.93 with $p = 0.004$) (Table 2 and figure 3C). At day 7 post ligation, average SUVs in the ischemic hind limb strongly decreased (ratio 3.27 with $p = 0.013$). The average ratio continued to decrease and reached a value comparable to baseline 14 days post ligation (Table 2 and Figure 3C).

Postmortem analysis of collateral artery size, macrophage infiltration, and DNA fragmentation

To evaluate post femoral artery ligation changes in collateral artery size in harvested adductor muscles we stained for αSMA (Fig. 4A, B). We found a significant increase in mean collateral artery diameter at day 7 and day 14 post ligation while collateral artery diameter of the contralateral control limb remained stable over time (Figure 4C).

Figure #3. Average LDPI ratios (ischemic/non-ischemic) of both groups, $n = 7$ in the ^{99m}Tc -sestamibi group, $n \geq 5$ in the ^{99m}Tc -PyP group (A). Average ratios determined from SUVs calculated in the ^{99m}Tc -sestamibi scanned group with $n \geq 6$ for every time point (B). Average ratios determined from SUVs calculated in the ^{99m}Tc -PyP scanned group with $n \geq 3$ for every time point (C). All data are represented as mean $\pm$ S.E.M. Asterisk indicates significantly different from non-ischemic in the ^{99m}Tc -sestamibi group while # is used for significantly different from non-ischemic in the ^{99m}Tc -PyP group.

To assess (clearance of) muscular damage in the adductor muscle of the ischemic hind limb a CD68 monocyte/macrophage staining was performed (Figure 5A, B). Peak macrophage infiltration in the peri-muscular fascia in the adductor muscle of the ischemic limb was observed at day 3 post ligation with numbers significantly higher compared to the non-ischemic limb. Macrophage infiltration decreased strongly at day 7 after ligation and remained decreased at day 14 after ligation. ANOVA analysis revealed no significant differences in CD68 positivity at day 7 and 14 after ligation between ischemic and non-ischemic limbs (Figure 5C).

To further assess muscular damage in the adductor muscle of the ischemic hind limb we performed a TUNEL staining (Figure 6A, B). The TUNEL positivity in the peri-muscular fascia of the ischemic limb followed a similar trend compared to the ^{99m}Tc -PyP uptake. Post-operatively TUNEL positivity was non-significantly increased in the ischemic limb compared to the control limb, while 3 days after ligation surgery we observed a significant increase in TUNEL positivity ($p < 0.001$) in the ischemic limb. On day 7 and 14, TUNEL positivity decreased again to non-significant levels compared to the control limb (Figure 6C).

Figure 4. Increasing collateral diameter over time in ischemic adductor muscles. Representative pictures of collateral arteries are shown immediately after ligation (A) and at day 14 post ligation (B). Compared to the non-ischemic limb, a significant increase in collateral diameter was found at day 7 and 14 post ligation (C).

Figure 5. Monocyte/macrophage infiltration in the peri-muscular fascia over time. Panel A (non-ischemic) and B (ischemic) show representative images of the CD68 staining 3 days after ligation surgery. Significantly higher macrophage infiltration compared to the non-ischemic limb was found on day 3 ($p < 0.001$) (C). On all other days no significant difference was found.

Figure 6. DNA fragmentation detected by TUNEL staining in ischemic and non-ischemic adductor muscles. Panel A (non-ischemic) and B (ischemic) show representative images of the TUNEL staining 3 days after ligation surgery. Significantly higher TUNEL positivity in the ischemic limb compared to the non-ischemic limb was found on day 3 after ligation ($p < 0.001$) (C). On all other days no significant difference was found.

DISCUSSION

This study demonstrates that perfusion recovery as assessed by $^{99\text{m}}\text{Tc}$ -sestamibi SPECT appears much faster than LDPI data suggest. The therapeutic window in an ischemic hind limb model for the induction of arteriogenesis might therefore be drastically shorter than indicated by standard LDPI analysis.

Perfusion recovery analysis using nuclear imaging has previously been investigated in a hind limb ischemia model in rats^[15] and mice^[13]. While these studies used a single time point to indicate perfusion recovery, they succeeded to show that the scintigraphic enhancement of perfusion was accompanied by morphological changes (histology) and increased vascularity (angiography) indicating ongoing perfusion recovery^[13,15]. In this study, we subjected two groups of mice to LDPI and SPECT scans to assess perfusion recovery at 5 different time points: baseline, post-operatively, day 3, 7 and 14, thereby providing a unique insight into the temporal changes in perfusion recovery throughout the depth of the limb. Using LDPI we found an ongoing recovery in perfusion 14 days post ligation of the femoral artery, while the $^{99\text{m}}\text{Tc}$ -sestamibi SPECT data show nearly complete recovery of blood perfusion 7 days after ligation. Additionally, these findings were supported by postmortem histological analysis. In harvested adductor muscle tissue we found a significantly increased collateral artery diameter at day 7 and 14 post ligation.

LDPI and SPECT measure different physiological parameters. LDPI measures blood velocity whereas ^{99m}Tc -sestamibi SPECT imaging detects steady state distribution of ^{99m}Tc -sestamibi and is therefore a representation of present blood volume and perfusion through uptake of the tracer by living and perfused cells. Hence, regardless of the low flow velocity, perfused collaterals will be detected by ^{99m}Tc -sestamibi SPECT scans while LDPI might overlook or underestimate this perfusion. Moreover, ^{99m}Tc -sestamibi SPECT imaging is able to indicate the perfusion over the entire volume of the hind limb whereas LDPI only reaches a tissue depth of a few hundred micrometers^[8]. The former is obviously preferred as the majority of perfusion recovery occurs subcutaneously.

Additionally, skin and muscle perfusion are differently regulated and perhaps react differently to ligation induced ischemia. Beta-2 (β_2) adrenergic receptors residing on muscular arterioles cause vasodilation in response to sympathetic nervous induced norepinephrine release while alpha-1 and -2 adrenergic receptors on cutaneous arterioles cause vasoconstriction. A close correlation between sympathetic activation (causing norepinephrine release) and myocardial ischemia has already been shown^[24,25]. Considering the discrepancy between the LDPI and SPECT data, it is conceivable that ligation induced ischemia triggers the sympathetic nervous system to release norepinephrine, subsequently causing cutaneous vasoconstriction and thereby creating an apparent delay in reperfusion on LDPI.

The ^{99m}Tc -PyP SPECT data showed peak muscular damage 3 days post ligation and a drastic decrease in uptake 7 days after ligation. 14 days post ligation ^{99m}Tc -PyP SPECT data showed a nearly complete recovery. The increase in uptake of ^{99m}Tc -PyP is likely to come from its binding to hydroxyapatite crystals in damaged myocytes as well as its uptake in macrophages coordinating the clearance of damaged tissue. In support of this theory and our ^{99m}Tc -PyP SPECT data we found peak macrophage infiltration in the peri-muscular fascia in the adductor muscle of the ischemic hind limb at day 3 post ligation. Macrophage numbers continued to decrease after the peak at day 3 in the ischemic hind limb thereby explaining the decrease in uptake of ^{99m}Tc -PyP. The rapid clearance of muscular damage after peak levels on day 3 was further underlined by data obtained from our TUNEL staining. TUNEL positivity in the adductor muscle of the ischemic limb followed a similar trend as the ^{99m}Tc -PyP uptake. We observed a significantly higher TUNEL positivity in the ischemic limb compared to the non-ischemic limb on day 3 after ligation. TUNEL positivity on day 7 and 14 after ligation were non-significantly different compared to the non-ischemic limb. Interestingly, uptake of ^{99m}Tc -sestamibi roughly mirrored ^{99m}Tc -PyP uptake. This indicates that the lowest level of perfusion coincides with the highest level of muscular damage. Furthermore, our data show that the (near) completion of perfusion recovery precedes clearance of damaged myocytes and subsequent muscular recovery.

So far, extremity ischemia and subsequent neovascularization guided perfusion recovery in animal models has often been evaluated by predominantly anatomic (MRI),

invasive (angiography) or limited (LDPI) techniques. Moreover, accurate documentation of tissue ischemia and perfusion recovery by means of histology requires the collection, and often the destruction of tissue samples, thereby providing limited clinical relevance and prohibiting serial monitoring of the biologic processes in living animals^[26]. Nuclear perfusion and myocyte damage imaging might provide a useful and non-invasive alternative to evaluate serial changes in tissue perfusion. The feasibility and accuracy of nuclear perfusion imaging has already been demonstrated by Stacy et al. in a pig model of limb ischemia^[27].

The benefit of perfusion imaging in the clinic using SPECT has already been proven. ^{99m}Tc-sestamibi imaging in patients has revealed improved sensitivity for detecting differences in resting perfusion between the lower extremities of peripheral vascular disease patients with unilateral disease and improved sensitivity compared with Doppler ultrasound for the detection of peripheral vascular disease^[28,29]. Moreover, ^{99m}Tc-sestamibi was also used for the evaluation of regional blood supply of thigh and calf muscles in early stages of atherosclerosis^[30,31]. A reduced stress and rest perfusion of lower limb muscles could be documented in clinically asymptomatic patients with atherosclerotic changes of lower limb vessels. Therefore, this technique could help early detection of the ischemic changes in asymptomatic patients, thereby enabling early intervention and prevention of disease progression.

Furthermore, ^{99m}Tc-PyP has been used to estimate the ischemic skeletal muscle mass in ischemia-reperfusion injury^[32]. In another study, ^{99m}Tc-PyP SPECT was employed to measure the amount of regional skeletal muscle necrosis in patients^[33]. It was speculated that the volume of necrosis determined by this method could predict the clinical outcome.

Our SPECT data shed a new light on studies relying on LDPI results as a read out parameter to indicate a therapeutic effect or window. The LDPI data we obtained largely resemble those reported in other studies^[10,34] and therefore point toward a similar course of perfusion recovery. However, the presented SPECT data, validated with postmortem tissue analysis, indicate recovered perfusion only 7 days post-operatively and clearance of muscular damage 14 days post-operatively.

CONCLUSION

In conclusion, in this study, we showed that LDPI strongly overestimates the created therapeutic window in the ischemic hind limb model. Overestimation of the therapeutic window might lead to misinterpretation of the effect of arteriogenesis stimulating agents or application at the wrong time point as relevant perfusion recovery might have already taken place while LDPI still indicates the need for perfusion improvement. Future studies testing neovascularization stimulating agents in a hind limb model for profound ischemia

should take into account that the window of opportunity is short and that this window can be ascertained using molecular perfusion/myocyte damage tracers like ^{99m}Tc -sestamibi and/or ^{99m}Tc -PyP.

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CHAPTER 5

LEFT VENTRICULAR FUNCTION MEASUREMENTS IN A MOUSE MYOCARDIAL INFARCTION MODEL A COMPARISON BETWEEN 3D-ECHOCARDIOGRAPHY AND ECG-GATED SPECT

Geert Hendrikx
Matthias Bauwens
Roel Wiertz
Felix M. Mottaghy
Mark J. Post

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ABSTRACT

Aim: To assess the accuracy of ECG-gated micro (μ)-SPECT in a mouse myocardial infarction (MI) model in comparison to 3D-echocardiography.

Methods: In a mouse (Swiss mice) MI model we compared the accuracy of technetium-99m sestamibi (^{99m}Tc -sestamibi) myocardial perfusion, electrocardiogram (ECG) gated μ SPECT to 3D-echocardiography in determining left ventricular function. 3D-echocardiography and myocardial perfusion ECG-gated μ SPECT data were acquired in the same animal at baseline ($n = 11$) and 7 ($n = 8$) and 35 ($n = 9$) days post ligation of the left anterior descending coronary artery (LAD). Sham operated mice were used as a control (8, 6 and 7 mice respectively). Additionally, after day 35 μ SPECT scans, hearts were harvested and 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining and autoradiography was performed to determine infarct size.

Results: In both infarcted and sham-operated mice we consistently found comparable values for the end-diastolic volume (EDV), end-systolic volume (ESV) and ejection fraction (EF) obtained by 3D-echocardiography and ECG-gated μ SPECT. Excellent correlations between measurements from 3D-echocardiography and ECG-gated μ SPECT were found for EDV, ESV and EF ($r = 0.9532$, $r = 0.9693$ respectively and $r = 0.9581$) in infarcted mice. Furthermore, comparable infarct size values were found at day 35 post MI by TTC staining and autoradiography ($27,71 \pm 1,80\%$ and $29,20 \pm 1,18\%$ with $p = 0,43$).

Conclusion: We demonstrated that ECG-gated μ SPECT imaging provides reliable left ventricular function measurements in a mouse MI model. Obtained results were comparable to the highly accurate 3D-echocardiography. This, in addition to the opportunity to simultaneously image multiple biological processes during a single acquisition makes μ SPECT imaging a serious option for studying cardiovascular disease in small animals.

INTRODUCTION

Patients suffering from cardiac diseases nowadays benefit from better understanding, diagnosis, new therapy strategies and continuously improving imaging equipment. In vivo cardiac imaging in mouse models for cardiac disease has proven to be essential in this niche of translational medicine. Monitoring left ventricular (LV) function is a key factor in this research. In patients LV function can be measured noninvasively using echocardiography, magnetic resonance imaging (MRI), equilibrium radionuclide angiography, myocardial perfusion electrocardiogram (ECG) gated single photon emission computed tomography (SPECT), positron emission computed tomography (PET)(14). Despite the challenging aspects (i.e. the small heart size and rapid heart rate) of cardiac imaging in the mouse, there are multiple dedicated preclinical imaging modalities available that enable detailed analysis of cardiac function. Successful studies have been reported for MRI(2, 7, 10, 17, 22), micro (μ) CT(3-6, 11), 2 dimensional (2D)(21) and 3-dimensional (3D) echocardiography(10), PET imaging(18) and μ SPECT imaging(8, 9, 23).

While MRI is considered gold-standard for cardiac imaging in the mouse(6, 10) echocardiography has developed into one of the most commonly used techniques to measure murine cardiac function in cardiac research laboratories(19). Especially current echocardiography systems capable of acquiring 3D data are of superior quality compared to 2D echocardiography and have already for more than a decade been reported to approach the accuracy of MRI and histological examination for determining LV parameters and infarct size, respectively(10). As previously reported, a new generation of μ SPECT scanners has been developed, enabling high-resolution functional cardiac imaging(6, 13). These new generation scanners provide clear advantages over echocardiography as quantitative radiotracer distribution revealing multiple biochemical processes can be gathered within one single acquisition. Simultaneously acquiring data on biochemical processes like myocardial perfusion or neovascularization and functional cardiac data is a cost and time effective way to diagnose cardiac disease and to monitor therapy.

Here, we compared technetium-99m sestamibi (^{99m}Tc -sestamibi) myocardial perfusion ECG-gated μ SPECT to routine 3D echocardiography for the assessment of LV function in a mouse model of myocardial infarction (MI). 3D-echocardiography and myocardial perfusion ECG-gated μ SPECT data were acquired in the same animals at baseline and 7 and 35 days post ligation of the left anterior descending coronary artery (LAD). Infarct size was measured 35 days after surgically induced MI by 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining and autoradiography.

MATERIALS AND METHODS

Animal experiments

Animals were held under the guidelines of the animal care facility (Maastricht University) with unrestricted access to food and drinking water. All animal experiments were approved by the Committee for Animal Welfare of the Maastricht University conform the Directive 2010/63/EU of the European Parliament.

A flowchart overview (Fig. 1) shows the number of used Swiss mice per group and technique (i.e. 3D-echocardiography and ECG-gated μ SPECT). Non-interpretable 3D-echocardiography and ECG-gated μ SPECT scans were excluded from analysis. Non-interpretability of ECG-gated μ SPECT images was usually a result of a distorted ECG-signal hampering image reconstruction at true EDV and ESV dimensions. This, together with animal drop-out in the MI group caused a variable number of animals to be available for paired analysis of left ventricular function parameters (Fig. 1 and Table 1). In total 7 mice died in the MI group during the study. Of them, 1 died immediately after the baseline echo (and before the first μ SPECT scan), 5 died within a few days after the MI procedure but before the day 7 scans and 1 died during the day 35 echo. Furthermore, we were unable to perform 1 3D-echocardiography scan in the MI group at day 7 and 2 ECG-gated μ SPECT scans in the MI group at day 7, accounting for the difference in available animals at day 7 and day 35.

Table 1. Number of Swiss mice used in the study.

Procedure	Paired analysis*					
	MI	Sham	MI	Baseline	MI	Baseline
	Baseline		Day 7		Day 35	
3D-echocardiography	11	8	8	6	9	7
ECG-gated μSPECT	11	8	8	6	9	7

MI: myocardial infarction. *Paired analysis was performed on interpretable images from animals that successfully underwent both 3D-echocardiography and ECG-gated μ SPECT at the baseline, day 7 or day 35 time point.

Experimental protocol

All mice were subjected to baseline 3D-echocardiography measurements followed by a baseline ECG-gated SPECT scan 4 days before surgical intervention. Surgical intervention marked the start of the experiment (i.e. day 1). 3D-echocardiography measurements followed by ECG-gated SPECT scans were repeated at day 7 and 35 after surgical intervention.

Left anterior descending coronary artery (LAD) ligation

Experimental MI and sham surgery were performed as previously described(15). In short, after induction of anesthesia (ketamine/medetomidin 50-75 mg/kg + 0,3-1 mg/kg intra-peritoneally) mice were fixed in supine position, intubated and connected to a respiratory pump (210 cycles/min and a tidal volume of 220 µl). The heart was then exposed via an incision in the fourth intercostal space of the left thorax. After opening the pericardium the left ascending coronary artery (LAD) was ligated just proximal to its main bifurcation with a 6-0 Prolene suture. The thorax was closed and the skin was sutured using 5-0 silk sutures. Animals recovered from surgery at 30 °C. Sham-operated animals were subjected to similar surgery, except that no ligature was placed.

3D-echocardiography

Mice were anesthetized with isoflurane (induction 2,5 %; maintenance 1,5 %) and their chest was shaved. Subsequently, the mice were placed in supine position on a heating pad maintaining the body temperature at 37 °C via a rectally inserted feedback thermoprobe. Aquasonic ultrasound gel (Parker Laboratories, Fairfield, USA) was applied to the chest and the cardiac dimensions were measured with the Vevo 2100 echocardiography system (Visual Sonics, Amsterdam, The Netherlands). The endocardial left ventricular dimensions were contoured in a slice by slice fashion in short axis view in Vevo 2100 software (version 1.5.0). The volume of each contoured short axis slice was automatically calculated by the software and summed to determine the total volume. To improve reproducibility end diastolic volume (EDV) and end systolic volume (ESV) were assessed twice by the same observer. The results of both assessments were averaged. Stroke volume (SV) and ejection fractions (EF) were subsequently calculated according to equation 1 and 2.

$$\text{Equation 1: } SV = EDV - ESV$$

$$\text{Equation 2: } EF = \frac{SV}{EDV} \times 100\%$$

ECG-gated µSPECT imaging

ECG-gated µSPECT imaging was performed at baseline and 7 and 35 days after LAD ligation. Mice were anesthetized with isoflurane (induction 2,5 %; maintenance 1,5 %), a tail vein catheter was placed and the mice were transferred to the VECTOR system (MILabs, Utrecht, The Netherlands) fitted with a 0.35 mm multipinhole collimator. The animals were positioned in supine position on a heated bed with integrated ECG monitoring. Throughout µSPECT acquisitions the body temperature was maintained at 37 °C.

Prior to image acquisition, the field of view was centered to the heart and ^{99m}Tc-sestamibi (30 – 240 MBq) was injected via the tail vein catheter in a maximum volume of 200 µl. The large range of injected ^{99m}Tc-sestamibi activity did not influence image quality

as non-interpretable scans were evenly distributed over high and low injected dose scans. Immediately after injection 4 consecutive time frames of 15 minutes each were acquired. The acquired list mode data was reconstructed with MILabs reconstruction software (version 2.51) using the POS-EM algorithm (6 iterations and 16 subsets, reconstructed at a voxel size of 0.4 mm) and retrospective cardiac gating (9 time-bins per cardiac cycle). After image reconstruction the time frames were summed and voxel values were averaged. Subsequent image reorientation and left ventricular volume measurements were performed in the PMOD view tool (PMOD technologies, Zürich, Switzerland). Left ventricular volumes were determined through slice by slice delineation and summation in short axis.

