Molecular imaging of atherosclerosis and thrombus formation

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Molecular Imaging of Atherosclerosis and Thrombus Formation

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Abbreviations

^{18}F -FDG	^{18}F fluorodeoxyglucose
α_2 -AP	α_2 -antiplasmin
AnxA5	Annexin A5
ApoE ^{-/-}	Apolipoprotein E-deficient
Bi- α_2 AP-CA	Bimodal α_2 -antiplasmin-based contrast agent
Bi-con-CA	Bimodal control contrast agent
BMM	Bone marrow-derived macrophage
CA	Contrast agent
CCR5	Chemokine receptor 5
CE-MRI	Contrast-enhanced MRI
CLEAR	Constant level of appearance
CLIO	Crosslinked iron oxide
CNR	Contrast-to-noise ratio
CPM	Count per minute
CT	Computed tomography
CTA	Computed tomography angiography
DMEM	Dulbecco's modified Eagle's medium
DSA	Digital subtraction angiography
EDTA	Ethylene diamine tetra acetate
ESI-MS	Electrospray ionization mass spectrometry
FXIII	Factor XIII
FXIIIa	Activated factor XIII
FITC	Fluorescein isothiocyanate
FOV	Field-of-view
Gd	Gadolinium
Gd-DTPA	Gadolinium-diethylenetriaminepentaacetic acid
HBSS	Hanks balanced salt solution
HE	Hematoxylin/eosine
HPLC	High pressure liquid chromatography
ICAM 1	Intercellular adhesion molecule 1
ICC	Intraclass correlation coefficients
ICP-MS	Inductively coupled plasma mass spectrometry
IFN- γ	Interferon- γ
IHC	Immunohistochemistry
IMT	Intima-media thickness
IR-TSE	Inversion recovery turbo spin echo
IVMRI	Intravascular magnetic resonance imaging
IVUS	Intravascular ultrasound
LCM	L929 cell conditioned medium

LDL	Low-density lipoprotein
LDLr ^{-/-}	LDL receptor deficient
LPS	Lipopolysaccharide
LR	Likelihood ratio
LRNC	Lipid-rich necrotic core
MDA2	Monoclonal oxidation-specific antibody against malondialdehyde(MDA)-lysine epitopes
MMP	Matrix metalloproteinase
MRA	Magnetic resonance angiography
MRI	Magnetic resonance imaging
MALDI-MS	Matrix-assisted laser desorption/ionization mass spectrometry
NIR	Near-infrared
NSA	Number of signal averages
NSF	Nephrogenic systemic fibrosis
NZW	New Zealand white
OCT	Optical coherence tomography
oxLDL	Oxidized low density lipoprotein
PCD	Programmed cell death
PET	Positron emission tomography
PIXE	Proton induced x-ray emission
PS	Phosphatidylserine
QD	Quantum dot
r ₁	Longitudinal relaxivity
RANTES	Regulated upon activation, normal T cell expressed and secreted
RARE	Rapid acquisition with relaxation enhancement
REML	Restricted maximum likelihood
REST	Regional saturation
RF	Radiofrequency
ROI	Regions of interest
RPMI	Roswell park memorial institute
rSI	Relative signal intensities
SD	Standard deviation
SE	Signal enhancement
SI	Signal intensities
SMC	Smooth muscle cells
SNR	Signal-to-noise ratio
SPECT	Single photon emission computed tomography
SPPS	Solid phase peptide synthesis
TE	Echo time
TFE	Turbo field echo
TI	Inversion time

TPLSM	Two-photon laser-scanning microscopy
TR	Repetition time
TSE	Turbo spin echo
USPIO	Ultrasmall superparamagnetic particles of iron oxide
VCAM 1	Vascular cell adhesion molecule 1

CHAPTER 1

General introduction

Atherosclerosis and thrombus formation

Atherosclerosis is a complex, progressive inflammatory disease of the large arteries. Rupture of atherosclerotic lesions and subsequent thrombus formation are the main underlying causes of cardiovascular diseases such as myocardial infarction and stroke. Cardiovascular diseases are a major cause of morbidity and mortality in the western society (1-5).

A healthy vessel wall is built up out of three layers. The inner or intimal layer, adjoining the lumen, consists of a monolayer of endothelial cells (luminal side) and the internal elastic lamina (peripheral side). The medial layer is composed of smooth muscle cells (SMCs), while the outer or adventitial layer consists of connective tissue with interspersed fibroblasts and SMCs (1). The formation of early lesions (intimal xanthoma or intimal thickening) is considered to be the initial step in the atherosclerotic process and is predisposed to parts of the arteries which are exposed to low or oscillatory shear stress, such as curvatures or branches in the arterial tree (6).

Endothelial cells exposed to low shear stress become activated, which subsequently results in increased permeability of the endothelial surface and upregulation of adhesion molecules such as vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-selectin (7). During this initiation step, lipoproteins such as low-density lipoprotein (LDL) infiltrate into the intima where they undergo modifications such as oxidation, lipolysis, proteolysis, and aggregation. In addition, monocytes attach to the adhesion molecules and migrate into the subendothelial space, initiating an inflammatory response. Subsequently, the monocytes differentiate into macrophages which take up the modified lipoproteins, forming lipid-laden foam cells. Together with T cells, they form fatty streaks which usually are already present in young people. During the progression of atherosclerotic lesions, T cells secrete inflammatory and anti-inflammatory cytokines and chemokines. The inflammatory cytokines and chemokines activate macrophages and vascular cells, thereby amplifying the inflammatory response. Subsequently, more leukocytes (such as T cells, monocytes, macrophages), will be attracted which will even further amplify the inflammatory response. Due to apoptosis, necrosis, increased proteolytic activity, and lipid accumulation, a lipid-rich necrotic core will form which is initially separated from the lumen by a fibrous cap. The fibrous cap consists of a layer of smooth muscle cells, migrated from the medial layer, and extracellular matrix proteins. Proteases secreted by activated leukocytes can degrade the extracellular matrix while pro-inflammatory cytokines such as interferon- γ (IFN- γ) inhibit matrix production by SMCs thereby thinning the fibrous cap. In addition, other processes such as apoptosis of SMCs and intraplaque hemorrhage contribute to the transition of a stable to a so-called 'vulnerable lesion'. Eventually, plaque erosion or rupture can

initiate coagulation, platelet adherence and thrombus formation. The formed thrombi narrow or occlude the lumen at the site of erosion or rupture, or might occlude more distal areas due to emboli formation. These events may eventually result in thrombotic complications such as myocardial infarction, stroke, pulmonary embolism, or deep venous thrombosis (1,2,8-14).

Classification of atherosclerotic lesions

Based on the morphology, atherosclerotic lesions can be classified as early (subdivided in intimal xanthoma or intimal thickening) or advanced progressive atherosclerotic lesions (subdivided in pathological intimal thickening, fibrous cap atheroma, thin fibrous cap atheroma, calcified nodule, and fibrocalcified atherosclerotic lesions) (15). A consensus document by Naghavi et al. described the so-called ‘vulnerable lesions’ as atherosclerotic lesions leading to complications such as myocardial infarction and stroke. Major criteria in defining vulnerable lesions are active inflammation, thin cap with large lipid core, endothelial denudation with superficial platelet aggregation, and severe stenosis (3).

Molecular imaging of atherosclerotic lesions

Molecular imaging is a multidisciplinary field that exploits cellular and molecular information for the detection of disease-related targets using specific target-directed molecular imaging agents. These molecular imaging agents are commonly composed out of three parts: 1) a specific ligand for the target of interest, 2) an imaging agent dependent on the preferred imaging modality, and 3) a linker between the specific ligand and the imaging agent. Dependent on the target of choice, these molecular imaging agents have to meet important criteria such as high specificity and affinity for the chosen target, the ability to overcome biological barriers, and to have tolerable pharmacokinetics (16-18). Several imaging modalities can be used for the molecular imaging of atherosclerosis and thrombus formation. Non-invasive imaging modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET) enable non-invasive assessment of atherosclerotic lesions and will be discussed in more detail in the next chapter (**chapter 2**). Imaging modalities such as two-photon laser-scanning microscopy (TPLSM) can aid in the validation of molecular imaging agents suitable for molecular imaging of atherosclerosis since TPLSM can provide subcellular information.

Hypothesis

Considerable progress has been made in the visualisation of atherosclerosis and thrombus formation during the last few years, predominantly due to technical improvements. We hypothesized that molecular imaging strategies using specific site-directed molecular imaging agents can contribute and further improve the assessment of atherosclerosis and thrombus formation by obtaining molecular information of both atherosclerosis and thrombus formation.

Thesis outline

This thesis focuses on the molecular imaging of atherosclerosis and thrombus formation.

In the **second chapter**, an overview of the atherosclerotic process and a comparison of the available imaging modalities for the visualization of atherosclerotic lesions is provided. In addition, an overview is presented on current molecular imaging agents for atherosclerosis and thrombus formation.

Gadofosveset is a relatively new FDA approved contrast agent (CA) for MRI. Large fractions of the circulating Gadofosveset reversibly bind to albumin thereby prolonging the clearance half-life. Since albumin is present in atherosclerotic lesions and the albumin content is associated with plaque size and microvessel density, albumin-binding CAs might improve atherosclerotic plaque assessment. Therefore, the potential of Gadofosveset in visualizing atherosclerotic lesions was determined by comparing this CA with the more commonly used Gd-DTPA in a rabbit model (**chapter 3**).

Vulnerable lesions are macrophage-rich. Therefore, macrophages might be suitable targets for the molecular imaging of macrophage-rich atherosclerotic lesions. We found that mRNA levels and protein expression of chemokine receptor 5 (CCR5), which is one of the chemokine receptors expressed on macrophages, are up regulated in ruptured human carotid atherosclerotic lesions while it is only limited present in stable lesions. Therefore, molecular imaging agents based on the CCR5 ligand called RANTES (regulated upon activation, normal T cell expressed and secreted), were evaluated *in vitro* using murine bone-marrow derived macrophages. In addition, the feasibility of the RANTES-based molecular imaging agents for the visualization of murine atherosclerotic lesions was demonstrated with TPLSM and PET (**chapter 4**).

A molecular imaging agent that might be used for the visualization of atherosclerosis as well as thrombosis is presented in **chapter 5**. In this chapter, annexin A5 was used to target phosphatidylserine (PS), which is exposed on the

surface of dying cells as well as on activated blood platelets. The synthesized molecular imaging agent, based on a quantum dot (QD) loaded with gadolinium-wedges, was used to visualize apoptotic Jurkat cells and activated platelets in *in vitro* formed thrombi with TPLSM and MRI. In addition, the feasibility of this molecular imaging agent for visualizing cell death in a murine carotid artery was demonstrated.

Thrombus visualization might also be achieved using fibrin-targeted molecular imaging agents, as presented in **chapter 6**. Our approach was based on the presence of activated factor XIII (FXIIIa) during thrombus formation. FXIIIa is capable of crosslinking α_2 -antiplasmin to fibrin during the early phases of thrombus formation. Therefore, a bimodal α_2 -antiplasmin-based molecular imaging agent was synthesized for the *in vitro* and *in vivo* visualization of thrombus formation.

Finally, the results obtained in this thesis and their implications on the assessment of atherosclerosis and thrombus formation are discussed in **chapter 7**.

References

1. Lusis AJ. Atherosclerosis. *Nature* 2000;407:233-41.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105:1135-43.
3. Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 2003;108:1664-72.
4. Lloyd-Jones D, Adams R, Carnethon M, et al. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;119:e21-181.
5. Weintraub HS. Identifying the vulnerable patient with rupture-prone plaque. *Am J Cardiol* 2008;101:3F-10F.
6. Ridger V, Krams R, Carpi A, Evans PC. Hemodynamic parameters regulating vascular inflammation and atherosclerosis: A brief update. *Biomed Pharmacother* 2008;62:536-40.
7. Chatzizisis YS, Coskun AU, Jonas M, Edelman ER, Feldman CL, Stone PH. Role of endothelial shear stress in the natural history of coronary atherosclerosis and vascular remodeling: molecular, cellular, and vascular behavior. *J Am Coll Cardiol* 2007;49:2379-93.
8. Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med* 1999;340:115-26.
9. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-95.
10. Packard RR, Libby P. Inflammation in atherosclerosis: from vascular biology to biomarker discovery and risk prediction. *Clin Chem* 2008;54:24-38.
11. Aukrust P, Halvorsen B, Yndestad A, et al. Chemokines and cardiovascular risk. *Arterioscler Thromb Vasc Biol* 2008;28:1909-19.
12. Virmani R, Kolodgie FD, Burke AP, et al. Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* 2005;25:2054-61.
13. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* 2008;8:802-15.
14. Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 2009;27:165-97.
15. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000;20:1262-75.
16. Weissleder R, Mahmood U. Molecular imaging. *Radiology* 2001;219:316-33.
17. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev* 2003;17:545-80.
18. Jaffer FA, Weissleder R. Molecular imaging in the clinical arena. *Jama* 2005;293:855-62.

CHAPTER 2

Development and validation of novel imaging technologies to assist translational studies in atherosclerosis

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Abstract

In the past decade, significant progress has been made to visualise atherosclerotic diseases. Until recently, imaging technologies mainly focused on lumen and vessel wall visualisation. Current advances and knowledge on the molecular mechanisms of initiation and progression of atherosclerosis has emphasized the need for imaging technologies and probes that can image function and biology rather than anatomy. This field of molecular imaging is now in rapid development with new imaging agents that aim at visualising processes involved in atherosclerosis such as inflammation, macrophage activation, protease activity, angiogenesis, apoptosis, lipid accumulation and thrombus formation.

Introduction

Atherosclerosis and its thrombotic complications are the leading cause of mortality and morbidity in the Western World. This multi-factorial disease is characterized by accumulation of lipids, cells and extracellular matrix in the wall of large arteries. The concept that this accumulation over the years irrevocably leads to stenosis was changed by the seminal paper of Glagov et al. (1), showing compensatory vessel enlargement and lumen preservation at sites of atherosclerotic lesions. Clinical imaging modalities such as angiography, relying on arterial lumen visualisation to image and diagnose atherosclerosis, are unable to detect these expanding lesions. Developments in atherosclerotic imaging are increasingly guided by growing insights into the molecular mechanisms of lesion development. It is widely accepted that atherosclerosis is an inflammatory disease and that lesion components rather than size determine the adverse events such as myocardial infarction and stroke (2). Consequently, clinical imaging modalities focus on imaging inflammation and the critical plaque components that characterize the so-called vulnerable lesion.

Atherosclerosis – current concepts

The vulnerable lesion

The vulnerable lesion was recently re-defined in a consensus document (3) in which this term was recommended to identify all thrombosis-prone plaques and plaques with a high probability of undergoing rapid progression. Criteria for defining the vulnerable lesion were (amongst others): active inflammation (macrophage/T-lymphocyte infiltration), thin cap with large lipid core, endothelial denudation with platelet aggregation, intraplaque hemorrhage and >90% stenosis. Importantly, these new criteria define several types of vulnerable lesions; for example, rupture-prone lesions with large lipid core and thin cap, ruptured lesions with sub-occlusive thrombus and early organization or lesions with intraplaque hemorrhages due to leaky vasa vasorum. Each will have its own challenges for visualisation by imaging technologies.

Inflammation

It is clear from the criteria for vulnerable lesions that inflammation is an essential feature. More importantly, inflammation and cytokine pathways are crucial in the development and progression of atherosclerotic lesions. The influx of inflammatory cells, macrophages, and T-lymphocytes in atherosclerotic plaques increases with

plaque progression and is increased at sites of plaque rupture. Immune cells are involved in every stage of the disease and cytokine pathways are already activated in early lesions (2). Imaging agents that would be able to visualise these early pathways could open avenues towards early detection (and possibly treatment) of atherosclerotic lesions.

Thrombosis

Rupture of a vulnerable lesion and subsequent thrombus formation accounts for ~70% of fatal acute myocardial infarctions and/or sudden coronary deaths. It is important to realize that plaque rupture does not always imply a fatal event. In patients who died of non-cardiovascular causes, plaque rupture was present in 10% of atherosclerotic lesions (4). Nonfatal lesions can contain areas of (repeated) plaque rupture and thrombosis. If a thrombus remains mural rather than occlusive and its lysis is incomplete, re-endothelialization followed by fibrous thrombus organization results in exponential plaque growth (5). The same exponential plaque growth can be observed after intraplaque hemorrhage (6). Thus, imaging technologies that use thrombus detection to depict the vulnerable lesion should also focus on visualisation of non-fatal thrombus-formation and intraplaque hemorrhage.

Imaging technologies and atherosclerosis – using the concepts

This section reviews several imaging modalities capable of visualising atherosclerotic lesions (table 2.1).

Lumen visualisation

Invasive x-ray angiography remains the current gold standard technique for the diagnosis of stenotic vessel disease. Although lesions that have adapted to positive (outward) remodeling will be underestimated, plaques with severe stenosis can be diagnosed with angiography. Stenotic lesions are considered vulnerable, as increased surface shear stress could induce thrombosis and sudden occlusion (3). Non-invasive imaging technologies to visualise stenotic lesions include ultrasound, computed tomography angiography (CTA) and magnetic resonance angiography (MRA) (7,8).

Table 2.1: Comparison summary table

Names of specific technologies with associated companies and company websites	Ultrasound		Intravascular ultrasound		Ultra-fast computed tomography		Nuclear (PET/SPECT)		Magnetic resonance imaging		Intravascular magnetic resonance imaging		Angioscopy		Optical computed tomography		Thermography	
	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
	-Philips Medical Systems http://www.medical.philips.com -Siemens Medical Solutions http://www.medical.siemens.com -General Electric Healthcare http://www.gehealthcare.com -Fukuda Denshi http://www.fukuda.co.uk -SonoSite http://www.sonosite.com -Toshiba Medical Systems http://medical.toshiba.com -Hitachi Medical Systems http://www.hitachimed.com	-Volcano Corporation http://www.volcanocorp.com -Boston Scientific http://www.bostonscientific.com -General Electric Healthcare -Toshiba Medical Systems -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Toshiba Medical Systems -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Philips Medical Systems -Siemens Medical Solutions -General Electric Healthcare -Hitachi Medical Systems	-Surgi-Vision http://www.surgi-vision.com -TopSpin Medical (research / clinical trials) http://www.topspin.co.il	-Baxter Healthcare Corporation http://www.baxter.com	-Novacam (research) http://www.novacam.com	-Thermocore http://www.thermocoremed.com				
Invasive Resolution	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes	No	Yes
	50-500 μm	$\pm 100 \mu\text{m}$	50-200 μm	$\pm 100 \mu\text{m}$	4-7 mm (PET), 8-14 mm (SPECT)	$\pm 300 \mu\text{m}$	10^{-11} to 10^{-12} M (PET), 10^{-10} to 10^{-11} M (SPECT)	10^{-3} to 10^{-5} M	10^{-3} to 10^{-5} M	$\pm 160 \mu\text{m}$	$\pm 160 \mu\text{m}$	$\pm 10 \mu\text{m}$	$\pm 10 \mu\text{m}$	$\pm 0.5 \text{ mm}$				
Sensitivity	Not well characterized	-	Not well characterized	-	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good
Operator dependency	Poor	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good	Good
Additional comments	-Cheap -Patient friendly -Initial studies showed possibilities for molecular imaging -Used to image superficial arteries	-Used to image superficial and deep arteries -Detection plaque components (fibrous and calcified tissue) between fibrous and lipid-rich plaques -Possible vessel wall damaging -Catheter positioning by X-ray angiography	-Identification of calcified areas -Use of ionizing radiation -Use of iodinating contrast agents for reliable discrimination between lumen and vessel wall -Not very promising for molecular imaging	-Highly sensitive molecular imaging technique -Use of ionizing radiation	-Identification and quantification plaque components -Patient friendly -Initial studies showed possibilities for molecular imaging -Contra indications MRI -Scan duration -Possible RF-heating -Possible vessel wall damaging coronary arteries not yet feasible	-Used to image superficial and deep arteries -Identification of plaque components -Contra indications MRI -Scan duration -Possible RF-heating -Possible vessel wall damaging	-Direct visualisation -Requires blood free zone -Only surface visualisation -Catheter positioning by X-ray angiography required	-Identification of plaque components -Low penetration depth -Requires blood-free zone -Catheter positioning by X-ray angiography required	-Sensitive for inflammation -No detection of plaque components -Contact with arterial wall required									
References	(9-12, 48, 49)	(21-23, 27, 48)	(8,13,48,49)	(48, 49)	(7,14-16, 18-20, 48, 49)	(22, 27)	(22)	(22, 24-26, 48)	(22, 28, 29)									

Components of the vulnerable lesion

Both invasive and non-invasive imaging technologies have been used to study the components of vulnerable lesions. B-mode duplex ultrasound allows non-invasive imaging of the large superficial arteries and has become routine in the screening of patients with carotid artery disease. Ultrasound can measure lumen stenosis, arterial wall thickness, as well as intima-media thickness (IMT). Population studies have shown mean carotid IMT predicts risk of both cerebrovascular and myocardial events (9). Using acoustic impedance, ultrasound can also provide information on carotid plaque morphology. Intraplaque hemorrhage and lipids are represented by echolucent signal, while an echo-rich signal is characteristic for fibrous tissue (10). Calcifications are known to cause acoustic shadowing, through which distal plaque components and the outer vessel boundary may become invisible. Using a semi-automated ultrasound system and histology as standard, Devuyst et al. showed that it was possible to measure fibrous cap thickness of carotid lesions (11). Also, by analyzing integrated ultrasonic backscatter of carotid artery lesions, differentiation between thick and thin atherosclerotic fibrous caps proved to be possible (12). Accuracy for these measurements, however, is dependent on the experience of the researcher (10).

Computed tomography is an alternative imaging modality with an excellent sensitivity for calcified areas, but non-calcified lesions can only be detected using contrast agents (13). Modern multislice or multidetector CT scanners with high rotation speed (< 400 ms), resulted in faster imaging and improved spatial resolution compared to conventional CT. Electron-beam CT with an acquisition time of less than 100 ms, fast enough to eliminate motion artifacts related to the cardiac contractions, is predominantly used to detect and to quantify coronary calcifications.

Magnetic resonance imaging (MRI) is often cited as most promising for the routine clinical detection of vulnerable lesions. It is non-invasive, does not involve ionizing radiation, can be repeated serially and has high soft tissue contrast (14-16). Carotid plaque components such as lipid-rich necrotic core (LRNC) with possible hemorrhage, calcification and fibrous tissue can be distinguished with MRI by combining information from different MR pulse sequences (figure 2.1, (17)). Moreover, quantifying plaque components with MRI correlated well with histological findings (18). Intra-plaque hemorrhage could be detected using a T_1 -weighted turbo field echo sequence with 84% sensitivity and specificity (19). Contrast-enhanced MRI (CE-MRI) improved the differentiation between LRNC and fibrous tissue (16). Quantification of vessel wall thickness and area was achieved with MRI in both carotid and coronary arteries (20). However, in contrast to the carotid artery, differentiation of plaque components is still challenging in coronary arteries owing to their small size and extensive motion. Improvements in pulse

sequences, radiofrequency coils and CE-MRI might further improve coronary plaque imaging.

Invasive imaging technologies are able to directly visualise the arterial wall and plaque components, also in non-superficial arteries. Intravascular ultrasound (IVUS) is an application of ultrasound using an intravascular transducer that enables direct lesion imaging. IVUS is able to visualise angiographically invisible, non-stenotic lesions (21). Also, acoustic properties allow determination of fibrous and calcified lesions, but less so for lipid-rich lesions (22). IVUS elastography is an application in which local elastic properties of coronary atherosclerotic lesions can be measured. First results indicate that this method could improve the detection of a vulnerable lesion based on the measurement of locally increased strain (23).

Angioscopy allows direct visualisation of the vessel wall surface using an intravascular camera. It is capable of discriminating the normal arterial wall from the atherosclerotic lesion. Yellow-colored lesions indicate the presence of a thin fibrous cap, suggesting a vulnerable lesion. A fibrin-rich overlying thrombus is seen as an irregular red structure, protruding in the lumen. Limitations are that only the surface of the vessel wall can be visualised and a blood-free zone is required for acquisition (22).

Figure 2.1: Transverse MR images and corresponding histologic slice obtained at the same carotid artery level. Weightings included T_1 -weighted turbo field echo (TFE) and intermediate-weighted, T_2 -weighted, T_1 -weighted double-inversion-recovery black-blood technique, and partial T_2 -weighted turbo spin echo (TSE). Hemorrhage (h) was well differentiated from fibrous tissue (f) on the T_1 -weighted TFE image. On the T_2 -weighted TSE image, fibrous tissue had high signal intensities whereas calcification (c, arrows) had very low signal intensities. Calcification also had very low relative signal intensities on intermediate-weighted, T_1 -weighted, and partial T_2 -weighted TSE images (copyright ©Radiological Society of North America, 2005) (17). For full color figure see chapter 9.

Optical coherence tomography (OCT) is a catheter-based imaging modality that uses an infrared light source to generate images with resolutions up to 10 μm . OCT can visualise several plaque components such as lipid core, thrombus and calcification, and can measure fibrous cap thickness (24). In comparison with IVUS, OCT has equal sensitivity for detecting plaques and fibrous and calcified deposits and is superior for identification of intimal hyperplasia and lipid-rich lesions (25). Macrophage abundance (*ex vivo*) was also determined with OCT and correlated well with histological measurements (26). OCT has the limitation of requiring a blood free-zone for *in vivo* visualisation, which is usually achieved by saline flushes allowing only short acquisition times, excluding imaging long artery segments (24). Only limited data is available of OCT studies in patients.