2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) staining

Hearts were excised at day 35 post LAD ligation (n = 13) and day 35 post sham surgery (n = 8), rinsed in PBS solution and sectioned in 1 mm sections using a mouse heart slicer matrix (Zivic instruments, Pittsburgh, USA). Subsequently, the slices were stained with 2 % TTC (Sigma-Aldrich, Zwijndrecht, The Netherlands) in PBS for 10 minutes at 37 °C, rinsed in cold PBS and digital pictures were acquired for quantification of infarct size. Myocardial borders of the left ventricle were traced manually in Leica Qwin Pro software (Leica Microsystems, Zürich, Switzerland) and expressed as a percentage of the total left ventricular area. The infarct size was subsequently calculated as the sum of the infarcted segments in each individual slice and expressed as the percentage of total left ventricular size.

Autoradiography

After TTC staining, heart sections were placed on a glass slide and exposed to a storage phosphor screen. For subsequent phosphor imaging we used the phosphor imaging settings of the Typhoon FLA 7000 laser scanner (GE Healthcare Bio-sciences, Uppsala, Sweden). Myocardial borders of the left ventricle were traced manually in Leica Qwin Pro software (Leica Microsystems, Zürich, Switzerland) and expressed as a percentage of the total left ventricular area. The infarct size was subsequently calculated as the sum of the infarcted segments in each individual slice and expressed as the percentage of total left ventricular size.

Statistics

All data were represented as mean $\pm$ S.E.M. Functional measurements by 3D-echocardiography and ECG-gated SPECT in the same animal were compared using a two-tailed paired t-test and a Bland-Altman plot. Linear regression analysis was performed to compare infarct size measurements by TTC to autoradiography measurements. $p < 0.05$ was considered statistically significant. Data analysis was performed in Graphpad Prism 5 (GraphPad, La Jolla, USA).

RESULTS

Left ventricular function measurements in infarcted and sham operated mice over time

ECG-gated 3D-echocardiography and μ SPECT datasets were acquired in infarcted and sham-operated mice. Left ventricular short and long axis 3D-echocardiography and μ SPECT images acquired in the same animal at day 35 post sham (Fig. 2: column 1 and 2) and MI surgery (Fig. 2: column 3 and 4). Representative 3D-echocardiography images show myocardial dilation and decreased contraction at day 35 post MI while this is not the case in the sham operated animal.

Figure 2. Left ventricular short and long axis 3D-echocardiography and ECG-gated μ SPECT images of EDV and ESV dimensions at day 35 post MI or sham surgery. Representative short (column 1 and 3) and long axis (column 2 and 4) slices of the left ventricle of a sham operated (column 1 and 2) and an infarcted mice (column 3 and 4). In 3D-echocardiography slices, EDV and ESV dimensions are encircled in green. In ECG-gated μ SPECT slices, EDV and ESV dimensions are encircled in bright orange. ^{99m}Tc -sestamibi signal intensity is color coded and relative to the injected dose per animal.

Representative μ SPECT images of the uptake of ^{99m}Tc -sestamibi in viable myocardium at day 35 days post MI are displayed (Fig. 2) as seen in PMOD software. While in the sham operated animal the uptake is visible in the entire myocardium, the uptake is strongly decreased in the antero-lateral part of the myocardium 35 days after MI surgery. Moreover, enlarged EDV and ESV dimensions were observed 35 days post MI.

Images from 3D-echocardiography and ECG-gated μ SPECT were quantified to assess the mechanical function of the heart (Fig. 3A-C, Table 2). As expected in MI, cardiac function continued to decline while left ventricular EDV and ESV dimensions continued to increase during the 35 days post MI. Both imaging modalities showed similar EDV, ESV and resulting EF values over time (Fig. 3 and Table 2). No statistical differences were found between measurements from both modalities.

Figure 3. Comparison of left ventricular parameters over time in infarcted mice as measured using 3D-echocardiography and ECG-gated μ SPECT. End-diastolic volume (EDV), endo-systolic volume (ESV) and Ejection Fraction (EF), were measured at baseline (A), day 7 (B) and day 35 (C) after MI surgery. Mechanical function of the heart after MI continued to decline over the course of 5 weeks. Compared to 3D-echocardiography ($n \geq 8$ at every time point) no significantly different values were found for EDV, ESV and EF values measured with μ SPECT ($n \geq 8$ at every time point). Data are represented as mean $\pm$ S.E.M.

In sham operated mice, cardiac function was stable over time. Again EDV, ESV and EF values measured by both modalities showed similar results (Table 3 and Fig. 4). No statistical differences were found between measurements from both modalities.

Additionally we observed strong linear relationships for EDV, ESV and EF values measured by both imaging modalities ($r = 0.9532$, $r = 0.9693$ and $r = 0.9581$ respectively) (Fig. 4A, C and E). Bland-Altman plots further underlined the good agreement between measurements of both modalities (Fig. 5 B,D and F).

Table 2. Results of left ventricular function measurements in infarcted mice.

Parameter	Mean 3D- echocardiography value ± S.E.M.	Mean ECG-gated μSPECT value ± S.E.M.
Baseline EDV (μl)	101,2 ± 6,6	95,0 ± 6,0
Baseline ESV (μl)	47,5 ± 3,6	43,2 ± 2,9
Baseline EF (%)	53,3 ± 1,4	54,5 ± 1,4
Day 7 EDV (μl)	183,3 ± 12,8	184,2 ± 12,1
Day 7 ESV (μl)	142,7 ± 11,8	139,5 ± 9,8
Day 7 EF (%)	22,6 ± 1,1	24,5 ± 1,2
Day 35 EDV (μl)	224,8 ± 18,4	218,0 ± 25,4
Day 35 ESV (μl)	190,8 ± 16,5	180,9 ± 20,4
Day 35 EF (%)	15,7 ± 1,1	16,9 ± 1,0

Figure 4. Left ventricular parameters over time in sham operated mice. End-diastolic volume (EDV), endo-systolic volume (ESV) and Ejection Fraction (EF), were measured at baseline (A), day 7 (B) and day 35 (C) after sham surgery. Mechanical function of the heart after sham surgery was stable over the course of 5 weeks. Compared to 3D-echocardiography ($n \geq 6$ at every time point) no significantly different values were found for EDV, ESV and EF values measured with μ SPECT ($n \geq 6$ at every time point). Data are represented as mean $\pm$ S.E.M.

Infarct size measurements using TTC staining and autoradiography

Two techniques were used to determine the left ventricular infarct size immediately after the day 35 post MI SPECT scan. Comparable infarct size values were found at day 35 post MI for both techniques ($27,71 \pm 1,80$ and $29,20 \pm 1,18$ with $p = 0,43$ for TTC staining and autoradiography, respectively) (Fig. 6).

Table 3. Results of left ventricular function measurements in sham operated mice.

Parameter	Mean 3D- echocardiography value ± S.E.M.	Mean ECG-gated μSPECT value ± S.E.M.
Baseline EDV (μl)	106,9 ± 5,2	96,5 ± 4,2
Baseline ESV (μl)	47,9 ± 3,2	45,3 ± 2,5
Baseline EF (%)	55,3 ± 1,7	53,0 ± 2,2
Day 7 EDV (μl)	110,1 ± 5,7	100,6 ± 5,0
Day 7 ESV (μl)	52,0 ± 3,1	48,5 ± 3,6
Day 7 EF (%)	52,8 ± 1,3	51,9 ± 1,7
Day 35 EDV (μl)	116,3 ± 4,3	107,1 ± 5,1
Day 35 ESV (μl)	51,8 ± 2,7	47,7 ± 4,1
Day 35 EF (%)	55,6 ± 1,3	55,8 ± 2,2

Figure 5. Correlations between left ventricular parameters measured by 3D-echocardiography and ECG-gated μSPECT. Plots of all EDV (A), ESV (C) and EF (E) values measured at three time points in the MI group by 3D-echocardiography and ECG-gated μSPECT. Bland-Altman plots of all EDV (B), ESV (D) and EF (F) values with limits of agreement shown as dotted lines.

Figure 6. Comparison of TTC-staining and autoradiography infarct size measurements. Similar slices with manually traced left ventricular (LV) area (A,C) and infarct area (B,D) in TTC staining (A, B) and autoradiography (C, D). Average infarct size $\pm$ S.E.M. at day 35 post MI as calculated by both techniques (E). Correlation between infarct size measurements at day 35 post MI and sham surgery by TTC-staining and autoradiography (F).

DISCUSSION

With the introduction of 3D-echocardiography more than a decade ago a highly accurate technique for noninvasive, rapid phenotyping of the mouse heart was developed, replacing conventional 2D-echocardiography which relied on geometric assumptions for calculation of left ventricular mass and volume and fell short in case of calculations on asymmetrically shaped infarcted hearts(10, 14). Currently, 3D-echocardiography is a widely available high-throughput technique, known to approach the accuracy of pre-clinical MRI(10). In this study, we monitored left ventricular function at different time points in a mouse model for MI using 3D-echocardiography. Additionally, on the same day and in the same mice we performed ECG-gated μ SPECT using the perfusion tracer ^{99m}Tc -sestamibi, assuring optimal comparability of the performance of both techniques.

Most studies measuring left ventricular function in the mouse used C57Bl6 mice which are known to have better post MI preservation of cardiac function(21). We opted for Swiss mice in this study as they are known to have better post MI survival rates than C57Bl6 mice in spite of severe cardiac function deterioration(21). The latter was important as we wanted to compare the accuracy of ECG-gated μ SPECT compared to the widely available and highly accurate 3D-echocardiography in a progressively deteriorating infarction model.

Earlier studies, comparing μ SPECT with μ CT(6) and 3D-echocardiography with MRI(10) were conducted in a similar mouse model for MI, albeit in a different mouse strain (C57Bl6). Whereas Dawson et al.(10) reported EF values of approximately 63% at baseline for MRI and 3D-echocardiography, Befera et al.(6) reported EF values of approximately 70% for both ^{99m}Tc -tetrofosmin ECG-gated μ SPECT and iodinated liposomal blood pool agent μ CT at baseline. Other studies using noninvasive imaging to determine left ventricular function also reported baseline EF values that were distinctly higher than the EF values we obtained using 3D-echocardiography and ECG-gated μ SPECT(7-10, 12, 17, 18, 22), although some studies reported values that were within a more comparable range of our baseline EF values obtained by both imaging modalities(1-3, 5, 11, 16, 23). Strain differences may account for the variability in reported EF values post MI as was shown in an earlier study(21).

Baseline EF values we found using ECG-gated μ SPECT were comparable to 3D-echocardiography values in both the MI and sham group ($54,47 \pm 1,44$ versus $53,26 \pm 1,43$ and $52,99 \pm 2,23$ versus $55,27 \pm 1,68$, respectively) (Table 2 and 3). Comparability of EF values, obtained with both modalities, continued to exist 7 and 35 days after MI and sham surgery. Moreover, the EDV and ESV values determined by both modalities were comparable at any time point in the MI and sham group (Table 2 and 3 and Fig. 2 and Fig. 3). The results we show here illustrate the reliability of functional cardiac μ SPECT measurements in healthy and infarcted mouse hearts. This reliability is further emphasized by the strong correlations between all EDV, ESV and EF values obtained by both modalities (Fig. 4). Considering the fact that functional 3D-echocardiography measurements approach the gold-standard for functional cardiac imaging in the mouse (i.e. MRI)(10), current generation μ SPECT scanners have to be regarded as a serious option for functional cardiac imaging in small laboratory animals. It has to be noted that ECG-gated μ SPECT had a higher number of non-interpretable scans compared to 3D-echocardiography in our study. Non-interpretability of ECG-gated μ SPECT images was usually a result of a distorted ECG-signal hampering image reconstruction at true EDV and ESV dimensions. Assuring a stable connection of the ECG leads to the animal throughout the entire scan is important for obtaining a clear ECG signal. Moreover, accurate endocardial contouring in the presence of scar formation (signified here by low uptake of ^{99m}Tc -sestamibi) and high liver uptake requires a trained observer as enlarged left ventricular dimensions in combination with low scar uptake and high liver uptake might easily lead to under- or overestimation

of the volume. For functional left ventricular measurements solely 3D-echocardiography might remain the preferred option due to its relatively low costs and its image acquisition being independent of the injection of a radioactive tracer. However, ECG-gated μ SPECT offers the opportunity to simultaneously monitor multiple biochemical processes through multi-isotope imaging. This enables the assessment of myocardial viability, perfusion and ongoing neovascularization in combination with reliable functional cardiac measurements during a single scan. Especially in therapy studies (both pre-clinical and clinical) directed at improving post infarct cardiac performance, the one-stop shop principle of SPECT imaging will be the better option as it limits the amount of procedures study subjects have to undergo to a single scan while all important data can still be obtained.

The gold standard to measure infarct size is TTC staining(20). At day 35 post MI we compared infarct size measured with TTC staining to autoradiography (i.e. the absence of ^{99m}Tc -sestamibi). Our results demonstrated a good correlation between infarct size values at day 35 post MI and sham surgery measured by both methods (Fig. 5). The infarct sizes values were in the same range as reported earlier(24). Infarct size measurements are operator and strain dependent and may vary per laboratory. Our results, however, indicate that autoradiography on heart sections with ^{99m}Tc -sestamibi is an easy and reliable method to determine the infarct size. Therefore, in studies where ^{99m}Tc -sestamibi is used for perfusion imaging, additional TTC-staining based infarct measurements are redundant.

CONCLUSION

We demonstrated that functional cardiac measurements using ECG-gated μ SPECT imaging agreed well with values measured using the highly accurate 3D-echocardiography. The one-stop shop principle of μ SPECT imaging makes it a serious option for studying cardiovascular disease in small animals. In the near future it might even prove to be the preferred option in both pre-clinical and clinical studies directed at unraveling the complex and multi-factorial nature of cardiovascular disease.

Additionally, we showed that autoradiography imaging of ^{99m}Tc -sestamibi heart slices is a reliable alternative to gold standard TTC staining for infarct size measurements, making additional TTC-staining redundant in studies where ^{99m}Tc -sestamibi is used as a perfusion tracer.

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CHAPTER 6

MOLECULAR IMAGING OF ANGIOGENESIS AFTER MYOCARDIAL INFARCTION BY ^{111}In - DTPA-CNGR AND $^{99\text{m}}\text{Tc}$ -SESTAMIBI DUAL- ISOTOPE MYOCARDIAL SPECT

Geert Hendrikx

Marijke De Saint-Hubert

Ingrid Dijkgraaf

Matthias Bauwens

Kim Douma

Roel Wiertz

Ivo Pooters

Nynke M.S. Van den Akker

Tilman M. Hackeng

Mark J. Post

Felix M. Mottaghy

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ABSTRACT

Background: CD13 is selectively upregulated in angiogenic active endothelium and can serve as a target for molecular imaging tracers to non-invasively visualise angiogenesis in vivo. Non-invasive determination of CD13 expression can potentially be used to monitor treatment response to pro-angiogenic drugs in ischemic heart disease. CD13 binds peptides and proteins through binding to tripeptide asparagine-glycine-arginine (NGR) amino acid residues.

Previous studies using in vivo fluorescence microscopy and magnetic resonance imaging indicated that cNGR tripeptide-based tracers specifically bind to CD13 in angiogenic vasculature at the border zone of the infarcted myocardium.

In this study, the CD13-binding characteristics of an ^{111}In -labeled cyclic NGR peptide (cNGR) were determined. To increase sensitivity, we visualised ^{111}In -DTPA-cNGR in combination with $^{99\text{m}}\text{Tc}$ -sestamibi using dual-isotope SPECT to localize CD13 expression in perfusion deficient regions.

Methods: Myocardial infarction (MI) was induced in Swiss mice by ligation of the left anterior descending coronary artery (LAD). ^{111}In -DTPA-cNGR and $^{99\text{m}}\text{Tc}$ -sestamibi dual-isotope SPECT imaging was performed 7 days post ligation in MI mice and in control mice. In addition, ex vivo SPECT imaging on excised hearts was performed and biodistribution of ^{111}In -DTPA-cNGR was determined using gamma counting. Binding specificity of ^{111}In -DTPA-cNGR to angiogenic active endothelium was determined using the Matrigel model.

Results: Labelling yield of ^{111}In -DTPA-cNGR was 95-98% and did not require further purification. In vivo, ^{111}In -DTPA-cNGR imaging showed a rapid clearance from non-infarcted tissue and a urinary excretion of 82% of the injected dose (I.D.) 2 hours after intravenous injection in MI mice. Specific binding of ^{111}In -DTPA-cNGR was confirmed in the Matrigel model and, moreover, binding was demonstrated in the infarcted myocardium and infarct border zone.

Conclusions: Our newly designed and developed angiogenesis imaging probe ^{111}In -DTPA-cNGR allows simultaneous imaging of CD13 expression and perfusion in the infarcted myocardium and the infarct border zone by dual-isotope micro-SPECT imaging.

BACKGROUND

The formation of new capillaries from existing microvessels occurs as a natural healing process following myocardial infarction (MI) [1]. This process, called angiogenesis, is triggered by ischemia and results in partial restoration of blood perfusion in the ischemic zone. Importantly, the extent of angiogenesis is associated with post-infarct remodelling and has positive implications for the prognosis of MI patients [2]. Several therapy approaches, aimed at stimulating angiogenesis in the infarcted area, including gene therapy, intramyocardial administration of pro-angiogenic factors, administration of bone marrow derived stem cells and transmyocardial revascularization have been tested [3-12]. To date, results in animal models have been encouraging [3-5]. Nonetheless, the clinical translation of these therapies has proven to be difficult and the clinical benefit for MI patients has been disappointing and controversial [6-12].

In order to improve treatment options, assessing the efficacy of pro-angiogenic therapies and monitoring the temporal development of angiogenesis, non-invasive methods (e.g. molecular imaging) are warranted. A sensitive and specific molecular imaging marker for angiogenesis however, is still missing.

Angiogenic vessels demonstrate upregulation of CD13, a membrane-bound aminopeptidase, on activated endothelial cells [13, 14]. The cyclic tripeptide Asn-Gly-Arg (cNGR) homes to CD13 expressed on endothelial cells of angiogenic tumour vasculature and angiogenic vessels in the infarcted area and border zones of the myocardium [14-19]. Moreover, evidence was obtained that cNGR does not bind to CD13 positive macrophages in the infarcted myocardium and infarct border zone. This finding was ascribed to cNGR, possibly targeting a subset of post-translationally modified CD13 that might be specific to smaller and perhaps newly formed vessels [17]. Furthermore, using polymerase chain reaction, peak expression of CD13 was shown 7 days after MI in a mouse myocardial infarction model [15]. Non-invasive imaging of CD13 in mice has further been demonstrated with cNGR-labelled paramagnetic quantum dots for molecular magnetic resonance imaging (MRI) [20]. Although angiogenic activity could selectively and non-invasively be depicted in the infarcted heart, molecular imaging with MRI requires high contrast agent dosage, which hampers translation to clinical trials.

The arginine-glycine-aspartic-acid (RGD) motif is known to label angiogenic active endothelium through binding to $\alpha_v\beta_3$ integrin [21]. In several pre-clinical studies in different neovascularisation models, RGD conjugated agents have been employed [2, 22]. However, competition studies in tumour angiogenesis with the NGR and RGD motif demonstrated a 3-fold higher target homing ratio (tumour/control organ) for NGR than for RGD [16].

In this study, we designed and developed a CD13 molecular imaging probe that allowed sensitive visualization using micro-SPECT imaging. In order to target CD13 we used the cNGR tripeptide, having a 10-fold higher targeting efficacy than the linear entity [23],

to design a non-invasive angiogenesis imaging probe. Thereto, the cNGR tripeptide was conjugated with diethylene triamine pentaacetic acid (DTPA), radiolabeled with ^{111}In and evaluated in a mouse model of myocardial infarction in combination with $^{99\text{m}}\text{Tc}$ -sestamibi in dual-isotope micro-SPECT imaging. In order to determine binding specificity of our newly designed probe we employed the validated subcutaneous Matrigel plug assay as a model for in vivo angiogenesis.