In the large superficial arteries, MRI was capable of detecting plaque components. In deeper arteries, such as iliac atherosclerotic arteries, intravascular magnetic resonance imaging (IVMRI) has been used to detect plaque components. High sensitivity and specificity were achieved for lipid, fibrous tissue and calcium (27). However, the risk of damaging the vessel wall and the potential of radiofrequency (RF) heating using intravascular coils are drawbacks in IVMRI.

Inflammation

A recent innovation in catheter-based visualisation of plaque composition is intravascular thermography (28). Vulnerable lesions emit more heat due to their inflammatory content and intravascular thermography was able to measure local elevations in temperature (differentiation of 0.05°C). This temperature heterogeneity was related to the degree of histologically detected inflammation (28) and matrix metalloproteinase (MMP) activity (29).

The ability to detect vulnerable lesions by virtue of a functional difference (temperature) is on the edge of using biological processes and pathways as molecular targets for imaging technologies.

Molecular imaging and atherosclerosis – taking the concepts one step further

Molecular imaging is an emerging field that uses knowledge of the molecular pathways and cellular process of atherosclerosis to find, use and exploit targeted and, if possible, 'activatable' imaging agents (table 2.2). Molecular imaging can be used to visualise important biological processes in atherosclerosis with specific molecular contrast agents (figure 2.2). Molecular contrast agents consist of a specific ligand for the target of interest, linked to a contrast agent which can be detected by the imaging modality of choice. Additionally, for MRI, amplification

strategies are usually used, such as gadolinium-loaded liposomes, dendrimers, micelles, nanoparticles or quantum dots.

Adhesion molecules

Atherosclerosis is initiated by dysfunction/damage of the endothelial layer, by factors such as modified low-density lipoproteins (LDL), free radicals, hemodynamic stress and hypertension. Activated endothelial cells express adhesion molecules, such as E-selectin, P-selectin, intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM). Several imaging agents have been developed to detect these adhesion molecules. In particular, antibodies or specific peptides have been conjugated to nanoparticles or contrast agents to target VCAM or E-selectin. Using these imaging agents, it was possible to detect signal enhancement in murine models for inflammation and atherosclerotic lesions of ApoE^{-/-} mice *in vivo* (30-32).

(Activated) macrophages

Activated macrophages are important cellular effectors in atherosclerosis and their presence or activity is a major criterion to define vulnerable lesions. Macrophages are known to phagocytose iron and 'ultrasmall superparamagnetic particle of iron oxide' (USPIO)-enhanced MRI has been used for *in vivo* detection of macrophages in human plaques. In subsequent histological analysis, it was shown that USPIOs

Figure 2.2: Schematic representation of the atherosclerotic process indicating several molecular targets. Abbreviations: ICAM, intercellular adhesion molecule; LDL, low-density lipoprotein; LRNC, lipid-rich necrotic core; MMPs, matrix metalloproteinases; oxLDL, oxidized low density lipoprotein; PS, phosphatidylserine; VCAM, vascular cell adhesion molecule. For full color figure see chapter 9.

primarily accumulated in plaque macrophages, resulting in areas with focal signal loss in MRI images (33,34).

An alternative approach is to use the high metabolic activity of macrophages as a target. Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are excellent imaging technologies for this purpose as both can detect low concentrations (picomolar range); however, spatial resolution is lower than for other imaging technologies. ^{18}F fluorodeoxyglucose (^{18}F -FDG) is a glucose analogue and marks active glycolysis. PET imaging demonstrated higher uptake of ^{18}F -FDG in symptomatic lesions compared to asymptomatic lesions and, more specifically, ^{18}F -FDG accumulation was seen in the macrophage-rich lesions (35).

Protease activity

Proteolytic enzymes such as matrix metalloproteinases (MMPs) and cathepsins have been implicated in fibrous cap destruction. This biological pathway has been used to develop and test protease-sensitive molecular imaging agents. Administration of ^{123}I -labelled MMP inhibitor CGS 27023A to ApoE $^{-/-}$ mice four weeks after carotid ligation and high cholesterol diet, visualised carotid lesions *in vivo* using a planar gamma camera (36). A novel imaging technology exploits the near-infrared bandwidth to obtain efficient fluorescent imaging agents for *in vivo* applications. In the atherosclerosis field, protease-activatable imaging agents for cathepsins have been developed. In one clinical study, a cathepsin-B protease-activatable NIRF probe was able to image macrophage and cathepsin-B-rich atherosclerotic lesions (37).

Apoptosis

Apoptosis or programmed cell death plays an important role not only in physiology, but also in pathology. In atherosclerosis, apoptotic cell death has been shown to contribute to necrotic core formation and smooth muscle cell death in the fibrous cap. Apoptosis has been imaged in the carotid atherosclerotic plaque of four patients using radiolabeled annexin A5. In two patients with recent transient ischemic attacks, an elevated signal was detected in the carotid region. No signal was found in the other two patients who had more remote symptoms. In subsequent histological analysis, annexin A5 signal was correlated to a vulnerable lesion phenotype characterized by macrophage presence and intraplaque hemorrhage (38).

Lipids

A large lipid core is a major criterion to define vulnerable lesions and lipid accumulation has been used to image atherosclerosis. Using autoradiography with a ^{99m}Tc - or ^{125}I -bound oxidation-specific antibody, atherosclerotic lesions in LDL-receptor-deficient Watanabe heritable hyperlipidemic rabbits were visualised both *in vivo* and *ex vivo* (39). In older studies, nuclear imaging using ^{123}I or ^{131}I -labeled LDL was also successful in detecting human atherosclerosis (40).

Angiogenesis

Angiogenesis is associated closely with plaque progression, and microvascular leakage is a likely source of intraplaque hemorrhage. The $\alpha_v\beta_3$ -integrin is expressed on endothelial cells during angiogenesis, but not on non-activated endothelial cells. Linking the short $\alpha_v\beta_3$ -integrin-specific RGD peptide sequence to paramagnetic nanoparticles, enabled *in vivo* visualisation of angiogenesis in atherosclerotic lesions with MRI in a rabbit model (41).

Thrombosis

Thrombosis is the pathological hallmark of cardiovascular events such as myocardial infarction, and several imaging agents have been developed to target the thrombus or its components. As for cathepsin-B, a thrombin-cleavable, near-infrared labeled peptide was able to image mice hematomas and intravascular thrombi with near infrared reflectance imaging (42). A specific contrast agent used for *in vivo* visualisation of thrombi is EP-2104R (EPIX Pharmaceuticals). This contrast agent, composed of a small peptide linked to four gadolinium-chelate moieties, selectively binds fibrin. *Ex vivo* formed human thrombi, delivered *in vivo* in swine lungs and coronary arteries, showed enhanced MRI signal intensities in the presence of this agent (43). This promising contrast agent is currently under investigation in patients with thrombotic events.

Other processes in the functional context of thrombus formation that have been used as targets for molecular imaging are factor XIII (one of the factors in the coagulation-cascade) (44), activated platelets (through conformational changes of the glycoprotein IIb/IIIa receptor) (45), and fibrin itself (through antifibrin antibodies coupled to nanoparticles) (46).

Table 2.2: Targets for molecular imaging of atherosclerosis and thrombus formation.

Target	Imaging agent	Image modality	Stage of development	Advantages (+) Disadvantages (-)	Who is working on the target (Institute or company)	Refs
Adhesion Molecules						
VCAM-1	Monoclonal antibody linked to CLIO ² -Cy5.5	Confocal microscopy / MRI	Animal model	+ Promising marker for early stage inflammation	Tsourkas/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(30)
VCAM-1	CVHSPKKC-peptide linked to CLIO-Cy5.5	Confocal microscopy / MRI	Animal model	+ Promising marker for early stage inflammation + Internalization	Kelly/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(31)
E-selectin	Mimetic of sialyl-Lewis ^x linked to Gd-DTPA ^b	MRI	Animal model	+ Transient interaction	Boutry/Muller, (University of Mons-Hainaut)	(32)
Macrophages						
Macrophages	USPIO ^c (Sinerem, Guerbet, ferumoxtran-10)	MRI	Clinical pilot study	+ Imaging of macrophage-rich areas + Internalization	Koo/Engelshoven, (University Hospital Maastricht); Trivedi/Gillard, (Addenbrooke's Hospital)	(33,34)
Macrophages	¹⁸ F-fluorodeoxyglucose	PET-CT	Human	- Negative contrast agent - Not specific for plaque macrophages	Rudd/Weissberg, (Addenbrooke's Hospital, University of Cambridge)	(35)
Protease activity						
MMP	MMP inhibitor linked to ¹²⁵ I	Gamma camera	Animal model	- Not specific for atherosclerosis	Schäfers/Riemann/Levkau, (Hospital of the Westfälische Wilhelms-Universität)	(36)
Cathepsin B	Enzyme sensitive activatable probe	Near-infrared reflectance imaging / fluorescence-mediated tomography	Animal model	+ High Local expression levels - Present in many cell types	Chen/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(37)
Apoptosis						
Phosphatidylserine	Annexin A5 linked to ^{99m} Tc	SPECT	Clinical pilot study	+ Apoptotic cell imaging	Kletselaer/Reutelingsperger/Hofstra, (University hospital of Maastricht)	(38)

Table 2.2: Targets for molecular imaging of atherosclerosis and thrombus formation. Continued.

Target	Imaging agent	Image modality	Stage of development	Advantages (+) Disadvantages (-)	Who is working on the target (Institute or company)	Refs
Lipids						
α LDL ^e	MDA2 ^f linked to ^{99m} Tc or ¹²⁵ I	Gamma camera / autoradiography	Animal model	+ High levels present in vulnerable lesions	Tsimikas/Witztum (University of California)	(39)
LDL ^g	LDL linked to ¹²⁵ I or ¹³¹ I	Gamma Camera	Human	+ Accumulates in atherosclerotic lesions	Virgolini/Sinzinger, (Wilhelm Auerwald- Atherosclerosis Research Group Vienna)	(40)
Angiogenesis						
$\alpha_v\beta_3$ -Integrin	RGD-peptide linked to paramagnetic nanoparticle	MRI	Animal model	- Not specific for atherosclerosis	Winter/Lanza/Wickline, (Washington University School of Medicine)	(41)
Thrombosis						
Thrombin	Thrombin cleavable peptide linked to Cy5.5	Near-infrared reflectance imaging	In vitro / animal model	+ Imaging of enzyme activity	Jaffer/Tung/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(42)
Fibrin	EP-2104	MRI	Animal model	+ Only present in thrombi + Amount of binding sites + In vivo human studies currently performed.	Spuentrup/Günther, (University Hospital, Aachen Technical University)	(43)
Factor XIII activity	α_2 -antiplasmin based peptide linked to Alexa fluor 680	Near-infrared reflectance imaging	Animal model	+ Acute thrombus visualization	Jaffer/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(44)
Activated platelets	Pro-Ser-Pro-Gly-Asp-Trp-peptide linked to Cy5.5	Fluorescence microscopy	In vitro	+ Prominent component of thrombus	Tung/Weissleder, (Massachusetts General Hospital, Harvard Medical School)	(45)
Fibrin	Monoclonal antibody linked to paramagnetic nanoparticle	MRI	In vitro and In vivo/situ	+ Only present in thrombi + Amount of binding sites	Flacke/Wickline/Lanza, (Washington University Medical School)	(46)

^a CLIO: Crosslinked Iron Oxide; ^b Ga-DTPA: Gadolinium-diethylenetriaminepentaacetic acid (Gadolinium based paramagnetic contrast agent);^c USPIO: Ultrasmall superparamagnetic particles of iron oxide; ^d MMP: Matrix metalloproteinases; ^e oxLDL: Oxidized low density lipoprotein;^f MDA2: Monoclonal oxidation-specific antibody against malondialdehyde (MDA)-lysine epitopes; ^g LDL: Low density lipoprotein.

Translational studies

Several imaging technologies have the potential to be used in daily clinical practice in the near future. The identification of carotid plaque components by MRI has been extensively studied and further optimised in small patient cohorts (figure 2.1 (17)). It was recently shown that measurements of lesion features (such as thin or ruptured fibrous cap, intra-plaque hemorrhage, larger maximum proportion of the wall occupied by a LRNC, and maximum wall thickness) by MRI were associated with the risk for subsequent cerebrovascular events in patients who initially had an asymptomatic carotid stenosis (47). The use of MR contrast agents will further improve visualisation of atherosclerotic lesions. As an example, the use of USPIOs enabled identification of macrophage-rich human atherosclerotic lesions (33,34).

In addition, it is expected that molecular imaging of atherosclerotic lesions with nuclear imaging techniques, for example using radiolabeled ^{18}F -FDG (35) or annexin A5 (38), can also be translated to the clinic relatively straightforward. Nevertheless, large multicenter trials in daily clinical practice are necessary to confirm the feasibility of these imaging technologies for clinical use.

Conclusions

In the past decade, significant progress has been made to visualise atherosclerotic disease. Until recently, imaging technologies mainly focused on visualisation of the lumen and vessel wall components. Using non-invasive imaging techniques, differences in mean values of detected plaque components can stratify lesions of a patient group as either 'more stable' or the vulnerable lesion phenotype. For this purpose, MRI is a promising imaging modality, but validation on the individual patient level in large cohorts and daily clinical practice is needed. Furthermore, the density of binding sites together with the use of contrast amplification mechanisms (e.g. gadolinium-loaded liposomes, nanoparticles, dendrimers, micelles or quantum dots) will determine whether or not MRI will be able to visualise atherosclerotic markers.

Differentiation between different plaque components in the coronary arteries is still challenging and can possibly be achieved using MRI (using improved pulse sequences and/or coils) or intravascular imaging modalities such as IVUS, IVMRI and OCT. Patient studies have shown that these imaging technologies can provide information on plaque components. However, given their invasive nature (and high costs), it is likely that use of these imaging technologies will be limited to a selected group of patients with well-defined symptoms.

Current advances and knowledge on the molecular mechanisms of initiation and progression of atherosclerosis have emphasized the need for imaging technologies and targets that can image function and biology rather than anatomy. This field of molecular imaging is now in rapid development with new imaging agents that aim at visualising processes such as inflammation, macrophage activation, protease activity, angiogenesis, apoptosis, lipid accumulation and thrombus formation. Moreover, the search for novel targets is still in progress. It is likely that current genomic, proteomic or metabolomic screening methods will provide novel, specific ligands that can ultimately be used as imaging agents. Herein, MRI is a valuable imaging technology that could provide synchronized molecular and anatomical imaging with high spatial resolution. Alternatively, if sensitivity of MRI for molecular imaging is not sufficient, a co-registration with PET, as a sensitive molecular imaging modality but with less spatial resolution, could possibly improve diagnostic potential.

The benefits of molecular imaging of atherosclerosis are many, as it could enable earlier detection (imaging specific initiating pathways); define patient status (vulnerable versus stable lesions); monitor (novel) therapies; facilitate disease prognosis; and define novel intermediate endpoints ('imaging-endpoints' instead of major adverse clinical events). Clinical translation of molecular imaging technologies will further unravel the underlying molecular mechanism of atherosclerotic disease and will undoubtedly aid in a personalized disease therapy in the future.

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References

- 1 Glagov, S. et al. (1987) Compensatory enlargement of human atherosclerotic coronary arteries. *N Engl J Med* 316 (22), 1371-1375
- 2 Hansson, G.K. (2005) Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 352 (16), 1685-1695
- 3 Naghavi, M. et al. (2003) From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 108 (14), 1664-1672
- 4 Arbustini, E. et al. (1991) Coronary atherosclerotic plaques with and without thrombus in ischemic heart syndromes: a morphologic, immunohistochemical, and biochemical study. *Am J Cardiol* 68 (7), 36B-50B
- 5 Virmani, R. et al. (2000) Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 20 (1262-1275)
- 6 Virmani, R. et al. (2005) Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol* 25 (10), 2054-2061
- 7 Corti, R. (2006) Noninvasive imaging of atherosclerotic vessels by MRI for clinical assessment of the effectiveness of therapy. *Pharmacol Ther* 110 (1), 57-70
- 8 Nieman, K. et al. (2001) Coronary angiography with multi-slice computed tomography. *Lancet* 357 (9256), 599-603
- 9 O'Leary, D.H. et al. (1999) Carotid-artery intima and media thickness as a risk factor for myocardial infarction and stroke in older adults. *Cardiovascular Health Study Collaborative Research Group. N Engl J Med* 340 (1), 14-22
- 10 Nordestgaard, B.G. et al. (2003) Echolucent rupture-prone plaques. *Curr Opin Lipidol* 14 (5), 505-512
- 11 Devuyst, G. et al. (2005) Ultrasound measurement of the fibrous cap in symptomatic and asymptomatic atheromatous carotid plaques. *Circulation* 111 (21), 2776-2782
- 12 Waki, H. et al. (2003) Ultrasonic tissue characterization of the atherosclerotic carotid artery: histological correlates or carotid integrated backscatter. *Circ J* 67 (12), 1013-1016
- 13 Nikolaou, K. et al. (2004) MRI and other non-invasive imaging modalities for atherothrombotic plaque. In *Handbook of the vulnerable plaque* (Waksman, R. and Serruys, P.W., eds.), pp. 97-124, Taylor & Francis
- 14 Fayad, Z.A. and Fuster, V. (2000) Characterization of atherosclerotic plaques by magnetic resonance imaging. *Ann N Y Acad Sci* 902, 173-186
- 15 Yuan, C. and Kerwin, W.S. (2004) MRI of atherosclerosis. *J Magn Reson Imaging* 19 (6), 710-719
- 16 Leiner, T. et al. (2005) Magnetic resonance imaging of atherosclerosis. *Eur Radiol* 15 (6), 1087-1099
- 17 Cappendijk, V.C. et al. (2005) Assessment of human atherosclerotic carotid plaque components with multisequence MR imaging: initial experience. *Radiology* 234 (2), 487-492
- 18 Saam, T. et al. (2005) Quantitative evaluation of carotid plaque composition by *in vivo* MRI. *Arterioscler Thromb Vasc Biol* 25 (1), 234-239
- 19 Moody, A.R. et al. (2003) Characterization of complicated carotid plaque with magnetic resonance direct thrombus imaging in patients with cerebral ischemia. *Circulation* 107 (24), 3047-3052
- 20 Spuentrup, E. and Botnar, R.M. (2006) Coronary magnetic resonance imaging: visualization of the vessel lumen and the vessel wall and molecular imaging of arteriothrombosis. *Eur Radiol* 16 (1), 1-14
- 21 Schoenhagen, P. and Nissen, S. (2002) Understanding coronary artery disease: tomographic imaging with intravascular ultrasound. *Heart* 88 (1), 91-96
- 22 MacNeill, B.D. et al. (2003) Intravascular modalities for detection of vulnerable plaque: current status. *Arterioscler Thromb Vasc Biol* 23 (8), 1333-1342
- 23 Carlier, S.G. et al. (2002) Imaging of atherosclerosis. *Elastography. J Cardiovasc Risk* 9 (5), 237-245

- 24 Jang, I.K. et al. (2005) *In vivo* characterization of coronary atherosclerotic plaque by use of optical coherence tomography. *Circulation* 111 (12), 1551-1555
- 25 Jang, I.K. et al. (2002) Visualization of coronary atherosclerotic plaques in patients using optical coherence tomography: comparison with intravascular ultrasound. *J Am Coll Cardiol* 39 (4), 604-609
- 26 Tearney, G.J. et al. (2003) Quantification of macrophage content in atherosclerotic plaques by optical coherence tomography. *Circulation* 107 (1), 113-119
- 27 Larose, E. et al. (2005) Characterization of human atherosclerotic plaques by intravascular magnetic resonance imaging. *Circulation* 112 (15), 2324-2331
- 28 Casscells, W. et al. (1996) Thermal detection of cellular infiltrates in living atherosclerotic plaques: possible implications for plaque rupture and thrombosis. *Lancet* 347 (9013), 1447-1451
- 29 Krams, R. et al. (2005) *In vivo* temperature heterogeneity is associated with plaque regions of increased MMP-9 activity. *Eur Heart J* 26 (20), 2200-2205
- 30 Tsourkas, A. et al. (2005) *In vivo* imaging of activated endothelium using an anti-VCAM-1 magnetooptical probe. *Bioconjug Chem* 16 (3), 576-581
- 31 Kelly, K.A. et al. (2005) Detection of vascular adhesion molecule-1 expression using a novel multimodal nanoparticle. *Circ Res* 96 (3), 327-336
- 32 Boutry, S. et al. (2005) Magnetic resonance imaging of inflammation with a specific selectin-targeted contrast agent. *Magn Reson Med* 53 (4), 800-807
- 33 Kooi, M.E. et al. (2003) Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by *in vivo* magnetic resonance imaging. *Circulation* 107 (19), 2453-2458
- 34 Trivedi, R.A. et al. (2004) *In vivo* detection of macrophages in human carotid atheroma: temporal dependence of ultrasmall superparamagnetic particles of iron oxide-enhanced MRI. *Stroke* 35 (7), 1631-1635
- 35 Rudd, J.H. et al. (2002) Imaging atherosclerotic plaque inflammation with (18F)-fluorodeoxyglucose positron emission tomography. *Circulation* 105 (23), 2708-2711
- 36 Schafers, M. et al. (2004) Scintigraphic imaging of matrix metalloproteinase activity in the arterial wall *in vivo*. *Circulation* 109 (21), 2554-2559
- 37 Chen, J. et al. (2002) *In vivo* imaging of proteolytic activity in atherosclerosis. *Circulation* 105 (23), 2766-2771
- 38 Kietselaer, B.L. et al. (2004) Noninvasive detection of plaque instability with use of radiolabeled annexin A5 in patients with carotid-artery atherosclerosis. *N Engl J Med* 350 (14), 1472-1473
- 39 Tsimikas, S. et al. (1999) Radiolabeled MDA2, an oxidation-specific, monoclonal antibody, identifies native atherosclerotic lesions *in vivo*. *J Nucl Cardiol* 6 (1 Pt 1), 41-53
- 40 Virgolini, I. et al. (1991) Autologous low-density lipoprotein labelling allows characterization of human atherosclerotic lesions *in vivo* as to presence of foam cells and endothelial coverage. *Eur J Nucl Med* 18 (12), 948-951
- 41 Winter, P.M. et al. (2003) Molecular imaging of angiogenesis in early-stage atherosclerosis with alpha(v)beta3-integrin-targeted nanoparticles. *Circulation* 108 (18), 2270-2274
- 42 Jaffer, F.A. et al. (2002) *In vivo* imaging of thrombin activity in experimental thrombi with thrombin-sensitive near-infrared molecular probe. *Arterioscler Thromb Vasc Biol* 22 (11), 1929-1935
- 43 Spuentrup, E. et al. (2005) Molecular magnetic resonance imaging of coronary thrombosis and pulmonary emboli with a novel fibrin-targeted contrast agent. *Circulation* 111 (11), 1377-1382
- 44 Jaffer, F.A. et al. (2004) Molecular imaging of factor XIIIa activity in thrombosis using a novel, near-infrared fluorescent contrast agent that covalently links to thrombi. *Circulation* 110 (2), 170-176
- 45 Tung, C.H. et al. (2005) A branched fluorescent peptide probe for imaging of activated platelets. *Mol Pharm* 2 (1), 92-95
- 46 Flacke, S. et al. (2001) Novel MRI contrast agent for molecular imaging of fibrin: implications for detecting vulnerable plaques. *Circulation* 104 (11), 1280-1285
- 47 Takaya, N. et al. (2006) Association between carotid plaque characteristics and subsequent ischemic cerebrovascular events: a prospective assessment with MRI--initial results. *Stroke* 37 (3), 818-823

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- 48 Davies, J.R. et al. (2005) Targeting the vulnerable plaque: the evolving role of nuclear imaging. *J Nucl Cardiol* 12 (2), 234-246
- 49 Massoud, T.F. and Gambhir, S.S. (2003) Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev* 17 (5), 545-580

CHAPTER 3

Contrast-enhanced magnetic resonance vessel wall imaging in a rabbit model of atherosclerosis: comparison of Gadofosveset and Gd-DTPA

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Abstract

Purpose: To investigate the potential of Gadofosveset for contrast-enhanced magnetic resonance imaging of plaque in a rabbit model of atherosclerosis.

Materials and methods: All experiments were approved by the animal ethics committee. Thirty-one New Zealand White rabbits were included in four study groups: atherosclerotic animals receiving Gadofosveset (n=10) or Gd-DTPA (n=7), and control animals receiving Gadofosveset or Gd-DTPA (both n=7). Aortic atherosclerosis was induced through endothelial denudation combined with a cholesterol-enriched diet. Control rabbits were sham operated and received a regular diet. After eight weeks, pre- and postcontrast T₁-weighted MR images of the aortic vessel wall were acquired. Relative signal enhancement was determined with dedicated software. Statistical analysis was performed using a generalized linear mixed model. CD31 and albumin immunohistochemical staining was used to assess microvessel density and albumin content of the vascular wall. Group differences were analyzed using a χ^2 -test. Gadofosveset spatial distribution and content within the vessel wall was determined with proton induced x-ray emission (PIXE) analysis.

Results: Postcontrast signal enhancement was significantly larger for atherosclerotic than for control animals using Gadofosveset (P=0.022). Gd-DTPA could not discriminate between normal and atherosclerotic vessel walls (P=0.428). PIXE analysis showed higher amounts of Gd-DTPA in both atherosclerotic and normal rabbit aortas compared to Gadofosveset. Immunohistochemical staining showed presence of albumin and increased microvessel density in the vascular wall of atherosclerotic rabbits.

Conclusion: These results suggest that Gadofosveset can be used to differentiate between atherosclerotic and normal rabbit vessel walls.

Introduction

Atherosclerotic plaques develop over decades, leading to advanced lesions characterized by a lipid core separated from the lumen by a fibrous cap (1). Clinical events (e.g. myocardial infarction) are caused by rupture or erosion of the fibrous cap accompanied by thromboembolization and occlusion of a diseased or distal vessel (2, 3). The risk of a clinical event is determined by plaque composition and degree of stenosis (4, 5). Histologic examination-based studies demonstrated that macrophage-rich plaques with a thin fibrous cap, large lipid core, and abundance of (leaky) microvessels tend to be more vulnerable (4). Intraplaque albumin content is positively associated with plaque size and micro-vessel density, which could be related to leakage of albumin into the lesion through leaky microvessels (6).

Digital subtraction angiography (DSA) and ultrasound are important diagnostic modalities to determine degree of stenosis in atherosclerosis, but DSA is unable to visualize plaque composition. In addition, identification of these components with ultrasound remains highly operator-dependent and has low reproducibility, despite recent improvements (7, 8). MRI can non-invasively assess lumen diameter and plaque components (9-11). However, the limited spatial resolution and relatively low signal-to-noise ratio (SNR) of MRI makes it difficult to detect plaque components in small arteries (e.g. coronary arteries). MRI contrast agents improve SNR and CNR and therefore might improve plaque imaging. Several gadolinium (Gd) chelates, primarily Gd-DTPA, have been used for plaque imaging. After Gd-DTPA administration, increased signal enhancement occurs for fibrous tissue, improving the contrast between fibrous tissue and the lipid core. Kinetic modeling of Gd-DTPA enhanced dynamic MRI data can be used to quantify neovascularisation density (12-16).

Gadofosveset (MS-325, Vasovist®, Bayer Schering Pharma AG, Germany) consists of a diphenylcyclohexyl group attached to a gadolinium chelate by a phosphodiester linkage. Because a large fraction (>75%) of circulating Gadofosveset is bound to serum albumin, it has a longer elimination half-life than Gd-DTPA. Bound Gadofosveset gives a strong signal enhancement due to its high relaxivity (17, 18). In an experimental MR angiography (MRA) study, Lin et al (19) observed signal enhancement in an occluded atherosclerotic pig carotid vessel wall after Gadofosveset administration. In the present study we aimed to investigate the potential of Gadofosveset for plaque imaging in a rabbit model of atherosclerosis.

Materials and methods

Animal experiment protocols

Gadofosveset was donated by Bayer Schering Pharma AG, Germany. B.M. is an employee of Bayer Schering Pharma and provided us with critical comments on this article. B.M. had no influence on animal experiments or data analysis.

Thirty-one male New Zealand White rabbits (Charles River, Romans, France) (12 weeks old, minimum weight 2.0 kg) were randomized into one of four study groups. Animals were randomly assigned to receive either a 1.0% cholesterol enriched diet (atherosclerotic animals, $n=17$) or normal rabbit chow (controls, $n=14$) for two weeks. Cholesterol-fed rabbits underwent an intervention consisting of a balloon denudation of the abdominal aorta, while control rabbits underwent sham surgery. During the balloon denudation or sham surgery, rabbits were anesthetized with ketamine (0.5 ml/kg) and xylazine (0.25 ml/kg). When sedated, animals were intubated with an endotracheal tube and actively ventilated using oxygen with 2% isoflurane inhalation gas. The left carotid artery was exposed and a 4F catheter introducer sheath was inserted (Avanti Plus, Cordis Europe, Belgium) until the aortic arch was reached. Next, a small guidewire (Radiofocus Guidewire M, Terumo Europe, Belgium) was inserted through the sheath until it was situated in the descending aorta. Then, the guidewire was replaced with a small 3F Fogarty embolectomy catheter (Edwards Lifesciences, USA), which was positioned 1 cm proximally to the aortic bifurcation. From this starting point, the catheter was inflated and withdrawn 5 cm three times. After removal of the catheter, the carotid artery was ligated and the wound was sutured. The sham surgery of control rabbits (receiving normal chow) consisted of a ligation of the left carotid artery. No catheterization material was introduced.

After surgery, animals received their assigned diets for eight weeks and underwent both pre- and postcontrast MR imaging of the abdominal aorta. Between pre- and postcontrast MRI experiments, 10 atherosclerotic and 7 control rabbits received an intravenous injection of Gadofosveset (0.05 mmol/kg), whereas 7 atherosclerotic and 7 controls rabbits received Gd-DTPA (Magnevist®, 0.5 mmol/kg) (both Bayer Schering Pharma AG, Germany). The doses of both contrast agents were chosen to compensate relaxivity differences between the contrast agents (free Gadofosveset $6.0 \text{ mM}^{-1}\text{s}^{-1}$, bound Gadofosveset $33.0 \text{ mM}^{-1}\text{s}^{-1}$, Gd-DTPA $4.5 \text{ mM}^{-1}\text{s}^{-1}$). We calculated that with these doses, T_1 values of blood immediately after contrast administration were comparable for both contrast agents. The optimal postcontrast imaging time was determined based on pilot experimental data (see below). At four times the elimination half-life of the administered contrast agent, postcontrast imaging was performed. For Gadofosveset and Gd-DTPA these periods were 11 hours and 90 minutes, respectively.

Technical outliers were defined as rabbits in which development of atherosclerotic plaque was markedly less than in other animals, even though identical experimental protocols were used. These animals (n=2) were excluded from analysis. Animals were sacrificed after the final experiment with an overdose of sodium pentobarbital (Euthesate, Willows Francis Veterinary, Crawley, England). All experiments were approved by the local animal ethics committee.

Pilot experiment

To determine the optimal postcontrast imaging time, a pilot experiment was conducted, in which a single atherosclerotic rabbit was continuously imaged for 13 hours after administration of Gadofosveset (0.05 mmol/kg). In this experiment, the mean signal enhancement of the vessel wall increased to 3.1 after 72 minutes, after which it decreased to 2.4 after 13 hours of imaging, when the rabbit was sacrificed. As a trade off between maximum signal enhancement of the vessel wall versus minimal signal enhancement of the lumen, we chose a postcontrast imaging delay of 11 hours for the Gadofosveset groups (equalling four times the elimination half-life). Postcontrast imaging of Gd-DTPA receiving animals would have to be performed at a similar multiple of elimination half-life, taken into account the differences in elimination half-life of the contrast agents. Therefore, the postcontrast imaging delay for Gd-DTPA groups was 90 minutes, four times the elimination half-life of the contrast agent.

MR imaging

MR imaging was performed by using a 1.5T MRI scanner (Philips Medical Systems, the Netherlands) and a five-element synergy receiver cardiac coil. A two-dimensional fast field echo sequence was used to identify the aortic bifurcation. A three-dimensional, double inversion recovery (black blood) turbo spin echo (TSE) pulse sequence was performed. For the two-dimensional fast field echo sequence, the following scan parameters were used: repetition time (TR) 17 ms, echo time (TE) 3.8 ms, flip angle 60 °C. The three-dimensional, double inversion recovery (black blood) turbo spin echo (TSE) pulse sequence was performed with the following scan parameters: echo train length 5, echo train spacing 14 ms, TR 570 ms, TE 14 ms, inversion time (TI) 252 ms, number of signal averages (NSA) 2, 9 slices of 3.0 mm thickness, field-of-view 90x90 mm, matrix size 304x304, resulting in an in-plane resolution of 0.3x0.3 mm. In this sequence, a constant level of appearance (CLEAR) technique was used to compensate for signal inhomogeneities. TI was not adjusted during postcontrast imaging because after four times the elimination half-life, T_1 values of blood restored to their original values. A regional saturation (REST) slab was placed over the bowels to prevent

bowel motion artefacts in the abdominal aorta. The central imaging slice was placed 3.5 cm above the aortic bifurcation.

MR image analysis

MR image analysis was performed with the software program VesselMASS (Leiden University Medical Center, the Netherlands). On the images, operator defined regions of interest (ROI) were drawn by two independent readers blinded for study group (M.L., R.M., both with more than three years of experience) (20). In the MR image analysis, an operator-defined region-of-interest (ROI) was drawn encompassing the arterial vessel wall. This ROI was copied from pre- to postcontrast images, adjusting only for minor displacements that occurred between the two scans. Mean signal intensities (SI) of both pre- and postcontrast images were adjusted for scaling factors and gain. Mean precontrast SI of adjacent muscle tissue, again adjusted for scaling factors and gain, was also determined in an operator-defined ROI. Pre- and postcontrast relative vessel wall signal intensities (rSI) were determined by dividing mean signal intensities (pre- or postcontrast) by the precontrast mean SI of adjacent muscle tissue. Signal enhancement (SE) was calculated by dividing the postcontrast SI by the precontrast SI. One reader (M.E.K., with more than nine years of experience), who was blinded to the study groups and MRI results, determined overall image quality. Images in which contours of the vessel wall could not be distinguished from lumen or surrounding tissue were excluded from analysis.

Histological analysis

After euthanasia, the aorta was dissected from bifurcation to approximately 7 cm proximally. The aortas of 7 atherosclerotic and 7 control rabbits were fixed in formalin for 24 hours, after which they were cut into 3 mm thick tissue slices (taking into account shrinkage that occurred during the fixation process) and embedded in paraffin. For PIXE analysis and immunohistochemical staining, tissue slices of the remaining rabbits were embedded in Tissue-Tek (Electron Microscopy Sciences, USA) and were snap frozen in dry-ice cooled isopentane. Paraffin and cryosections were cut into 4 and 10 μm thick slices, respectively. Slices of poor quality, i.e. broken or incomplete sections, were excluded from analysis. All sections were Lawson and hematoxylin/eosine (HE) stained and microscopically analyzed. Digital photographs of Lawson stained sections were subsequently analyzed with ImageJ 1.37 (National Institute of Health, Bethesda, Md) to assess plaque size. All sections were immunohistologically stained for endothelial cells with CD31 (clone JC70A, Dako North America Inc., USA) and assessed for microvessel density. Presence and localization of albumin was assessed with

immunohistochemical staining using a human serum albumin antibody (AB8940, Abcam Inc., USA). The number of microvessels in completely intact samples was assessed and scored on a 5-point scale: none (0), few (1: 1-10 vessels per cross-section), minor (2: 11-20 vessels), average (3: 21-30 vessels) and abundant (4: > 30 vessels). Albumin content was measured by dividing the total area that stained positive by total plaque area, and multiplying this by 100. Thus, the albumin content was expressed as the percentage of total plaque area that stained positive. Analysis of the albumin staining was quantified using computerized morphometry (Leica QWin V3, Cambridge, United Kingdom).

For both immunohistochemical stainings, proper positive and negative controls were used and sections were stained in one batch. For CD31, positive controls consisted of human carotid atherosclerotic plaques, whereas for albumin, positive controls consisted of human breast carcinoma. In both stainings, negative controls consisted of omission of the first antibody.

Proton Induced X-ray Emission analysis

Proton Induced X-ray Emission (PIXE) analysis was used to investigate contrast agent distribution within the vascular wall (21). Two cryosections of two individual rabbits of each group were cut into 10 μm thick slices, placed on pioloform film and compared with adjacent HE-stained samples. PIXE measurements were performed with a 3 MeV proton beam from a 3.5 MV SingletronTM. To encompass the entire width of the vessel wall, a scanning pattern of 128x128 points and a proton beam size of approximately $8 \times 8 \mu\text{m}^2$ was used. Because elemental concentrations are measured over the entire grid, the grid was placed as accurately as possible to encompass the entire vessel wall. The detection limit of gadolinium and elemental concentrations expressed in $\mu\text{g/g}$ were calculated. Subsequently, distribution maps were created.

Statistical analysis

Intraclass correlation coefficients (ICC) between the two MR image readers were calculated to determine interobserver agreement. Pre- and postcontrast rSI measurements were analysed with a generalised linear mixed model using the statistical software program SPSS (version 15.0; SPSS Inc., Chicago, USA) (22). In the generalized linear mixed model, individual rabbits were treated as random factor and contrast agent and intervention as fixed effects. For the analysis of postcontrast rSI, precontrast rSI was added to the model as a covariate in order to correct for differences in baseline measurements among groups. Model parameters were considered significant if P-values of their corresponding Wald tests were $P < 0.05$. For those parameters whose $P < 0.1$, an additional likelihood ratio

test (LR) was carried out to determine their final significance. The final model parameter estimates were computed via the restricted maximum likelihood (REML) method. The LR tests were carried out with maximum likelihood estimates.

Mean scores for microvessel density were assessed for both atherosclerotic and normal aortas by dividing total microvessel density score by number of slices assessed. A χ^2 -test was performed to evaluate differences in microvessel densities and percentages of vessel wall areas positively stained for albumin. P-values of <0.05 were considered to indicate a statistically significant difference.

Results

Although identical experimental protocols were applied, two atherosclerotic rabbits that received Gadofosveset showed minimal plaque development (mean plaque size $0.8 \pm 0.7 \text{ mm}^2$ versus $3.3 \pm 1.7 \text{ mm}^2$ in other atherosclerotic rabbits). We considered these rabbits as technical outliers and excluded them from further analysis.

MRI results

A total of 558 images were acquired. Due to poor vessel wall delineation in MR images, 52 images (9%) out of 13 different animals were excluded. Typical examples of MR images per study group are presented in figure 3.1.

Interobserver agreement as expressed by the ICC for pre- and postcontrast rSI of the vessel wall was 0.951 and 0.986, respectively ($P < 0.001$ for both).

Figure 3.2a shows box plots of relative signal intensity (rSI) baseline values for each rabbit, clustered according to group. The animals in the atherosclerotic groups showed higher precontrast rSI than the control animals. To check whether precontrast rSI values were significantly different among rabbits, baseline measurements were analysed via a mixed model with random intercept and contrast and illness as fixed effects. A significant higher precontrast rSI was found for atherosclerotic rabbits: The mean precontrast rSI difference between atherosclerotic and normal animals was 0.264 ($P < 0.001$). The mean precontrast rSI difference between contrast groups (Gadofosveset or Gd-DTPA) was 0.042 ($P = 0.196$, note that no contrast has been added at this stage).

Box plots for observed postcontrast SE showed that, with exception of five animals, mean postcontrast SE increased with at least a factor of 1.5 after contrast administration (figure 3.2b). Five animals that showed limited increase in postcontrast SE were control animals that received Gadofosveset.

A mixed model with random intercept and random slope for the precontrast rSI was fit to the postcontrast measurements. Final fixed effects parameters are given in table 3.1.

To create figure 3.3, observed postcontrast rSI values for each rabbit were plotted against their corresponding precontrast rSI measurements. Additionally, the regression lines, as predicted by the fixed effects only (yet estimated under the mixed model), were displayed.

Figure 3.1. Overview of typical pre- (A) and postcontrast (B) MR images in atherosclerotic animals receiving Gadofosveset. Insets show magnified views of inferior vena cava (left) and aorta (right). Detailed MR images in all study groups (C) show signal enhancement after contrast administration in all groups except controls receiving Gadofosveset. Abbreviations: aorta (AO), vena cava (VC), and Gadofosveset (GFV).

Figure 3.2. Box plot of precontrast rSI values (A) and postcontrast signal enhancement (B) for each rabbit, clustered by groups. Abbreviation: Gadofosveset (GFV). For full color figure see chapter 9.

Table 3.1. Final model parameters for fixed effects. P-value for Wald test is presented. For illness and contrast, atherosclerosis and Gd-DTPA served as references, respectively.

Covariate	Regression coefficient	Std. error	Wald p-value
intercept	1.397	0.243	< 0.001
illness	-0.205	0.255	0.428
contrast	-1.326	0.244	< 0.001
pre-contrast rSI	1.477	0.178	< 0.001
interaction illness / contrast	0.801	0.352	0.032

The significant interaction between lesion development and contrast suggests that there was a differential effect of the presence of atherosclerosis on postcontrast rSI, depending on the contrast agent used. To illustrate the interaction effect, pair-wise comparisons based on estimated group mean postcontrast rSI values (table 3.2) were performed by holding the precontrast rSI value constant at its mean value of 1.176. A significant difference could be shown between atherosclerotic and control rabbits receiving Gadofosveset (mean postcontrast rSI difference of 0.595, $P=0.022$). No significant difference was shown between atherosclerotic rabbits and control rabbits receiving Gd-DTPA (mean postcontrast rSI difference 0.205, $P=0.428$). Moreover, a differential contrast effect could be observed, with higher postcontrast rSI difference between Gadofosveset and Gd-DTPA for control animals (mean postcontrast rSI difference of 1.326, $P<0.001$) than for atherosclerotic rabbits (mean postcontrast rSI difference 0.526, $P=0.047$). These observed differences remained statistically significant even when the two technical outliers were included in the analysis (data not shown).

One control rabbit that received Gadofosveset showed increased signal enhancement after 11 hours. A similar experimental protocol was followed and no evident plaques, albumin content or increased microvessel density was observed in the histological slices in this animal. Therefore, we have no explanation for this outlier. Despite this outlier, a significant difference in signal enhancement after Gadofosveset administration was observed between atherosclerotic and control rabbits.

Figure 3.3. Observed postcontrast rSI as a function of precontrast rSI for individual rabbits according to study group. The regression lines are based on the predicted postcontrast rSI values for fixed effects only. Abbreviation: Gadofosveset (GFV). For full color figure see chapter 9.

In summary, postcontrast mean signal enhancement was significantly greater for atherosclerotic than for control animals with Gadofosveset. For non-diseased control rabbits, Gd-DTPA yielded greater postcontrast MR signal enhancement than Gadofosveset. These results suggest that Gadofosveset, in contrast to Gd-DTPA, is able to help distinguish between atherosclerotic and normal vessel walls.

Histology

In atherosclerotic rabbits, macrophage-rich and collagen-poor lesions were formed (figure 3.4a, mean plaque size $3.3 \pm 1.7 \text{ mm}^2$). All control animals (which did not undergo denudation) were free of atherosclerotic disease.

In the analysis of microvascular density and albumin content, 15% of the histological samples were not intact and excluded. An increase in microvascular density was observed, mainly in the adventitia and in five sections of two separate atherosclerotic animals also in the neointima (figure 3.4c and 3.5c). In control animals, occasional adventitial microvessels were seen (figure 3.4c). There was a significant difference in mean microvessel density scores ($\pm$ standard error) between atherosclerotic and normal vessels: 2.6 ± 1.1 and 0.7 ± 0.4 ($P < 0.001$), respectively.

The percentage of the area that stained red in sections that were immunohistochemically stained for albumin was significantly higher in atherosclerotic rabbits than in control animals: $43.9 \pm 4.2\%$ versus $9.4 \pm 2.3\%$, respectively ($p < 0.001$, figure 3.5).

PIXE results

PIXE analysis showed diffuse presence of gadolinium across the entire vessel wall (figure 3.6). In atherosclerotic rabbits that received Gadofosveset, the mean amount of gadolinium was $79 \text{ }\mu\text{g/g}$ dry tissue (range $16\text{--}248 \text{ }\mu\text{g/g}$). In control rabbits that received Gadofosveset, the mean gadolinium content was $64 \text{ }\mu\text{g/g}$ (range $6\text{--}602 \text{ }\mu\text{g/g}$). In contrast, mean gadolinium content in groups that received Gd-DTPA was higher: $408 \text{ }\mu\text{g/g}$ (range $97\text{--}2967 \text{ }\mu\text{g/g}$) in atherosclerotic rabbits and $776 \text{ }\mu\text{g/g}$ (range $91\text{--}1875 \text{ }\mu\text{g/g}$) in control rabbits. When corrected for clearance and dose, the amount of gadolinium in the study groups was similar.

Figure 3.4. Histological examples of the aortic vessel wall of atherosclerotic and control rabbits. (A) HE stained anatomical overview. (B) HE stained detail of vessel wall. (C) CD31 immunoreactivity of adventitial microvessels visualized with Vector Red. (D) Positive CD31 red staining of endothelium. Abbreviations: intima (int), media (m), adventitia (adv). For full color figure see chapter 9.

Table 3.2. Estimated group mean rSI values on which pairwise comparison was based at a precontrast rSI of 1.176.

Study group	Estimated mean	St. error
Atherosclerosis – Gadofosveset	2.402	0.173
Atherosclerosis – Gd-DTPA	2.928	0.188
Controls – Gadofosveset	1.807	0.177
Controls – Gd-DTPA	3.133	0.178

Figure 3.5. Immunohistochemical staining for albumin in vessel walls. Albumin is present in the atherosclerotic vessel wall lesions (A) and not in control animals (B). CD31 immunoreactive neointimal microvessels (arrows) in atherosclerotic animals might serve as a port of entry (C). Intraluminal staining is caused by albumin-containing blood remnants and demonstrates positive staining for albumin in A and B. For full color figure see chapter 9.

Figure 3.6. PIXE analysis. Hematoxylin-Eosin stained sections served as orientation point (square) for the analysis of the distribution of gadolinium. Colors are indicative of the amount of gadolinium present. Scaling of bars is in micrograms per gram dry tissue. (*) Inferior vena cava. For full color figure see chapter 9.

Discussion

Our study demonstrated that Gadofosveset showed specific MR signal enhancement in the atherosclerotic vessel wall, whereas Gd-DTPA could not discriminate between normal and atherosclerotic rabbit vessel walls. Therefore, the use of Gadofosveset for differentiation between atherosclerotic and normal vessel walls is promising.

In an experimental MRA study, Lin et al. described an increased signal intensity in an occluded atherosclerotic pig carotid after Gadofosveset administration. Increased neovascularisation was suggested as a possible explanation (19). In our study, we confirmed and quantified the signal enhancement after Gadofosveset administration in a large group of atherosclerotic rabbit vessel walls and demonstrated limited enhancement in nondiseased vessel walls. The presence of albumin, which entered the lesions through leaky microvessels, might play an important role in this phenomenon.

After administration, Gadofosveset (>75%) is bound to serum albumin, resulting in an increased elimination half-life and a prolonged time window for uptake in atherosclerotic plaques. Binding of Gadofosveset to albumin increases r_1 relaxivity from 6.0 (free) to 33.0 mM⁻¹s⁻¹ (bound), resulting in marked MR signal enhancement even at low doses (17, 23). Albumin is the most abundant protein present in human circulation, accounting for approximately 60% of the measured serum proteins (24). Normal arterial vessel walls do not contain albumin, since albumin leaves the circulation only through the fenestrated endothelium of capillaries. Albumin can enter the atherosclerotic vessel wall as demonstrated by Zhang et al, who showed a positive correlation between plaque size, microvessel density, and plaque albumin content (6). The albumin content present in atherosclerotic lesions has been found to be significantly higher than plasma albumin concentration (25). Gadofosveset binds to albumin and might enter the plaque in a similar way as native albumin. Alternatively, the relatively small free Gadofosveset might be able to diffuse through the 'leaky' microvasculature or cross the endothelium into the plaque. Here it encounters albumin, resulting in binding of Gadofosveset to albumin and increased relaxivity, creating a measurable amount of MR signal enhancement, even when low concentrations of free Gadofosveset have entered the atherosclerotic vessel wall. Since no endothelial dysfunction is present in normal arterial vessel walls, bound Gadofosveset is expected to be unable to enter the arterial vessel wall. Free Gadofosveset could be able to diffuse through the endothelium into the normal vessel wall but does not encounter albumin to increase its relaxivity. In both cases, no apparent postcontrast signal enhancement will be detected in normal vessel walls, especially when low doses of Gadofosveset are used, as in the present study.

Postcontrast signal intensity was increased in the vessel wall of atherosclerotic and normal animals receiving Gd-DTPA. These results are in agreement with results of a study published by Barkhausen et al., who could not distinguish between Watanabe rabbit wall segments with and those without plaque burden directly after administration of Gd-DTPA (26). In their study, signal intensities restored 60 minutes after contrast administration, whereas signal intensities were still increased in our NZW rabbits after 90 minutes, even though similar doses were applied. Differences in pharmacokinetic behaviour of the contrast agent in Watanabe and NZW rabbits might explain this discrepancy.

Studies by Barkhausen et al. (26) and Zheng et al. (27) demonstrated the specific enhancement of atherosclerotic plaques in Watanabe rabbits after administering gadofluorine. No signal enhancement was observed in the vessel wall of control rabbits. Gadofluorine has a prolonged elimination half-life compared to Gd-DTPA. In combination with the increased permeability of atherosclerotic vessel walls this could explain the specific signal enhancement of plaques observed in these studies. Although Gadofosveset and gadofluorine could thus be used for similar applications, Gadofosveset is currently commercially available and allows for more rapid human applications and studies. In addition, novel contrast agents are being developed and evaluated for their use in MR plaque imaging. For example, Brushett et al. demonstrated the potential use of motexafin gadolinium in MR plaque imaging of deep-seated arteries (28).

In our study, PIXE analysis showed comparable concentrations of gadolinium in both atherosclerotic and normal vessel walls in rabbits that received Gadofosveset. The albumin content was significantly higher in atherosclerotic rabbits than in control rabbits. This supports our hypothesis that small amounts of Gadofosveset are taken up in normal as well as atherosclerotic vessel walls, but that binding to albumin and the subsequent increase in relaxivity causes the higher signal enhancement observed in atherosclerotic animals. In atherosclerotic rabbits, increased numbers of microvessels were present in the adventitia and occasionally in the neointima. These microvessels could function as a port of entry for albumin and Gadofosveset. The small amount of Gadofosveset in normal vessel wall increased the signal intensity only slightly. The increased postcontrast signal intensities in all rabbits that received Gd-DTPA were confirmed by the large amount of gadolinium present in both atherosclerotic and normal vessel walls.