METHODS

cNGR-DTPA synthesis

NAc-Cys(4MeBzl)-Asn(Xanthyl)-Gly-Arg(Tosyl)-Cys(4MeBzl)-Gly-Gly-Lys(2 chloro-Z)-peptide was synthesized by *t*Boc (tert-butyloxycarbonyl) solid-phase peptide synthesis, on 4-methylbenzhydryl amine resin, as described before [15, 24]. After coupling of the last amino acid, the Boc-protecting group was cleaved and the peptide was N^a acetylated (10 mL DMF + 230 μL acetic anhydride + 190 μL pyridine, 2 x 2 min). 0.35 g resin-bound peptide was cleaved and simultaneously deprotected in anhydrous HF (1 h, 0°C) with 4 v-% *p*-cresol as a scavenger and the product was precipitated with ice-cold diethyl ether, collected on a glass filter, eluted from the filter using $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{TFA}$ (50:50:0.1 v/v/v), and lyophilized to give crude material containing NAc-Cys-Asn-Gly-Arg-Cys-Gly-Gly-Lys-NH₂. Subsequently, 31.1 mg crude NAc-Cys-Asn-Gly-Arg-Cys-Gly-Gly-Lys-NH₂ was dissolved in 50 mL 0.1 M TRIS buffer (pH 8), containing 1 M Gn·HCl. The resulting solution was stirred for 3 hours at room temperature to form the internal disulfide bond. Reaction progress was monitored by analytical HPLC and after reaction completion, oxidized NAc-Cys-Asn-Gly-Arg-Cys-Gly-Gly-Lys-NH₂ was purified by semi-preparative HPLC.

Cys-DTPA was coupled to the C-terminal lysine residue of cNGR peptide through a bifunctional succinimidyl ester-maleimide linker, sulfo-SMCC [25]. Therefore, 6.3 mg (7.6 μmol) *cyclic*(NAc-Cys-Asn-Gly-Arg-Cys-Gly-Gly-Lys; cNGR) and 3.5 mg (8.0 μmol ; 1.1 eq) sulfo-SMCC were dissolved in 2 mL 0.2 M phosphate buffer (pH 7.5) containing 6 M Gn·HCl. After 3.5 hours, the reaction mixture was purified by semi-preparative HPLC. Finally, 4.1 mg (4.1 μmol) SMCC-cNGR and 3.5 mg (6.2 μmol ; 1.5 eq) Cys-DTPA were dissolved in 400 μL 6 M Gn·HCl, 0.2 M phosphate buffer (pH 6.5). The resulting solution was stirred at 37 °C and reaction progress was monitored by analytical HPLC. After reaction completion, the reaction mixture was purified by preparative HPLC, resulting in 4.2 mg DTPA-SMCC-cNGR (yield 63.4 %; overall yield 34.3 %).

2.2 Animal studies

Animal studies

MI was induced in 10-12 weeks old male Swiss mice by ligation of the left anterior descending coronary artery (LAD) ($n = 7$), as previously described [26]. The angiogenesis

imaging protocol was performed 7 days after the LAD ligation. A group of non-operated male Swiss mice ($n = 5$) was used as control. A variable number of animals was available for analysis per experiment. For SPECT scans 7 MI mice and 5 control mice were used while for biodistribution purposes ≥ 5 MI mice and ≥ 4 control mice were used. Furthermore, a group of 6 10-12 weeks old male Swiss mice were bilaterally, subcutaneously implanted in the flanks with Matrigel. These Matrigels were supplemented with either the pro-angiogenic human fibroblast growth factor-2 (hFGF2, final concentration in Matrigel: $0.15\mu\text{g/ml}$) or phosphate buffered saline (PBS) solution as a control, to specifically induce the presence of angiogenic active endothelium. All animals were held under the guidelines of the animal care facility (Maastricht University). All animal experiments were approved by the Committee for Animal Welfare of Maastricht University.

Imaging probes

¹¹¹In-DTPA-cNGR

$10\mu\text{g}$ DTPA-cNGR was dissolved in 0.5 ml of NaAc buffer (0.5 M , pH6) and radiolabeled with $^{111}\text{InCl}_3$ ($400\text{--}600\text{ MBq}$, in $\pm 0.2\text{ ml}$ 0.1 M HCl, Mallinckrodt, Petten, The Netherlands) at room temperature for 30 min . Product purity was checked by HPLC (Shimadzu Corporation, Columbia, Maryland, US) using an Aeris WIDEPOR 3.6u XB-C18 column $250\times 4.6\text{ mm}$ (Phenomenex, Utrecht, The Netherlands) eluted with a gradient from 0.1% trifluoroacetic acid (TFA) in water ($0\text{--}3\text{ min}$) to acetonitrile (ACN) over the course of 20 min at a flow rate of 1 ml/min .

^{99m}Tc-sestamibi

^{99m}Tc -sestamibi was prepared with freshly eluted pertechnetate from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator (UltraTechneKow, Mallinckrodt Medical, Petten, The Netherlands). A fraction of the eluate was added to the lyophilized sestamibi-kit (Mallinckrodt Medical, Petten, The Netherlands) and the product was prepared according to manufacturer guidelines. Quality controls were satisfactory for every synthesis (radiochemical purity $> 98\%$).

Micro-SPECT

Imaging protocols

Mice were anesthetized with isoflurane (induction 2.5% ; maintenance; 1.5%), a catheter was placed in the tail vein and the animals were positioned in the micro-SPECT camera (MiLabs, Utrecht, the Netherlands).

SPECT reconstructions

U-SPECT-II reconstruction software version 2.38 (MiLabs, Utrecht, the Netherlands) was used to reconstruct images [27]. ^{99m}Tc and ^{111}In images were reconstructed by selecting the photopeak (PP) and background (BG) windows as indicated in figure 1.

Table 1. Overview of the number of animals that were subjected to different SPECT scans. *6 Swiss mice were implanted bilaterally subcutaneously with Matrigels (hFGF2 supplemented or PBS), hence, 6 pairs of Matrigels were scanned.

Scan	Scan protocol	Injected activity (MBq)	Number of Swiss mice used
In vivo; MI mice	8 consecutive time frames (15 minutes each)	30 – 100 MBq ¹¹¹ In-DTPA-cNGR co-injected with 100 – 150 MBq ^{99m} Tc-sestamibi	7
In vivo; control mice	8 consecutive time frames (15 minutes each)	30 – 100 MBq ¹¹¹ In-DTPA-cNGR co-injected with 100 – 150 MBq ^{99m} Tc-sestamibi	5
Ex vivo; heart slices of MI mice	1 time frame (240 minutes)	-	Out of 7 MI mice, 4 were used for ex vivo scanning purposes
Ex vivo; explanted Matrigels	1 time frame (180 minutes)	90 – 110 MBq ¹¹¹ In-DTPA-cNGR	6*

Figure 1. Energy spectra of ^{99m}Tc, ¹¹¹In and the PP and BG windows used during image reconstruction.

SPECT quantification

To allow quantification of radiotracer uptake in vivo, conversion factors (CFs) were determined for the 0.6 mm collimator system in a representative phantom (20 ml) for ^{99m}Tc and/or ¹¹¹In. During the SPECT reconstruction the total amount of counts was distributed over the different voxels, resulting in a counts/voxel expression for the average concentration of counts. Taking into account the voxel volume (Vv), the CFs are calculated using equation (a):

$$\text{Conversion Factor (MBq)} = \frac{\text{Activity concentration } \left(\frac{\text{MBq}}{\text{ml}}\right)}{\frac{\text{SPECT counts per voxel}}{\text{Vv (ml)}}} \tag{a}$$

Using this formula we were able to determine the following CFs: $CF_{Tc} = 635$ MBq and $CF_{In} = 648$ MBq.

The PMOD 2.95 cardiac tool PCARD (PMOD Technologies, Zürich, Switzerland) was used to segment the heart in the 17-segment model according to the American Heart Association (AHA) guidelines. For both the ^{99m}Tc -sestamibi and ^{111}In -DTPA-cNGR images the uptake was measured for each segment and expressed as mean standardized uptake value (SUV_{mean}) using equation (b).

$$SUV = \left[\text{SPECT counts per voxel} \times \frac{CF}{V_v} \right] (\text{MBq/ml}) \times \left[\frac{\text{Body weight (g)}}{\text{Injected dose (MBq)}} \right] \quad (b)$$

For body weight we assumed that 1 gram of body weight equalled 1 ml.

In order to quantify the absolute uptake of ^{111}In -DTPA-cNGR in the different heart pieces gamma counting was performed using an automated NaI(Tl) gamma counter (Wallac Wizard, Turku, Finland). Additionally, we dissected the vital organs of the mice to study overall biodistribution. The uptake of ^{111}In -DTPA-cNGR was determined 2h post-injection (p.i.) for both MI ($n \geq 5$) and control animals ($n \geq 4$). Data were expressed as percentage of injected dose per gram tissue (%ID/g). Urinal excretion was expressed as %ID.

Following ex vivo SPECT scans, the explanted Matrigels were kept for gamma-counting. Muscular tissue from the hind limb was harvested as a reference. The activity per Matrigel was corrected for ID and weight of the matrigel.

Statistics

Data were expressed as averages for each group $\pm$ standard error of the mean (SEM). To test significance we performed unpaired one-tailed student's T-test and $p < 0.05$ was considered statistically significant. Data were analysed using Microsoft Excel (version 2010).

RESULTS

Chemistry

After cleavage from the solid support with HF, the linear NGR peptide was cyclised by the formation of an internal disulfide bond. This resulted in a mass reduction of 2 by the loss of 2 protons. MALDI-ToF analysis of the linear peptide showed a mass of 835.47 (calculated: 834.97; $C_{30}H_{54}N_{14}O_{10}S_2$ $[M+H]^+$). For the oxidised peptide a mass of 833.30 was observed on ESI-MS (calculated: 832.95; $C_{30}H_{52}N_{14}O_{10}S_2$ $[M+H]^+$). Subsequently, a linker that contains both an NHS-ester and a maleimide reactive group was coupled to the N^ϵ -group of lysine. Finally, the maleimide group was used to couple Cys-DTPA. cNGR-SMCC-DTPA was

obtained in an overall yield of 34.3 %. ESI-MS of cNGR-SMCC-DTPA: m/z $C_{63}H_{102}N_{20}O_{24}S_3$ $[M+H]^+$ calculated monoisotopic mass: 1618.65; found: 1619.72.

Synthesis of ^{111}In -DTPA-cNGR

The labelling yield of ^{111}In -DTPA-cNGR was 95-99% for every synthesis and did not require further purification.

Micro-SPECT imaging and analysis

Transversal tomographic images of the mouse hearts with MI showed decreased uptake of ^{99m}Tc -sestamibi in the anterolateral region of the left ventricle 7 days after LAD occlusion (Figure 2), indicating a perfusion defect. The uptake of ^{111}In -DTPA-cNGR mirrored that of ^{99m}Tc -sestamibi in that the probe was taken up in areas of low perfusion. These uptake patterns were revealed on images of the in vivo scans and even more pronounced on images of the ex vivo scans (Figure 2). In non-infarcted hearts, the uptake of ^{99m}Tc -sestamibi was equally distributed over the whole myocardium while uptake of ^{111}In -DTPA-cNGR was limited (data not shown).

Figure 2. In vivo and ex vivo transversal tomographic images of an infarcted mouse-heart. **a** In vivo images of ^{111}In -DTPA-cNGR uptake, mainly in areas of ^{99m}Tc -sestamibi absence. Numerical values on the scale bars indicate the SUV_{max} and SUV_{min} . **b** Ex vivo images of ^{111}In -DTPA-cNGR uptake, mainly in areas of ^{99m}Tc -sestamibi absence. **c** Polar perfusion maps combined with the 17 segment model show that enhanced ^{111}In -DTPA-cNGR uptake (orange in areas 13, 16 and 17) occurred mainly in areas with low ^{99m}Tc -sestamibi uptake (green in areas 13, 16 and 17), signifying the anterolateral region in the infarcted myocardium.

Standardized uptake values (SUVs) of ^{99m}Tc -sestamibi were significantly decreased in the anterolateral region of the left ventricle in infarcted hearts compared to healthy control

mice (Table 2 and Figure 3a). In MI mice, 6 segments displayed significantly higher ^{111}In -DTPA-cNGR uptake compared to healthy control hearts (Table 1 and Figure 3b), of which 3 had significantly lower $^{99\text{m}}\text{Tc}$ -sestamibi uptake. The 6 segments that displayed significantly enhanced ^{111}In -DTPA-cNGR uptake were all located in the infarcted myocardium or in the infarct border zone.

Table 2. Overview of the SUVs of $^{99\text{m}}\text{Tc}$ -sestamibi and ^{111}In -DTPA-cNGR in healthy control mice (n=5) and MI mice (n=7). Data are displayed as mean $\pm$ S.E.M. Statistical significance was indicated as * ($p < 0.05$).

Segments	Standardized uptake values (SUV)			
	$^{99\text{m}}\text{Tc}$ -sestamibi		^{111}In -DTPA-cNGR	
	Healthy control	Myocardial infarction	Healthy control	Myocardial infarction
1. Basal anterior	1,87 $\pm$ 0.34	1,94 $\pm$ 0.36	0,16 $\pm$ 0.05	0,24 $\pm$ 0.04
2. Basal anteroseptal	1,63 $\pm$ 0.28	1,72 $\pm$ 0.33	0,16 $\pm$ 0.05	0,21 $\pm$ 0.03
3. Basal inferoseptal	1,54 $\pm$ 0.29	1,38 $\pm$ 0.28	0,13 $\pm$ 0.06	0,20 $\pm$ 0.02
4. Basal inferior	1,77 $\pm$ 0.28	1,64 $\pm$ 0.24	0,15 $\pm$ 0.05	0,23 $\pm$ 0.03
5. Basal inferolateral	1,68 $\pm$ 0.30	1,78 $\pm$ 0.31	0,14 $\pm$ 0.04	0,23 $\pm$ 0.03*
6. Basal anterolateral	1,95 $\pm$ 0.33	2,11 $\pm$ 0.39	0,18 $\pm$ 0.06	0,26 $\pm$ 0.04
7. Mid anterior	1,80 $\pm$ 0.30	1,18 $\pm$ 0.26	0,15 $\pm$ 0.05	0,21 $\pm$ 0.02
8. Mid anteroseptal	1,67 $\pm$ 0.28	1,73 $\pm$ 0.27	0,14 $\pm$ 0.06	0,20 $\pm$ 0.02
9. Mid inferoseptal	1,80 $\pm$ 0.34	1,82 $\pm$ 0.30	0,12 $\pm$ 0.06	0,18 $\pm$ 0.03
10. Mid inferior	1,72 $\pm$ 0.28	1,85 $\pm$ 0.31	0,12 $\pm$ 0.05	0,17 $\pm$ 0.03
11. Mid inferolateral	1,71 $\pm$ 0.25	1,60 $\pm$ 0.32	0,11 $\pm$ 0.04	0,20 $\pm$ 0.03
12. Mid anterolateral	2,03 $\pm$ 0.32	1,15 $\pm$ 0.37	0,15 $\pm$ 0.05	0,24 $\pm$ 0.01*
13. Apical anterior	1,64 $\pm$ 0.30	0,49 $\pm$ 0.10*	0,11 $\pm$ 0.03	0,21 $\pm$ 0.02*
14. Apical septal	1,82 $\pm$ 0.34	1,55 $\pm$ 0.25	0,12 $\pm$ 0.04	0,20 $\pm$ 0.03
15. Apical inferior	1,67 $\pm$ 0.28	1,36 $\pm$ 0.35	0,10 $\pm$ 0.03	0,18 $\pm$ 0.03*
16. Apical lateral	1,60 $\pm$ 0.30	0,64 $\pm$ 0.24*	0,14 $\pm$ 0.04	0,22 $\pm$ 0.02*
17. Apex	1,47 $\pm$ 0.34	0,39 $\pm$ 0.17*	0,10 $\pm$ 0.03	0,20 $\pm$ 0.03*

Biodistribution of ^{111}In -DTPA-cNGR

Considerable kidney uptake of ^{111}In -DTPA-cNGR was observed, which was significantly higher in healthy control mice than in MI mice (Figure 4, 4.04 ± 0.41 vs. 2.73 ± 0.42 , $p = 0.04$). The urine (measured as %ID) contained the highest radioactivity. The difference between healthy control and MI mice was not significant (92.5 ± 1.17 vs. 82.29 ± 4.87 , $p = 0.08$). Aspecific uptake in other organs was limited and did not reveal any significant differences between MI mice and healthy control mice (Figure 4).

The uptake of ^{111}In -DTPA-cNGR was significantly higher in the apical region of the infarcted hearts than in non-infarcted hearts (%ID/g of 0.16 ± 0.0542 vs. 0.02 ± 0.0050 , $p = 0.03$). The left ventricular and atrial regions were not significantly different between groups (Figure 5, $p = 0.09$ and $p = 0.07$ respectively). Moreover, uptake of ^{111}In -DTPA-cNGR in healthy myocardium of the left ventricle was not significantly different between MI mice and healthy control mice (Figure 5).

Figure 3. Overview of cardiac 17 segment models for ^{99m}Tc-sestamibi and ¹¹¹In-DTPA-cNGR SUVs in healthy control hearts and MI hearts. **a** Cardiac 17 segment models of healthy control mice and MI mice indicating color-coded average ^{99m}Tc-sestamibi SUVs per segment **b** Cardiac 17 segment models of healthy control mice and MI mice indicating color-coded average ¹¹¹In-DTPA-cNGR SUVs per segment. Statistical significance was indicated as * ($p < 0.05$).

Binding of ¹¹¹In-DTPA-cNGR to angiogenic active endothelium

To visualise specific binding of ¹¹¹In-DTPA-cNGR to angiogenic active endothelium, we bilaterally implanted Matrigel plugs in the flanks of 6 Swiss mice. On the in vivo SPECT images, the hFGF2 supplemented Matrigels were unidentifiable as the kidneys, displaying high uptake, were in too close proximity. Therefore, we explanted each Matrigel and performed ex vivo scans. The mean SUV of explanted hFGF2 supplemented Matrigels was higher compared with controls ($8.238 \times 10^{-4} \pm 5.823 \times 10^{-4}$ vs. $2.2235 \times 10^{-4} \pm 7.001 \times 10^{-5}$, $p = 0.1527$ and ratio 3.7 supplemented vs. non-supplemented) (see Additional file 1). Moreover, gamma counter data of explanted Matrigels indicated a higher uptake of ¹¹¹In-DTPA-cNGR between hFGF2 supplemented and control Matrigels ($p = 0.0748$). Compared with harvested control muscle tissue a significantly higher uptake of ¹¹¹In-DTPA-cNGR was noticed ($p = 0.0497$) in hFGF2 supplemented Matrigels (Figure 6).

Figure 4. Comparison of ¹¹¹In-DTPA-cNGR uptake in different organs between MI (n ≥ 5) and healthy control mice (n ≥ 4). Significantly higher uptake of ¹¹¹In-DTPA-cNGR was observed in kidneys of healthy control mice compared to MI mice (p = 0.04). Statistical significance was indicated as * (p < 0.05).

Figure 5. Uptake of ¹¹¹In-DTPA-cNGR was significantly higher in the apical region of infarcted hearts compared to healthy control hearts. Uptake of ¹¹¹In-DTPA-cNGR was not significantly different in other regions of the myocardium.

Figure 6. Uptake of ^{111}In -DTPA-cNGR was higher in hFGF2 supplemented Matrigels compared with controls. The uptake of ^{111}In -DTPA-cNGR in hFGF2 supplemented Matrigels was significantly higher compared with control muscle tissue while uptake of ^{111}In -DTPA-cNGR in control Matrigels was non-significantly different compared to control muscle tissue. Data are displayed as mean $\pm$ S.E.M. Statistical significance was indicated as * ($p < 0.05$).

DISCUSSION

Angiogenesis is associated with post-infarct remodelling and has important implications for prognosis following MI [2]. While previous research has mainly focused on developing pro-angiogenic therapies to stimulate angiogenesis in the infarcted myocardium [28, 29], a clinically applicable method for the evaluation of these therapies is still lacking. Most clinical studies thus far have relied on indirect evidence of myocardial angiogenesis. For example, changes in stress-induced ischemia and myocardial perfusion were assessed by treadmill exercise testing and dual-isotope gated SPECT thallium-201/99mTc-sestamibi perfusion SPECT imaging at baseline, day 29, day 57 and day 180 after administration of the pro-angiogenic recombinant-fibroblast growth factor 2 (rFGF2) in a clinical study [9]. In another study, using a single intracoronary infusion of rFGF2, patients experienced increased quality of life (assessed by Seattle Angina Questionnaire) and exercise tolerance (assessed by treadmill exercise testing). Moreover, in this study MRI demonstrated increased regional wall thickening and a reduction in the extent of the ischemic area compared to baseline [30].