Study limitations

Measurement of the vessel wall signal intensities at MR imaging can be affected by partial volume effects due to the limited in-plane resolution in relation to the small vessel wall thickness. However, the error that can occur because of this effect was comparable for all four study groups, and we therefore assume that it had no

influence on the final study outcomes. In addition, the longitudinal inhomogeneity of the plaque thickness is much smaller than in the axial plane. Therefore, we chose a higher spatial resolution in the axial direction than in the longitudinal direction.

The violation of the linear model assumption regarding groups' homogeneity of variances could be regarded as a statistical limitation of the conducted analysis. Accordingly, P-values of the statistical tests should be interpreted cautiously. Log transformation of post- and precontrast rSI values, intended to make group's variances more uniform, was carried out and ensuing analysis (with the same mixed model) resulted in fully concordant results (data not shown). As such, they substantiated the statistical significance of the comparisons of the groups and the conclusions drawn.

Practical applications

It has been shown by a large number of studies that MRI is a diagnostic modality to non-invasively image carotid plaque components, such as the lipid core, the fibrous cap, calcifications, and intraplaque haemorrhage (8-11). MR plaque imaging of the small, deeper situated coronary arteries, remains extremely challenging due to the limited spatial resolution and low SNR of MRI. The use of Gadofosveset in contrast-enhanced MR plaque imaging could enable specific visualization of atherosclerosis in these arteries. Future studies are warranted to determine whether Gadofosveset is a valuable tool for plaque visualization in humans.

Conclusion

Gadofosveset showed specific MR signal enhancement in the atherosclerotic rabbit vessel wall, whereas Gd-DTPA could not discriminate between the normal and atherosclerotic vessel wall. Therefore, Gadofosveset-enhanced MR plaque imaging might be used to differentiate between atherosclerotic and normal vessel walls.

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References

1. Thom T, Haase N, Rosamond W, et al. Heart disease and stroke statistics: 2006 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation*. 2006; 113: e85-151.
2. Lusis AJ. Atherosclerosis. *Nature*. 2000; 407: 233-41.
3. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. 2005; 352: 1685-95.
4. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol*. 2000; 20: 1262-75.
5. Virmani R, Kolodgie FD, Burke AP, et al. Atherosclerotic plaque progression and vulnerability to rupture: angiogenesis as a source of intraplaque hemorrhage. *Arterioscler Thromb Vasc Biol*. 2005; 25: 2054-61.
6. Zhang Y, Cliff WJ, Schoefl GI, Higgins G. Immunohistochemical study of intimal microvessels in coronary atherosclerosis. *Am J Pathol*. 1993; 143: 164-72.
7. Kern R, Szabo K, Hennerici M, Meairs S. Characterization of carotid artery plaques using real-time compound B-mode ultrasound. *Stroke*. 2004; 35: 870-5.
8. Fayad ZA, Fuster V. Clinical imaging of the high-risk or vulnerable atherosclerotic plaque. *Circ Res*. 2001; 89: 305-16.
9. Fayad ZA, Fuster V. Characterization of atherosclerotic plaques by magnetic resonance imaging. *Ann N Y Acad Sci*. 2000; 902: 173-86.
10. Yuan C, Kerwin WS. MRI of atherosclerosis. *J Magn Reson Imaging*. 2004; 19: 710-9.
11. Leiner T, Gerretsen S, Botnar R, et al. Magnetic resonance imaging of atherosclerosis. *Eur Radiol*. 2005; 15: 1087-99.
12. Wasserman BA, Casal SG, Astor BC, Aletras AH, Arai AE. Wash-in kinetics for gadolinium-enhanced magnetic resonance imaging of carotid atheroma. *J Magn Reson Imaging*. 2005; 21: 91-5.
13. Aoki S, Aoki K, Ohsawa S, Nakajima H, Kumagai H, Araki T. Dynamic MR imaging of the carotid wall. *J Magn Reson Imaging*. 1999; 9: 420-7.
14. Yuan C, Kerwin WS, Ferguson MS, Polissar N, Zhang S, Cai J, Hatsukami TS. Contrast-enhanced high resolution MRI for atherosclerotic carotid artery tissue characterization. *J Magn Reson Imaging*. 2002; 15: 62-7.
15. Cai J, Hatsukami TS, Ferguson MS, et al. *In vivo* quantitative measurement of intact fibrous cap and lipid-rich necrotic core size in atherosclerotic carotid plaque: comparison of high-resolution, contrast-enhanced magnetic resonance imaging and histology. *Circulation*. 2005; 112: 3437-44.
16. Kerwin WS, O'Brien KD, Ferguson MS, Polissar N, Hatsukami TS, Yuan C. Inflammation in carotid atherosclerotic plaque: a dynamic contrast-enhanced MR imaging study. *Radiology*. 2006; 241: 459-68.
17. Corot C, Violas X, Robert P, Gagneur G, Port M. Comparison of different types of blood pool agents (P792, MS325, USPIO) in a rabbit MR angiography-like protocol. *Invest Radiol*. 2003; 38: 311-9.
18. Goyen M, Shamsi K, Schoenberg SO. Vasovist-enhanced MR angiography. *Eur Radiol*. 2006; 16 Suppl 2: B9-14.
19. Lin W, Abendschein DR, Haacke EM. Contrast-enhanced magnetic resonance angiography of carotid arterial wall in pigs. *J Magn Reson Imaging*. 1997; 7: 183-90.
20. Adame IM, Van der Geest RJ, Wasserman BA, Mohamed MA, Reiber JHC, Lelieveldt BPF. Automatic segmentation and plaque characterization in atherosclerotic carotid artery MR images. *Magma*. 2004; 16: 227-34.
21. Roijers RB, Dutta RK, Mutsaers PHA, Gijbels MJJ, De Winther MPJ, De Goeij JJM, Van der Vusse GJ. Early Calcifications in Human Coronary Arteries As Determined with a Proton Microprobe. *Anal Chem*. 2008; 80: 55-61.

22. McCullagh P, Nelder J. Generalized linear models. 1989; London: Chapman and Hall.
23. Caravan P, Cloutier NJ, Greenfield MT, et al. The interaction of MS-325 with human serum albumin and its effect on proton relaxation rates. *J Am Chem Soc.* 2002; 124: 3152-62.
24. Nicholson JP, Wolmarans MR, Park GR. The role of albumin in critical illness. *Br J Anaesth.* 2000; 85: 599-610.
25. Smith EB, Staples EM. Distribution of plasma proteins across the human aortic wall: barrier functions of endothelium and internal elastic lamina. *Atherosclerosis.* 1980; 37: 579-90.
26. Barkhausen J, Ebert WJ, Heyer C, Debatin JF, Weinmann H. Detection of atherosclerotic plaque with Gadofluorine-enhanced magnetic resonance imaging. *Circulation.* 2003; 108: 605-9.
27. Zheng J, Ochoa E, Misselwitz BM, Yang D, El Naga I, Woodard PK, Abendschien D. Targeted contrast agent helps to monitor advanced plaque during progression: a magnetic resonance imaging study in rabbits. *Invest Radiol.* 2008; 43: 49-55.
28. Brushett C, Qiu B, Atalar E, Yang X. High-resolution MRI of deep-seated atherosclerotic arteries using motexafin gadolinium. *J Magn Reson Imaging.* 2008; 27: 246-50.

CHAPTER 4

Molecular imaging of murine atherosclerosis using RANTES-based imaging agents: a feasibility study

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In preparation

Abstract

Background: Plaque rupture is the main cause of life-threatening cardiovascular events such as myocardial infarction and stroke. Rupture prone plaques are typified by the abundant presence of inflammatory cells, which have likely been recruited to the plaque through chemokine receptors. In this chapter we investigated the potential of chemokine receptor targeted molecular imaging agents for advanced atherosclerosis in ApoE^{-/-} mice.

Material and Methods: Micro array analysis was performed to assess the mRNA levels of several chemokine receptors. To validate protein expression levels, immunohistochemistry (IHC) was performed on stable (N=6) and ruptured (N=11) human carotid atherosclerotic lesions. RANTES-based (CCR5 ligand) imaging agents were synthesized by coupling rhodamine (RANTES-rhodamine), a DTPA-chelate for capturing gallium-68 (RANTES-DTPA-⁶⁸Ga), or both (bimodal-RANTES) to the N-terminus of the RANTES-protein. CCR5-expressing bone marrow-derived macrophages (BMMs) and CCR5 negative control cells (3T3) were exposed to RANTES-rhodamine or RANTES-DTPA-⁶⁸Ga. Uptake was determined with two-photon laser-scanning microscopy (TPLSM) and gamma counting, respectively. Additionally, RANTES-based agents were intravenously administered in ApoE^{-/-} mice to determine half-life, biodistribution and uptake in murine atherosclerotic lesions.

Results: Strong upregulation of CCR5 mRNA and protein expression levels (mainly in macrophages) were found in ruptured compared to stable human carotid lesions. In BMMs *in vitro*, TPLSM demonstrated RANTES-rhodamine internalization. RANTES-DTPA-⁶⁸Ga uptake was significantly increased in non-stimulated (counts per minute $\pm$ s.e.: 4776 $\pm$ 316) and LPS-stimulated (3636 $\pm$ 25) BMMs compared to the control 3T3 fibroblast cells (1211 $\pm$ 100). In ApoE^{-/-} mice, TPLSM on excised atherosclerotic lesions after *in vivo* injection of the imaging agents showed macrophage internalization of RANTES-rhodamine and bimodal-RANTES. Both agents displayed a relatively short half-life (4 minutes for RANTES rhodamine and 10.6 minutes for RANTES-DTPA-⁶⁸Ga) and accumulated mainly in liver and spleen. Additionally, microPET imaging showed increased standard uptake values in atherosclerotic lesions compared to vessels of control C57Bl6 mice when using the bimodal-RANTES agent.

Conclusion: CCR5 expression is upregulated in ruptured compared to stable human atherosclerotic lesions. Furthermore, RANTES-based imaging agents enabled the *ex vivo* detection of macrophages within murine atherosclerotic lesions, indicating that molecular imaging using RANTES-based imaging agents is a promising strategy for the assessment of inflammatory foci within atherosclerotic lesions.

Introduction

Atherosclerosis is an inflammatory disease that is a major cause of death and morbidity in the Western World (1-4). Prominent features within the inflammatory process are infiltration of monocytes and T cells into the atherosclerotic lesion and the formation of foam cells from macrophages (5-7). Over time, atherosclerotic lesions mature and may eventually rupture, causing life-threatening cardiovascular events such as myocardial infarction and stroke. Rupture-prone atherosclerotic lesions are characterized by the abundant presence of inflammatory cells (2). These cells have likely been recruited to the atherosclerotic lesion through chemokine receptors (8). It is widely accepted that chemokines and chemokine receptors are of fundamental importance in atherogenesis (6-8). Chemokines are small chemotactic cytokines which are subdivided into four families based on the cysteine configuration (CC, CXC, CX3C, XC) and are known to guide cell migration and stimulate the adaptive immune response (6,8). Chemokine receptors are expressed on plaque-residing cells such as macrophages, smooth muscle cells, and T-cells (8,9).

Although several molecular imaging agents have been developed to visualize molecular characteristics of atherosclerotic lesions (10-15), the use of chemokine receptors as targets is yet limited. However, given the accessibility, abundance and existing knowledge on ligands, chemokine receptors are promising targets for molecular imaging of atherosclerosis. Therefore, in this study, we used a differential gene expression approach to select for chemokine receptors that are abundantly expressed in ruptured human atherosclerotic lesions. Microarray analysis showed the highest positive fold change for CCR5, suggesting that this chemokine receptor could be a suitable target for the molecular imaging of atherosclerosis. Subsequently, unique imaging agents based on RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted (CCR5 ligand)) were synthesized using solid phase peptide synthesis by tBoc chemistry protocols (16) and native chemical ligation (17). These imaging agents were tested and validated in *in vitro* and *ex vivo* studies.

In this study we present the proof of principle that it is feasible to select for chemokine receptors that are abundantly expressed in ruptured lesions, and subsequently design and validate RANTES-based imaging agents that may be suitable for the assessment of inflammatory foci within atherosclerotic lesions.

Materials and Methods

CCR5 in human atherosclerotic lesions

To assess the mRNA levels of several chemokine receptors in different atherosclerotic stages, tissue sections containing human carotid atherosclerotic lesions were evaluated by micro array analysis using GeneChip Human Genome U133 Plus 2.0 array (Affymetrix Inc, USA). All samples were obtained by carotid endarterectomy and immediately processed to minimize RNA degradation. Each alternative slice was processed for histological evaluation (formalin fixed and subsequently paraffin-embedded) and classified according to the Virmani classification criteria (18). In total, 12 ruptured and 3 stable lesions were used. RNA isolation was performed using guanidine thiocyanate lysis followed by cesium chloride gradient centrifugation. After the extracting procedure, the RNA was further purified using the Nucleospin RNAII (Macherey-Nagel GmbH & Co. KG). Only samples with a good RNA quality were used for microarray analysis. Microarray data was analyzed using Statistical Analysis of Microarrays (SAM). All tissues were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage, and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>).

Additionally, CCR5 protein expression was determined in paraffin-embedded stable (N=6) and ruptured (N=11) human atherosclerotic lesions using immunohistochemistry (IHC). IHC was performed using conventional methods. Briefly, a mouse monoclonal CCR5 antibody (1:70, clone R-C10, Abcam, Cambridge, UK) was used as primary antibody. The omission of the primary antibody was used as a negative control. Visualization was achieved with vectastain red (alkaline phosphatase substrate kit I, Vector Laboratories, Burlingame, CA). In addition, macrophages were immunohistochemically stained using a primary antibody against CD68 (1:100, Clone KP1, Dako Cytomation, Glostrup, Denmark). Visualization was achieved with DAB staining. The presence of CCR5 was evaluated using an arbitrary qualitative score system by VH (intraobserver variability <10%).

Synthesis of the RANTES-based contrast agents

Materials: (2-(6-Chloro-1H-benzotriazole-1-yl)-1,1,3,3-tetramethylammonium hexafluoro-phosphate (HCTU) and S-trityl-mercaptopropionic acid were obtained from Peptides International (Louisville, KY). Boc-amino acids were obtained from NovaBiochem (San Diego, CA), Boc-Arg (p-toluenesulfonyl)-OH and Boc-Asn(xanthyl)-OH were obtained from Midwest (Fishers, IN). N,N,-diisopropylethylamine (DIEA) was obtained from Biosolve (Foster City, CA). 4-

methyl-benzhydrylamine (MBHA) resin and Boc-thiaproline-OH were obtained from Anaspec (Fremont, CA). N,N-Dimethylformamide (DMF) and HPLC-grade acetonitrile were purchased from Biosolve (Valkenswaard, the Netherlands). Trifluoroacetic acid (TFA) was obtained from Halocarbon (River Edge, NJ). HF was purchased from Sigma-Aldrich (St Louis, USA).

Peptide Synthesis: Peptides were prepared by manual solid phase peptide synthesis (SPPS) typically on a 0.2-0.3 mmol scale using the *in situ* neutralization/benzotriazole-N,N',N'-tetramethyl-uronium-hexafluoro-phosphate (HBTU) activation procedure for Boc chemistry (16). Each synthetic cycle consisted of N α -Boc-removal by a 1-2 minutes treatment with neat TFA, a 1 minute DMF-flow wash, a 10 to 20 minutes coupling time with 1.0 mmol preactivated Boc-amino acid in the presence of excess diisopropyl ethanolamine (DIEA), followed by a second DMF-flow wash. N α -Boc amino acids (1.1 mmol) were preactivated for 3 minutes with 1.0 mmol HCTU (0.5 M in DMF) in the presence of excess DIEA (3 mmol). After coupling of Gln residues, a dichloromethane flow wash was used before and after deprotection using TFA, to prevent possible high temperature (TFA/DMF)-catalyzed pyrrolidonecarboxylic acid formation (16). Side-chain protected amino acids were: Boc-Arg (p-toluenesulfonyl)-OH, Boc-Asn/Gln(xanthyl)-OH, Boc-Asp(O-cyclohexyl)-OH, Boc-Cys(4-methylbenzyl)-OH, Boc-Glu(O-cyclohexyl)-OH, Boc-His(dinitrophenyl)-OH, Boc-Lys(2-Cl-Z)/(Fmoc)-OH, Boc-Ser(benzyl)-OH, Boc-Thr(benzyl)-OH, and Boc-Tyr(2-Br-Z)-OH. Other amino acids were used without side-chain protection.

After chain assembly, the peptides were deprotected and cleaved from the resin by treatment with anhydrous HF for 1 hour at 0°C with 4% p-cresol as a scavenger. Dinitrophenyl (Dnp) protecting groups on His remained on the thioester fragments because the Dnp-removal would reduce C-terminal thioester bonds. Dnp was gradually removed by thiols during the ligation reaction yielding unprotected His. After cleavage, peptides were precipitated with ice-cold diethylether, dissolved in aqueous acetonitrile and lyophilized.

RANTES Polypeptide Fragment Synthesis: RANTES 1-33 (N-term), and RANTES 34-69 (C-term) were synthesized on thioester (MPAL) resin and MBHA resin, respectively. Thioester resin was prepared as described (19). After the C-terminus was completed, the fully protected peptide-resin was treated with 20% piperidine/DMF (4x3 minutes) to remove Fmoc from Lys⁶⁹. Depending on the imaging agent rhodamine-succinimide ester was added (RANTES-rhodamine); tBoc-Thz-OH (for RANTES-DTPA) or levulinic acid (for bimodal RANTES) was coupled using DIC/NHS. After deprotection and cleavage from the resin, the polypeptides were HPLC-purified, lyophilized and stored at -20°C until use.

Folding of RANTES: RANTES was dissolved in 0.1 M Tris, pH 8.0 containing 3 M guanidin and 1 mM cystine/8 mM cysteine as a redox couple. Mixture was left stirring overnight at 4°C, and purified using preparative HPLC. In case of thiaproline deprotection folded RANTES was dissolved in 0.1 M Acetate, pH 5.0 containing 6 M guanidin, and 100 mM hydroxylamine. After 6 hours 95% was deprotected and maleimide-activated labels were directly added. After 2 hours RANTES imaging agents were purified on HPLC.

Bimodal rhodamine-DTPA synthon: tBoc-Lys(Fmoc)-Cys(4-MeBzl) dipeptide was synthesized on MBHA resin. Fmoc was removed as described above, and rhodamine was coupled by adding rhodamine succinimidyl ester. tBoc was removed by 2 x 1 minutes treatment with TFA, and N α -aminooxyacetyl was added by reaction with HCTU-activated aminooxyacetic acid. Peptide-resin was cleaved using HF and purified using HPLC. DTPA was coupled by reaction of the peptide with a molar equivalent of DTPA-maleimide (20) for 2 hours, after which the bimodal label was HPLC purified. Oxime formation with RANTES-levulinic acid was performed as described (21).

High Pressure Liquid Chromatography (HPLC): Analytical reversed-phase HPLC was performed on a Varian Prostar system using Vydac C-18 columns (5 μ m, 0.46 x 15 cm). Semipreparative reversed-phase HPLC was performed on a Waters HPLC system using Vydac C-18 columns (10 μ m, 1.0-2.5 x 25 cm). Linear gradients of acetonitrile in water/0.1% TFA were used to elute bound peptides. The flow rates used were 1 ml/min (analytical), and 5 ml/min (semipreparative).

Mass Spectrometry: Electrospray ionization mass spectrometry (ESI-MS) was performed on an API-150 single quadrupole mass spectrometer (Applied Biosystems). Peptide masses were calculated from the experimental mass to charge (m/z) ratios from all the observed protonation states of a peptide. Matrix-assisted laser-desorption ionization mass spectrometry (MALDI-MS) was performed on a ABI 4800 MALDI TOF/TOF proteomics analyzer (Applied Biosystems) using α -cyano-4-hydroxycinnamic acid (Aldrich, Milwaukee, WI) as a matrix. Theoretical masses of peptides and proteins were calculated using MacProMass software (Beckman Research Institute, Duarte, CA).

Native Chemical Ligation: The ligation of unprotected synthetic peptide segments was performed as follows: 0.1 M tris buffer, pH 8, containing 6 M guanidine, 1% (v/v) benzylmercaptan and 1% (v/v) thiophenol was added to dry peptides resulting in a final peptide concentration of 1-3 mM at a pH $\approx$ 7 (lowered due to addition of thiols and TFA from the lyophilized peptide). The ligation reaction was performed in

a heating block at 37°C and was vortexed periodically to equilibrate the thiol additives.

Gallium labeling: RANTES-DTPA and bimodal-RANTES were solved in 0.9% sodium chloride (2mg/ml) and labeled with gallium-68 (^{68}Ga) at pH 4. ^{68}Ga was added to RANTES-DTPA (molar ratio; 1 mol RANTES : 1.17 μmol ^{68}Ga) and bimodal RANTES (molar ratio; 1 mol RANTES : 41.17 μmol ^{68}Ga). After labeling the pH was brought to 7 using PBS.

***In vitro* experiments**

Murine bone marrow-derived macrophages (BMMs) were isolated from ApoE^{-/-} mice by flushing femurs and tibiae. Cells were cultured in RPMI medium containing L-glutamine, HEPES, 10% FCS, and 100 IU / ml penicillin/streptomycin. To induce differentiation into macrophages, 15% L929 cell conditioned medium (LCM) was added (22). 3T3 fibroblasts cells were used as negative control cells since these do not express CCR5 (23). These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, Glutamax-1, and 100 IU / ml penicillin/streptomycin. To determine CCR5 expression, IHC was performed on formalin fixed 3T3 control cells and BMMs as described above.

For *in vitro* imaging with two-photon laser-scanning microscopy (TPLSM), BMMs and 3T3 cells were exposed to increasing concentrations of RANTES-rhodamine (0, 50, 250, 500, 1000, 1750, 2500, and 5000 nM) during 20 minutes at 37°C. After washing the cells with phosphate buffered saline (PBS), Syto 44 (final concentration 0.2 $\mu\text{g}/\text{ml}$) (Invitrogen, Eugene, OR) and CD11b-Alexa 488 (only for BMM, final concentration 8.0 $\mu\text{g}/\text{ml}$) (BD Biosciences Pharmingen, San Diego, CA) were added to visualize nucleic acids (mostly cell nuclei) and macrophages, respectively. Cells were visualized using a BioRad 2100MP microscope (Hemel Hempstead, UK) in TPLSM mode (24). A Spectra Physics Tsunami Ti: Sapphire laser (Mountain View, CA), mode locked at 800 nm, with a 140 fs pulse width was used for excitation. Imaging speed at 512x512 was 50 lines per second. Magnification was achieved using a Nikon water dipping lens (60x), in combination with an upright Nikon E600FN microscope (Tokyo, Japan). Images were obtained combining the fluorescence detected by 3 photomultipliers. Filter settings of these photomultipliers were as follows: blue (Syto 44 for nucleic acids): 420-470 nm; green (CD11b-Alexa 488 for BMMs): 500-560 nm (3T3 cells) and 510-540 nm (BMMs); red (RANTES-rhodamine detection): 560 nm and higher. Filters were chosen such as to optimize signal intensity in each channel while minimizing spectral leak-through. Shown are the merged images without further image processing.

To investigate the potential of RANTES-DTPA as a microPET-tracer, RANTES-DTPA was labeled with the positron-emitter gallium-68 (RANTES-DTPA-⁶⁸Ga). Uptake of the radioactive-labeled RANTES protein was investigated using BMMs (with and without 4 hours of lipopolysaccharide (LPS) stimulation). 3T3 fibroblasts exposed to RANTES-DTPA-⁶⁸Ga and BMMs exposed to bare gallium-68 (⁶⁸Ga) were used as negative controls. Cells were exposed to the radioactive tracers during 20 minutes at 37°C after which the cells were harvested, washed, and centrifuged. To determine RANTES-⁶⁸Ga uptake, radioactivity in obtained cell pellets (n=3 for each group) was measured using a gamma counter.

***Ex vivo* experiments: RANTES-rhodamine**

All animal experiments were approved by the local ethical review committee. Maintenance and care of the animals were in compliance with the guidelines set by the institutional animal care committee, following national procedures.

Mice were anesthetized by inhalation of air with 2% isoflurane inhalation gas. Half-life and biodistribution of RANTES-rhodamine were determined in ApoE^{-/-} mice (N=3). RANTES-rhodamine (0.5 µmol/kg body weight) was administered intravenously. Blood samples were collected before contrast administration and at different time points after contrast administration: 1, 5, 10, 15, 30, 60, and 90 minutes. Blood samples were measured with TPLSM by detecting the mean fluorescence of the red channel (filter setting: 560 nm and higher) with Image-Pro Plus version 6.1 (Media Cybernetics Inc., Bethesda, USA). An exponential fit through the obtained values was used to estimate the half-life of RANTES-rhodamine in blood (GraphPad Prism 4.0, La Jolla, USA). To determine the biodistribution of RANTES-rhodamine, mice were sacrificed and kidney, liver, spleen, fat, intestine, heart, and lung were excised, embedded in 2% agarose, and subsequently studied with TPLSM. Filter settings were optimized (maximal intensities while avoiding saturation and leak-through) for each tissue.