A clinical trial was performed in which patients with exertional angina were treated with the pro-angiogenic recombinant human vascular endothelial growth factor (rhVEGF) [31]. In this trial, no improvement in exercise treadmill test time or myocardial perfusion, as assessed by rest-stress thallium-201/99mTc-sestamibi (gated) SPECT scanning protocol, was detected. However, patients experienced a significant improvement in angina class at day 120 after the first administration of rhVEGF [31]. Furthermore, in a small phase 1 clinical study using VEGF gene transfer, all patients had significant reduction in angina and objective evidence of reduced ischemia was documented using dobutamine 99mTc-sestamibi SPECT imaging. Postoperative left ventricular ejection fraction (LVEF) was either unchanged or improved. Moreover, the Rentrop scoring system, indicating the coronary collateral filling as assessed by coronary angiography, improved in all patients [32].

The pro-angiogenic effect of transmyocardial laser revascularisation (TMLR) was assessed in a small clinical study (7 patients) by Rimoldi et al. While angina scores (Canadian Cardiovascular Society) decreased significantly, exercise tolerance (treadmill testing), 12 minute walk distance and the LVEF (assessed by radionuclide ventriculography) remained unchanged. Moreover, despite subjective improvement in some patients, myocardial blood-flow (as assessed by 15O-labelled water) during dobutamine in lasered segments failed to show any significant increase [33].

Clearly, improved myocardial perfusion or function can be used as indirect evidence for angiogenesis in the infarcted myocardium. Reduced angina scores, increased treadmill exercise times and improved myocardial perfusion as measured by angiography, SPECT, PET and MRI all seem to indicate recovery [34]. However, despite the ability of these methods to indicate recovery it often takes several months before any improvement can be detected. Therefore, these methods, used in current MI patient care, lack the sensitivity to monitor, or predict, pro-angiogenic treatment. To make direct angiogenesis imaging available for MI patient care, sensitive and specific non-invasive direct angiogenesis imaging probes are required.

To our knowledge this is the first time CD13 expression was visualised with a nuclear cNGR probe in a mouse model of MI using dual-isotope micro-SPECT imaging. Simultaneous perfusion-imaging enabled us to visualise the angiogenesis process in the border zone of the infarct and the infarcted tissue itself. According to previous studies from our lab, angiogenesis is expected to occur in the infarct area and border zone of the infarct [15, 20]. Additionally, in co-localisation experiments using the fluorescently labelled endothelial cell marker anti-CD31-PE, the fluorescently labelled angiogenic active endothelial cell marker anti-CD105-FITC and fluorescently labelled cNGR conjugates, evidence for preferential binding of cNGR conjugates to angiogenic active endothelial cells of capillaries and larger vessels with an inner diameter up to 15 μm was provided [15, 20]. Moreover, despite the abundant presence of CD13-positive macrophages, fluorescently labelled cNGR did not bind to anti-CD13-FITC labelled macrophages [15]. In the current

study, we used the exact same animal model and the same cNGR moiety, assuring identical experimental conditions. Extrapolating from these data, we therefore consider binding of our ^{111}In -DTPA-cNGR imaging probe to be specific for angiogenic endothelium.

Specific binding of our probe is further emphasised by enhanced binding of ^{111}In -DTPA-cNGR to angiogenic active endothelium in a specific model for angiogenesis. Unfortunately (hFGF2 supplemented) Matrigels, implanted in the flanks for optimal stimulation of angiogenesis, were unrecognisable on in vivo SPECT scans as high renal uptake hampered image analysis. Therefore we explanted the Matrigels and performed ex-vivo SPECT scans that indicated higher uptake of ^{111}In -DTPA-cNGR in hFGF2 supplemented Matrigels compared with control Matrigels. Moreover, the uptake of ^{111}In -DTPA-cNGR in hFGF2 supplemented Matrigels was higher compared with control Matrigels as indicated by gamma-counting. hFGF2 supplemented Matrigels displayed significantly higher uptake than control muscle tissue, indicating active angiogenesis.

To use this imaging probe in a clinical setting the probe should display a high specific target uptake and fast clearance from non-specific organs. The biodistribution characteristics of our probe were studied 2h post injection and demonstrated a rapid clearance in the urine which is a favourable clearance pathway. This is in contrast with results for the hydrophilic RGD probe, mainly studied for tumour angiogenesis imaging, that demonstrates unfavourable binding to kidneys, spleen, lungs, liver and gastrointestinal system thereby hampering the image quality [35].

We simultaneously imaged and quantified cardiac uptake of $^{99\text{m}}\text{Tc}$ and ^{111}In in the AHA 17 segment model. The isotope uptake per segment was quantified using isotope specific CFs, assessed in phantom studies. Furthermore, isotope uptake was expressed in the clinically used SUV parameter, which normalises for injected dose and body weight. Although normalisation for body weight might be considered irrelevant for a rapidly excreted tracer that does not reach a uniform distribution throughout the body, we believe that the body weight bias in our quantification is limited due to the fact that all the animals used in this study were of similar age and body weight.

We found target-specific binding of our new angiogenesis imaging probe as evidenced by the significantly enhanced SUVs in 6 segments in the infarcted myocardium and the infarct border zone. To even further improve dual-isotope micro-SPECT imaging of perfusion defects and mirroring ^{111}In -DTPA-cNGR uptake in the hearts of MI mice we are currently pursuing strategies to further enhance probe-target interaction through multivalent binding which may result in higher image quality. The proof of principle of this approach has already been demonstrated in studies from Li et al. and Dijkgraaf et al, in which tumour uptake increased with increasing RGD valency [35, 36].

Despite displaying enhanced, specific uptake in the infarcted myocardium and infarct border zone, we observed several remarkable and so far inexplicable features of our probe. We found the overall uptake to be higher in every section of the infarcted hearts

compared with healthy control hearts. This may be due to enhanced post-infarct overall stress in the heart, but we cannot confirm such a finding at this moment. Moreover, kidney uptake was significantly higher in healthy control mice compared to MI mice. Although we currently have no direct link between the infarct and these findings, these discoveries we made using SPECT scans were overlooked in earlier studies employing two-photon microscopy an MRI and may be of considerable importance for future studies employing the cNGR molecule as a molecular angiogenesis imaging probe.

In this study we opted for ^{111}In due to the fact that pre-clinical SPECT scanners outclass pre-clinical PET scanner in terms of spatial resolution. Of course the use of ^{111}In in a clinical situation may be less favourable due to its long physical half-life of 2.8 days and resolution limitations. While pre-clinical SPECT scanners outclass pre-clinical PET scanners in terms of spatial resolution, in the clinical setting this is reversed. Therefore, angiogenesis imaging via the cNGR tripeptide in MI patients would benefit from the use of a clinical PET system. Future research should therefore focus on the development of a high resolution cNGR-based PET imaging probe, for example by replacing ^{111}In by the PET isotope ^{68}Ga .

In conclusion, in this study we designed and developed a molecular angiogenesis imaging probe that allowed us to sensitively visualise CD13 in the border zone of infarcted myocardium and infarcted myocardium itself using dual-isotope SPECT imaging. Analysis of acquired SPECT images in the AHA 17 segment model revealed target-specific uptake of our new angiogenesis imaging probe in areas of decreased perfusion. Furthermore, we noticed rapid clearance via the urinary tract and low uptake in non-specific organs. However, for the cNGR tripeptide to be considered as a clinical angiogenesis imaging tool, specific target uptake has to increase. Therefore, we are currently pursuing a multi-valent cNGR motif with supposed longer half-life and higher target affinity.

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SUPPLEMENTAL DATA

Supplemental Figure 1. The SUVs of ^{111}In -DTPA-cNGR in ex vivo scanned Matrigels. The mean SUV of explanted hFGF2 supplemented Matrigels was higher compared to controls ($8.238 \times 10^{-4} \pm 5.823 \times 10^{-4}$ vs. $2.2235 \times 10^{-4} \pm 7.001 \times 10^{-5}$, $p = 0,1527$ and ratio 3.7 supplemented vs. non-supplemented).

CHAPTER 7

USE OF CYCLIC BACKBONE NGR-BASED SPECT TO INCREASE EFFICACY OF POST MYOCARDIAL INFARCTION ANGIOGENESIS IMAGING

Geert Hendrikx

Rick van Gorp

Matthias Bauwens

Tilman M. Hackeng

Felix M. Mottaghy

Mark J. Post

Ingrid Dijkgraaf

Submitted

ABSTRACT

Purpose: To improve the efficacy of a cyclic Asn-Gly-Arg (cNGR)-based imaging probe for noninvasive SPECT imaging of angiogenesis.

Methods: The CD13 targeting cNGR moiety was synthesized and cyclized by Native Chemical Ligation (NCL) instead of disulfide bridging, leading to a cyclic peptide backbone: cyclo NGR (coNGR). Beside this new monomeric coNGR, we designed and synthesized a tetrameric imaging agent: tetrameric coNGR. Both imaging agents carried a diethylene triamine pentaacetic acid (DTPA) chelator enabling radiolabeling with the SPECT radionuclide Indium-111 (^{111}In). Using the perfusion tracer $^{99\text{m}}\text{Tc}$ -sestamibi we performed dual isotope SPECT imaging in myocardial infarction (MI) and sham operated Swiss mice 7 days after surgical intervention. Myocardial uptake was examined in the cardiac 17 segment model and compared to a previously tested cNGR-based SPECT imaging agent for angiogenesis. CD13 immunohistochemistry was performed to validate cNGR uptake patterns. Moreover, blood stability was examined using HPLC and biodistribution patterns were examined using gamma counting.

Results: Labeling yield of coNGR and tetrameric coNGR exceeded 90% and did not require further purification. Both coNGR-based imaging agents displayed considerably higher standardized uptake values (SUVs) in and around the infarcted areas (signified by decreased $^{99\text{m}}\text{Tc}$ -sestamibi uptake) but also in the non-infarcted myocardium compared to the previously tested disulfide-cyclized cNGR imaging agent. These uptake patterns colocalized with CD13 immunopositivity on excised hearts. Blood stability tests indicated better stability for both novel imaging agents after 50 min blood incubation compared to the disulfide-bonded cNGR imaging agent. One hour after injection, the tracers had cleared from remote organs.

Conclusions: The new angiogenesis SPECT imaging agents, monomeric coNGR and tetrameric coNGR, with their backbone cyclized by NCL, displayed a significantly higher uptake in the infarcted myocardium compared to a previously tested disulfide-cyclized cNGR imaging agent and might allow for sensitive imaging of angiogenesis in the infarcted myocardium.

INTRODUCTION

Angiogenesis is an endogenous healing process which serves to restore tissue blood supply in response to ischemic injury[1]. The extent of angiogenic activity is correlated with infarct healing and post myocardial infarction (MI) remodeling[2]. Augmentation of experimental angiogenesis has several beneficial effects on post MI remodeling, including reduced apoptosis of hypertrophied cardiomyocytes in the border zone, attenuated collagen deposition and scar formation in the non-infarcted zone and improved long-term ventricular function[2, 3]. However, while positive results were shown in animal models of MI, the benefit for MI patients has yet to be shown. So far, results from double-blind placebo-controlled trials were disappointing[4-7]. These negative outcomes were most likely attributable to a combination of factors including patient selection, choice of delivery platforms for therapeutic agents and, importantly, lack of sensitive clinical detection methods for angiogenesis[8].

Frequently employed imaging agents for non-invasive nuclear imaging of cardiovascular angiogenesis in animal models are based on the $\alpha_v\beta_3$ integrin targeting L-Arg-Gly-L-Asp acid (RGD) motif and vascular endothelial growth factor (VEGF)[9]. Several single-photon emission computed tomography (SPECT) and positron emission tomography (PET) based studies reported positive results (reviewed in [10, 11]). However, in competition studies the RGD motif was found to have a lower target homing ratio (target to control tissue) compared to the cyclic-NGR (cNGR) motif which binds to selectively upregulated CD13 on angiogenically active endothelial cells[12]. Given the high target homing ratio of the NGR motif, employing a cNGR based imaging agent might result in better image quality. In our institute, the CD13 targeting cNGR motif has been explored as a molecular angiogenesis imaging agent for fluorescence microscopy[13], magnetic resonance imaging (MRI)[14], and more recently for single photon emission computed tomography (SPECT) imaging[15].

In a mouse myocardial infarction (MI), enhanced uptake was found 7 days post infarction in the infarct and infarct border zone for all of the cNGR-based imaging agents[13-15]. Although each study established specific binding of their respective cNGR-based imaging agent in the infarct area and infarct border-zone, none of these approaches had the potential for clinical translation. While localizing the fluorescently labeled cNGR imaging agent would require an invasive procedure, the cadmium-selenium core of the cNGR labeled paramagnetic quantum dots (pQD) in combination with their accumulation in the liver and spleen would hamper translation of the MRI angiogenesis imaging agent into clinical trials. Furthermore, the target specific uptake of the In-111 (^{111}In) labeled diethylene triamine pentaacetic acid (DTPA)-cNGR (^{111}In -DTPA-cNGR) was rather low[15].

In this study, we investigated the potential of two modified versions of the previous SPECT imaging agent for angiogenesis. Whereas the ring structure of the cNGR peptide within our former SPECT imaging agent for angiogenesis was cyclized using a disulfide-

bond (henceforth called “cNGR”), we now cyclized the ring structure with a peptide bond by using Native Chemical Ligation (NCL) resulting in a new monomeric tracer: DTPA-cyclo(CNGRG) (“coNGR”). Theoretically this would lead to a more stable molecule and higher target uptake in vivo. Furthermore, we were able to couple four coNGR peptide moieties to a DTPA chelator thereby creating a tetrameric coNGR imaging agent: DTPA-[cyclo(CNGRG)]₄ (“tetrameric coNGR”). This multimerization approach was previously shown to be successful for the RGD peptides [16, 17]. Using dual-isotope SPECT imaging, these new angiogenesis imaging agents were tested in a mouse model for MI in combination with the perfusion tracer ^{99m}Tc-sestamibi (Technescan sestamibi).

MATERIALS AND METHODS

Synthesis of coNGR and tetrameric coNGR

Synthesis of linear H-[Cys¹-Asn²-Gly³-Arg⁴-Gly⁵]-MpaL-NH₂

Linear CNGRG-MpaL thioester peptide was synthesized by manual solid-phase peptide synthesis on Methylbenzhydrylamine (MBHA)-polystyrene resin (ChemPep (Wellington, FL, USA; 0.2–0.4 mmol scale) using the in situ neutralization/activation procedure for Boc-/Bzl peptide synthesis as described previously [18, 19]. Instead of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 2-(6-Chloro-1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HCTU, Peptides International (Louisville, KY, USA) was used as coupling reagent. The peptide was cleaved from the resin by treatment with anhydrous HF (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 0 °C, using 4 v-% *p*-cresol (Sigma-Aldrich) as a scavenger. The crude product was analyzed on a Waters™ (Milford, MA, USA, Etten-Leur, The Netherlands) ultra-high performance liquid chromatography mass spectrometry (UPLC-MS) XEVO-G2QToF system. After lyophilization, the peptide was purified by semi-preparative HPLC using Vydac C₁₈ HPLC columns (10 mm x 250 mm, 12 mL/min flow rate or 22 mm x 250 mm, 20 mL/min flow rate) connected to a Waters Deltaprep System consisting of a Waters Prep LC Controller and a Waters 2487 Dual wavelength Absorbance Detector (λ = 214 nm). To elute the peptide, an appropriate gradient of buffer B in buffer A, where buffer A is 0.1% trifluoroacetic acid (TFA) in H₂O/CH₃CN (95/5, v/v) and buffer B is 0.1% TFA in CH₃CN/H₂O (90/10, v/v), was used. Fractions containing the desired product were identified by UPLC-MS, pooled, and lyophilized.

Cyclization of H-[C-N-G-R-G]-MpaL-NH₂ by NCL

For cyclization, the peptide was dissolved in 50 mM (NH₄)₂CO₃ (pH 7.80) at a maximum concentration of 1 mg/mL. The reaction was performed at 37 °C and was followed over time by UPLC-MS analysis. Generally, cyclization was complete after 1 h. After cyclization, the peptide was purified as described above.

Tetrameric coNGR synthesis

To synthesize a tetrameric cyclic NGR peptide, a scaffold peptide with 4 NGR-coupling sites was necessary. Therefore, a lysine wedge was synthesized on MBHA resin (0.2 mmol scale). First, Boc-Lys(Fmoc)-OH (Fmoc = 9-fluorenylmethoxycarbonyl) was coupled to the solid support, followed by coupling of two times Boc-Lys(Boc)-OH. After chain assembly of the N^{α} -Boc protected lysine wedge, 313 mg peptidyl-resin was treated with piperidine 20 v-% in DMF (4x3 min) for N^{ϵ} -Fmoc group removal. Then, Boc-thiazolidine-4-carboxylic acid (thiaproline; 259 mg) was coupled using 2 mL 0.5 M HCTU and 400 μ L N,N -Diisopropylethylamine (DiPEA). After treatment of the peptidyl-resin with TFA (2 x 1 min) for N^{α} -Boc-deprotection of the 4 lysine residues and coupling of succinimidyl 4-(N -maleimidomethyl)cyclohexane-1-carboxylate (SMCC; 1.47 g in 6 mL DMF), the peptidyl-resin was cleaved from the resin using HF as described above. Subsequently, the crude Lys(Thz)-(Lys)₂-(SMCC)₄ product was purified on semi-preparative RP-HPLC and fractions containing the desired product were identified by UPLC-MS, pooled, and lyophilized as described above. As the cyclo-(CNGRG) peptide is cyclized via NCL, a free thiol group is available for coupling to the tetra-maleimide-functionalized lysine-wedge, Lys(Thz)-(Lys)₂-(SMCC)₄. For synthesis of the tetrameric c(CNGRG) peptide, 5.3 mg c(CNGRG) and 1.7 mg of wedge were dissolved in 1 mL 0.1 M phosphate buffer pH 6.5, containing 6 M GuHCl. After 3 h at 37 °C, the reaction mixture was purified on semi-preparative HPLC as described above.

Maleimide-DTPA coupling

Finally, maleimide-Diethylenetriaminepentaacetic acid (DTPA) was coupled to coNGR and tetrameric coNGR. Therefore, maleimide-DTPA (1.5 eq) and coNGR or coNGRG₄ were dissolved in 0.1 acetate pH 4, MeONH₂, EDTA, 6 M GuHCl. Both reactions were performed at 37 °C and followed on UPLC-MS. After reaction completion, the reaction mixture was purified on semi-preparative HPLC.

Animal studies

In 10-12 week old male Swiss mice we induced MI (n = 8) by ligation of the left anterior descending coronary artery (LAD) as described before[20] to test the tetrameric coNGR imaging agent. A group of sham operated Swiss mice (n = 6) was used as control. Furthermore, we used a group of 5 Swiss mice to test the covalent-cNGR imaging agent. SPECT imaging was performed 7 days after MI or sham surgery. An overview of the number of animals used per imaging agent is given in Table 1. All animals were held under the guidelines of the animal care facility (Maastricht University). All animal experiments were approved by the Committee for Animal Welfare of Maastricht University.

Table 1. Overview of the number of animals used per imaging agent. The injected activity is displayed as mean $\pm$ S.E.M. *A variable number of animals was available for biodistribution experiments.

Procedure	Angiogenesis tracer	Injected dose (MBq)	^{99m} Tc-sestamibi (MBq)	SPECT	Bio-distribution
MI	Tetrameric coNGR	38,5 $\pm$ 1,3	28,6 $\pm$ 5,6	N = 8	N = 8
Sham	Tetrameric coNGR	47,3 $\pm$ 2,2	56,8 $\pm$ 8,2	N = 6	N $\geq$ 5*
MI	coNGR	57,4 $\pm$ 0,9	48,0 $\pm$ 2,0	N = 5	N $\geq$ 3*

Imaging probes

¹¹¹In- coNGR and ¹¹¹In-tetrameric coNGR

Thirty micrograms of coNGR or tetrameric coNGR was dissolved in 0.5 ml of NaAc buffer (2.0 M, pH6) and radiolabeled with ¹¹¹InCl₃ (400 – 600 MBq in $\pm$ 0.5 ml 0.1 M HCl, Mallinckrodt, Petten, The Netherlands) at room temperature for 30 min. Product purity was checked by HPLC (Shimadzu Corporation, Columbia, MD, USA) using an Aeris WIDEPOR 3.6u XB-C18 column 250 x 4.6 mm (Phenomenex, Utrecht, The Netherlands) eluted with a gradient from 0.1% trifluoroacetic acid in water (0 to 3 min) to acetonitrile over the course of 20 min at a flow rate of 1 ml/minute. Product purity was over 90% every time (HPLC chromatograms are displayed in Supplemental Figure 1).

Stability tests

Both compounds were separately added to 1 x 10 ml human blood in heparin. Blood stability was subsequently tested over 50 min at the following time intervals: 0, 10, 30, and 50 min. For each time point blood plasma was separated from proteins and analyzed by HPLC using the above mentioned method. Results of the stability tests are displayed in Supplemental Table 2.

^{99m}Tc-sestamibi

Freshly prepared ^{99m}Tc-sestamibi (Technescan sestamibi) was ordered from GE Healthcare (Eindhoven, The Netherlands).

Micro SPECT imaging

Mice were anesthetized with isoflurane (induction 2.5%; maintenance 1.5%), a catheter was placed in a tail vein and animals were positioned in the SPECT camera (MILabs, Utrecht, The Netherlands). Prior to image acquisition, a bolus injection of ^{99m}Tc-sestamibi and the angiogenesis imaging agent was given in a maximal volume of 200 μ l. Table 1 provides an overview of the average injected dose. Immediately after injection, 4 consecutive time frames of 15 min each were acquired. For image quantification we used the last of these frames.

SPECT image reconstruction

Acquired list mode data was reconstructed using MILabs reconstruction software (version 2.51) employing the POS-EM algorithm (6 iterations and 16 subsets, reconstructed at a voxel size of 0.4 mm). ^{99m}Tc and ^{111}In images were reconstructed by selecting photopeak and background energy levels as described before[15].

Image quantification

To allow quantification of imaging agent uptake, in vivo isotope-specific conversion factors (CF) were determined for the 0.6 mm collimator in a representative phantom with a known activity. Using a previously described method[15] we found the following conversion factors (CF): $\text{CF}_{^{99m}\text{Tc}}$: 612 MBq/ml and $\text{CF}_{^{111}\text{In}}$: 643 MBq/ml.

The PMOD 3.7 cardiac tool PCARD (PMOD technologies, Zürich, Switzerland) was used to segment the heart in the 17 segment model. The uptake per segment was subsequently expressed as a mean Standardized Uptake Value (SUV_{mean}), also using a previously described method[15]. For body weight we assumed that 1 g equaled 1 ml.

Biodistribution

After imaging, the vital organs were harvested and kept for gamma counting (Wallac Wizard, Turku, Finland). Acquired data were expressed as percentage injected dose per gram tissue (%I.D./g).

CD13 immunohistological staining

Hearts were dissected and fixated in HEPES-buffered formaldehyde containing 150 mM saline for 24 h at 4°C. Hereafter hearts were placed in 70% ethanol for one month before embedding in paraffin. Paraffin-embedded hearts were cut at 4 μm thickness. Sections were deparaffinized and rehydrated after which endogenous peroxidase was blocked by 0.3% H_2O_2 in methanol. After antigen retrieval (DAKO, target retrieval solution) sections were blocked in 5% goat serum for one hour. Next, sections were incubated overnight with primary monoclonal antibody against CD13 (1:500, Sigma). After washing, sections were incubated with BrightVision poly horseradish peroxidase (HRP) Goat anti-Rabbit antibody (immunologic). HRP was visualized by NOVARED substrate kit (VECTOR Laboratories Inc., Burlingame, CA, USA) and sections were counterstained with hematoxyline. Finally, sections were covered by cover glass with entellan and visualized using Leica Application Suite X (Leica Microsystems, Wetzlar, Germany).

Statistics

All data were expressed as mean $\pm$ S.E.M. To test for significant differences we performed an unpaired student's t-test with $p < 0.05$ being considered statistically significant. Data were analyzed using Microsoft Excel (version 2010).

RESULTS

Chemistry

Analytical mass data of linear CNGRG-MpaL, cyclic(CNGRG) (coNGR), Lys(Thz)-(Lys)₂-(SMCC)₄, DTPA-c(CNGRG) (coNGR), and DTPA-[c(CNGRG)]₄ (tetrameric coNGR; co(NGR)₄) are given in Table 2. Analytical HPLC chromatograms and mass spectrometry data are given as Supplementary Data. All masses of the constructs fell within the range of monoisotopic and average theoretical masses.

Table 2. Analytical mass data of the cNGR-based tracer constructs.

Compound	Monoisotopic mass	Molecular weight	Measured
CNGRG-MpaL	705.31	705.81	705.32
coNGR	487.20	487.54	487.22
Lys(Thz)-(Lys) ₂ -(SMCC) ₄	1520.77	1521.84	1520.83
DTPA-coNGR	1170.50	1171.25	1170.49
DTPA-co(NGR) ₄	4139.86	4142.70	4140.76

SPECT imaging and analysis of the coNGR imaging agent uptake

Using the perfusion tracer ^{99m}Tc-sestamibi we were able to visualize the infarcted myocardium 7 days after LAD ligation. In order to determine tracer uptake we used the 17 segment model to determine the standardized uptake values (SUVs). On ^{99m}Tc-sestamibi images we observed 8 segments with significantly lower uptake compared to sham operated animals (Supplemental Table 2). The highest uptake of the monomeric coNGR imaging agent was observed in the area in and around the apex which corresponds to the infarct area (Figure 1A, 2, 3, and Supplemental Table 3).

In all perfusion deficient areas, uptake of both coNGR imaging agents was significantly increased compared to previously tested cNGR imaging agent (Figure 1A). The average SUV in all infarcted areas for both NCL-cyclized-cNGR imaging agents is 0.88 while the disulfide cyclized-cNGR imaging agent only reached 0.21 in infarcted areas (Figure 1A and Supplemental Table 3). However, we also noticed an increased uptake of the monomeric coNGR imaging agent in all non-infarcted areas compared to the previously tested cNGR imaging agent (Figure 1A and Supplemental Table 3).

SPECT imaging and analysis of the tetrameric coNGR imaging agent uptake

In mice that were injected with the tetrameric coNGR imaging agent we found significantly lower uptake of ^{99m}Tc-sestamibi in 7 segments compared to sham operated animals (Supplemental Table 2). The uptake pattern of the tetrameric coNGR imaging agent can be seen in Figure 4 and 5. The highest uptake of the tetrameric coNGR imaging agent was

Figure 1. Overview of the SUVs for the three angiogenesis imaging agents in MI animals. Bar graphs comparing the uptake of coNGR to the previously tested disulfide-bonded cNGR imaging agent[15] (A). In panel B the uptake of monomeric coNGR and tetrameric coNGR is compared. The infarcted areas (significantly decreased ^{99m}Tc -sestamibi uptake) in the coNGR group (A) and tetrameric coNGR group (B) are encircled by the dashed line. * $p < 0.05$ is considered statistically significant.

Figure 2. Polar perfusion maps displaying the uptake pattern of ^{99m}Tc -sestamibi and the monomeric coNGR imaging agent in MI mice. Polar perfusion maps from each mouse in the MI group. The uptake in the polar perfusion maps is color coded and relative to the injected dose per animal.

observed in the area in and around the apex which corresponds to the infarct area (Figure 1B, 4, 5, and Supplemental Table 3).

In all but the basal anterior segment we found a significantly higher uptake of the tetrameric coNGR imaging agent when compared with the uptake of the cNGR imaging agent (Supplemental Table 3). Additionally, we compared the uptake of the tetrameric coNGR imaging agent in MI mice to the uptake of the monomeric coNGR imaging agent in MI mice to examine whether multimerization indeed results in higher uptake. Surprisingly, we found the monomeric coNGR imaging agent to be taken up significantly higher in 11 out of 17 segments than the tetrameric coNGR imaging agent (Figure 1B and Supplemental Table 3). In infarcted areas, the monomeric coNGR imaging agent reached an average SUV of 0.88 whereas the tetrameric coNGR imaging agent only reached an average SUV of 0.49.

Figure 3. Representative in vivo fusion images of the uptake of ^{99m}Tc -sestamibi and the monomeric coNGR in MI mice. In “grey” the uptake of ^{99m}Tc -sestamibi in the myocardium can be seen while in “hot metal”-color the uptake of the monomeric coNGR imaging agent is displayed. In panel A and B slices of an infarcted heart are shown. The infarct area is visualized by the decreased uptake of ^{99m}Tc -sestamibi (in gray) which is clearly visible in the anterolateral region of the heart (arrowheads). Enhanced uptake of coNGR is clearly visible in the infarct area (arrows). N.B. the uptake is color coded and relative to the injected dose per animal. SA: short axis, VLA: vertical long axis.

Figure 4. Polar perfusion maps displaying the uptake pattern of ^{99m}Tc -sestamibi and tetrameric coNGR in MI and sham operated mice. Polar perfusion maps from each mouse in the MI group injected with tetrameric coNGR. N.B. the uptake in the polar perfusion maps is color coded and relative to the injected dose per animal.

Biodistribution of coNGR compared to tetrameric coNGR

One hour after injection, urinary excretion in MI mice was 72.5 ± 5.2 for coNGR and 54.9 ± 9.1 for tetrameric coNGR (%I.D./g $\pm$ S.E.M., $p = 0.23$). Furthermore, uptake of coNGR appeared to be significantly higher in the blood, muscle, lungs, intestines and skin 1 hour post injection compared to uptake of tetrameric coNGR. Organs in the rest of the body displayed a low uptake of coNGR that did not differ from the uptake of tetrameric coNGR (Figure 6).

CD13 immunohistochemistry

To validate uptake patterns of the NGR-based tracers we evaluated CD13 expression through immunohistological staining. A low level of CD13 expression was observed within the myocardium of sham operated control animals (Figure 7 A,D). While the level of CD13

Figure 5. Representative in vivo fusion images of the uptake of ^{99m}Tc -sestamibi and tetrameric coNGR. In “grey” the uptake of ^{99m}Tc -sestamibi in the myocardium can be seen while in “hot metal” the uptake of the tetrameric coNGR imaging agent is displayed. Panels A and C: infarcted heart. The infarct area is signified by the decreased uptake of ^{99m}Tc -sestamibi which is clearly visible in the anterolateral region of the heart (arrowheads). Enhanced uptake of tetrameric coNGR is clearly visibly in the infarct area (arrows). Panel B and D: sham operated heart. Uniform uptake of ^{99m}Tc -sestamibi can be seen in combination with low overall uptake of tetrameric coNGR. N.B. the uptake is color coded and relative to the injected dose per animal. SA: short axis, VLA: vertical long axis.

Figure 6. Biodistribution of coNGR and tetrameric coNGR in MI mice. Substantial kidney uptake of coNGR and tetrameric coNGR was observed ($n \geq 3$ for coNGR and $n = 8$ for tetrameric coNGR injected mice). coNGR had significantly higher uptake than tetrameric coNGR in blood, lungs, intestines, and skin whereas the uptake in other organs was similar.

expression in the non-infarcted myocardium of MI animals appeared marginally higher compared to the expression level in control animals (Figure 7 C,F), the level of CD13 expression was dramatically higher in the infarcted myocardium of MI animals (Figure 7 B,E).

Figure 7. Representative pictures of CD13 expression in the myocardium of sham and MI operated animals. Low baseline expression of CD13 was observed on photomicrographs (magnification 40X) of the myocardium of sham operated animals (A). Dramatically increased CD13 expression was observed on photomicrographs (magnification 40X) of the infarcted myocardium of MI operated animals (B). On photomicrographs (magnification 40X) of the non-infarcted myocardium a marginally higher expression of CD13 was noticed (C). The photomicrographs (magnification 200X) in panel D, E, and F represent a magnification of the region indicated in panel A, B, and C respectively. Note that the expression of CD13 is not restricted to blood vessels, but that the endothelium of blood vessels is positive in the infarcted heart as opposed to the sham (arrows).

DISCUSSION

Since angiogenesis is associated with post-MI remodeling it has important implications for the prognosis of MI patients[21]. Accurate monitoring of angiogenesis can therefore be a substantial asset to MI patient risk stratification and can potentially be used to examine the effect of pro-angiogenic therapy. Numerous pre-clinical studies have sought for ways to sensitively monitor post-MI angiogenesis through nuclear imaging. Positive results were reported for various RGD-based and other tracer constructs. Nevertheless, despite the abundance of available tracer constructs, only a few small scale clinical studies report imaging of angiogenesis in patients[22-24]. All of these tracers were RGD-based. Beside the lack of approved therapeutic programs for patients, a possible explanation for the lack of large volume patient angiogenesis imaging studies is the unconvincing outcome

of these small scale clinical studies. The reported superior target homing ratio for NGR compared to RGD peptides[12] is a promising new approach that should be translated after further adaptations into a clinical study.

In this study, we explored the potential of two cyclic NGR peptide-based SPECT imaging agents for angiogenesis in a mouse MI model. After successful radiolabeling of the imaging agents with ^{111}In we performed dual isotope SPECT imaging and quantified the uptake of $^{99\text{m}}\text{Tc}$ -sestamibi and the ^{111}In labeled angiogenesis imaging agents coNGR and tetrameric coNGR in the 17 segment model.

In a previous study in the same mouse model and using an identical study set up we found target-specific uptake of the disulfide-bonded monomeric cNGR-based SPECT imaging agent in areas with decreased perfusion[15]. However, the uptake of this tracer was rather low. Considering this, we set out to increase the uptake of the NGR-based tracer by altering the nature and the valency of the tracer construct. The ring structure of the coNGR moiety was established using NCL, yielding a cyclic backbone structure assuring a more stable ring structure compared to disulfide-bond based rings. Blood stability tests indeed indicated a considerably higher percentage of intact coNGR after 50 min blood incubation compared to cNGR (Supplemental Table 1). As expected the uptake of the monomeric coNGR around the infarcted area was significantly higher than the previously tested cNGR. In the 8 infarcted areas, we found an average SUV of 0.88 for the coNGR imaging agent whereas we only found an average SUV of 0.21[15] in the infarcted areas for cNGR.

A different strategy that proved successful for RGD peptides in the past with regard to improving imaging agent uptake is multimerization[16, 17]. Here, we succeeded to synthesize and radiolabel a tetrameric cyclic NGR-based SPECT tracer for angiogenesis: tetrameric coNGR. Like for the coNGR imaging agent we synthesized the ring-structures using NCL. Blood stability tests using tetrameric coNGR also indicated a considerably higher percentage of intact imaging agent after 50 min blood incubation compared to the previously tested cNGR (Supplemental Table 1). Tetrameric coNGR showed indeed a higher uptake than cNGR in the infarct area, but lower than coNGR. Increasing the stability of the cyclic NGR ligand leading to a higher stability of the imaging agent, seems therefore a viable strategy to improve imaging efficacy through enhanced sensitivity. However, it should be noted that these stable agents were also taken up more by the non-infarcted area. As a result, it is therefore not immediately obvious that the distinction between infarct and healthy tissue will be facilitated. Systematic studies have to be performed in order to address that question.

Unlike the reported results with the RGD-peptides, multimerization does not result in higher target uptake for cyclic NGR-based imaging as the uptake of monomeric coNGR exceeded the uptake of tetrameric coNGR. The explanation for this surprising finding can only be speculative at this stage. It is conceivable that the reduced uptake of tetrameric

coNGR is a result of steric hindrance whereas CD13 receptor-binding of the single coNGR moiety in the coNGR imaging agent is not affected by steric hindrance. Furthermore, choosing a proper spacer unit can be important. This was illustrated by Thumshirn et al. who reported a lower affinity for a tetrameric RGD compound with a aminohexanoic spacer compared to the dimeric analogue while their tetrameric RGD compound with a Hegas spacer showed enhanced target affinity compared to the monomeric and dimeric analogue[25]. Changing the currently used SMCC spacer for a longer and less rigid spacer unit might further improve the uptake of our coNGR imaging agents by enhancing their binding affinity.

The enhanced uptake of the monomeric and tetrameric coNGR imaging agent in the healthy myocardium of infarcted hearts points towards an overall angiogenic response of the heart in MI animals. This was further underlined by histological findings as the level of CD13 expression seemed to be marginally higher in the non-infarcted myocardium of MI animals compared to the expression level in the myocardium of sham operated control animals. Additionally, for both coNGR and tetrameric coNGR the highest level of uptake was found in and around the infarcted areas of MI animals which also correlates to the histological findings as the highest level of CD13 expression was found in and around the infarcted areas. It is therefore highly likely that the uptake in the infarcted area as well as in the healthy myocardium is specific and related to increased CD13 expression.

We assumed that uptake of ^{99m}Tc -sestamibi is independent of co-injection of monomeric coNGR and tetrameric coNGR angiogenesis imaging agents. Therefore, we did not include sham operated mice in the coNGR group and compared the uptake of ^{99m}Tc -sestamibi in the coNGR group to the uptake of ^{99m}Tc -sestamibi in the tetrameric coNGR group. Furthermore, we are aware of the different time point at which we compared the uptake of the coNGR and tetrameric coNGR imaging agents to the uptake of the cNGR imaging agent (analysis of uptake between 45 and 60 min after injection for the currently examined imaging agents in comparison to an averaged time frame of 120 min after injection for the previously examined disulfide-cNGR imaging agent). However, based on the clearance of agents in 50 min, we can safely assume that the difference in binding was not determined by the variation in circulation time.

CONCLUSION

We found higher uptake for both of newly developed SPECT tracers for angiogenesis (coNGR and tetrameric coNGR) in the infarcted myocardial areas compared to the previously examined disulfide-bonded cNGR imaging agent. However, unlike with RGD-based imaging agents, target affinity of cyclic NGR-based imaging agents does not seem

to increase with increasing valency. Instead, the key to enhance cyclic NGR-based imaging agent uptake was to stabilize the ring structure through NCL.

These novel NGR-based SPECT imaging agents are promising tools to visualize and monitor angiogenesis in the setting of myocardial infarction.

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SUPPLEMENTAL DATA

HPLC quality control

The labelling yield of both tracers (an HPLC chromatogram of our previously used tracer is added in the bottom panel C) exceeded 90% and did not require further purification (Supplemental Figure 1).

Supplemental Figure 1. HPLC chromatograms for coNGR, tetrameric coNGR and the previously examined cNGR imaging agent.

Blood stability

Supplemental Table 1 indicates the blood stability of coNGR and tetrameric coNGR after 50 min of blood incubation. The percentage of intact tetrameric coNGR is slightly higher compared to coNGR. As a reference we also performed a blood stability test for the previously described cNGR imaging agent. Blood stability of cNGR appeared to be weaker compared to both tracers that were tested in the current study with only a minor amount of cNGR present after 50 min of blood incubation.

Supplemental Table 1. Blood stability of cyclic NGR-based SPECT tracers. Blood stability of the coNGR and tetrameric coNGR imaging agent after 50 min of blood incubation. As a reference we also included the blood stability of the tracer cNGR imaging agent we used in our previous study[15].

Tracer	Intact product (%)
coNGR	20.22
Tetrameric coNGR	25.31
cNGR	7.55

Supplemental Table 2. Overview of the SUVs of ^{99m}Tc -sestamibi in the tetrameric coNGR group and the coNGR group. Data are displayed as mean $\pm$ S.E.M. * $p < 0.05$ is considered statistically significant from SUVs in sham operated animals.

Segments	Standardized uptake values (SUV) of ^{99m}Tc -sestamibi		
	Tetrameric coNGR group		coNGR group
	Sham (n = 6)	MI (n = 8)	MI (n = 5)
1. Basal anterior	2.91 $\pm$.026	2.60 $\pm$ 0.70	2.46 $\pm$ 0.45
2. Basal anteroseptal	2.72 $\pm$ 0.32	3.68 $\pm$ 0.42	2.28 $\pm$ 0.39
3. Basal inferoseptal	2.89 $\pm$ 0.33	3.97 $\pm$ 0.49	2.33 $\pm$ 0.38
4. Basal inferior	3.07 $\pm$ 0.33	3.06 $\pm$ 0.54	2.60 $\pm$ 0.56
5. Basal inferolateral	3.03 $\pm$ 0.30	1.66 $\pm$ 0.47*	2.23 $\pm$ 0.63
6. Basal anterolateral	2.89 $\pm$ 0.20	1.60 $\pm$ 0.55	2.14 $\pm$ 0.52
7. Mid anterior	3.00 $\pm$ 0.22	1.35 $\pm$ 0.31*	1.41 $\pm$ 0.28*
8. Mid anteroseptal	3.10 $\pm$ 0.28	3.56 $\pm$ 0.47	2.46 $\pm$ 0.39
9. Mid inferoseptal	3.16 $\pm$ 0.26	3.57 $\pm$ 0.68	2.27 $\pm$ 0.53
10. Mid inferior	3.03 $\pm$ 0.29	2.21 $\pm$ 0.52	1.64 $\pm$ 0.67
11. Mid inferolateral	3.06 $\pm$ 0.34	0.68 $\pm$ 0.07*	0.75 $\pm$ 0.24*
12. Mid anterolateral	2.77 $\pm$ 0.22	0.62 $\pm$ 0.07*	0.42 $\pm$ 0.09*
13. Apical anterior	2.93 $\pm$ 0.22	1.04 $\pm$ 0.25*	0.71 $\pm$ 0.30*
14. Apical septal	3.23 $\pm$ 0.24	2.68 $\pm$ 0.30	1.67 $\pm$ 0.39*
15. Apical inferior	2.88 $\pm$ 0.32	2.06 $\pm$ 0.24	1.30 $\pm$ 0.31*
16. Apical lateral	2.44 $\pm$ 0.20	0.67 $\pm$ 0.11*	0.59 $\pm$ 0.26*
17. Apex	2.71 $\pm$ 0.25	1.25 $\pm$ 0.32*	0.98 $\pm$ 0.35*

Supplemental Table 3. Overview of the SUVs of the cNGR imaging agents in MI mice. The uptake of the new coNGR and tetrameric coNGR imaging agents is compared to the uptake of the previously examined (disulfide-bonded) cNGR imaging agent[15] in MI mice. Data are displayed as mean $\pm$ S.E.M. A statistically significant difference compared to SUVs in the disulfide-cNGR group is indicated by an asterisk (*) or a dagger sign(†). The section sign (§) indicates a statistically significant difference compared to SUVs in the tetrameric coNGR group.