Uptake of RANTES-rhodamine in atherosclerotic lesions in the aortic arch was determined in 43-52 weeks aged male ApoE^{-/-} mice (n=3). RANTES-rhodamine was administered intravenously (0.8-1.6 µmol/kg body weight). After a circulation period of 75 minutes, mice were sacrificed and the aortic arch was excised, immersed in Hanks Balanced Salt Solution (HBSS, pH 7.4 containing 144 mM NaCl, 14.9 mM HEPES, 5.5 mM glucose, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 1.2 mM MgSO₄), dissected longitudinally, and visualized from the luminal side ("en face") with TPLSM. Syto 44 (final concentration 0.8 µg/ml) and CD11b-Alexa 488 (final concentration 8.0 µg/ml) were added to visualize nucleic acids and macrophages, respectively. Filter settings were: 420-470 nm (blue, Syto 44 for nucleic acids), 500-550 nm (green, CD11b-Alexa 488 for macrophages), and 560 nm

and higher (red, RANTES-rhodamine detection). The filters were chosen to optimize signal intensities and minimize leak-through.

***Ex vivo* experiments: bimodal-RANTES**

To determine the uptake of bimodal-RANTES, this imaging agent was administered (0.2 $\mu\text{mol/kg}$ body weight, activity: 0.5-1.5 mCi) to aged ApoE^{-/-} mice (n=2, age: 58-59 weeks) and wild type C57Bl6 control mice (n=2, age: 12 weeks). The bimodal-RANTES imaging agents were allowed to circulate for 120 minutes. From one wild type C57Bl6 control mouse, blood samples were collected on various time points to determine the clearance of the bimodal-RANTES imaging agent. After sacrificing the mice, 50-200 μl blood was withdrawn from the heart after which the remaining blood was removed from the vasculature by cardiac perfusion with 5 ml HBSS. Subsequently, liver, spleen, kidney, lung, heart, small intestine, abdominal fat, and the entire arterial tree were excised. At 205 ± 6 minutes after injection of bimodal-RANTES, the arterial tree was imaged *ex vivo* using a Focus 220 microPET scanner (Concorde Microsystems Inc.) during 10 minutes. Radioactivity in blood samples and excised organs was quantified with a gamma counter (WALLAC 1480 Wizard 3" automatic gamma counter, PerkinElmer, USA) and corrected for radioactive decay to determine the clearance (GraphPad Prism 4.0, La Jolla, USA) and biodistribution of the bimodal-RANTES agent. Counts per minute (CPMs) were corrected for the injected dose and sample weight. MicroPET images were analyzed using the software program ASIpro VMtm (CTI Concorde Microsystems, LLC, Knoxville, Tennessee). Regions of interest (ROIs) were drawn at several locations within the arterial tree. Measured activities were corrected for the injected activity. Standardized uptake values (SUVs) were calculated by dividing the measured activities by the quotient of the injected dose and the weight of the mouse. The percentage of SUV increase in the ApoE^{-/-} mice was determined by dividing the difference between the mean SUV in ApoE^{-/-} and wild type C57Bl6 control mice by the mean SUV in the control mice and multiplying this by a factor of 100.

After microPET imaging, the excised aortic arch was imaged "en face" with TPLSM as described before. To visualize macrophages, the excised aortic arch was incubated with CD11b-Alexa 488 (final concentration 8.0 $\mu\text{g/ml}$) for 30 minutes. Filter settings were: 500 and less nm (blue, autofluorescence), 500-560 nm (green, CD11b-Alexa 488 for macrophages), and 560 nm and higher (red, bimodal-RANTES detection). Subsequently, all arterial tissue was embedded in paraffin for histological analysis. CCR5 immunohistochemistry was performed as described above.

Statistics

Data are presented as mean $\pm$ standard error. Significance analysis of microarrays (SAM) was used to determine statistically significant mRNA differences between stable and ruptured human lesions. Significant differences were considered to have a SAM q-value of $< 5\%$. A Mann-Whitney U test was performed for the scores obtained from the IHC stained carotid endarterectomy samples. For the *in vitro* gamma counting experiments one-way ANOVA was used with Bonferroni correction for multiple group comparison. Differences with a P-value less than 0.05 were considered significant.

Results

Mapping of chemokine receptor expression in human atherosclerotic lesions

Micro-array analysis demonstrated that mRNA levels of CCR1, CCR2 and CCR5 were significantly upregulated in ruptured atherosclerotic lesions compared to stable lesions. The highest positive fold change was found for CCR5 (5.4) suggesting that this chemokine receptor could be a suitable target for the molecular imaging of atherosclerosis. Lower fold changes were found for CCR1 (3.1) and CCR2 (2.9) (table 4.1).

Additionally, immunohistochemistry showed that CCR5 was mainly expressed in macrophages present in ruptured atherosclerotic lesions. Mean scores for CCR5 expression levels were significantly higher ($P = 0.003$) in ruptured (mean score: 1.4 ± 0.1) compared to stable atherosclerotic lesions (mean score: 0.7 ± 0.2) (figure 4.1).

Table 4.1: Fold change in mRNA levels between stable and ruptured human atherosclerotic lesions of several chemokine receptors.

Study group	Fold Change	q-value %
Chemokine (C-C motif) receptor 1	3.1	4.1*
Chemokine (C-C motif) receptor 2	2.9	4.1*
Chemokine (C-C motif) receptor 3	1.2	61.6
Chemokine (C-C motif) receptor 4	1.1	58.9
Chemokine (C-C motif) receptor 5	5.4	4.1*
Chemokine (C-C motif) receptor 6	1.4	41.1
Chemokine (C-C motif) receptor 7	1.7	15.8
Chemokine (C-C motif) receptor 8	-	100.0
Chemokine (C-C motif) receptor 9	0.7	29.3
Chemokine (C-C motif) receptor 10	0.7	25.6

*Significant differences (q-value $< 5\%$)

Figure 4.1: Immunohistochemistry stainings for CCR5 (A, B, D, and E) and macrophages (CD68) (C and F) on human atherosclerotic lesions. Stable lesions (A-C) show no or hardly any CCR5 staining while ruptured lesions (D-F) strongly express CCR5. Mean CCR5 score was significantly higher in ruptured lesions (1.4 ± 0.1) compared to stable lesions (0.7 ± 0.2) (G). Blue: haematoxylin staining. Red: CCR5 staining. Brown: macrophage staining. Bars: 100 μ m (A and D) or 50 μ m (B, C, E, and F). For full color figure see chapter 9.

Synthesis of RANTES-rhodamine

Since the imaging studies were anticipated in mice, murine RANTES was synthesized by total chemical synthesis using solid phase peptide synthesis by tBoc chemistry protocols (16) and native chemical ligation (17). A C-terminal Fmoc-protected lysine was added to facilitate attachment of imaging probes at a single specific site (figure 4.2).

Figure 4.2: Murine and human RANTES. A: Human RANTES solution NMR structure showing antiparallel β -sheet and α -helical secondary structure, and Cys¹⁰-Cys³⁴ and Cys¹¹-Cys⁵⁰ disulfide bonds (25). B: Murine (P30882) (26) and human (P13501) (27) RANTES sequence with differences indicated in red. The underlined Lys-Cys sequence represents the native chemical ligation site. A C-terminal Lys(Fmoc)⁶⁹ handle is attached as a linker to attach (multimodal) imaging probes. For full color figure see chapter 9.

RANTES was synthesized in two fragments; Ser¹-Lys³³ with a C-terminal thioester (COSR), and Cys³⁴-Ser⁶⁸-Lys⁶⁹(Fmoc). For RANTES-rhodamine the Lys⁶⁹(Fmoc) residue was deprotected on the resin yielding a single amine moiety for attachment of an activated (succinimidyl ester) rhodamine label (figure 4.3A). Fragments were cleaved from the resin and joined by native chemical ligation through an initial thioester bridge with a subsequent spontaneous rearrangement in a peptide bond (figure 4.3B). Oxidative folding of the 1-69(rhodamine) polypeptide chain yielded a mass of 8410.5 Da on electrospray ionization mass spectrometry corresponding well with RANTES with two internal disulfide bonds and a C-terminal rhodamine attached (figure 4.3D).

In vitro experiments

Immunohistochemistry demonstrated that CCR5 was strongly expressed on non-stimulated as well as 4 hours LPS-stimulated BMMs, while no CCR5 expression was detected in the control 3T3 fibroblast cells (figure 4.4).

TPLSM demonstrated RANTES-rhodamine internalization in BMMs (figure 4.5) at 250 nM, whereas RANTES-rhodamine internalization in 3T3 cells was detectable at concentrations $\geq$ 1750 nM. In both cell types, RANTES-rhodamine was internalized dose dependently.

Figure 4.3: Total chemical synthesis of RANTES-rhodamine. A: Strategy: deprotection of Lys(Fmoc) yielded a single amine moiety for attachment of rhodamine(label)-succinimidyl ester. B: Native Chemical Ligation between RANTES fragments proceeded through an initial thioester intermediate that spontaneously rearranged into a full peptide backbone. C: ESI-MS spectra of folded RANTES-rhodamine yielding a molecular mass of 8410.5 Da. D: Final RANTES product with a rhodamine dye attached to the C-terminus. For full color figure see chapter 9.

Figure 4.4: Immunohistochemistry (IHC) stainings for CCR5 of 3T3 fibroblast cells and bone marrow-derived macrophages (BMMs). 3T3 cells show no or hardly any CCR5 staining while BMMs with and without 4 hours of LPS stimulation, strongly express CCR5 (red). Bars: 40 μ m. For full color figure see chapter 9.

Ex vivo experiments: RANTES-rhodamine

RANTES-rhodamine rapidly cleared from the murine blood. Based on one phase exponential fitting curves, the half-life of RANTES-rhodamine was estimated to be 4 minutes. TPLSM on excised organs showed that RANTES-rhodamine accumulated mainly in the kidney, spleen, and liver. In addition, some fluorescence was found in lung, intestine, and fat tissue (figure 4.6) while RANTES-rhodamine presence in excised heart tissue was negligible (not shown).

Figure 4.5: Two-photon laser-scanning microscopy (TPLSM) of 3T3 control cells and bone marrow-derived macrophages (BMMs) exposed to 250 nM RANTES-rhodamine. Left: No RANTES-rhodamine uptake was detected in the 3T3 control cells. Right: Clear RANTES-Rhodamine internalization is shown in the BMMs exposed to RANTES-rhodamine (white arrows). Red: RANTES-rhodamine. Blue: nucleic acid stain using Syto 44. Green: macrophage staining using CD11b-Alexa 488. For full color figure see chapter 9.

TPLSM on excised aortic arches of aged ApoE^{-/-} mice (43-52 weeks) after intravenous administration of RANTES-rhodamine demonstrated specific uptake in the atherosclerotic lesions while no RANTES-rhodamine was detected in non-atherosclerotic areas. Co-staining with CD11b-Alexa 488 demonstrated that RANTES-rhodamine was predominantly internalized in macrophages. In addition, some RANTES-rhodamine uptake was also visible in elongated smooth muscle cells (SMCs), which have been described to express CCR5 (9). No RANTES-rhodamine was detected in non-atherosclerotic parts of the aorta (figure 4.6).

Synthesis of RANTES-DTPA

To quantitate RANTES binding to macrophages, we designed a strategy to attach diethylene triamine pentaacetic acid (DTPA) to RANTES as a common chelator for ⁶⁸Ga isotopes resulting in a RANTES PET-probe. Analogous to RANTES-rhodamine, Lys⁶⁹(Fmoc) was deprotected on the resin, after which a thiaproline residue was coupled to the ε-amino group of the lysine residue. The thiaproline branching off the lysine side chain represented an encrypted cysteine that did not interfere with the formation of the native disulfide bonds during folding of RANTES (figure 4.7A left). After native chemical ligation and folding of RANTES, thiaproline was converted to a cysteine using methoxylamine, yielding a single sulfhydryl moiety for attachment of a maleimide-functionalized DTPA label (figure 4.7A) (22).

***In vitro* experiments RANTES-DTPA**

CCR5 expressing BMM cells and 3T3 control cells were incubated with RANTES-DTPA- ^{68}Ga or free ^{68}Ga . Gamma counting yielded a significant increase in counts per minutes (CPM) for the BMMs exposed to RANTES-DTPA- ^{68}Ga (CPM: 4776 ± 316) compared to the 3T3 cells (CPM: 1211 ± 100 , $P < 0.001$) and the BMMs exposed to free ^{68}Ga (CPM: 1508 ± 137 , $P < 0.001$). LPS stimulation (4 hours) had no additive effect on RANTES-DTPA- ^{68}Ga uptake in BMMs (CPM: 3636 ± 25), compared to the non-stimulated BMMs (CPM: 4776 ± 316) (figure 4.8).

Figure 4.6: Top left panel: Clearance of RANTES-rhodamine as measured with two-photon laser-scanning microscopy (TPLSM) on different time points after RANTES-rhodamine administration (1, 5, 10, 15, 30, 60, and 90 minutes, $n=3$). Top right panel: TPLSM examination of kidney, spleen, liver, fat, lung and intestine demonstrated that the RANTES-rhodamine predominantly accumulated in the kidney, spleen, and liver. Red: RANTES-rhodamine. Blue / white: autofluorescence. Bottom panels: Ex vivo TPLSM of a murine aortic atherosclerotic lesion demonstrating in vivo RANTES-rhodamine uptake in macrophages (white arrows) and a non-atherosclerotic part (showing mainly smooth muscle cells) of the same mouse without any RANTES-rhodamine uptake. Red: RANTES-rhodamine. Blue: nucleic acid stain using Syto 44. Green: macrophage staining using CD11b-Alexa 488. For full color figure see chapter 9.

Figure 4.7: Total chemical synthesis of RANTES-DTPA. **A:** Strategy: deprotection of Lys(Fmoc) yielded a single amine moiety for attachment of a thiapropionyl residue. After oxidative folding of RANTES thiapropionyl was converted into cysteine, and DTPA maleimide was attached. **B:** Final RANTES product with a DTPA-moiety on the C-terminus. **C:** Analytical RP-HPLC of RANTES-DTPA. RANTES was eluted with a linear gradient of 0-60% acetonitrile in water/0.1% TFA in 30 minutes. **D:** MALDI-MS spectrum of folded RANTES-DTPA yielding a molecular mass of 8764.5 Da (8100.7 Da represents unlabeled RANTES). Chelation of ^{68}Ga to RANTES-DTPA yielded a contrast agent for positron emission tomography (PET) imaging. Note that DTPA conjugate structure is enlarged in respect to RANTES structure for reasons of clarity. Label represents 7.5% of the total contrast agents' mass. For full color figure see chapter 9.

Figure 4.8: Gamma counting results obtained from bone marrow-derived macrophages (BMMs), 4 hours LPS stimulated BMMs, and 3T3 control cells, exposed to ^{68}Ga labeled RANTES (RANTES-DTPA- ^{68}Ga) as well as BMMs exposed to ^{68}Ga alone. Counts per minute were significantly increased in BMMs exposed to RANTES-DTPA- ^{68}Ga . LPS stimulation had no additive effect on RANTES-DTPA- ^{68}Ga uptake. * Indicates $P < 0.05$; ** indicates $P < 0.001$.

Synthesis of bimodal-RANTES

A bimodal RANTES agent containing a fluorescent probe and DTPA-chelate was generated to enable molecular imaging with TPLSM and microPET. An oxime linkage was chosen between RANTES and the bimodal label (figure 4.9A) (21). For this strategy a levulinic acid was coupled to the Lys⁶⁹ residue to obtain an aldehyde on RANTES (figure 4.9B), and a bimodal rhodamine-DTPA label was prepared on a Lys-Cys backbone on which the N-terminus was functionalized with an aminooxyacetyl group (figure 4.9D). Oxime formation between the aldehyde and aminooxyacetyl moieties yielded an oxime bond between RANTES and labels (figure 4.9F). After labeling, 10-50% free ⁶⁸Ga was found in the final solution with HPLC (data not shown).

Figure 4.9: Total chemical synthesis of bimodal-RANTES A: Strategy: deprotection of Lys (Fmoc) yielded a single amine moiety for attachment of a levulinic acid. After oxidative folding RANTES was reacted with aminooxyacetyl-bimodal label to yield an oxime bond. B: RANTES with a levulinic acid on the C-terminus. C: MALDI-MS spectrum of folded RANTES-levulinic acid yielding a molecular mass of 8093.7 Da. D: Bimodal rhodamine-DTPA synthon on Lys-Cys backbone functionalized with an aminooxyacetyl group. E: MALDI-MS spectrum of bimodal rhodamine-DTPA synthon yielding a molecular mass of 1396.6 Da. F: Final bimodal RANTES imaging agent with an oxime bond between RANTES and bimodal label. G: MALDI-MS spectrum of bimodal RANTES-rhodamine-DTPA yielding a molecular mass of 9474.1 Da. For full color figure see chapter 9.

Ex vivo results: bimodal-RANTES

120 minutes after intravenous injection of bimodal-RANTES into ApoE^{-/-} mice, organs were harvested and analyzed. The highest activities were found in the spleen and liver, indicating that the bimodal-RANTES accumulated predominantly in spleen and liver. Limited bimodal-RANTES was found in the intestines and fat tissue (figure 4.10). Additionally, a relatively high activity could be found in the blood at 120 minutes after contrast administration. This relatively high activity was also found in the clearance experiment at 90 minutes after contrast administration. Based on the observations in one mouse, the half-life of bimodal-RANTES was estimated to be 10.6 minutes for the first phase after which it seems to reach a plateau instead of returning back to the base value (± 0 CPM/gram) (figure 4.10). Additional experiments need to be performed to validate this observation.

After intravenous injection of bimodal-RANTES, increased SUVs were observed in the excised aortic arch ($236\% \pm 95\%$) and the carotid arteries (right carotid: $125\% \pm 56\%$, left carotid $205\% \pm 25\%$) of the ApoE^{-/-} mice compared to wild type C57Bl6 control mice in the *ex vivo* microPET images. Histology of the corresponding parts in the arterial tree confirmed the presence of atherosclerotic lesions and CCR5 expression in all vessels except for the right carotid artery of one mouse. No atherosclerotic lesions and CCR5 expression was found in the C57Bl6 wild type mice. Additionally, relatively minor SUV increase ($85\% \pm 48\%$) was found at the kidney bifurcation, where we did not observe any atherosclerotic lesion formation (figure 4.11).

Figure 4.10: Biodistribution and clearance of bimodal-RANTES. Left: Counts per minute (CPMs), corrected for injected dose and sample weight, measured in lung, heart, kidney, spleen, intestine, liver, fat, and blood of ApoE^{-/-} (n=2) and control mice (n=2). Right: Clearance of bimodal-RANTES as measured in different blood samples using a gamma counter (n=1).

Figure 4.11: Bimodal-RANTES results in aged $ApoE^{-/-}$ mice ($n=2$) and wild type control mice ($n=2$). Top left: Ex vivo microPET image and photograph of the arterial tree. Top right: Hematoxylin/eosine (HE) staining and CCR5 immunohistochemistry (IHC), indicating the presence of CCR5 in the murine atherosclerotic lesions. Bars represent 100 μm (HE staining) and 300 μm (CCR5 IHC). Middle: HE staining of a thoracic part of the arterial tree without atherosclerotic lesions. This part corresponds with limited activity detected with PET. Bar represent 300 μm . Bottom left: Mean standard uptake value (SUV) increase measured in the different parts of the arterial tree. SUV values measured in 58-59 weeks aged $ApoE^{-/-}$ mice were strongly increased in the aortic arch ($236\% \pm 95\%$), right carotid artery ($125\% \pm 56\%$) and left carotid artery ($205\% \pm 25\%$), while only a minor SUV increase was measured in the abdominal aorta ($85\% \pm 48\%$). Bottom right: TPLSM confirmed the uptake of the bimodal-RANTES agent in macrophages that were present in the atherosclerotic lesions. Red: bimodal-RANTES agent. Green: macrophage staining using CD11b-Alexa 488. For full color figure see chapter 9.

Discussion

The present study includes a number of necessary steps to design and apply a novel imaging agent for the imaging of atherosclerosis, i.e. target identification, evaluation of expression levels, ligand synthesis, coupling of imaging agents (fluorescent probe and nuclear tracer), and *in vitro* and *ex vivo* experiments. We demonstrated that CCR5 mRNA and protein expression levels are strongly upregulated in ruptured compared to stable human carotid atherosclerotic lesions. Additionally, we showed that the intravenously injected bimodal RANTES-based

imaging agent was taken up by macrophages in murine atherosclerotic lesions leading to increased standard uptake values in microPET images of atherosclerotic lesions compared to control vessels. TPLSM confirmed uptake of the RANTES-based agents in macrophages. These results demonstrate the potential of RANTES-based imaging agents for the assessment of murine atherosclerotic lesions.

Immunohistochemistry demonstrated that CCR5 expression levels were significantly upregulated ($P = 0.003$) in ruptured compared to stable human carotid atherosclerotic lesions and that CCR5 expression predominantly colocalized with plaque macrophages. The mRNA levels of CCR1 and CCR2 were elevated in ruptured human carotid lesions as well, albeit to a lesser extent than CCR5. CCR5 is expressed on various cell types involved in atherosclerosis such as monocytes, macrophages, T cells, and smooth muscle cells (SMC) (8,9). Studies using murine models demonstrated that deficiency of CCR5 in apolipoprotein E-deficient mice ($\text{ApoE}^{-/-}\text{CCR5}^{-/-}$) did not affect early atherosclerosis (28) but impeded late-stage atherosclerosis (29). In addition, Braunersreuther et al. demonstrated that reduced plaque formation in $\text{ApoE}^{-/-}\text{CCR5}^{-/-}$ mice was associated with a reduced content of monocytes, macrophages, and T lymphocytes and with an increased SMC content, suggesting a more stable plaque phenotype (30). In addition, administration of RANTES-based chemokine antagonists such as MET-RANTES (31), and [44AANA47]-RANTES (32) to $\text{LDLr}^{-/-}$ mice also limited atherosclerotic plaque formation and induced a more stable plaque phenotype. Furthermore, it was demonstrated that RANTES levels were increased during episodes of unstable angina pectoris and that increased RANTES levels predicted future cardiovascular events (33). In addition, patients who are homozygous for a 32-bp deletion in the CCR5 gene showed reduced risk for advanced coronary artery disease (34) and myocardial infarction (35).

Collectively, these studies indicate that CCR5 is involved in the formation of advanced atherosclerotic lesions. Therefore, CCR5 might be a suitable target for the assessment of atherosclerosis using molecular imaging techniques. Several molecular imaging agents have already been used for the visualization of atherosclerotic lesions (10-15). However, the use of chemokine receptors as targets is yet limited. A previous study showed increased uptake of $^{99\text{m}}\text{Tc}$ labeled MCP-1 (CCR2 ligand) by atherosclerotic lesions in a rabbit model at which MCP-1 uptake strongly correlated with macrophage density (36). In addition, Veillard et al. administered a radiolabeled CCR5 antagonist (^{125}I -Met-RANTES) and demonstrated *ex vivo* elevated radioactivity in murine $\text{LDLr}^{-/-}$ aortic atherosclerotic lesions compared to the radioactivity obtained from the aorta of wild type mice (31).

As an extension of these studies we now show the potential of a bimodal RANTES-based agent for molecular imaging of murine atherosclerotic lesions with microPET. Moreover, TPLSM showed that the imaging agents internalized in macrophages in murine atherosclerotic lesions, leading to accumulation of the

imaging agents, and to biological signal amplification. Biodistribution of the RANTES-based imaging agents demonstrated that these agents predominantly accumulated in the liver and spleen. RANTES-rhodamine was cleared rapidly from the blood, at a half-life of approximately 4 minutes. Measurements in the different blood samples after injection of the bimodal-RANTES agent demonstrated that activity was still detectable in the blood at 90 minutes after contrast administration. The administered bimodal-RANTES solution still contained a high amount of free gallium (10-50%). Therefore, the prolonged activity in the blood might be explained by binding of free gallium to plasma proteins such as transferrin (37). A recent study demonstrated that transferrin receptors are co-localized with CD-68-positive macrophages in human carotid lesions. In addition, expression levels of transferrin receptors were higher in plaques of symptomatic patients (38). This suggests that binding of free gallium to transferrin may lead to enhanced imaging intensities caused by gallium uptake through transferrin receptors in the atherosclerotic lesions.

Whether RANTES-based imaging agents will provide the possibility to distinguish between several atherosclerotic stages remains to be determined since macrophages are present in all stages of the atherosclerotic process. Macrophage localization within the atherosclerotic lesions could help in distinguishing stable and vulnerable lesions since prominent inflammation in the shoulders of atherosclerotic lesions is one of the risk factors for plaque rupture (39). In the present study, CCR5 mRNA and protein expression levels were clearly different in the ruptured and stable human atherosclerotic lesions. Additionally, a feasibility study demonstrated the potential of RANTES-based imaging agents for the detection of atherosclerotic lesions *ex vivo*. Future *in vivo* studies on mice and/or rabbits are needed to confirm these initial results.

In conclusion, we are the first to show upregulated expression of CCR5 at a mRNA and protein level in ruptured compared to stable human atherosclerotic lesions. Moreover, we developed RANTES-based molecular imaging agents and demonstrated the potential of these molecular imaging agents in detecting CCR5-expressing cells in atherosclerotic lesions with TPLSM (RANTES-rhodamine) and microPET (bimodal-RANTES). Therefore, RANTES-based imaging agents may be used to assess atherosclerotic lesions.

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References

1. Libby P. Inflammation in atherosclerosis. *Nature* 2002;420:868-74.
2. Naghavi M, Libby P, Falk E, et al. From vulnerable plaque to vulnerable patient: a call for new definitions and risk assessment strategies: Part I. *Circulation* 2003;108:1664-72.
3. Weintraub HS. Identifying the vulnerable patient with rupture-prone plaque. *Am J Cardiol* 2008;101:3F-10F.
4. Lloyd-Jones D, Adams R, Carnethon M, et al. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;119:e21-181.
5. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med* 2005;352:1685-95.
6. Galkina E, Ley K. Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 2009;27:165-97.
7. Weber C, Zernecke A, Libby P. The multifaceted contributions of leukocyte subsets to atherosclerosis: lessons from mouse models. *Nat Rev Immunol* 2008;8:802-15.
8. Charo IF, Ransohoff RM. The many roles of chemokines and chemokine receptors in inflammation. *N Engl J Med* 2006;354:610-21.
9. Potteaux S, Combadiere C, Esposito B, et al. Role of bone marrow-derived CC-chemokine receptor 5 in the development of atherosclerosis of low-density lipoprotein receptor knockout mice. *Arterioscler Thromb Vasc Biol* 2006;26:1858-63.
10. Miserus RJHM, Heeneman S, Engelshoven JMAv, Kooi ME, Daemen MJAP. Development and validation of novel imaging technologies to assist translational studies in atherosclerosis. *Drug Disc. Today: Techn.* 2006;3:195-204.
11. Jaffer FA, Weissleder R. Molecular imaging in the clinical arena. *Jama* 2005;293:855-62.
12. Choudhury RP, Fuster V, Fayad ZA. Molecular, cellular and functional imaging of atherothrombosis. *Nat Rev Drug Discov* 2004;3:913-25.
13. Briley-Saebo KC, Mulder WJ, Mani V, et al. Magnetic resonance imaging of vulnerable atherosclerotic plaques: current imaging strategies and molecular imaging probes. *J Magn Reson Imaging* 2007;26:460-79.
14. Langer HF, Haubner R, Pichler BJ, Gawaz M. Radionuclide imaging: a molecular key to the atherosclerotic plaque. *J Am Coll Cardiol* 2008;52:1-12.
15. Tahara N, Imaizumi T, Virmani R, Narula J. Clinical feasibility of molecular imaging of plaque inflammation in atherosclerosis. *J Nucl Med* 2009;50:331-4.
16. Schnolzer M, Alewood P, Jones A, Alewood D, Kent SBH. Insitu Neutralization in Boc-Chemistry Solid-Phase Peptide-Synthesis - Rapid, High-Yield Assembly of Difficult Sequences. *International Journal of Peptide and Protein Research* 1992;40:180-193.
17. Dawson PE, Muir TW, Clarklewis I, Kent SBH. Synthesis of Proteins by Native Chemical Ligation. *Science* 1994;266:776-779.
18. Virmani R, Kolodgie FD, Burke AP, Farb A, Schwartz SM. Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions. *Arterioscler Thromb Vasc Biol* 2000;20:1262-75.
19. Hackeng TM, Griffin JH, Dawson PE. Protein synthesis by native chemical ligation: Expanded scope by using straightforward methodology. *Proceedings of the National Academy of Sciences of the United States of America* 1999;96:10068-10073.
20. Dirksen A, Langereis S, de Waal BFM, van Genderen MHP, Hackeng TM, Meijer EW. A supramolecular approach to multivalent target-specific MRI contrast agents for angiogenesis. *Chemical Communications* 2005:2811-2813.
21. Dirksen A, Hackeng TM, Dawson PE. Nucleophilic catalysis of oxime ligation. *Angewandte Chemie-International Edition* 2006;45:7581-7584.

22. KanTERS E, Pasparakis M, Gijbels MJ, et al. Inhibition of NF-kappaB activation in macrophages increases atherosclerosis in LDL receptor-deficient mice. *J Clin Invest* 2003;112:1176-85.
23. Wang J, Alvarez R, Roderiquez G, Guan E, Norcross MA. Constitutive association of cell surface CCR5 and CXCR4 in the presence of CD4. *J Cell Biochem* 2004;93:753-60.
24. Megens RT, Reitsma S, Schiffrers PH, et al. Two-photon microscopy of vital murine elastic and muscular arteries. Combined structural and functional imaging with subcellular resolution. *J Vasc Res* 2007;44:87-98.
25. Skelton NJ, Aspiras F, Ogez J, Schall TJ. Proton Nmr Assignments and Solution Conformation of Rantes, a Chemokine of the C-C Type. *Biochemistry* 1995;34:5329-5342.
26. Schall TJ, Simpson NJ, Mak JY. Molecular-Cloning and Expression of the Murine Rantes Cytokine - Structural and Functional Conservation between Mouse and Man. *European Journal of Immunology* 1992;22:1477-1481.
27. Schall TJ, Jongstra J, Dyer BJ, et al. A Human T-Cell-Specific Molecule Is a Member of a New Gene Family. *Journal of Immunology* 1988;141:1018-1025.
28. Kuziel WA, Dawson TC, Quinones M, et al. CCR5 deficiency is not protective in the early stages of atherogenesis in apoE knockout mice. *Atherosclerosis* 2003;167:25-32.
29. Quinones MP, Martinez HG, Jimenez F, et al. CC chemokine receptor 5 influences late-stage atherosclerosis. *Atherosclerosis* 2007;195:e92-103.
30. Braunersreuther V, Zernecke A, Arnaud C, et al. Ccr5 but not Ccr1 deficiency reduces development of diet-induced atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 2007;27:373-9.
31. Veillard NR, Kwak B, Pelli G, et al. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* 2004;94:253-61.
32. Braunersreuther V, Steffens S, Arnaud C, et al. A novel RANTES antagonist prevents progression of established atherosclerotic lesions in mice. *Arterioscler Thromb Vasc Biol* 2008;28:1090-6.
33. Kraaijeveld AO, de Jager SC, de Jager WJ, et al. CC chemokine ligand-5 (CCL5/RANTES) and CC chemokine ligand-18 (CCL18/PARC) are specific markers of refractory unstable angina pectoris and are transiently raised during severe ischemic symptoms. *Circulation* 2007;116:1931-41.
34. Szalai C, Duba J, Prohaszka Z, et al. Involvement of polymorphisms in the chemokine system in the susceptibility for coronary artery disease (CAD). Coincidence of elevated Lp(a) and MCP-1 -2518 G/G genotype in CAD patients. *Atherosclerosis* 2001;158:233-9.
35. Gonzalez P, Alvarez R, Batalla A, et al. Genetic variation at the chemokine receptors CCR5/CCR2 in myocardial infarction. *Genes Immun* 2001;2:191-5.
36. Hartung D, Petrov A, Haider N, et al. Radiolabeled Monocyte Chemotactic Protein 1 for the detection of inflammation in experimental atherosclerosis. *J Nucl Med* 2007;48:1816-21.
37. Li Y, Liu B, Ge Z, Yang B. Spectroscopic analysis of the interaction between gallium(III) and apoovotransferrin. *J Photochem Photobiol B* 2008;91:137-42.
38. Li W, Xu LH, Forssell C, Sullivan JL, Yuan XM. Overexpression of transferrin receptor and ferritin related to clinical symptoms and destabilization of human carotid plaques. *Exp Biol Med (Maywood)* 2008;233:818-26.
39. Fishbein MC. The vulnerable and unstable atherosclerotic plaque. *Cardiovasc Pathol* 2008.

CHAPTER 5

Optical and magnetic resonance imaging of cell death and platelet activation using Annexin A5- functionalized quantum dots

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Abstract

A quantum dot-based nanoparticle is presented, allowing visualization of cell death and activated platelets with fluorescence imaging and MRI. The particle exhibits intense fluorescence and a large MR relaxivity (r_1) of 3000-4500 $\text{mM}^{-1}\text{s}^{-1}$ per nanoparticle, due to a newly designed construct increasing the gadolinium-DTPA load. The nanoparticle is suitable for both anatomic and sub-cellular imaging of structures in the vessel wall and is a promising bimodal contrast agent for future *in vivo* imaging studies.

Introduction

Contrast-enhancing nanoparticles are essential in the field of molecular imaging. Molecular imaging focuses on early detection of various diseases and supports personalized therapy. While conventional imaging modalities such as computer tomography and magnetic resonance imaging (MRI) allow visualization of anatomical and (patho)physiological consequences of the disease, molecular imaging is based on the molecular and cellular fingerprints of the disease, requiring disease-specific molecular targets as well as target-specific nanoparticles. Furthermore, these nanoparticles should allow *in vivo* visualization with sufficient perceptibility.

Programmed cell death (PCD) is a process of organized cell suicide, and occurs in various forms, of which apoptosis is the best known (1). PCD plays important roles in physiology as well as pathology of multi-cellular organisms. During homeostasis a balance exists between cell proliferation and cell death. This equilibrium is disturbed under certain pathological conditions. Acute myocardial infarctions (2), heart failure (3), or unstable atherosclerotic plaques (4,5) show increased PCD, whereas in cancer this balance shifts towards cell proliferation (6). Anti-cancer therapies are being developed in which PCD is induced in cancer cells to reverse tumor growth (7-9). Because of the role of PCD in numerous diseases, imaging of this process would be of great value to diagnosis and guidance of therapy.

One of the most explored molecular imaging targets of PCD is phosphatidylserine (PS). This aminophospholipid is translocated from the inner to the outer leaflet of the cellular membrane in dying cells, triggering phagocytosis (10). Cell surface exposure of PS occurs during most types of cell death (8), but is not limited to dying cells. For example, blood platelets expose PS in an advanced stage of activation (11,12), providing a physiological surface for blood coagulation processes. Hence, molecular imaging of PS can also be of value to diagnosis and management of thrombotic diseases.

Annexin A5 (AnxA5) is a well explored molecular imaging probe to visualize cell surface exposure of PS (13). AnxA5 binds to PS with a K_d in the nM-range in the presence of Ca^{2+} -ions (14). It can be coupled to a variety of reporter compounds making it suitable to use in combination with various imaging modalities including optical (15,16), nuclear (17,18), and magnetic resonance imaging (MRI) (19,20). *In vivo* studies in animals and patients demonstrated that AnxA5 can be used to non-invasively detect the vulnerability of atherosclerotic plaques (21,22), size and location of the infarct region in acute myocardial infarctions (2), the extent of liver apoptosis (23,24), and the localization of mural thrombi in abdominal aortic aneurysms (25). In addition, imaging cell death with AnxA5 allows early assessment of the efficacy of anti-cancer therapy (9). These studies utilized radioactively

labeled AnxAS in combination with single photon emission computed tomography (SPECT) or positron emission tomography (PET). SPECT and PET imaging do not yield anatomical information, and have a relatively poor resolution: 8-14 mm for SPECT and 4-7 mm for PET (17, 26). These limitations can be overcome by MRI using targeted contrast agents. MRI has a better resolution ($\pm 300 \mu\text{m}$) (26) and provides anatomical information, enabling a more accurate localization and characterization of the target site. However, sensitivity of this imaging modality for contrast agents is still relatively low.

In this paper a new, AnxAS-functionalized and bimodal nanoparticle is presented that is suitable for MRI and fluorescence imaging allowing analysis at the anatomical as well as sub-cellular level. The nanoparticle is based on a quantum dot (QD), which is intensely fluorescent. The emission wavelength of QDs can be varied by altering the size of the semiconductor crystal core. Emission spectra of QDs are very narrow: emission peak width is about 20 nm at half maximum. Furthermore, QDs with different emission wavelengths have the same excitation wavelength. These properties allow multicolor analysis of samples in a single procedure (27). A recent publication by Le Gac et al. (28) showed that QDs (Quantum Dot Corporation, now Invitrogen) resist photo-bleaching and allow therefore imaging of fast, cellular processes over a longer period of time as compared to organic fluorescent dyes.

Long term *in vivo* effects of QDs are unknown so far. QDs used in this study are potentially toxic substances because the semiconductor crystal core contains cadmium and selenium. Toxicity of QDs depends on multiple factors, e.g. size, charge, concentration, outer coating bioactivity, and stability of the QD (29). A recent study reported that mice tolerated intravenous injection of QDs well up to 133 days after administration. Neither signs of necrosis at sites of deposition nor signs of QD breakdown were observed (30). The QDs used in that study are from the same supplier as the QDs of the current study.

The present study visualizes QDs by two-photon laser-scanning microscopy (TPLSM). The laser power of TPLSM required to have sufficient QD fluorescence is considerably lower as compared to organic fluorescent dyes, due to the high quantum yield of QDs. This limits further potential photo-bleaching of the probe and photo-damage of the sample. Furthermore, high resolution images with low background fluorescence can be acquired. This is due to several factors: first, TPLSM excitation occurs only at the focal point preventing out-of-focus contribution (31,32). Second, as indicated above, the required laser power is low, thereby also reducing background (auto)fluorescence. Third, because emission spectra of the QDs are narrow (27), stringent emission filters can be applied to reduce background light as much as possible. Hence, it is possible to image structures hidden deeper in tissues more accurately with TPLSM without provoking tissue damage, for example atherosclerotic plaque imaging in arteries (31).

Materials and Methods

Annexin A5 functionalized QDs

In order to accommodate conjugation chemistry for coupling AnxA5 to larger structures without impairing the PS binding property, a cysteine residue was engineered in the N-terminal tail, which is located apical to the PS binding sites (cys2-AnxA5) (33). Maleimide-activated biotin (Pierce, Rockford, IL) was coupled to cys2-AnxA5 according to the recommendations of the manufacturer. The cys2-M1234 variant, which does not bind PS (34), was coupled to maleimide-activated biotin similarly and served as a negative control. In addition, biotin was coupled to Gd-DTPA as described by Langereis et al. (35), and biotinylated Gd-wedge was synthesized similar to a method described previously (36).

Functionalized quantum dots (AnxA5-QDs) were prepared in a 1:1 stoichiometry by mixing equimolar amounts of streptavidin-coated QDs (stock concentration 1 μ M, Invitrogen, Eugene, OR, with emission peaks at either 525 or 585 nm) with biotinylated AnxA5 in binding buffer (10 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂ and 1 mg/mL Bovine Serum Albumin). The AnxA5-functionalized QDs were incubated with a minor excess of Gd-DTPA or Gd-wedge in order to saturate the unoccupied biotin binding sites of streptavidin. This procedure yields complexes with average stoichiometries of QD:AnxA5:Gd of 1:1:30 for AnxA5-QD-Gd and 1:1:240 for AnxA5-QD-Gd-wedge. These stoichiometries are derived from the information supplied by Invitrogen that each QD is coated with 8-12 streptavidin molecules.

Cryo-transmission electron microscopy

Specimen preparation for cryo-EM was done according to established procedures (37). Briefly, a thin film was prepared from the sample in the Vitrobot™, an automated vitrification robot with temperature and humidity control (patented by the University of Maastricht; patent licensed to FEI company, Hillsboro, OR/USA). The thin film was formed by applying 3 μ l of the sample/suspension to a Quantifoil grid in the chamber of the Vitrobot at 22°C and at a relative humidity >97%. Prior to sample application, the Quantifoil grid (R 2/2, Quantifoil GmbH, Jena/BRD) was glow-discharged to make the surface hydrophilic. Excess liquid was blotted away (one blot for one second) and the thin film thus formed was shot into melting ethane. The grids with vitrified thin films were transferred to the microscope using a Gatan 626 cryo-transfer/cryo-holder system (Gatan, Pleasanton, CA, USA). Micrographs were taken with the specimen at -170°C, using low dose conditions in a CM12 transmission microscope (Philips, Eindhoven, The Netherlands) operating at 120 kV.

***In vitro* cell experiments**

Jurkat cells (ATCC, Middlesex, UK) were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Gibco BRL), supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin and 10% heat-inactivated fetal bovine serum, and incubated in a humidified atmosphere at 37 °C and 5% CO₂. Cells were split every two or three days to 10⁵/mL. For experiments, cells were concentrated to 10⁶/mL. Apoptosis was induced by adding anti-Fas (anti-human antibody, clone 7C11, Immunotech, Marseille, France) to the culture medium at a final concentration of 200 ng/mL, and incubated in a humidified atmosphere at 37°C and 5% CO₂ for three hours. Next, cells were washed with binding buffer, and stained, as indicated, with either of the two nanoparticles at a concentration of 40 nM for 10 minutes. Afterwards, cells were washed again with binding buffer and imaged with TPLSM or MRI.

For TPLSM experiments, cells were also incubated with a nucleic acid stain, either 2 µM Syto41, a nucleic acid dye for viable and dead cells, (peak emission wavelength 450 nm, stock concentration 5 mM, Invitrogen, Eugene, OR/USA), or 2.5 µg/mL Propidium Iodide (peak emission wavelength 620 nm, stock 250 µg/mL, Molecular Probes, Leiden, the Netherlands) a nucleic acid dye for cells with a disrupted cellular membrane, i.e., dead cells. For internalization experiments, cells were incubated with 40 nM AnxA5-QD525-Gd, along with 200 ng/mL anti-Fas. After 5.5 hours cells were washed extensively in ethylene diamine tetra acetate (EDTA) to remove AnxA5 present on the outside of cells, and after washing with binding buffer, cells were stained with 40 nM AnxA5-QD585-Gd. Next, cells were washed with binding buffer and imaged with TPLSM using a standard Bio-Rad 2100MP (Hemel Hempstead, UK) in TPLSM mode. The excitation source was a Spectra Physics Tsunami Ti:Sapphire laser (Mountain View, CA/USA), tuned and mode-locked at 800 nm. The Tsunami laser produces light pulses of about 140 fs width at a repetition rate of 82 MHz. Laser light reached the sample through the Nikon water immersion lenses (60x water dipping, numerical aperture (NA) 1.0, working distance (WD) 2 mm or 60x water immersion, NA 1.2 and WD 0.22 mm), incorporated in an upright Nikon E600FN microscope (Tokyo, Japan). Further magnification was achieved by optical zoom. Three photomultipliers detected the fluorescence. Each photomultiplier accepted a different, tunable wavelength region, which were color-coded: blue (for Syto41, 420-470 nm); green (for QD525 and FITC 510-540 nm); and red (for QD585 570-600 nm, for PI 580 nm and higher, and for acridine red 590 nm and higher). The three obtained images were combined into a single image. Only combined images are shown. No additional image processing was performed. An imaging speed of 0.1 Hz with a pixel dwell time of 39 µs was used, while power at the sample was between 0.25 and 2.25 mW.

Magnetic resonance imaging was performed at room temperature with a 1.5T whole-body system (Intera, Philips Medical Systems, Best, the Netherlands) using an inversion recovery, turbo spin echo (IR-TSE) MR pulse and a 23 mm diameter surface radio-frequency coil (Philips Medical Systems, Best, the Netherlands). The imaging parameters for the single slice IR-TSE sequence were: repetition time (TR), 1580 ms; inversion time (TI), 546 ms; echo time (TE), 13 ms; field-of-view (FOV), 40x40 mm²; matrix size, 192x192; slice thickness, 1.5 mm; echo train length, 6; number of signal averages (NSA), 16. The obtained in-plane resolution was 208x208 μm^2 .

***In vitro* clot experiments**

Blood was collected with informed consent from healthy volunteers by venapuncture and anticoagulated in 0.1 volume of 129 mM trisodium citrate. For TPLSM, clots were incubated with nanoparticles after clot-formation. Clot formation was induced by adding 15 mM CaCl₂, and gentle mixing. Clots were allowed to form for one hour at 37°C in coagulation tubes (BD Vacutainer SST II Advance, BD Diagnostics, Plymouth, UK). After coagulation, clots were washed in binding buffer, and next incubated with 40 nM AnxA5-QD-Gd-wedge, prepared as described above, or with 500 nM AnxA5-FITC (NeXins Research BV, Kattendijke, the Netherlands), for one hour at 37°C. Clots were also stained with Acridine Red (AR), a cytoplasm stain, (Chroma-Gesellschaft, Köngen, Germany) or 2 μM Syto41. In one instance, whole blood was also pre-incubated with FITC-labeled anti-fibrinogen (anti-human antibody, WAK FA AF FITC-2, WAK Chemie Medical, Steinbach, Germany). Afterwards, clots were again washed in binding buffer and enclosed in 2% agarose gel for imaging.

For MRI, whole blood was incubated with 40 nM or 200 nM AnxA5-QD525-Gd-wedge prior to clot formation to label as many platelets as possible, because incubation of the clots after formation did not result in sufficient labeling for detection. To compensate for the anticoagulant effect of AnxA5, whole-blood was incubated with thrombin and collagen, triggering additional PS-exposure on platelets. Clot-formation was induced by adding 15 mM CaCl₂, 1 nM thrombin, and 20 $\mu\text{g/mL}$ collagen in the presence of either 40 or 200 nM AnxA5-QD-Gd-wedge. Clots were allowed to form for one hour at 37°C in coagulation tubes as described above. Clots were placed in 2% agarose gel without washing, to minimize the wash out of labeled platelets. Magnetic resonance imaging was performed at room temperature at 1.5T using a 47 mm diameter surface radio-frequency coil (Philips Medical Systems, Best, the Netherlands) and slightly different parameters for the IR-TSE sequence: TR, 1500 ms; TI, 610 ms; TE, 13 ms; FOV, 40x40 mm²; matrix size, 192x192; slice thickness, 1.5 mm; echo train length, 6; NSA, 32. The obtained in-plane resolution was 208x208 μm^2 .

Ex vivo experiment

An 18-week old male Swiss mouse was anesthetized by pentobarbital (110 mg/kg i.p.). The carotid artery was exposed while keeping it moist with saline and ligated at three sites: one proximal to the bifurcation of the artery and two distal to the bifurcation (one on each branch of the carotid artery). A flexible wire of 0.53 mm diameter was inserted in the common carotid artery via the external carotid artery and traversed three times to remove or damage the endothelium. Next, the blood flow was restored in the common carotid and internal branch; the external branch was tied off proximally of the incision with a fourth ligation (38). 45 minutes after injury the mouse was sacrificed by dissection of the diaphragm and cardiac perfusion with 5 mL of binding buffer in order to remove blood from the vasculature via liver-puncture. Then the carotid arteries were carefully excised, and connective and adipose tissue were removed. Next, the arteries were mounted into a home-built perfusion chamber (39) (IDEE BV, Maastricht, the Netherlands) in Hanks Balanced Salt Solution (HBSS, pH 7.4), containing 144 mM NaCl, 14.9 mM HEPES, 5.5 mM glucose, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, and 1.2 mM MgSO₄. The artery was slowly perfused with 200 nM AnxA5-QD-Gd-wedge (500 μ L in 30 minutes) using a microinjection pump (Predicor Infors, Basel, Switzerland). To stain cell nuclei and elastin in the vascular wall, the arteries were perfused with 500 μ L HBSS containing syto41 (final concentration 2 μ M) and eosin (final concentration 0.5 μ M) in a similar way, while simultaneously removing unbound nanoparticles. A static transmural pressure of 40 mmHg was applied (using a modified Big Ben sphygmomanometer, Riester, Germany), and the arteries were imaged with TPLSM. Afterwards, the arteries were removed from the perfusion chamber, embedded in 2% agarose gel, and imaged with MRI using a Bruker Biospec 7.0T scanner (Bruker Biospin GmbH, Ettlingen, Germany). Carotid images were obtained using a 35 mm diameter quadrature coil (Bruker Biospin GmbH, Ettlingen, Germany) and a multi-slice spin echo sequence with the following parameters: TR, 800 ms; TE, 10.2 ms; FOV, 27.7x27.7 mm²; matrix size, 312x312; slice thickness, 0.5 mm; NSA, 125. The obtained in-plane resolution was 89x89 μ m².

MRI data analysis

In all MR images, regions of interest (ROIs) were drawn, and mean values and standard deviations were calculated using JiveX (version 4.1, VISUS Technology Transfer GmbH, Bochum, Germany) or Para-vision 4.0 (Bruker Biospin GmbH, Ettlingen, Germany). ROIs were placed in the center of the Jurkat cell samples and whole-blood clots. For the analysis of the carotid arteries ROIs were placed over the entire vessel wall of the carotid arteries, and divided into quadrants to allow analysis and comparison of different regions of the vascular wall.

Results

The first version of the nanoparticle consisted of biotinylated AnxA5 coupled to streptavidin coated QDs in a 1:1 stoichiometry. QDs had emission peaks of either 525 nm (QD525, green) or 585 nm (QD585, red). AnxA5 contained a single biotin, located apical to the PS binding sites. The remaining binding sites of streptavidin were saturated with biotinylated Gadolinium-DTPA (35), an MRI contrast-agent (biotinylated Gd-DTPA, figure 5.1A; AnxA5-QD-Gd, figure 5.2A).

To increase the perceptibility of the nanoparticle in MRI, it was aimed to increase the load of Gd-DTPA. Therefore, a biotinylated construct was designed consisting of a lysine-wedge with eight Gd-DTPA complexes attached to the periphery (36) (Gd-wedge, figure 5.1B). Addition of this wedge to the described QDs resulted in the nanoparticle represented in figure 5.2B (AnxA5-QD-Gd-wedge).

Low temperature transmission electron microscopy (cryo-TEM, figure 5.3) showed that functionalized QDs in suspension were spherical, monodisperse, and non-aggregated. The spheric diameter was measured to be 6.7 ± 1.0 nm (mean $\pm$ standard deviation (SD)), $n = 124$. AnxA5 and Gd-DTPA are not visible on the EM image; however, a gap of at least 1 nm between any two adjacent QDs indicates the presence of molecules on the surface of QDs. These results are similar to results in literature (40).

In MRI, Gd-DTPA accelerates longitudinal relaxation (T_1 -relaxation) which can be quantified by its longitudinal relaxivity (r_1) expressed in $\text{mM}^{-1}\text{s}^{-1}$. The r_1 -values of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge were estimated based on the r_1 per Gd-DTPA complex, which were subsequently multiplied by the number of Gd-DTPA complexes per nanoparticle. For biotinylated Gd-DTPA coupled to avidin r_1 was $17.5 \text{ mM}^{-1}\text{s}^{-1}$ per Gd-DTPA (35), and for biotinylated Gd-wedge coupled to avidin r_1 was $15.6 \text{ mM}^{-1}\text{s}^{-1}$ per Gd-DTPA (36). Based on information supplied by the manufacturer (≈ 10 streptavidin molecules per QD), it can be calculated that AnxA5-QD-Gd and AnxA5-QD-Gd-wedge have r_1 -values per nanoparticle of 420-630 $\text{mM}^{-1}\text{s}^{-1}$ and 3000-4500 $\text{mM}^{-1}\text{s}^{-1}$, respectively.