Standardized uptake values (SUV) of cNGR imaging agents			
	cNGR	coNGR	Tetrameric coNGR
Segments	MI (n = 7)	MI (n = 5)	MI (n = 8)
1. Basal anterior	0.24 $\pm$ 0.04	0.73 $\pm$ 0.12*,§	0.37 $\pm$ 0.06
2. Basal anteroseptal	0.21 $\pm$ 0.03	0.75 $\pm$ 0.12*,§	0.37 $\pm$ 0.05†
3. Basal inferoseptal	0.20 $\pm$ 0.02	0.81 $\pm$ 0.15*,§	0.40 $\pm$ 0.05†
4. Basal inferior	0.23 $\pm$ 0.03	0.75 $\pm$ 0.15*,§	0.41 $\pm$ 0.06†
5. Basal inferolateral	0.23 $\pm$ 0.03	0.76 $\pm$ 0.14*	0.46 $\pm$ 0.07†
6. Basal anterolateral	0.26 $\pm$ 0.04	0.88 $\pm$ 0.18*,§	0.40 $\pm$ 0.07†
7. Mid anterior	0.21 $\pm$ 0.02	0.73 $\pm$ 0.07*,§	0.32 $\pm$ 0.04†
8. Mid anteroseptal	0.20 $\pm$ 0.02	0.66 $\pm$ 0.10*,§	0.36 $\pm$ 0.03†
9. Mid inferoseptal	0.18 $\pm$ 0.03	0.72 $\pm$ 0.14*	0.59 $\pm$ 0.06†
10. Mid inferior	0.17 $\pm$ 0.03	0.83 $\pm$ 0.16*	0.56 $\pm$ 0.08†
11. Mid inferolateral	0.20 $\pm$ 0.03	0.92 $\pm$ 0.17*,§	0.44 $\pm$ 0.07†
12. Mid anterolateral	0.24 $\pm$ 0.01	0.95 $\pm$ 0.13*,§	0.40 $\pm$ 0.05†
13. Apical anterior	0.21 $\pm$ 0.02	0.99 $\pm$ 0.17*,§	0.54 $\pm$ 0.07†
14. Apical septal	0.20 $\pm$ 0.03	0.73 $\pm$ 0.17*	0.80 $\pm$ 0.09†
15. Apical inferior	0.18 $\pm$ 0.03	0.77 $\pm$ 0.12*	0.82 $\pm$ 0.08†
16. Apical lateral	0.22 $\pm$ 0.02	0.96 $\pm$ 0.10*,§	0.54 $\pm$ 0.06†
17. Apex	0.20 $\pm$ 0.03	0.95 $\pm$ 0.13*	0.76 $\pm$ 0.10†

CHAPTER 8

GENERAL DISCUSSION

Despite the increasing survival rate after a cardiovascular event[1], cardiovascular diseases (CVD) remain the leading cause of death globally[2]. The major cause of CVD is atherosclerosis, a process in which slowly progressing lesions form and cause luminal narrowing of arteries[3, 4]. The underlying pathology is based on a chronic inflammatory condition, which is triggered by endothelial dysfunction and the subsequent deposition and oxidation of low-density lipoprotein (LDL) particles in the arterial wall. Oxidized LDL particles (oxLDL) in turn trigger the expression of adhesion molecules that drives the infiltration of immune cells. Successive accumulation of immune cells, apoptotic cells, debris, and cholesterol crystals culminates in the formation of an atherosclerotic plaque with a necrotic core and a (thin) fibrotic cap[4]. Atherosclerosis gives rise to coronary artery disease (CAD), cerebrovascular disease, and peripheral vascular disease (PVD)[4, 5]. Upon gradual expansion or sudden plaque rupture and subsequent thrombosis, the most common clinical manifestations are acute coronary syndrome (ACS), myocardial infarction, stroke, intermittent claudication, and critical limb ischemia[4, 6].

Fortuitously, the body is equipped with sophisticated rescue mechanisms that serve to protect ischemic tissue from becoming permanently damaged. These protective mechanisms are collectively coined neovascularization. Neovascularization is an innate physiologic response by which tissues respond to various stimuli through diametrical growth of pre-existing collateral arteries (arteriogenesis) and new vessel formation from existing vessels (angiogenesis)[7]. As a consequence of their importance, angiogenesis and arteriogenesis are considered a therapeutic goal. However, thus far results from successful pre-clinical studies[8-12] were not recapitulated in clinical trials[6, 13-18]. Among the proposed reasons for these clinical failures are ineffective delivery, insensitive and irreproducible readout parameters, and an unresponsive patient population[19]. The ability to accurately monitor these two processes in the very early stages of disease would improve the diagnosis and risk stratification of patients suffering from cardiovascular disease. Moreover, sensitive neovascularization imaging paves the way for tailored therapy as clinicians would be able to detect the therapeutic effect with high sensitivity.

Single photon emission computed tomography (SPECT) is a powerful non-invasive tool that can be used to obtain (quantitative) information about radiotracer distribution in humans and laboratory animals. Its application in the cardiovascular field is omnipresent and continuously expanding. While both hardware and software components are constantly improving, the quest for new and better radiotracers is perhaps the most vibrant area of SPECT imaging. The pre-clinical research described in this dissertation, revolved around SPECT imaging of arteriogenesis and angiogenesis. The pre-clinical endeavors to design a new arteriogenesis specific SPECT tracer are described in **chapter 3**. In **chapter 4** we report that laser Doppler perfusion imaging does not accurately reflect the actual perfusion recovery through arteriogenesis in a mouse ischemic hind limb model. A longitudinal follow up study showing good correlations between left ventricular cardiac

function measurements from ECG-gated μ SPECT and 3D-echocardiography in a mouse MI model is described in **chapter 5**. In **chapters 6 and 7** we report on successful imaging of angiogenesis using new SPECT tracers for angiogenesis.

SPECT TRACERS FOR ARTERIOGENESIS IMAGING

Collateral artery growth through arteriogenesis is the most important mechanism in the functional replacement of an occluded artery in PVD[20, 21]. Additionally, its importance in obstructive coronary artery disease is also well recognized and described. The extent of myocardial damage following complete coronary occlusion is dependent on the degree of development of the collateral circulation at that time. However, this propensity to develop collaterals is known to be extremely heterogeneous in man[22]. Despite the extensive network of collateral arteries and related opportunities for arteriogenic repair we currently lack radiotracers to specifically image this process. Instead, the ankle-brachial index is currently the recommended method for diagnosis of PVD in patients[23] while in pre-clinical studies, perfusion tracers are frequently used to monitor general perfusion (recovery) in animal models of PVD[24]. In coronary artery disease, non-nuclear imaging (i.e. microspheres and angiography) has been used for detection and quantification. However, these methods are restricted to late stage arteriogenesis and lack sensitivity or the quantitative capacity compared to nuclear techniques such as SPECT or PET. Early detection of arteriogenesis through nuclear imaging would be a valuable asset to current diagnostic tools which provides specific information about the most efficient mechanism to restore interrupted blood flow.

It is generally accepted that arteriogenesis is initiated by enhanced shear stress over pre-existing collateral arteries causing them to adapt a temporary inflammatory status which is essential for their diametrical growth. A fairly extensive set of molecular circuits that underlie this process have been unraveled[25], hence there is no scarcity in theoretical targets for nuclear imaging. The shear stress elevation triggers the endothelial cells to assume a proliferative phenotype that involves the upregulation of adhesion molecules, in particular intercellular adhesion molecule 1 (ICAM-1)[26]. Additionally, endothelial cells increase the expression of cytokines and chemokines which are presented on the luminal surface in order to recruit monocytes from the blood into the peri-collateral space[27, 28]. Among these upregulated chemokines, C-X-C motif ligand 1 (CXCL1) appears to act as an important player. Recently it was shown that shear stressed endothelium binds and takes up CXCL1[29], most likely via the upregulated number of binding sites present in the glycocalyx[30, 31]. CXCL1, tethered to the endothelial glycocalyx is known to interact with the monocyte-based G-protein coupled receptor C-X-C motif receptor 2 (CXCR2)[32] and facilitate monocyte recruitment to the peri-collateral space[29]. Considering

the prominent role of ICAM-1 and CXCL1 in the process of arteriogenesis they appear to be suitable targets for radiotracer guided imaging of arteriogenesis. However, targeting these proteins was challenging. We targeted ICAM-1 in a mouse model of PVD using a fluorescently labelled antibody. Unfortunately binding of the antibody was not restricted to the area of collateral artery growth. Instead, we were able to detect binding of the antibody to the vascular wall of ligated, sham-operated, and even healthy control animals. This finding is perhaps related to the fact that ICAM-1 is constitutively expressed on the endothelial cell surface. Hence, due to a lack of specific binding we decided to not pursue targeted imaging of ICAM-1 any further as an option for arteriogenesis imaging.

Shear stressed endothelium is shown to upregulate its binding sites for CXCL1 in the glycocalyx[29]. Hence, the availability of enhanced binding sites for CXCL1 makes exogenously administered CXCL an appealing strategy for targeted imaging of arteriogenesis. CXCL1 was synthesized via native chemical ligation and supplemented with a DTPA chelator and an OG488 fluorescent tag. Unfortunately, chemokines such as CXCL1 are known to form dimers[33]. This is most likely the reason as to why we were unable to extract a pure radiolabeled compound from radiolabeling procedures and abrogate experiments directed at using DTPA-CXCL1-OG488 as an arteriogenesis specific tracer .

Evasins are chemokine binding proteins that neutralize the bioactivity of chemoattractants such as CXCL1[34, 35] and are therefore interesting molecules for targeted imaging of arteriogenesis. To target CXCL1, evasin3 was synthesized via native chemical ligation and attached to a diethylene triamine pentaacetic acid (DTPA) chelator and an Oregon-green 488 (OG488) fluorescent tag. We opted for this double-labelling approach as it would enable us to accurately identify the spatiotemporal binding characteristics of our tracer through nuclear imaging, fluorescence microscopy, and post-mortem histology. In a rat model of PVD we injected indium-111 (^{111}In) labeled DTPA-evasin3-OG488 and performed SPECT imaging. Unfortunately we were unable to detect specific binding of ^{111}In -DTPA-evasin3-OG488 in the area of collateral artery formation 3 and 24 hours after ligation surgery. Whole body scans revealed high activity in the kidneys and bladder suggesting rapid clearance of the tracer. Despite these unfavorable initial results we think structural modification to the evasin3-based tracer through PEGylation might lead to a prolonged circulation time and perhaps specific binding in the area of collateralization.

Furthermore, we explored the option for thymosin $\beta 4$ (T $\beta 4$) to serve as a basis for a radiotracer. We succeeded in coupling T $\beta 4$ to a NOTA-benzyl-NCS chelator, enabling radiolabeling with the positron emission tomography (PET) isotope gallium-68 (^{68}Ga). Since T $\beta 4$ is involved in both angiogenesis and arteriogenesis we set out to test ^{68}Ga -NOTA-T $\beta 4$ in mouse models for angiogenesis and arteriogenesis. We used the Matrigel-plug assay to induce angiogenesis. Unfortunately, in fibroblast growth factor 2 (FGF2) Matrigels implanted in the flanks, we were unable to observe specific uptake of our tracer on reconstructed PET images. Apparently the uptake of ^{68}Ga -NOTA-T $\beta 4$ is insufficient

to indicate angiogenesis in the Matrigel-plug assay. This is an interesting finding since uptake of T β 4 was suggested to occur through internalization or binding to an unknown cell surface receptor during vessel formation[36], the latter being enhanced in hFGF2 supplemented Matrigels compared to control Matrigels. However, the lack of specific uptake was further underlined by gamma-counting of explanted Matrigels since FGF2 supplemented Matrigels did not display higher uptake of ^{68}Ga -NOTA-T β 4 than non-FGF2 supplemented Matrigels. Additionally, we used a hind limb ischemia model to induce arteriogenesis. We injected animals intravenously with ^{68}Ga -NOTA-T β 4 at 5 different time points (i.e. baseline, post-ligation, day 3, 7, and 14 after ligation) to study binding of ^{68}Ga -NOTA-T β 4 over time. However, on PET images we were unable to detect specific uptake of ^{68}Ga -NOTA-T β 4 in the area of collateralization because of high non-specific uptake in the rest of the body. This high non-specific uptake of our tracer is most likely related to the fact that T β 4 is involved in a substantial amount of processes in the body through its function in actin polymerization[37, 38].

QUANTITATIVE SPECT IN CARDIOVASCULAR DISEASE

In chapter 4 and 5 we compared the accuracy of SPECT measurements to established and frequently used methods in pre-clinical cardiovascular research. In a mouse ischemic hind limb model we used the SPECT perfusion tracer technetium-99m ($^{99\text{m}}\text{Tc}$)-sestamibi to study perfusion recovery. In a direct comparison we showed that laser Doppler perfusion imaging (LDPI) drastically underestimated true perfusion recovery over the total volume of the limb when compared to $^{99\text{m}}\text{Tc}$ -sestamibi SPECT. Our SPECT data indicated the lowest level of perfusion directly after femoral ligation and (nearly) full perfusion recovery 7 days post-surgery while LDPI still indicated ongoing perfusion recovery. These findings were supported by post-mortem histology. In harvested adductor muscle tissue, we found a significantly increased collateral artery diameter at day 7 and 14 after ligation surgery. The most likely underlying cause of this underestimation is the limited penetration depth of the laser beam, which only permits skin perfusion measurements whereas the majority of perfusion recovery takes place subcutaneously. Furthermore, it is conceivable that ligation-induced ischemia triggers the sympathetic nervous system to release norepinephrine, subsequently causing cutaneous vasoconstriction (through α 1- and α 2-adrenergic receptors on cutaneous arterioles) and thereby creating an apparent delay in reperfusion on LDPI.

In addition to perfusion recovery, we measured the (clearing of) muscular damage in this ischemic hind limb model. We performed $^{99\text{m}}\text{Tc}$ -pyrophosphate (PyP) SPECT and observed that muscular damage peaked at the lowest level of perfusion on day 3 after ligation. Moreover, we noticed that perfusion recovery on day 7 preceded reduction of muscular

damage in the ischemic hind limb on day 14. These findings were further underlined by histology as we found peak monocyte/macrophage infiltration and DNA fragmentation in the perimuscular fascia by CD68 and TUNEL immunohistochemical staining respectively at day three post-surgery.

Studies that test neovascularization stimulating agents in hind limb ischemia models often rely on LDPI measurements as a readout for perfusion recovery. The therapeutic window which is created by ligation of the femoral artery might be drastically shorter than indicated by LDPI. Consequently, an overestimation of the therapeutic window might lead to misinterpretation of the effect of arteriogenesis stimulating agents or application at the wrong time point as relevant perfusion recovery might have already taken place while LDPI still indicates the need for perfusion improvement.

In chapter 5 we describe the observations we made in a comparative study between electrocardiogram (ECG)-gated SPECT and 3D-echocardiography in a mouse myocardial infarction model. We monitored left ventricular function at different time points in a mouse model for MI using 3D-echocardiography. Additionally, on the same day and in the same mice we performed ECG-gated μ SPECT using the perfusion tracer ^{99m}Tc -sestamibi, assuring optimal comparability of the performance of both techniques. We showed excellent correlations between cardiac function measurements performed by ECG-gated SPECT and 3D-echocardiography at baseline, 7 and 35 days after ligation of the left anterior descending coronary artery (LAD). Moreover, cardiac function measurements in sham operated animals were comparable at any time point between both methods.

The ejection fraction (EF) values we reported in this study were comparable to values that were reported in other studies and obtained by different imaging modalities[39-45], although considerably higher values were reported as well[46-53]. Strain differences may account for the variability in reported EF values post MI as was shown in an earlier study[54]. The majority of the studies that used non-invasive imaging to determine left ventricular function used C57Bl6 mice, whereas we deliberately opted for Swiss mice as they are known to have better post MI survival rates in spite of severe cardiac function deterioration[54].

Despite the strong correlations we found between the left ventricular function measurements from both modalities we noticed that ECG-gated μ SPECT had a higher number of non-interpretable scans compared to 3D-echocardiography in our study. Non-interpretability of ECG-gated μ SPECT images was usually a result of a distorted ECG-signal hampering image reconstruction at true end-diastolic volume (EDV) and end-systolic volume (ESV) dimensions. Assuring a stable connection of the ECG leads to the animal throughout the entire scan is important for obtaining a clear ECG signal. Moreover, accurate endocardial contouring in the presence of scar formation (signified here by low uptake of ^{99m}Tc -sestamibi) and high liver uptake requires a trained observer as enlarged left ventricular dimensions in combination with low scar uptake and high liver uptake might

easily lead to under- or overestimation of the volume. Notwithstanding these potential pitfalls, SPECT offers the opportunity to simultaneously monitor multiple biochemical processes through multi-isotope imaging. In combination with ECG gating, SPECT imaging provides researchers and clinicians with a one-stop-shop principle; offering the assessment of myocardial viability, perfusion, and ongoing neovascularization in combination with reliable left ventricular function measurements. Especially in therapy studies (both pre-clinical and clinical) directed at improving post infarct cardiac performance, the one-stop shop principle of SPECT imaging will be the better option as it limits the amount of procedures study subjects have to undergo to a single scan while all important data can still be obtained.

NOVEL SPECT TRACERS FOR POST MI ANGIOGENESIS IMAGING

Where in cancer the extent of angiogenesis has a negative influence on the prognosis[55, 56], the reverse holds true for myocardial remodeling following myocardial infarction (MI) [57]. While previous research has mainly focused on developing pro-angiogenic therapies to stimulate angiogenesis in the infarcted myocardium[19, 58], a clinically applicable method to accurately monitor the effect of these therapies is still lacking. Although there are a few clinical studies that performed angiogenesis imaging[59-61], most clinical studies thus far have relied on indirect evidence of myocardial angiogenesis. Clearly, improved myocardial perfusion or function can be used as indirect evidence for angiogenesis in the infarcted myocardium. Reduced angina scores, increased treadmill exercise times and improved myocardial perfusion as measured by angiography, SPECT, PET and MRI all seem to indicate recovery[62]. However, despite the ability of these methods to indicate recovery, it often takes several months before any improvement can be detected.

In chapter 6 we tested a novel SPECT tracer for angiogenesis in a mouse MI model. The cyclic tripeptide Asn-Gly-Arg (cNGR) was synthesized at our institution and coupled to a DTPA chelator which enabled radiolabeling with the SPECT isotope ^{111}In . Through simultaneous application of the perfusion tracer $^{99\text{m}}\text{Tc}$ -sestamibi and ^{111}In -DTPA-cNGR we were able to visualize ongoing angiogenesis in perfusion deficient areas 7 days post MI. Quantification in the 17 segment model showed significantly higher uptake in 6 segments that corresponded with the infarct and infarct border zone.