AnxA5-QD-Gd and AnxA5-QD-Gd-wedge nanoparticles were examined on their property to discriminate between living and dying cells using TPLSM. Jurkat cells were triggered to execute apoptosis by activation of Fas receptor using anti-Fas antibodies. Binding of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge occurred to early and late apoptotic cells but not to viable cells (figure 5.4) in a manner comparable to Oregon Green labeled AnxA5 (not shown) (41). During early apoptosis, small patches of green fluorescence were visible on the cellular membrane (figure 5.4A, B), whereas during late apoptosis the entire cellular membrane was brightly fluorescent (figure 5.4C, D). Late apoptotic phase is characterized by loss of plasma membrane integrity and concomitant nuclear uptake of propidium iodide (PI), showing a red, fragmented nucleus. In addition,

AnxA5-QD-Gd was able to be internalized by Jurkat cells (figure 5.5), as was reported for AnxA5 (41). A movie, showing a z-stack of a cell, similar to the cell in figure 5.5, is also available at <http://pubs.acs.org>. Internalization of AnxA5-QD-Gd-wedge by Jurkat cells was not investigated in this study.

Figure 5.1: Molecular structure of the biotinylated Gd-DTPA (A) and biotinylated Gd-wedge (B) structures with biotin (red) and Gd-DTPA (blue). For full color figure see chapter 9.

Figure 5.2: Schematic representation of the two nanoparticles. QDs contain approximately 1 AnxA5 and 10 streptavidin molecules. A: the nanoparticle with single biotinylated Gd-DTPA (AnxA5-QD-Gd); B: the nanoparticle with the biotinylated Gd-wedge, containing eight Gd-DTPA complexes each (AnxA5-QD-Gd-wedge). Green: QD, yellow: streptavidin, red dot: Gd-DTPA, red star: lysine-wedge, blue: AnxA5. For full color figure see chapter 9.

Figure 5.3: Cryo transmission electron microscopy image of AnxA5-QD525-Gd. Bar = 10 nm.

Binding of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge to dying cells depends on cell surface exposure of PS, since QDs functionalized with M1234, a variant of AnxA5 that does not bind to PS (34), do not show binding to apoptotic cells (figure 5.6). All together, these findings clearly demonstrate that AnxA5 coupled to a QD retains its full biological property to bind to PS exposed on the surface of dying cells.

Having established the AnxA5-specific binding properties of AnxA5-QD-Gd and AnxA5-QD-Gd-wedge by fluorescence studies, the functional bimodality of the nanoparticle had to be confirmed. Apoptotic Jurkat cells were incubated with buffer (control 1), Gd-wedge (control 2), AnxA5-QD-Gd or AnxA5-QD-Gd-wedge and subsequently measured with MRI (figure 5.7). The amount of Gd-wedge in sample 2 (control) was equal to the amount used in sample 4.

Cells incubated with AnxA5-QD-Gd (figure 5.7, sample 3) exhibit a signal intensity that is a factor 5.9 and 6.9 higher as compared to control 1 and control 2, respectively. Cells incubated with AnxA5-QD-Gd-wedge (4) exhibit a signal intensity that is a factor 5.8 higher than 3, demonstrating that AnxA5-QD-Gd-wedge has a much better T_1 -lowering effect than AnxA5-QD-Gd, as was expected from the differences in r_1 . This results in a higher MRI signal intensity in T_1 -weighted imaging using the same amount of nanoparticles.

Cultured cells are simple *in vitro* systems for testing molecular imaging agents. Results derived from such systems hardly give reliable predictions about behavior in the *in vivo* situation. Therefore, a more complex system was chosen to assess the robustness of this nanoparticle as a bimodal molecular imaging agent. Since activated platelets expose PS, a human whole-blood clot system was chosen.

Figure 5.4: TPLSM images showing cellular AnxA5-QD525 (green) distribution. All cells were counterstained with PI (red) to determine membrane leakage, i.e., determine the stage of apoptosis. Top-row: early apoptotic Jurkat cells labeled with A; AnxA5-QD-Gd and B; AnxA5-QD-Gd-wedge. Bottom-row: late apoptotic cells labeled with C; AnxA5-QD-Gd and D; AnxA5-QD-Gd-wedge. Bar = 10 μ m. For full color figure see chapter 9.

Figure 5.5: TPLSM image of a Jurkat cell, incubated with AnxA5-QD525-Gd (green), while simultaneously inducing apoptosis by anti-Fas antibodies. After 5.5 hours cells were washed in EDTA buffer to remove AnxA5 present on the outside of the cells, and subsequently stained with AnxA5-QD585-Gd (red). Yellow indicates co-localization of QD525 and QD585 (probably caused by QD525 on the cell exterior that had not completely been removed). Cells were counterstained with Syto41 (blue), a nucleic acid stain. Bar = 10 μ m. For full color figure see chapter 9.

Figure 5.6: TPLSM images showing the cellular distributions of QD585 (red) coupled to AnxA5 or a mutant of AnxA5, unable to bind to PS (M1234), on apoptotic Jurkat cells. All cells were counterstained with Syto41 (blue), a nucleic acid stain. A: AnxA5-QD-Gd. B: AnxA5-QD-Gd-wedge. C: M1234-QD-Gd-wedge. Bar = 50 μ m. For full color figure see chapter 9.

Human whole blood was incubated with AnxA5-QD-Gd-wedge; subsequently PS exposure by platelets was induced to trigger clot-formation. The concentration of nanoparticles within the clot was expected to be much lower as compared to cell pellets. Within the clot only platelets expose PS, and not, for example, captured erythrocytes. An additional complexity of this system comes from the facts that AnxA5 competes with coagulation factors for binding to PS and acts as an

anticoagulant preventing platelets from being incorporated into the thrombus (11,42). Hence, MRI signal intensities were not expected to achieve the levels of figure 5.7. Therefore, following experiments were performed with AnxA5-QD-Gd-wedge only.

In order to label as many platelets as possible, whole blood was incubated with AnxA5-QD-Gd-wedge prior to clot-formation. Coagulation in whole blood, mediated by thrombin generation and PS exposure on platelets, was triggered by adding Ca^{2+} to citrate-anticoagulated whole blood. Additional PS exposure was triggered by pre-activating the platelets in whole blood with thrombin and collagen (11), to counteract the anticoagulant effect of AnxA5. Whole-blood was incubated with buffer (control 1), Gd-wedge (control 2), 40 nM or 200 nM AnxA5-QD-Gd-wedge; subsequently clot-formation was induced and clots were measured with MRI (figure 5.8). The amount of Gd-wedge in sample 2 (control) was equal to the amount used in sample 4.

Signal intensities of the four clots are shown in figure 8B. Corresponding T_1 -values for clots 1, 2, 3, and 4 are 734 ± 85 , 740 ± 44 , 678 ± 89 , 628 ± 30 ms (mean $\pm$ SD), respectively. The signal intensity of clot 4 was approximately 47% and 36% higher than controls 1 and 2, respectively. These results demonstrate that MRI of PS exposure in a more complex system is feasible using the AnxA5-QD-Gd-wedge nanoparticle. A recent study showed that with SPECT imaging mural thrombus renewal in abdominal aortic aneurysms *in vivo* can be visualized using AnxA5 labeled with a radionuclide (25). This study proved that thrombus imaging *in vivo* is feasible using AnxA5.

Whole-blood clots were also analyzed with TPLSM. For TPLSM, clots were incubated with AnxA5-QD-Gd-wedge after clot-formation, because in contrast to MRI, a lower amount of AnxA5-QD-Gd-wedge was sufficient for visualization by TPLSM. These experiments could thus be performed without interfering with clot-formation.

Figure 5.7: Sections of a single MR image, showing pellets of apoptotic Jurkat cells incubated with AnxA5-QD-Gd or AnxA5-QD-Gd-wedge. A: T_1 -weighted MR-images of the four cell samples. B: Graph showing mean $\pm$ SD for signal intensities of the pixels in the regions of interest (ROIs), drawn within the samples shown in A. Cells incubated with: 1, buffer only (control 1); 2, $1.6 \mu\text{M}$ Gd-wedge only (control 2); 3, 40 nM AnxA5-QD-Gd; 4, 40 nM AnxA5-QD-Gd-wedge.

Distribution of AnxA5-QD-Gd-wedge in the whole-blood clots was similar to the distribution of FITC-labeled AnxA5 (figure 5.9A). AnxA5-QD-Gd-wedge specifically labeled activated platelets (figure 5.9B, C), as can be deduced from the size of the labeled structures, measuring ca. 1-2 μm . Larger cells incorporated in the clot and easily discernible with a cytoplasm-specific or nucleic acid-specific dye, were stained neither with AnxA5-FITC nor AnxA5-QD-Gd-wedge (figure 5.9A, B). Figure 5.9C shows whole blood that was pre-incubated with anti-fibrinogen-FITC and subsequently activated to form a clot. Staining the clot with AnxA5-QD-Gd-wedge reveals that PS exposing platelets are aligned along the fibrin network within the clot.

To demonstrate the feasibility of this AnxA5-functionalized bimodal nanoparticle as a target-specific nanoparticle for cell death in the vascular wall, a murine carotid artery was mechanically injured *in vivo* by endothelial denudation using a metal wire as described elsewhere (38). This injury results in rapid PS exposure by cells of the tunica intima, media, and adventitia (38). Both damaged and undamaged carotid arteries were excised and mounted in a perfusion chamber. A static transmural pressure of 40 mmHg was applied, and the arteries were labeled intraluminally with AnxA5-QD-Gd-wedge by slow perfusion. The arteries were imaged by TPLSM as described in a recent article (39). Figure 5.10A shows uptake of AnxA5-QD-Gd-wedge in the endothelial cells (ECs) in the tunica intima and in smooth muscle cells (SMCs) in the tunica media. Apparently, nanoparticles are able to penetrate through elastic laminae deep into the tunica media. It is unclear why the first SMC layer hardly shows any uptake of the nanoparticle, while the layer of SMCs between the outer two elastic laminae does show abundant labeling. Furthermore, no labeled platelets were visible, as no blood was present at time of labeling. The undamaged control artery hardly shows any labeling. Next, the arteries were removed from the perfusion chamber, and embedded in agarose gel for imaging with MRI. The amount of labeling of the damaged artery is sufficient to increase the MRI signal intensity compared to the control artery (figure 5.10B). To determine the increase in signal intensity, three transversal MRI slices of the carotid arteries were divided into quadrants. In these quadrants, ROIs were drawn to select the vascular wall. This is because the uptake of nanoparticle was not homogeneous throughout the vascular wall, as it was unevenly damaged by the wire. A total of twelve signal intensities was acquired per artery. The mean signal intensity increased from 83.2 ± 3.6 for the control artery to 91.9 ± 13.5 for the damaged artery (arbitrary units, mean $\pm$ SD), and ranging from 73.5 to 86.9 for the control artery and 73.4 to 125 for the damaged artery. This indicates that the increase in signal intensity differs per location in the vascular wall and ranges from 0 to 50% compared to the control, undamaged artery.

Table 5.1: Comparison of five different bimodal AnxA5-conjugated nanoparticles. NIRF = near infrared fluorescence

Nanoparticle	T ₁ or T ₂ -lowering	Size (nm)	r ₁	r ₂	stoichiometry AnxA5:particle	Optical
QD-AnxA5-Gd-wedge ^a	T1	~7	3000-4500 (mM ⁻¹ particle)s ⁻¹	5600-8400 (mM ⁻¹ particle)s ⁻¹	1:1	Visible, NIRF
Micellar QD-AnxA5 ^b	T1	< 10	~1860 (mM ⁻¹ particle)s ⁻¹	~2700 (mM ⁻¹ particle)s ⁻¹	>5:1 ^e	Visible, NIRF
AnxA5-fluorescein liposomes ^c	T1	100	~164,000 (mM ⁻¹ particle)s ⁻¹	~272,000 (mM ⁻¹ particle)s ⁻¹	>5:1 ^e	Visible
AnxA5-CLIO-Cy5.5 ^d	T2	50	19 (mM ⁻¹ Fe)s ⁻¹	48 (mM ⁻¹ Fe)s ⁻¹	3.5:1	NIRF
AnxA5-fluorescein micellar iron oxide ^c	T2	10	13.3 (mM ⁻¹ Fe)s ⁻¹	159.6 (mM ⁻¹ Fe)s ⁻¹	>5:1 ^e	Visible

^a this paper; ^b van Tilborg et al. (45); ^c van Tilborg et al. (20); ^d Schellenberger et al. (19); ^e personal communication.

Figure 5.8: A: T₁-weighted sections of a single MR image, showing whole-blood clots, activated with Ca²⁺, thrombin and collagen, and incubated with AnxA5-QD-Gd-wedge enclosed in 2% agarose gel. B: graph showing mean $\pm$ SD for the signal intensities of the pixels in ROIs, drawn within the samples shown in A. Clots were incubated with: 1, buffer only (control 1); 2, 8 μ M Gd-wedge only (control 2); 3, 40 nM AnxA5-QD-Gd-wedge; 4, 200 nM AnxA5-QD-Gd-wedge. Clot 3 was slightly damaged, causing the black spot in the clot. Clot 4 was larger probably due to the fact that the higher concentration of AnxA5 inhibited clot retraction through its anticoagulant action.

Figure 5.9: TPLSM images of whole-blood clots incubated with AnxA5 after clot-formation. A; green: AnxA5-FITC, blue: syto41, a nucleic acid stain; B; green: AnxA5-QD525-Gd, red: acridine red, a cytoplasmatic stain. C; red: AnxA5-QD585-Gd, green: anti-fibrinogen-FITC. Platelets show a bright cellular membrane, similar to apoptotic cells (compare to figures 5.4/5.5/5.6). Bar = 50 μ m. For full color figure see chapter 9.

Discussion

Reports about QD-labeled AnxA5 have been published recently. As mentioned before, Le Gac et al. (28) investigated photo-stability of streptavidin-coated, AnxA5-labeled QDs. In contrast to the biotinylated AnxA5 used in this study, the described AnxA5 contained three to four biotins, located at random sites on the AnxA5 polypeptide. This implies, as pointed out by the authors, that AnxA5 coupling to QDs may lead to masking of PS binding sites and aggregation of the QDs. The current study utilized cys2-AnxA5 which contains a single biotin attached to the N-terminal tail that is located opposite to the side of the AnxA5 molecule harboring the PS binding sites (43). Coupling of cys2-AnxA5 to streptavidin coated QDs neither induced aggregation nor caused a loss of biological activity. In addition, a recent study (44) showed that cells, labeled with AnxA5-QDs, can be imaged using an *in vivo* imaging system.

Another study investigated bimodal, paramagnetic micellar QDs that were functionalized with AnxA5 (45). Table 5.1 summarizes the most important characteristics of AnxA5-functionalized bimodal nanoparticles published in literature. Two other T_1 -weighted nanoparticles, loaded with Gd-DTPA, are compared with the novel nanoparticle described in the current study. Furthermore, two T_2 -weighted nanoparticles, containing iron oxide, are listed. AnxA5-QD-Gd-wedge has several advantages. First, it is the smallest nanoparticle so far. This can be advantageous when the imaging target is outside the vessel lumen, as in, for example, atherosclerotic plaques or tumors. Furthermore, of the T_1 -weighted nanoparticles, AnxA5-QD-Gd-wedge is the nanoparticle with the highest relaxivity relative to the nanoparticle surface area. AnxA5-QD-Gd-wedge allows visible and near-infrared imaging and shows high fluorescence intensities. Additionally, QDs can be localized by electron microscopy (40). Moreover, the 1:1 stoichiometry of the AnxA5-QD complex provides the most optimal configuration for maximizing the amount of bimodal nanoparticle bound to the PS exposing surface. Other advantages of AnxA5-QD-Gd-wedge are straightforward preparation (add and mix), and variability of the nanoparticle's biological functionality. By replacing AnxA5 by a different biotinylated targeting function this nanoparticle can be used for molecular imaging of different biological processes (46).

The results in this paper demonstrate that the bimodal AnxA5-QD-Gd-wedge nanoparticle can be used to analyze biological samples as well as vascular structures with MRI at the anatomical level and with TPLSM at the cellular level. AnxA5-QD-Gd-wedge has high potential for *in vivo* imaging in the near future.

Figure 5.10: A; TPLSM image of a wire-injured murine carotid artery, showing the various vascular wall layers. The tunica intima consists of endothelial cells (ECs); the tunica media mainly consists of smooth muscle cells (SMCs). Uptake of nanoparticles is visible in ECs and SMCs. Green: AnxA5-QD-Gd-wedge; red: eosin, labeling elastin laminae; blue, syto41, labeling cell nuclei. L: lumen; IEL: internal elastic lamina; EEL: external elastic lamina. Bar = 10 μ m. The imaging plane was slightly oblique, as opposed to a longitudinal direction of the artery. B; transversal MR image of two murine carotid arteries: undamaged (control, left) and damaged (right, same artery as shown in A). Bar = 0.5 mm. For full color figure see chapter 9.

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References

- 1 G. Kroemer, et al.; "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death", *Cell Death Differ* 2005, 12, 1463-7.
- 2 L. Hofstra, et al.; "Visualisation of cell death *in vivo* in patients with acute myocardial infarction", *Lancet* 2000, 356(9225) 209-12.
- 3 J. Narula, et al.; "Apoptosis in myocytes in end-stage heart failure", *N Engl J Med* 1996, 335(16) 1182-9.
- 4 J. M. Isner, et al.; "Apoptosis in human atherosclerosis and restenosis", *Circulation* 1995, 91(11) 2703-11.
- 5 Y. J. Geng and P. Libby; "Evidence for apoptosis in advanced human atheroma. Colocalization with interleukin-1 beta-converting enzyme", *Am J Pathol* 1995, 147(2) 251-66.
- 6 G. I. Evan and K. H. Vousden; "Proliferation, cell cycle and apoptosis in cancer", *Nature* 2001, 411(6835) 342-8.
- 7 W. Hu and J. J. Kavanagh; "Anticancer therapy targeting the apoptotic pathway", *Lancet Oncol* 2003, 4(12) 721-9.
- 8 M. F. Corsten, et al.; "Counting heads in the war against cancer: defining the role of annexin A5 imaging in cancer treatment and surveillance", *Cancer Res* 2006, 66(3) 1255-60.
- 9 R. L. Haas, et al.; "*In vivo* imaging of radiation-induced apoptosis in follicular lymphoma patients", *Int J Radiat Oncol Biol Phys* 2004, 59(3) 782-7.
- 10 J. Savill and V. Fadok; "Corpse clearance defines the meaning of cell death", *Nature* 2000, 407(6805) 784-788.
- 11 P. Thiagarajan and J. F. Tait; "Binding of annexin V/placental anticoagulant protein I to platelets. Evidence for phosphatidylserine exposure in the procoagulant response of activated platelets", *J Biol Chem* 1990, 265(29) 17420-3.
- 12 I. C. Munnix, et al.; "The glycoprotein VI-phospholipase Cgamma2 signaling pathway controls thrombus formation induced by collagen and tissue factor *in vitro* and *in vivo*", *Arterioscler Thromb Vasc Biol* 2005, 25(12) 2673-8.
- 13 H. H. Boersma, et al.; "Past, present, and future of annexin a5: from protein discovery to clinical applications", *J Nucl Med* 2005, 46(12) 2035-50.
- 14 C. P. Reutelingsperger and W. L. van Heerde; "Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis", *Cell Mol Life Sci* 1997, 53(6) 527-32.
- 15 M. van Engeland, et al.; "Annexin V-affinity assay: a review on an apoptosis detection system based on phosphatidylserine exposure", *Cytometry* 1998, 31(1) 1-9.
- 16 S. M. van den Eijnde, et al.; "In situ detection of apoptosis during embryogenesis with annexin V: from whole mount to ultrastructure", *Cytometry* 1997, 29(4) 313-20.
- 17 C. P. Reutelingsperger, et al.; "Visualization of cell death *in vivo* with the annexin A5 imaging protocol", *J Immunol Methods* 2002, 265(1-2) 123-32.
- 18 J. Toretzky, et al.; "Preparation of F-18 labeled annexin V: a potential PET radiopharmaceutical for imaging cell death", *Nucl Med Biol* 2004, 31(6) 747-52.
- 19 E. A. Schellenberger, et al.; "Magneto/optical annexin V, a multimodal protein", *Bioconjug Chem* 2004, 15(5) 1062-7.
- 20 G. A. van Tilborg, et al.; "Annexin A5-functionalized bimodal lipid-based contrast agents for the detection of apoptosis", *Bioconjug Chem* 2006, 17(3) 741-9.
- 21 L. L. Johnson, et al.; "99mTc-annexin V imaging for *in vivo* detection of atherosclerotic lesions in porcine coronary arteries", *J Nucl Med* 2005, 46(7) 1186-93.
- 22 B. L. Kietselaer, et al.; "Noninvasive detection of plaque instability with use of radiolabeled annexin A5 in patients with carotid-artery atherosclerosis", *N Engl J Med* 2004, 350(14) 1472-3.
- 23 H. G. Keen, et al.; "Imaging apoptosis *in vivo* using 124I-annexin V and PET", *Nucl Med Biol* 2005, 32(4) 395-402.

- 24 F. G. Blankenberg, et al.; "*In vivo* detection and imaging of phosphatidylserine expression during programmed cell death", *Proc Natl Acad Sci U S A* 1998, 95(11) 6349-54.
- 25 L. Sarda-Mantel, et al.; "99mTc-Annexin-V Functional Imaging of Luminal Thrombus Activity in Abdominal Aortic Aneurysms", *Arterioscler Thromb Vasc Biol* 2006, 26(9) 2153-9.
- 26 R. J. J. H. M. Miserus, et al.; "Development and validation of novel imaging technologies to assist translational studies in atherosclerosis", *Drug Disc. Today: Techn.* 2006, 3(2) 195-204.
- 27 A. Watson, et al.; "Lighting up cells with quantum dots", *Biotechniques* 2003, 34(2) 296-300, 302-3.
- 28 S. LeGac, et al.; "Quantum Dots Based Probes Conjugated to Annexin V for Photostable Apoptosis Detection and Imaging", *Nano Lett.* 2006, 6, 1863-9
- 29 R. Hardman; "A toxicologic review of quantum dots: toxicity depends on physicochemical and environmental factors", *Environ Health Perspect* 2006, 114(2) 165-72.
- 30 B. Ballou, et al.; "Noninvasive imaging of quantum dots in mice", *Bioconjug Chem* 2004, 15(1) 79-86.
- 31 M. van Zandvoort, et al.; "Two-photon microscopy for imaging of the (atherosclerotic) vascular wall: a proof of concept study", *J Vasc Res* 2004, 41(1) 54-63.
- 32 W. Denk, et al.; "Two-photon laser scanning fluorescence microscopy", *Science* 1990, 248(4951) 73-6.
- 33 R. Huber, et al.; "Crystal and molecular structure of human annexin V after refinement. Implications for structure, membrane binding and ion channel formation of the annexin family of proteins", *J Mol Biol* 1992, 223(3) 683-704.
- 34 J. P. Mira, et al.; "Inhibition of cytosolic phospholipase A2 by annexin V in differentiated permeabilized HL-60 cells. Evidence of crucial importance of domain I type II Ca²⁺-binding site in the mechanism of inhibition", *J Biol Chem* 1997, 272(16) 10474-82.
- 35 S. Langereis, et al.; "Probing the interaction of the biotin-avidin complex with the relaxivity of biotinylated Gd-DTPA", *Org Biomol Chem* 2004, 2(9) 1271-3.
- 36 A. Dirksen, et al.; "Strategy for the synthesis of multivalent peptide-based nonsymmetric dendrimers by native chemical ligation", *Chem Commun (Camb)* 2006, 15) 1667-9.
- 37 P. M. Frederik and D. H. Hubert; "Cryoelectron microscopy of liposomes", *Methods Enzymol* 2005, 391, 431-48.
- 38 S. Ravassa, et al.; "Annexin A5 Down-regulates Surface Expression of Tissue Factor: A novel mechanism of regulating the membrane receptor receptor", *J Biol Chem* 2005, 280(7) 6028-35.
- 39 R. T. A. Megens, et al.; "Two-Photon microscopy of vital murine elastic and muscular arteries. Combined structural and functional imaging with subcellular resolution", *J Vasc Res* 2007, 44, 87-98.
- 40 B. N. Giepmans, et al.; "Correlated light and electron microscopic imaging of multiple endogenous proteins using Quantum dots", *Nat Methods* 2005, 2(10) 743-9.
- 41 H. Kenis, et al.; "Cell surface-expressed phosphatidylserine and annexin A5 open a novel portal of cell entry", *J Biol Chem* 2004, 279(50) 52623-9.
- 42 S. Ramstrom, et al.; "Platelet phosphatidylserine exposure and procoagulant activity in clotting whole blood--different effects of collagen, TRAP and calcium ionophore A23187", *Thromb Haemost* 2003, 89(1) 132-41.
- 43 R. Huber, et al.; "The calcium binding sites in human annexin V by crystal structure analysis at 2.0 Å resolution. Implications for membrane binding and calcium channel activity", *FEBS Lett* 1990, 275(1-2) 15-21.
- 44 D. T. Dicker, et al.; "Heterogeneity in non-invasive detection of apoptosis among human tumor cell lines using annexin-V tagged with EGFP or Qdot-705", *Cancer Biol Ther* 2005, 4(9) 1014-7.
- 45 G. A. van Tilborg, et al.; "Annexin A5-Conjugated Quantum Dots with a Paramagnetic Lipidic Coating for the Multimodal Detection of Apoptotic Cells", *Bioconjug Chem* 2006, 17(4) 865-868.
- 46 A. Buehler, et al.; "cNGR: A Novel Homing Sequence for CD13/APN Targeted Molecular Imaging of Murine Cardiac Angiogenesis *In vivo*", *Arterioscler Thromb Vasc Biol* 2006, 26(12) 2681-7.