Despite finding target-specific binding of our novel angiogenesis imaging tracer we have been looking into strategies to further enhance tracer-target interaction. In chapter 7 we used Native Chemical Ligation (NCL) to establish the ring structure of the cyclic NGR moiety (henceforth “coNGR”), yielding a cyclic backbone structure that assures a more stable ring structure compared to disulfide-bond based rings. Blood stability tests indeed indicated a considerably higher percentage of intact coNGR after 50 min blood incubation compared

to the cNGR-based angiogenesis tracer (henceforth “cNGR”) that was used in chapter 6. As expected the uptake of coNGR around the infarcted area was significantly higher than the uptake of cNGR. In the 8 infarcted areas, we found an average standardized uptake value (SUV) of 0.88 for the coNGR tracer whereas we only found an average SUV of 0.21 in the infarcted areas for cNGR.

Increasing the valency of a tracer is a different strategy that proved to be successful with regard to improving tracer uptake in previous studies as tumor uptake was shown to increase with increasing valency of the commonly studied angiogenesis marker arginine-glycine-aspartic-acid (RGD) [63, 64]. Besides creating the more stable coNGR, we succeeded to synthesize and radiolabel a tetrameric cyclic NGR-based SPECT tracer for angiogenesis: tetrameric coNGR. Like for the coNGR imaging agent we synthesized the ring-structures using NCL. Blood stability tests using tetrameric coNGR also indicated a considerably higher percentage of intact imaging agent after 50 min blood incubation compared to the previously tested cNGR. The tetrameric coNGR indeed showed a higher uptake than cNGR in the infarct area (average SUV of 0.49 vs. 0.21 for tetrameric-coNGR vs. cNGR respectively), but lower than coNGR. Increasing the stability of the cyclic NGR ligand leading to a higher stability of the imaging agent, seems therefore a viable strategy to improve imaging efficacy through enhanced sensitivity. However, it should be noted that these stable agents were also taken up more by the non-infarcted area. The enhanced uptake of all cyclic NGR-based imaging agents in the healthy myocardium of infarcted hearts points towards an overall angiogenic response of the heart in MI animals. This was further underlined by histological findings as the level of CD13 expression seemed to be marginally higher in the non-infarcted myocardium of MI animals compared to the expression level in the myocardium of sham operated control animals. Additionally, for both coNGR and tetrameric coNGR the highest level of uptake was found in and around the infarcted areas of MI animals which also correlates to the histological findings as the highest level of CD13 expression was found in and around the infarcted areas. It is therefore highly likely that the uptake in the infarcted area as well as in the healthy myocardium is specific and related to increased CD13 expression.

The combination of low uptake in remote organs in combination with the high uptake in and around the infarcted areas in the myocardium makes coNGR and tetrameric-coNGR an interesting option for angiogenesis imaging in MI patients in the future. Nonetheless, the true merit of angiogenesis imaging in MI patients has to be proven as currently only a few small scale clinical studies have been performed[59-61]. Since there is no scarcity in available tracer constructs, it is conceivable that the field of clinical angiogenesis imaging is held up by the lack of therapeutic programs.

CONCLUSION

We demonstrated the feasibility of non-invasive in vivo SPECT imaging of angiogenesis using novel cNGR-based tracers. Furthermore, in this dissertation, we described pioneering work in the field of arteriogenesis imaging. Key players in the process of arteriogenesis were ruled out to serve as successful basis for a radiotracer and potential pitfalls for the development of an arteriogenesis specific radiotracer were described. Additionally, in pre-clinical models of PVD and MI we showed the merit and accuracy of SPECT imaging compared to established methods for perfusion and function measurements (i.e. LDPI and 3D-echocardiography). Results of these studies were supported by histological findings. Together, the research described in this dissertation helps to pave the way for clinical application of neovascularization imaging in patients suffering from cardiovascular disease. However, especially in the field of arteriogenesis imaging, several challenges have to be faced before radiotracer-guided neovascularization imaging becomes a viable strategy for risk stratification or therapy monitoring in cardiovascular disease patients.

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SUMMARY

The heart is a muscular pump that on average pumps 4 to 5 liters of blood per minute into the human circulatory system. The most important muscle in our body is about the size of a clenched fist and retains its size proportional to the rest of the body during growth. The heart is composed of four chambers, each of them being responsible for a different task. The right atrium receives blood with a low oxygen content from the systemic circulation, the right ventricle distributes this blood into the pulmonary circulation in which the blood is oxygenated. Subsequently, via the left atrium, the oxygenated blood is guided into the left ventricle and is pumped into the systemic circulation. Through firm and elastic conductance arteries the blood is carried to distal parts of the body. The most important cells in the vascular wall of these conductance arteries are the endothelial cells and smooth muscle cells. These specialized cells play an important role in the regulation and maintenance of an adequate blood circulation. Endothelial cells form the inner lining of blood vessels and prevent the blood from coagulating while smooth muscle cells control the diameter of the vessel through contraction and relaxation thereby modulating the blood supply to an organ. Once the blood reaches the capillary vessels of the organs the exchange of blood and nutrients can take place that is required to maintain the basal cell metabolism.

However, the situation described above holds true for a healthy circulation. Unfortunately, there are numerous situations in which one of the components of the circulation is compromised, subsequently leading to a restriction of the blood supply to an organ. One of the most common diseases of the vascular wall of conductance arteries is called 'atherosclerosis'. Atherosclerosis is a chronic inflammation of the vascular wall of an artery that involves the accumulation of fat and white blood cells. The pathogenesis of atherosclerosis starts in childhood, is stimulated by an unhealthy lifestyle (i.e. smoking, drinking, sedentary lifestyle etcetera), and results in an 'atherosclerotic plaque' over time. This plaque often results in a significant narrowing of the arterial lumen, thereby causing a severe restriction in the blood supply. However, it is not until an unstable plaque ruptures before atherosclerosis leads to life threatening events. Upon plaque rupture its content is set free into the blood, triggering the formation of a blood clot. This so called thrombotic event leads to a complete obstruction of the blood flow through an affected artery. In the heart this event is called a myocardial infarction, in the brain an ischemic cerebrovascular accident, and in the arteries supplying the legs this is termed peripheral vascular disease.

A multitude of treatment options for cardiovascular diseases caused by atherosclerosis are available, ranging from cholesterol lowering drugs to surgical interventions. However, the body itself is also equipped with several repair mechanisms to compensate for the loss of blood flow to an organ. In response to the deprivation of blood, different stimuli will be expressed and released by the affected tissue. The processes that are triggered by these stimuli are collectively referred to as 'neovascularization'. In adulthood, neovascularization comprises two distinct processes: angiogenesis and arteriogenesis.

Although both processes aim to restore the blood supply they differ in mechanism and in morphology. Angiogenesis involves the sprouting of new capillary arteries from other capillary arteries in response to a lack of oxygen. On the contrary, arteriogenesis is independent of oxygen levels and involves the enlargement of pre-existing collateral arteries into large conductance collaterals in response to enhanced fluid shear stress. In healthy individuals these collateral arteries transport an insignificant amount of blood. However, in case a conductance artery becomes atherosclerotically stenosed or occluded, the flow through these collateral arteries increases drastically. To cope with this increased blood flow a structural growth of the diameter of a collateral artery is required.

Both processes are strictly regulated and strongly dependent on complex interactions between cells in the blood, endothelial cells and smooth muscle cells. Nevertheless, the majority of the mechanisms that underlie these interactions are known by now. For example, the involvement of growth factors in both processes is evident. Therapies aimed at stimulation of neovascularization are therefore often based on the application of growth factors. However, clinical studies often failed to capitalize on the promising results reported from animal studies. An explanation for this problem might be found in the lack of sensitive imaging techniques that are able to detect the, often very subtle, effects of these therapies at an early stage.

This dissertation describes several studies in which we imaged neovascularization or the restoration of blood flow as a result of neovascularization in a sensitive and non-invasive way through 'single photon emission computed tomography' (SPECT). SPECT imaging is based on the detection of a radioactive tracer that is injected into the circulation and subsequently taken up by viable cells or bound by a biomarker. Biomarkers are measurable hallmarks of a disease or a medical condition. For example, angiogenesis is hallmarked by the selective upregulation of the aminopeptidase N peptide (also known as CD13) on angiogenically active endothelial cells. Arteriogenesis is hallmarked by the upregulated production and enhanced binding of the C-X-C motif ligand 1 (CXCL1) peptide by endothelial cells. The intercellular adhesion molecule 1 (ICAM-1) is a different protein that has been indicated to play an important role during arteriogenesis while the thymosin β 4 peptide was described to play a role in both angiogenesis and arteriogenesis.

The studies in this dissertation describe approaches to design and validate novel angiogenesis and arteriogenesis SPECT tracers that are based on or bind to the peptides mentioned above. Unfortunately, our efforts to design a new arteriogenesis SPECT tracer failed. However, this pioneering work has led to important new insights that have to be considered in future studies aimed at designing an arteriogenesis specific imaging tracer. We were able to successfully image angiogenesis using 3 newly designed tracers for SPECT imaging in a mouse myocardial infarction model. Additionally, we were able to sensitively monitor perfusion recovery in a mouse peripheral vascular disease model using SPECT imaging. This study showed that SPECT imaging is physiologically more relevant than the

traditionally used laser Doppler perfusion imaging. Furthermore, we performed functional cardiac measurements in the aforementioned myocardial infarction model and showed that functional measurements extracted from electrocardiogram (ECG) gated SPECT images were comparable to the frequently employed 3D echocardiography.

Chapter 2 offers a detailed overview and discussion of pre-clinical work aimed at validating new radiotracers for neovascularization, thereby outlining the context in which my research fits. Extensive research has been conducted concerning radiotracer imaging of angiogenesis which has resulted in the availability of ample tracer constructs while arteriogenesis radiotracer imaging is scarce and largely overlooked. So far, perfusion tracers have been used to indicate enhanced perfusion through arteriogenesis. Future studies aimed at designing an arteriogenesis tracer should keep in mind the high shear stress environment that has to be overcome as well as the notion that inflammatory factors might not operate well specific arteriogenesis markers within a transiently inflammatory environment.

Chapter 3 elaborates on the potential pitfalls encountered when designing an arteriogenesis specific radiotracer. Several agents that target biomarkers for arteriogenesis, were ruled out to serve as a successful arteriogenesis SPECT tracer. The fluorescently labeled ICAM-1 antibody and the gallium-68 labelled thymosin β 4 peptide displayed high non-specific binding in a mouse model for peripheral vascular disease. Radiolabeling of CXCL1 appeared to be extremely difficult as this peptide has the natural tendency to form dimers at very low concentrations. Unfortunately these problems appeared intractable and we had to discontinue our experiments. Furthermore, we were successful in radiolabeling evasin3 with the SPECT radionuclide indium-111 (^{111}In). However, injection of this evasin3 based radiotracer for arteriogenesis did not result in specific binding in a rat model for peripheral vascular disease.

In **chapter 4** perfusion recovery through arteriogenesis in a mouse model for peripheral vascular disease was investigated using laser Doppler perfusion imaging and SPECT. By using the SPECT perfusion tracer $^{99\text{m}}\text{Tc}$ -sestamibi we were able to investigate the perfusion recovery over the entire volume of the leg whereas the limited penetration depth of laser Doppler perfusion imaging only allows for skin perfusion measurements. Our SPECT data showed that perfusion recovery in this model takes place considerably faster than indicated by laser Doppler perfusion imaging. Furthermore, we examined muscular damage in this model using the SPECT tracer $^{99\text{m}}\text{Tc}$ -pyrophosphate and accurately showed that perfusion recovery precedes muscular recovery.

Nuclear imaging is a rapidly evolving area of research. Both hardware and software components are subjected to frequent updates. In recent years, fundamental research into complex diseases benefited greatly from the development of dedicated small laboratory animal magnetic resonance (MRI), positron emission tomography (PET), and SPECT scanners. Pre-clinical SPECT scanners nowadays progressed to a level that enables

functional measurements on the mouse heart that beats at a rate between 400 and 600 beats per minute. In **chapter 5** we demonstrated that these functional measurements were highly accurate. In a comparative study between 3D echocardiography and ECG gated ^{99m}Tc -sestamibi SPECT we showed that functional cardiac measurements of both methods are highly comparable in a mouse myocardial infarction model and in mice subjected to a sham (control) procedure. In the near future SPECT imaging might even prove the preferred option in both pre-clinical and clinical studies directed at unravelling the complex and multifactorial nature of cardiovascular disease since myocardial viability, perfusion and ongoing neovascularization in combination with reliable functional measurements can be acquired within one single scan.

In **chapter 6 and 7** we describe the successful application of 3 new angiogenesis SPECT tracers in a mouse myocardial infarction model. All tracers were based on the CD13 binding cyclic-NGR (cNGR) peptide. In previous studies our lab already showed that the cNGR peptide specifically binds to CD13 on newly formed capillary arteries using microscopy and MRI. However, the tracer constructs used for these studies had no opportunity for clinical translation. In **chapter 6** we show specific myocardial uptake of an ^{111}In labeled cNGR peptide in areas of decreased uptake of the perfusion tracer ^{99m}Tc -sestamibi. Despite the significantly higher uptake in infarcted areas in comparison with similar areas in healthy control animals and a fast clearance via the urinary tract, the uptake in infarct areas and the infarct border was rather low. The low uptake of this tracer would hamper clinical translation.

In **chapter 7** we describe two strategies to enhance the uptake of this cNGR based SPECT tracer. First, we created a more stable ring structure of the cNGR entity by incorporating stronger bonds through native chemical ligation. Enhanced stability of this tracer was confirmed by stability tests in blood. Second, we explored the capabilities of a multivalent tracer. In previous studies, albeit in tumor tissue, the application of multivalent tracers (tracers containing multiple entities that each bind a similar target) proved to be successful with regard to enhancing tracer uptake. Besides the new and more stable cNGR based SPECT tracer with a single cNGR entity, a new multivalent cNGR molecule containing 4 cNGR entities was synthesized for the study described in chapter 7. The ring structures of the cNGR entities for this tracer were synthesized by native chemical ligation as well. Enhanced blood stability of this tracer was also confirmed by stability tests. Following successful radiolabeling with ^{111}In , both new tracers were tested in mice with a myocardial infarction and mice that were subjected to a sham operation. Both ^{111}In labeled SPECT tracers for angiogenesis displayed a significantly higher uptake in and around infarcted areas in comparison to the cNGR based SPECT tracer that was used for the study described in chapter 6. However, this higher specific uptake was accompanied by higher non-specific uptake in non-infarcted myocardial tissue. Considering the favorable clearance and low

uptake in other organs we expect these tracers to be serious contenders for clinical translation.

In **Chapter 8** all findings are summarized and compared to published work of other groups in the general discussion. Finally, we will discuss how the acquired knowledge can contribute to the creation of economic value in clinical research or other fields.

Taken together, the studies described in this dissertation contribute the improvement of nuclear imaging of perfusion, function and neovascularization. I showed that SPECT imaging is an effective option for imaging perfusion recovery and parameters that indicate the functional status of the heart. Additionally, I demonstrated successful angiogenesis imaging by using novel cNGR based SPECT tracers in a mouse myocardial infarction model. Nevertheless, before specific arteriogenesis imaging can be realized we require full understanding of the transient inflammatory environment and solutions to overcome binding inefficiency of molecular contrast agents in a high shear stress environment.

SAMENVATTING

Het hart is een pomp die gemiddeld 4 tot 5 liter bloed per minuut rondpompt. De belangrijkste spier van ons lichaam is ongeveer zo groot als een vuist. Deze regel geldt altijd aangezien het hart met ons lichaam meegroeit. De vier holle ruimtes waaruit het hart bestaat hebben ieder hun eigen functie. In de rechterboezem stroomt zuurstofarm bloed binnen uit de rest van het lichaam, de rechterkamer pompt dit zuurstofarme bloed de kleine bloedsomloop in door de longen waar het bloed van zuurstof voorzien wordt. Daarna wordt via de linkerboezem het zuurstofrijke bloed naar de linkerkamer gepompt welke vervolgens zorgt voor de toevoer naar de rest van het lichaam via de grote bloedsomloop. Via stevige en elastische bloedvaten genaamd slagaders wordt het bloed richting de organen geleid. De wand van de slagaders bevat onder andere endotheelcellen en gladde spiercellen. Deze gespecialiseerde cellen spelen ieder een belangrijke rol in het regelen en onderhouden van een adequate bloedsomloop. Endotheelcellen kunnen gezien worden als een soort dekweefselcellaag van dunne en platte cellen die ervoor zorgen dat het bloed niet in de aderen stolt. De gladde spiercellen regelen middels samentrekken en ontspannen de diameter van het vat en daarmee de bloedtoevoer naar een orgaan. Eenmaal aangekomen bij de organen kan in de haarvaten (ookwel capillairen genoemd) de uitwisseling van voedingsstoffen en zuurstof plaatsvinden welke nodig zijn om de grondstofwisseling in de cellen in balans te houden.

Dit is hoe het in het gezonde vaatstelsel gaat. Echter zijn er ook situaties bekend waarin de bloedtoevoer dusdanig beperkt wordt dat men er hinder van ondervindt. Een van de meest voorkomende ziektes waarbij de vaatwand van de slagader een centrale rol speelt is 'atherosclerose'. Het woord is een samenvoeging van de Griekse woorden 'athere' wat brij of pap betekend en 'skleros' wat hard betekend, en wordt in de volksmond ookwel (onterecht) aderverkalking genoemd. Atherosclerose is een ontsteking van de vaatwand waarbij er een stapeling van vetten en witte bloedcellen in de vaatwand plaatsvindt. Dit is een proces wat al in de kindertijd begint, gestimuleerd wordt door een ongezonde levensstijl, en over de jaren heen resulteert in een zogenaamde 'atherosclerotische plaque'. Hoewel deze plaque in de slagaders de bloedtoevoer ernstig kan beperken zal de vernauwing zelf zelden dodelijk zijn. Het is echter het scheuren van deze plaque dat er voor zorgt dat er ernstige, vaak levensbedreigende, situaties kunnen ontstaan. Bij het scheuren van een plaque komt de inhoud, bestaande uit vet en witte bloedcellen, in contact met het bloed waardoor er bloedstolling optreedt. Het bloedpropje dat ontstaat zal vervolgens de gehele slagader afsluiten waardoor het weefsel dat verderop in de vaatboom ligt, verstoken blijft van bloed. Wanneer dit een slagader van het hart betreft spreken we van een 'myocardinfarct' (ookwel hartinfarct), in de hersenen spreken we van een 'cerebrovasculair accident' (ookwel beroerte) en wanneer dit in de slagaders van de benen gebeurt spreken we van 'perifeer arterieel vaatlijden' (ookwel etalagebenen).

Behandelingen voor hart- en vaatziekten waaraan atherosclerose ten grondslag ligt variëren van cholesterol-verlagende medicatie tot chirurgische ingrepen. Het lichaam

beschikt echter zelf ook over herstelmechanismen die de gevolgen van atherosclerose tegengaan. Verschillende stimuli, afgegeven door het weefsel dat verstoken blijft van bloed, worden in het lichaam beantwoord middels 'neovascularisatie'. Deze overkoepelende term (letterlijk nieuwgroei van bloedvaten) refereert naar twee verschillende processen, angiogenese en arteriogenese, die elk trachten de bloedtoevoer weer te herstellen. Daar waar het doel van beide processen overeenkomt, verschillen zij in hun totstandkoming. De term angiogenese (letterlijk bloedvat vorming) beschrijft het proces waarbij er uit bestaande capillairen nieuwe capillairen ontstaan als gevolg van een gebrek aan zuurstof. Arteriogenese (letterlijk slagader vorming) is daarentegen een proces dat onafhankelijk van een gebrek aan zuurstof plaatsvindt en de groei van 'collateraal arteriën' als gevolg van een verhoogde afschuifspanning langs de vaatwand beschrijft. Collateraal arteriën zijn bloedvaten die een verbinding vormen tussen de slagaders in ons lichaam. In de normale situatie zal het bloed de weg van de minste weerstand volgen waardoor er maar weinig bloed door de collateraal arteriën stroomt. Echter wanneer er door atherosclerose een blokkade optreedt in de slagaders zal het bloed door deze collateraal arteriën moeten stromen om toch naar het verderop gelegen weefsel te kunnen komen. Om adequaat met deze verhoogde bloedstroom om te kunnen gaan zullen de collateraal arteriën in diameter moeten groeien.

Beide processen zijn strikt gereguleerd en afhankelijk van een complexe interactie tussen cellen in het bloed en de eerder beschreven endotheelcellen en gladde spiercellen in de vaatwand. Veel van de onderliggende mechanismen die ten grondslag liggen aan zowel angiogenese als arteriogenese zijn nochtans bekend. Zo is bijvoorbeeld bekend dat verschillende groeifactoren een belangrijke rol spelen in beide processen. Therapieën gericht op het stimuleren van neovascularisatie zijn dan ook veelvuldig gebaseerd op toediening van groeifactoren. Waar deze therapieën in proefdieren vaker succesvol bleken heeft dit in de mens echter nog niet tot aantoonbare resultaten geleid. Een oorzaak hiervoor kan gezocht worden in beeldvormingstechnieken die momenteel niet de gewenste gevoeligheid hebben om de vaak subtiele effecten van deze therapieën in een vroeg stadium in beeld te brengen.

In deze dissertatie beschrijf ik een aantal studies waarin we hebben getracht de beeldvorming van neovascularisatie of het herstel in doorbloeding middels neovascularisatie op een zeer gevoelige manier met behulp van niet-invasieve beeldvorming in kaart te brengen. De niet-invasieve beeldvormingsmethode waarvan we tijdens deze studies gebruik hebben gemaakt is 'single photon emission tomography' (afgekort SPECT). SPECT maakt gebruik van een radioactief gemerkte stof (ookwel tracer) die aan de bloedbaan wordt toegediend en vervolgens wordt opgenomen door levende cellen of kan binden aan een zogenaamde 'biomarker'. Biomarkers zijn meetbare indicatoren die kenmerkend zijn voor een ziekte of medische conditie. Angiogenese wordt bijvoorbeeld gekenmerkt door een verhoogde productie van een bepaalde vorm van het 'aminopeptidase N' eiwit

(ookwel CD13 genoemd) door de endotheelcellen in de capillaire vaten die betrokken zijn bij angiogenese. Arteriogenese wordt onder andere gekenmerkt door de verhoogde productie en binding van het eiwit 'C-X-C motif ligand 1' (afgekort CXCL1) door en aan endotheelcellen in de wand van de collateraal arteriën. Een andere eiwit dat een belangrijke rol speelt tijdens arteriogenese is intercellular adhesion molecule 1 (kortweg ICAM1), terwijl het eiwit thymosine β 4 een belangrijke rol wordt toegedicht in zowel angiogenese als arteriogenese.

In de studies die besproken worden in deze dissertatie hebben we gepoogd een nieuwe arteriogenese tracer en angiogenese tracer te ontwikkelen die bindt aan of gebaseerd is op de bovengenoemde eiwitten. Hoewel de ontwikkeling van een nieuwe arteriogenese tracer tot dusver niet succesvol is gebleken heeft ons werk tot nieuwe inzichten geleid die in acht dienen te worden genomen bij toekomstige studies. Angiogenese hebben we met goed gevolg in beeld gebracht middels drie nieuw ontwikkelde tracers voor SPECT beeldvorming in een proefdiermodel van een hartinfarct. Eveneens hebben we SPECT beeldvorming gebruikt om het herstel van de bloedtoevoer in een proefdiermodel voor perifere arterieel vaatleiden nauwkeurig in beeld te brengen. SPECT beeldvorming bleek hierin nauwkeuriger dan de traditioneel gebruikte methode laser Doppler perfusie beeldvorming. Dat SPECT beeldvorming inderdaad geschikt is om buiten neovascularisatie ook nauwkeurige functionele metingen te verrichten hebben we aangetoond in het eerder genoemde hartinfarct model. De functiemetingen die aan de hand van SPECT beeldvorming gedaan werden bleken vergelijkbaar met de veelvuldig toegepaste 3D-echocardiografie.

Hoofdstuk 2 biedt een uitgebreide inkijk in wat er zowel pre-klinisch als klinisch aan onderzoek gedaan is naar nieuwe tracers voor angiogenese en arteriogenese beeldvorming. Het raamwerk waarbinnen ons onderzoek past is hierin weergegeven. Opmerkelijk is daarbij dat er momenteel geen nucleaire tracers zijn die specifieke beeldvorming van arteriogenese mogelijk maken terwijl er legio angiogenese tracers voorhanden zijn. Thans worden er perfusie tracers ingezet om de mate van arteriogenese te bepalen. Obstructies die de ontwikkeling van een arteriogenese tracer in de weg staan zijn wellicht gerelateerd aan de omgeving waarin deze tracer zou moeten binden. De hoge afschuifspanning langs de wanden van de collateraal arteriën in combinatie met de kortstondige ontstekings status van het weefsel vereisen wellicht een multivalente-binding van een arteriogenese tracer welke toegediend wordt in een nauwkeurig bepaalde tijdspanne.

Hoofdstuk 3 geeft een beter beeld van de moeilijkheden die het ontwikkelen van een arteriogenese tracer met zich mee brengt. Het fluorescent gemerkte antilichaam tegen ICAM-1 en een op het eiwit Thymosine β 4 gebaseerde tracer bonden niet-specifiek in een muis model voor perifere arterieel vaatleiden. Het gebruik van CXCL1 als basis voor een arteriogenese tracer bleek uiterst moeilijk. Reeds bij zeer lage concentraties worden er twee CXCL1 eiwitten aan elkaar gekoppeld. De vorming van deze zogenaamde dimeren bemoeilijkte de kwaliteitscontrole zodanig dat we niet konden bepalen of we een

zuivere radioactief gemerkte tracer hadden vervaardigd. Helaas bleken deze problemen onoplosbaar waardoor we verdere experimenten hebben moeten stoppen. Het radioactief merken van evasin3 (een eiwit dat aan CXCL1 bindt) was wel succesvol. Echter bleek toediening van het radioactief gemerkte evasin3 niet te resulteren in specifieke opname in een model voor perifeer arterieel vaatleiden in de rat.

In **hoofdstuk 4** hebben we in een rechtstreekse vergelijking tussen laser Doppler perfusie beeldvorming en SPECT aan kunnen tonen dat laser Doppler perfusie beeldvorming het herstel van de doorbloeding in een model voor perifeer arterieel vaatleiden in de muis sterk onderschat. Waar laser Doppler perfusie beeldvorming beperkt wordt door de penetratie diepte van de laser, konden we door gebruik te maken van de SPECT perfusie tracer ^{99m}Tc -sestamibi over de gehele diepte van de achterpoot de doorbloeding bepalen. Middels SPECT imaging konden we aantonen dat het herstel in doorbloeding veel sneller plaatsvindt dan wat de laser Doppler perfusie beeldvorming resultaten suggereerden. Tevens werd er in deze studie gekeken naar spierschade middels de SPECT tracer ^{99m}Tc -pyrophosphate waardoor we nauwkeurig vast konden stellen dat het herstel van de doorbloeding vooruitloopt op het herstel van spierschade.

De ontwikkelingen op het gebied van hardware en software voor nucleaire beeldvorming de afgelopen jaren zijn talrijk. Speciaal voor pre-klinische doeleinden zijn er 'magnetic resonance imaging' (afgekort MRI), 'positron emission tomography' (afgekort PET) en SPECT scanners met bijbehorende software ontworpen die het fundamenteel onderzoek naar complexe ziekten een enorm kwaliteits impuls hebben gegeven. Op het gebied van SPECT imaging is de techniek zo ver dat er functionele metingen verricht kunnen worden aan het hart van de muis dat tussen de 400 en 600 keer per minuut klopt. Dat deze functionele metingen ook zeer nauwkeurig zijn hebben we aangetoond in **hoofdstuk 5**. In een vergelijkende studie tussen 3D-echocardiografie en electrocardiogram (ECG)-getriggerde ^{99m}Tc -sestamibi SPECT bleken de functionele metingen aan het hart met elkaar vergelijkbaar te zijn in dieren met een sham (controle) operatie en in dieren met een permanente ligatie van de linker coronaire arterie (hartinfarct). Gezien de vaak multifactoriële aard van hart- en vaatziekten lijkt SPECT beeldvorming de ideale kandidaat omdat zowel functie, doorbloeding, leefbaarheid en herstelstatus van het hart middels angiogenese in slechts één scan betrouwbaar aan te tonen.

In **hoofdstuk 6 en 7** hebben we in een hartinfarct model in de muis 3 verschillende angiogenese tracers getest. Al deze tracers zijn gebaseerd op het CD13 bindende peptide cyclisch-NGR (cNGR). Hoewel ons lab in het verleden al middels microscopie en MRI aan heeft getoond dat het cNGR peptide specifiek bindt aan CD13 op nieuw gevormde capillairen waren er voor deze cNGR gebaseerde tracer constructies weinig mogelijkheden voor translatie naar de kliniek. In **hoofdstuk 6** tonen we specifieke opname van het cNGR peptide gemerkt met de SPECT radionuclide ^{111}In in gebieden met verlaagde opname van de perfusie tracer ^{99m}Tc -sestamibi. Hoewel de opname van deze angiogenese specifieke

tracer significant hoger was in vergelijking met de opname in gezonde controle dieren en een snelle klaring liet zien via de urine, was de opname in het gebied rondom het infarct en in het infarct weefsel zelf laag, wat nog steeds een obstakel is voor klinisch gebruik.

In **hoofdstuk 7** beschrijven we twee strategieën om de opname van deze cNGR-gebaseerde tracer te verhogen. Ten eerste hebben we de ringstructuur van de cNGR entiteiten stabiel(er) weten te maken door sterkere verbindingen aan te brengen middels natieve chemische ligatie. De verhoogde stabiliteit van deze middels natieve chemische ligatie vervaardigde tracer werd bevestigd door stabiliteitstesten in bloed. Ten tweede hebben we de mogelijkheden van een multivalente tracer geëxploreerd. In eerdere studies, voornamelijk in tumor weefsels, is reeds aangetoond dat multivalente moleculen (moleculen bestaande uit meerdere entiteiten die elk binden aan eenzelfde doelwit) een hogere affiniteit hebben voor hun doelwit. Voor de studie beschreven in hoofdstuk 7 werd naast de nieuwe en stabielere cNGR-gebaseerde SPECT tracer met 1 cNGR entiteit een nieuw multivalent cNGR molecuul gesynthetiseerd met 4 cNGR entiteiten. Voor deze tracer werden de ring structuren van de cNGR entiteiten eveneens vervaardigd middels natieve chemische ligatie. Ook voor deze tracer werd verhoogde stabiliteit in het bloed aangetoond. Na succesvolle radiolabeling met ^{111}In werden beide nieuwe tracers getest in muizen met een infarct en muizen die een sham operatie hebben ondergaan. Beide ^{111}In gelabelde tracers vertoonden een significant hogere opname in infarct gebieden in vergelijking met de cNGR-gebaseerde SPECT tracer die gebruikt werd voor de studie die beschreven wordt in hoofdstuk 6, echter ging dit enigszins te koste van hogere non-specifieke opname. Vanwege de gunstige klaring en lage opname in andere organen zijn beide tracers veelbelovend voor toepassing in klinische beeldvorming.

Het geheel aan bevindingen wordt samengevat in de algehele discussie in **hoofdstuk 8**. Tevens worden in dit hoofdstuk onze resultaten vergeleken en besproken aan de hand van door anderen gepubliceerd onderzoek. Tot besluit bespreken we nog hoe de verworven kennis uiteindelijk van tastbare waarde kan worden in het klinische onderzoek of in andere vakgebieden.

Concluderend kan worden gesteld dat de studies die beschreven staan in dit proefschrift een bijdrage leveren aan de nucleaire beeldvorming van perfusie, functie en neovascularisatie. Ik heb aan kunnen tonen dat SPECT imaging een betrouwbare optie is voor het in beeld brengen van perfusie herstel en het meten van parameters die een indicatie geven van de functie van het hart. Tevens heb ik laten zien dat angiogenese succesvol in beeld gebracht kan worden middels cNGR gebaseerde tracers in een infarct model in de muis. Echter voordat specifieke arteriogenese beeldvorming verwezenlijkt kan worden dient men rekening te houden met de hoge afschuifspanning langs de wanden van de collateraal arteriën en moet men een goed begrip hebben van de korstondig inflammatoire status van het weefsel waarin de groei van collateraal arteriën plaatsvindt.

VALORIZATION ADDENDUM

In the Netherlands alone, cardiovascular diseases account for roughly 1000 hospitalizations a day. The Dutch heart foundation calculated that in total 8.3 billion euro was spent on cardiovascular disease care in 2011, thereby illustrating the magnitude of the burden that cardiovascular diseases place on the national economy. The majority of this amount was spent on hospital care and care from medical specialists. However, despite the rise in life expectancy during the last decades, which can in a part be ascribed to novel, often interventional technologies, these technologies are typically applied in advanced stages of disease and are therefore cost-ineffective. Early diagnosis and early treatment or even prevention of heart and peripheral vascular disease, will allow better risk stratification of patients with disease-stage-adapted-therapy instead of escalating to the most aggressive and costly therapy. However, from clinical trials that have been executed it has become clear that clinical readout parameters such as peak walking distance in peripheral vascular disease trials or exercise tolerance test in coronary artery disease trials are not sufficiently sensitive and reproducible to document incremental improvements in function and perfusion. In addition, gathering indirect evidence for neovascularization (i.e. increased perfusion or function) through angiography, SPECT, PET or MRI often takes months before any improvement can be detected.

The work described in this dissertation contributes to the translation of non-invasive neovascularization imaging to the clinic, thereby ultimately providing patients with tailored therapy programs as well as reducing healthcare costs. All of our scientific endeavors were performed within the framework of a Center for Translational Molecular Medicine (CTMM), project Eminence. Within this project we collaborated closely with industry partners from MILabs and PIE Medical Imaging.

As is often the case for preclinical endeavors, the scientific community is the first to benefit from the novel findings described in this dissertation. Through online publication the majority of the scientific work we describe here is globally available. Nevertheless, how these new insights are eventually translated into value beyond the scientific domain is unfortunately difficult to measure. Still, we can speculate on the potential value creation for the technologies that we have investigated.

TOWARDS BETTER DIAGNOSIS AND CLINICAL NEOVASCULARIZATION THERAPY

Candidate agents for non-invasive arteriogenesis imaging

In chapter 2 and 3 we described pioneering work in the field of nuclear arteriogenesis imaging. To our knowledge no studies have been published describing nuclear arteriogenesis imaging or the development of a nuclear arteriogenesis tracer. The knowledge we generated on this topic can serve as a starting point for future studies investigating non-invasive arteriogenesis imaging. We narrowed the window of potential

candidate arteriogenesis tracers down by eliminating the most obvious candidates. This prevents scientists to pursue avenues that are unfruitful and costly.

Preclinical testbed

Over the past decades numerous hind limb ischemia animal models have been developed to mimic the situation of peripheral vascular disease and to study neovascularization stimulating agents. In these models, the blood flow in one (or more) arteries of the hind limb is obstructed, thereby creating a drop in blood perfusion throughout the whole limb. This drop in perfusion is temporary since the body is able to (fully) restore the perfusion by means of neovascularization. The time until perfusion restoration is completed is often called the therapeutic window (i.e. the time in which it is beneficial to stimulate neovascularization). In the past, numerous studies have employed the therapeutic windows, created in these hind limb ischemia models, to test neovascularization stimulating agents thereby using laser Doppler perfusion imaging (LDPI) as a readout. In chapter 4 we used SPECT perfusion imaging to show the inaccuracy of LDPI as a readout in these models. The latter still indicates the need for perfusion recovery while SPECT imaging indicated that the relevant perfusion recovery over the total volume of the hind limb has already taken place. Using LDPI it is easy to overestimate the therapeutic window, which in turn can lead to the misinterpretation of neovascularization stimulating agents or application at the wrong time point. This is an important finding for cardiovascular disease patients since almost all clinical studies employing neovascularization stimulating agents have failed to capitalize on the positive outcomes of their preclinical predecessors. Interpretation of stimulating agents in the correct time window (determined by SPECT perfusion imaging) can lead to important new insights about the mechanism of action and impact of such agents thereby eventually leading to different therapeutic regimens for cardiovascular disease patients that can be tested in clinical trials and are hopefully more predictive.

SPECT offers the opportunity to perform multi-isotope imaging thereby making it an attractive option for studying the often multifactorial nature of cardiovascular disease. In chapter 5 we showed that ECG-gated SPECT can deliver reliable cardiac functional measurements while simultaneously indicating myocardial perfusion in a mouse myocardial infarction model. Valuable input from our colleagues at MILabs helped us extract the most out of our SPECT data. During this joint project a strong relationship MILabs was established that provides both parties with new opinions, insights and tools for refining the technology.

In chapter 7 we built on insights we gained from the study described in chapter 6. Three new SPECT tracers for angiogenesis imaging were developed and tested in a mouse myocardial infarction model. In chapter 6 we demonstrated specific angiogenesis imaging in the infarcted myocardium using a disulfide cyclized cNGR angiogenesis tracer.

Considering the rather low uptake of this tracer we set out to improve this by altering the nature and the valency of our tracer. With the two new tracer constructs that resulted from these efforts we made a valiant step forward with respect to uptake in infarcted areas. Both new tracers showed significantly higher uptake in infarcted areas. Considering the high specific uptake in infarcted myocardium in combination with low uptake in remote organs we expect these tracers to eventually make the step to clinical trials. However, before these tracers can be tested in randomized controlled trials in humans several additional steps have to be taken. Large scale animal studies have to be conducted in different species (including toxicity studies) to provide sufficient proof of efficacy before production of the tracer under good manufacturing practice (GMP) conditions can be considered. Thereafter, the true benefit of clinical angiogenesis imaging has to be proven against traditional endpoints or indirect effects such as clinical state and tissue perfusion or function. In case clinical studies indicate positive results, these tracers will become available for everyday myocardial infarction patient care.

I believe the time efforts and costs that were made during my PhD trajectory are all justified by the work presented in this dissertation. Big things have small beginnings and like with all preclinical research, a lot of work still has to be performed before cardiovascular disease patients will benefit from the knowledge generated by our endeavors.

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Precies 4 jaar lang heb ik mij als PhD kandidaat in de veelzijdige onderzoekswereld van de Nucleaire Geneeskunde kunnen bewegen. Een periode die langzaam op gang leek te komen maar, zeker de laatste 2 jaar, om is gevlogen.

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CURRICULUM VITAE

Geert Hendrixx was born on October 18th 1987 in Weert, the Netherlands. After finishing his pre-university secondary education (VWO – Nature and Health track) at Bisschoppelijk College high school in Weert, he obtained his Bachelor's degree in Biomedical Sciences at Maastricht University while simultaneously completing the Honours Programme International Health. In 2012, Geert obtained his Master's degree in Cardiovascular Biology and Medicine at Maastricht University after a combined internship at the Babraham Institute (Cambridge, England) and the department of Physiology at Maastricht University. In the same year, he started his PhD trajectory at the department of Nuclear Medicine of the Maastricht University Medical Center under supervision Prof. Dr. Felix M. Mottaghy and Prof. Dr. Mark J. Post, which resulted in the work described in this thesis. Geert presented his work during several (inter)national conferences and obtained the Co-image of the year 2015 award from MILabs.

PUBLICATIONS

This dissertation is based on the following publications:

Hendrixx G., Vöö S., Bauwens M., Post M.J., Mottaghy F.M. SPECT and PET imaging of angiogenesis and arteriogenesis in pre-clinical models of myocardial ischemia and peripheral vascular disease. *European Journal of Nuclear Medicine and Molecular Imaging*. 2016. *E-publication ahead of print*.

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Other publications

De Saint-Hubert M., Bauwens M., Deckers N., Drummen M., Douma K., Granton P., **Hendrikx G.**, Kusters D., Bucerius J., Reutelingsperger C.P., Mottaghy F.M. In vivo molecular imaging of apoptosis and necrosis in atherosclerotic plaques using microSPECT-CT and microPET-CT imaging. *Molecular imaging and Biology*. 2014 Apr;16(2):246-54.

PRESENTATIONS

2016 Invited oral presentation. MILabs user meeting 2016, Utrecht, the Netherlands.

2015 Oral presentation. 31st Dutch Physiology days; Dutch Physiology Society. Groningen, the Netherlands.

2015 Oral presentation. 37th Annual Microcirculation Meeting; German Society for Microcirculation & Vascular Biology. Hannover, Germany.

2015 Invited oral presentation. MILabs user meeting 2015, Utrecht, the Netherlands.

2015 Invited oral presentation. CTMM Eminence Annual Meeting. Maastricht, the Netherlands.

2015 Oral presentation. Belgian Molecular Imaging Community Congress 2015. Brussels, Belgium.

2014 Invited oral presentation. CTMM Eminence Annual Meeting. Utrecht, the Netherlands.

2014 Two poster presentations. World Molecular Imaging Congress 2014. Seoul, South-Korea.

2014 Poster presentation. CARIM Symposium 2014. Maastricht, the Netherlands.

2014 Invited oral presentation. CTMM Eminence Annual Meeting. Maastricht, the Netherlands.