CHAPTER 6

Molecular MRI of early thrombus formation using a bimodal α_2 -antiplasmin-based contrast agent

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Abstract

Objectives: To investigate whether early thrombus formation can be visualized with *in vivo* MRI using a novel bimodal α_2 -antiplasmin-based contrast agent (CA).

Background: Thrombus formation plays a central role in several vascular diseases and non-invasive detection of early thrombus formation is a major challenge in clinical practice. During the early phases of thrombus formation, activated factor XIII (FXIIIa) covalently cross-links α_2 -antiplasmin to fibrin, indicating the potential of α_2 -antiplasmin-based CAs in detecting early thrombus formation.

Methods: A bimodal CA was synthesized by coupling gadolinium-DTPA and rhodamine to an α_2 -antiplasmin-based peptide. For the control CA, a glutamine residue essential for cross-linking was replaced by alanine. *In vitro* generated thrombi were exposed to both CAs and imaged by MRI and two-photon laser-scanning microscopy (TPLSM). Immunohistochemistry was performed on human pulmonary thrombo-emboli sections to determine α_2 -antiplasmin and FXIII presence in different thrombus remodeling phases. *In vivo* feasibility of the CA in detecting early thrombus formation specifically, was investigated with MRI.

Results: *In vitro* generated thrombi exposed to the α_2 -antiplasmin-based CA showed hyperintense MR signal intensities at the thrombus edge. No hyperintense signal was observed using the α_2 -antiplasmin-based CA in the presence of FXIII inhibitor dansylcadaverine, nor when using the control CA. TPLSM demonstrated that the α_2 -antiplasmin-based CA bound to fibrin. Immunohistochemistry demonstrated substantial α_2 -antiplasmin staining in fresh compared to lytic and organized thrombi. *In vivo* CA administration within seconds after inducing thrombus formation increased contrast-to-noise ratios (CNR: 2.28 ± 0.39 , $N=6$) at the site of thrombus formation compared to the control CA (CNR: -0.14 ± 0.55 , $P=0.003$, $N=6$) and α_2 -antiplasmin-based CA administration 24-48 hours after thrombus formation (CNR: 0.11 ± 0.23 , $P=0.006$, $N=6$).

Conclusions: A novel bimodal CA was developed, characterized, and validated. Our results showed that this bimodal CA enabled non-invasive *in vivo* MR visualization of early thrombus formation.

Introduction

Thrombotic complications such as myocardial infarction, stroke, deep venous thrombosis, and pulmonary thrombo-embolism, are major causes of morbidity and mortality (1,2). The resistance of thrombi to fibrinolytic therapy increases with thrombus age (3), and fibrinolytic agents can induce severe complications such as bleeding (4,5). This indicates the necessity to accurately diagnose early thrombus formation as it may improve selection of patients that will benefit from fibrinolytic therapy. Currently, non-invasive detection of early thrombus formation is a major problem in clinical practice. Several specific contrast agents (CAs) have been developed to visualize thrombi using molecular imaging techniques (6-8). Fibrin has frequently been used as a target for *in vivo* thrombus visualization using various MRI CAs, such as fibrin-targeted perfluorocarbon nanoparticles (9), and fibrin-specific peptide-based CAs (10-17). Recently, initial results of a human study using a fibrin-specific peptide-based CA (EP-2104R) were published, suggesting selective molecular MR imaging of thrombi (18). Nonetheless, these CAs have low sensitivity for the estimation of thrombus age.

In this study, we searched for other coagulation factors that could be used as a target for thrombus imaging. We focused on activated factor XIII (FXIIIa) as it cross-links fibrin chains and covalently cross-links α_2 -antiplasmin (α_2 -AP) to fibrin (19). In this latter process, the glutamine (Gln₂) substrate in the N-terminal domain of α_2 -AP is the primary substrate site for FXIIIa (20). Robinson et al. (21) have shown that the catalytic ability of FXIIIa in cross-linking α_2 -AP into formed thrombi declines with a short half-life. Therefore, an α_2 -AP-based CA might enable specific visualization of early thrombus formation.

Recently, a near-infrared fluorescent (NIRF) probe (A15) and an MRI CA (A14), both based on α_2 -AP, enabled visualization of *in vitro* formed thrombi with NIRF and *ex vivo* MRI (22). Subsequently, *in vivo* detection of FXIII activity was achieved by intravital fluorescence microscopy using the A15 probe (23,24). However, translation of this probe to human studies in the near future is unlikely due to the limited penetration depth of NIRF microscopy. Non-invasive thrombus visualization using imaging modalities such as MRI, may overcome this limitation.

In the present study, we analyzed the presence of FXIII and α_2 -AP in fresh, lytic and organized human pulmonary thrombo-emboli using immunohistochemistry. Subsequently, we investigated whether early thrombus formation can be visualized *in vitro* and *in vivo* with MRI using a bimodal α_2 -AP-based CA. Additionally, TPLSM was used to investigate whether the α_2 -AP-based CA co-localizes with the fibrin polymers.

Materials and Methods

Synthesis of the CAs

An α_2 -AP-based peptide sequence (GNQEQVSPLTLL) that binds covalently to fibrin (21,23) was synthesized using tertbutyloxycarbonyl (tBoc) solid-phase peptide synthesis (SPPS) (25). The peptide was bimodally labeled with rhodamine and a DTPA-chelate (Bi- α_2 AP-CA) by cross-linking maleimide-DTPA (26) and succinimidyl-rhodamine (Invitrogen, Molecular Probes, Breda, The Netherlands) to an additional C-terminal KW dipeptide, branched at the lysine ϵ -amino group with a cysteine. A control CA (Bi-con-CA) was obtained by replacing a glutamine residue essential for cross-linking by alanine ($Q^3 \rightarrow A^3$) to prevent binding to fibrin (figure 6.1). Bi- α_2 AP-CA and Bi-con-CA were characterized by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). Gadolinium chloride ($GdCl_3$) was added in a 0.9:1 molar ratio after which no MALDI spectrum was observed due to poor ionization.

Contrast agent validation

For r_1 relaxivity measurements, the gadolinium-labeled peptides were dissolved (concentrations ranging from 25-150 μ M) in phosphate buffered saline solution (PBS). Solutions were imaged at room temperature with an 1.5 Tesla MR-scanner (MR Intera, Philips Healthcare, Best, The Netherlands) using a commercially available head coil and a 7.0 Tesla Bruker Biospec scanner (Bruker Biospin GmbH, Ettlingen, Germany) using a 154 mm diameter quadrature transmit-receive radio-frequency coil. T_1 relaxation times were obtained using inversion recovery sequences with different inversion times (TI), ranging from 50-5000 ms. Additional parameters: repetition time (TR), 7500 ms; echo time (TE), 14 ms (1.5T) or 8.4 ms (7.0T); slice thickness, 3 mm; number of signal averages (NSA), 1; in-plane resolution, 0.55x0.55 mm. Gadolinium content was determined using inductively coupled plasma mass spectrometry (ICP-MS).

The effect of Bi- α_2 AP-CA on thrombus formation was determined with a thrombin generation assay using platelet poor plasma with different concentrations (0, 10, 20, and 40 μ M) of Bi- α_2 AP-CA.

In vitro thrombus imaging

Human blood was obtained by venous puncture from a healthy volunteer and collected in vacutainer tubes containing trisodium citrate (BD Vacutainer Systems, Preanalytical solutions, Plymouth, UK). Murine blood was obtained by right ventricle puncture. Human and murine thrombi were allowed to form during 90 minutes at 37°C in the presence of 30 μ l of 1.5 mg/ml Oregon Green 488-labeled

fibrinogen (Invitrogen, Molecular Probes, Breda, The Netherlands). Thereafter, thrombi were incubated with 150 μ M Bi- α_2 AP-CA or Bi-con-CA during 90 minutes at 37°C, followed by extensive washing with PBS. To determine the effect of FXIIIa on thrombus visualization, a human thrombus was formed and exposed to Bi- α_2 AP-CA in the presence of dansylcadaverine (final concentration 2 mM) (Fluka Chemie AG, Buchs, Switzerland), since dansylcadaverine is a competitive substrate for transglutaminases such as FXIII (27).

Visualization with TPLSM was performed as previously described (28). Briefly, a BioRad 2100MP (Hemel Hempstead, UK) was used in TPLSM mode. Fluorescence was detected by 3 photomultipliers. Filter settings: 420-470nm (blue), 510-540nm (green, fibrin network) and 570-590nm (red, rhodamine detection of the CAs).

After TPLSM and approximately 4 hours after CA incubation, thrombi were embedded in 2% agarose gel and imaged at 1.5T using a commercially available 47 mm diameter surface coil (Philips Healthcare, Best, The Netherlands). Images were acquired using a T_1 -weighted Inversion Recovery Turbo Spin Echo (IR-TSE) sequence with the following scan-parameters: TR/TE/TI, 1580/13/546 ms; echo train length, 6; NSA, 16; slice thickness, 1.5 mm; field of view (FOV), 40x40 mm; matrix size, 192x192.

Thrombus age classification

To assess whether FXIII and α_2 -AP can be seen as markers for early thrombus formation, tissue sections with pulmonary thrombo-emboli were evaluated by immunohistochemistry. Twenty-two paraffin-embedded blocks (15 from 8 autopsy patients and 7 from 3 lung biopsy tissues) containing pulmonary thrombo-emboli were used to analyze FXIII and α_2 -AP presence. All tissues were obtained from the Maastricht Pathology Tissue Collection (MPTC). Collection, storage, and use of tissue and patient data were performed in agreement with the "Code for Proper Secondary Use of Human Tissue in the Netherlands" (<http://www.fmwv.nl>).

Immunohistochemistry was performed using conventional methods. Briefly, mouse monoclonal anti-human FXIII alpha subunit (1:300; clone AC-1A1, Lab Vision, Fremont, CA), rabbit-anti human α_2 -AP polyclonal (1:200; Biogenesis-MorphoSys AG, Martinsried/Munich, Germany), and mouse monoclonal anti-human fibrin II beta chain (1:100; clone T2G1, Accurate Chemical, Westbury, NY) were used as primary antibodies. For the negative controls, no primary antibody was used. Visualization was achieved with vectastain red (alkaline phosphatase substrate kit I, Vector Laboratories, Burlingame, CA).

Thrombi were classified into fresh (<1 day; layered patterns of platelets, fibrin, erythrocytes, and intact granulocytes), lytic (1-5 days; areas of colligation necrosis and karyorrhexis of granulocytes), organizing thrombi (>5 days; ingrowth

of smooth muscle cells with or without deposition of connective tissue and capillary vessel ingrowth), or in thrombi containing more than one of these age classifications (29). Percentages of thrombi positive for FXIII, α_2 -AP, and fibrin staining were determined for the various thrombus age classifications. The degree of staining was scored for each thrombus age classification separately in no staining (score 0), slightly stained (score 1), moderately stained (score 2), and strongly stained (score 3) (by VH, intraobserver variability <10%).

Clearance and biodistribution

All animal experiments were approved by the local ethical review committee. Mice were anesthetized by inhalation of air with 2% isoflurane inhalation gas. Half-life of Bi- α_2 AP-CA (n=3) and Bi-con-CA (n=3) was determined by collecting blood samples before and at different time-points (1, 10, 15, 30, 60, and 90 minutes) after contrast administration (dose: 5.0 μ mol/kg body weight). Using TPLSM, mean fluorescence of the red channel was determined in all blood samples. Exponential fitting was used for half-life determination (GraphPad Prism 4.0, La Jolla, USA). After blood collection, mice were sacrificed using an excess of pentobarbital. Biodistribution of both CAs was determined with TPLSM by studying CA uptake in excised liver, spleen, kidney, heart, lung, and intestine. Filter settings: $\leq$ 500nm (blue), 500-560nm (green) and 560-610nm (red, rhodamine detection of the CAs).

***In vivo* thrombus imaging**

The right carotid artery was exposed by separating the sternocleidomastoid muscle from the trachea. Thrombus formation was induced by applying a strip of filter paper soaked in 10% ferric chloride (FeCl_3) on the carotid artery. After 5 minutes the filter paper was removed and the carotid artery was washed with PBS. Within seconds after FeCl_3 -induced thrombus formation, 5.0 μ mol/kg body weight Bi- α_2 AP-CA (n=6) or Bi-con-CA (n=6) was administered intravenously. In 6 other mice, Bi- α_2 AP-CA was administered 24-48 hours after FeCl_3 -induced thrombus formation.

MR imaging was performed at 7.0T using a 35 mm diameter quadrature transmit-receive radio-frequency coil (Bruker Biospin GmbH, Ettlingen, Germany). After scout scans, sagittal carotid MR images were obtained approximately 90 minutes after CA administration using a rapid acquisition with relaxation enhancement (RARE) inversion recovery pulse sequence. Parameters were: TR/TE/TI, 7500/8.9/1654 ms; 21-25 slices; slice thickness, 0.25 mm; interslice distance, 0.3-0.35 mm; NSA, 2; rare partitions, 4; FOV, 30x30 mm; matrix size, 312x312. Additionally, after Bi- α_2 AP-CA administration within seconds after thrombus formation, repeated MR measurements were performed (50, 65, 85, 100, 115, and 130 minutes, n=2).

For TPLSM, carotid arteries (n=4 for each group) were excised and mounted into a home-built perfusion chamber (IDEE BV, Maastricht, The Netherlands), immersed in Hanks Balanced Salt Solution, maintaining 60-80 mmHg transmural pressure (28). Syto 13 (Invitrogen, Eugene, OR) was added (final concentration: 2.5 μ M) to visualize nucleic acids. Filter settings: 420-470 nm (blue), 510-530 nm (green, nucleic acid staining) and 560-610 nm (red, rhodamine detection of the CAs).

MR contrast-to-noise ratios (CNRs) were calculated by dividing the difference between the mean MR SIs from a ROI positioned in the thrombus and that in neighboring muscle tissue by the noise measured in air. Noise values were corrected for magnitude effects by the Rayleigh factor. ROIs were drawn in ParaVision 4.0 (Bruker Biospin GmbH, Ettlingen, Germany).

Statistics

Data are presented as mean $\pm$ standard error. Statistical analyses were performed on the histology scores using the Mann-Whitney U test. For *in vivo* measurements the one-way ANOVA test was used. Due to Bonferroni correction for multiple group comparison, differences with a P-value less than 0.025 were considered significant.

Results

Synthesis of the CAs

MALDI-MS showed a molecular mass for Bi- α_2 AP-CA of 2832.9 g/mol, in agreement with the theoretical average mass of 2893.1 g/mol without Gd³⁺ bound (figure 6.1). The Bi-con-CA with a Q³ to A mutation had a molecular mass of 2775.4 g/mol, corresponding to the theoretical mass of 2776.1 g/mol without Gd³⁺ attached.

Contrast agent validation

The r_1 relaxivities of the Bi- α_2 AP-CA at 1.5T and 7.0T were 5.6 ± 0.4 and 3.0 ± 0.3 $\text{mM}^{-1}\text{s}^{-1}$, respectively. The r_1 relaxivities obtained for the Bi-con-CA were 6.5 ± 0.5 $\text{mM}^{-1}\text{s}^{-1}$ (1.5T) and 3.3 ± 0.2 $\text{mM}^{-1}\text{s}^{-1}$ (7.0T).

For all Bi- α_2 AP-CA concentrations (0, 10, 20, and 40 μ M) the endogenous thrombin potentials were comparable (708, 742, 733, and 717 nM·min, respectively). Additionally, no effects of Bi- α_2 AP-CA on thrombin peak heights or lag times of thrombin generation were observed. These results indicate that Bi- α_2 AP-CA did not interfere with thrombin generation and subsequent thrombus formation.

***In vitro* thrombus imaging**

TPLSM showed co-staining of the rhodamine-labeled Bi- α_2 AP-CA (red) and the OG488-labeled fibrin network (green) resulting in yellow areas of co-localization, indicating binding of Bi- α_2 AP-CA to fibrin in human and murine thrombi (figures 6.2B, H). In contrast, the control agent Bi-con-CA showed limited labeling and co-localization with the fibrin network (figures 6.2D, J). Hyperintense MR signals were found at the edge of thrombi incubated with Bi- α_2 AP-CA (figures 6.2A, G), which were absent using the Bi-con-CA (figures 6.2C, I). FXIIIa inhibition by dansylcadaverine decreased binding and co-localization of Bi- α_2 AP-CA with fibrin, thereby lowering the MR signal intensity at the edge of a human thrombus exposed to Bi- α_2 AP-CA (figures 2E-F). This finding confirms that FXIII presence is needed for Bi- α_2 AP-CA binding to fibrin.

IHC staining for FXIII and α_2 -AP on a human thrombus formed during three hours showed that FXIII was present throughout the thrombus (figure 6.3A), while α_2 -AP was strongly expressed at the edge (figure 6.3B). This is in line with the increased MR signal intensities at the edge of thrombi exposed to Bi- α_2 AP-CA.

Figure 6.1: Structure of the bimodal α_2 -antiplasmin-based contrast agent. Schematic representation of the α_2 -AP-based peptide that is bimodally labeled with rhodamine and a DTPA-chelate (Bi- α_2 AP-CA) and the corresponding mass spectra. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) shows a molecular mass of 2832.92 g/mol for the Bi- α_2 AP-CA. Substitution of only one amino acid ($Q^3 \rightarrow A^3$) results in a bimodal control CA (Bi-con-CA). For full color figure see chapter 9.

Thrombus age classification

We evaluated 44 thrombi for FXIII, 38 for α_2 -AP, and 47 for fibrin and categorized them in fresh, lytic, or organized. High percentages of positive fibrin staining were found in all categories, whereas positive α_2 -AP and FXIII staining was mainly present in fresh thrombi followed by lytic and organized thrombi. Additionally, mean α_2 -AP score was significantly higher in fresh thrombi compared to lytic and organized thrombi, and mean FXIII score was significantly higher in fresh compared to organized thrombi while no significant difference was found between fresh and lytic thrombi. Mean fibrin score was relatively high in all thrombus age classifications (table 6.1). Additionally, FXIII was expressed in inflammatory cells (mainly macrophages), representing the cellular component of FXIII. A typical example of a thrombus containing fresh and organized components is shown in figure 6.4.

Figure 6.2: In vitro results. TPLSM and MRI results for human (A-F) and murine (G-J) thrombi incubated with the bimodal α_2 -AP-based contrast agent (Bi- α_2 AP-CA) (A-B, E-H) and the bimodal control CA (Bi-con-CA) (C-D, I-J). For TPLSM, red color originated from the rhodamine-labeled CAs. Green color indicated the fibrin network due to use of fibrinogen-Oregon green 488. Yellow color illustrated co-localization of red and green. Hyperintense MR signals were found at the edge of thrombi incubated with Bi- α_2 AP-CA (A, G). Lower MR signal intensities were found at the edge of a thrombus exposed to dansylcadaverine and Bi- α_2 AP-CA (E). For full color figure see chapter 9.

Figure 6.3: Immunohistochemical stainings of thrombi. A) Immunohistochemical staining for factor XIII. B) Immunohistochemical staining for α_2 -antiplasmin. Positive immunohistochemical staining is depicted in red/pink color. The immunohistochemical staining for factor XIII (scanty spots) was present throughout the whole thrombus while the immunohistochemical α_2 -antiplasmin staining was mainly depicted at the edge of the thrombus (arrows). Bars: 50 μ m. For full color figure see chapter 9.

Clearance and biodistribution

Both CAs were rapidly cleared from the blood. Circulation half-lives of Bi- α_2 AP-CA and Bi-con-CA were 14.1 ± 8.4 and 16.3 ± 4.0 minutes, respectively. TPLSM on excised organs showed that both CAs were mainly cleared through the kidneys, while limited CA was found in other organs (figure 6.5), indicating that there is no or limited accumulation of the CAs on organ level.

In vivo thrombus imaging

Clear hyperintense signals from early thrombus formation were observed with *in vivo* MRI (CNR: 2.29 ± 0.39) when Bi- α_2 AP-CA was administered immediately after FeCl_3 -induced thrombus formation. No hyperintense MR signals were found using the control agent (Bi-con-CA) for fresh thrombus visualization (CNR: -0.14 ± 0.55 , $P=0.003$) nor when using Bi- α_2 AP-CA for the visualization of 24-48 hours old thrombi (CNR: 0.11 ± 0.23 , $P=0.006$) (figures 6.6A-D). Additionally, CNR-ratios as a function of time after Bi- α_2 AP-CA administration (50, 65, 85, 100, 115, and 130 minutes) were comparable (2.65 ± 0.11 , 2.43 ± 0.06 , 2.51 ± 0.15 , 2.43 ± 0.37 , 2.24 ± 0.21 , 2.01 ± 0.44 , respectively).

TPLSM of excised carotid arteries confirmed Bi- α_2 AP-CA presence in the thrombi formed in the lumen of the FeCl_3 -treated artery when Bi- α_2 AP-CA was administered within seconds after thrombus formation (figure 6.6E). Limited fluorescence was found using Bi-con-CA (figure 6.6F) or when Bi- α_2 AP-CA was administered 24-48 hours after FeCl_3 -induced thrombus formation (figure 6.6G). Occasionally, fluorescence was found in the medial layer (predominantly smooth muscle cells) of vessels that were treated with FeCl_3 24-48 hours prior to imaging. No fluorescence was detected in the non-treated contralateral carotid arteries. Histology confirmed presence of thrombi in all FeCl_3 -treated carotid arteries.

Table 6.1: Percentages and mean scores of α_2 -antiplasmin, factor XIII, and fibrin stainings in lung emboli tissue sections.

Thrombus age	α_2 -antiplasmin			factor XIII			fibrin	
	Staining	Score $\pm$ s.e.		Staining	Score $\pm$ s.e.		Staining	Score $\pm$ s.e.
Fresh (<1 day)	95%	1.4 $\pm$ 0.1	*]	92%	1.5 $\pm$ 0.1	*]	100%	2.1 $\pm$ 0.1
Lytic (1-5 days)	29%	0.3 $\pm$ 0.2		75%	0.8 $\pm$ 0.3		83%	2.0 $\pm$ 0.4
Organized (>5 days)	20%	0.2 $\pm$ 0.1		31%	0.3 $\pm$ 0.1		92%	1.9 $\pm$ 0.2

*Significantly different, s.e. indicates standard error

Figure 6.4: Pulmonary thrombo-emboli sections. Middle panels: overview of thrombo-emboli, from top to bottom: Hemathoxyline/Eosin stained, immunohistochemically stained for fibrin, factor XIII, and α_2 -antiplasmin. Bars: 300 μ m. Left panels: enlargements of boxed areas indicating an organized part of the thrombo-emboli. In the enlarged FXIII stained area clear cellular FXIII staining is visible. Bars: 50 μ m. Right panels: enlargements of boxed areas indicating a fresh segment (arrows). Bars: 50 μ m. For full color figure see chapter 9.

Figure 6.5: Clearance and kidney uptake of the contrast agents. Relative fluorescence measured in blood samples decreases with time after contrast administration (A). Half-life of the CAs are 14.1 ± 8.4 minutes for the bimodal α_2 -antiplasmin-based CA (Bi- α_2 -AP-CA) and 16.3 ± 4.0 minutes for the bimodal control CA (Bi-con-CA). Both CAs are mainly cleared through the kidney (B-C). Panels B-C: Red: Bimodal CA in kidney tubuli. For full color figure see chapter 9.

Discussion

Since α_2 -AP covalently crosslinks to fibrin during the early phases of thrombus formation, α_2 -AP-based CAs might enable non-invasive detection of early thrombus formation. We demonstrated that the α_2 -AP-based CA enabled MR imaging and TPLSM of *in vitro* generated thrombi and that FXIII presence is required for binding of the α_2 -AP-based CA to fibrin. Additionally, we showed that α_2 -AP and FXIII are predominantly present in fresh compared to lytic and organized human pulmonary thrombo-emboli, indicating the potential of α_2 -AP-based CAs in detecting early thrombus formation. Subsequently, we demonstrated that the α_2 -AP-based CA enabled *in vivo* visualization of early thrombus formation with MRI. TPLSM confirmed specific binding of the α_2 -AP-based CA to fibrin.

Previously, α_2 -AP-based peptides labeled with either Alexa680 (A15) or gadolinium (A14) were used *in vitro* for visualization with NIRF microscopy and MRI. Both probes were tested for their ability to visualize murine (22) and human thrombi (23). Additionally, it was shown that α_2 -AP-based peptides bind covalently to fibrin (21,23), indicating that the K_d -value approaches 0. These findings were confirmed by the present study since contrast addition 90 minutes after the initiation of thrombus formation resulted in hyperintense MR signal at the edge of human and murine blood-derived thrombi.

In previous murine studies, intravital fluorescence microscopy enabled visualization of carotid (23) and cerebral (24) thrombi using the A15 probe. This probe proved to be specific for the early phases of thrombus formation (23). Nevertheless, due to the limited penetration depth of NIRF microscopy it is unlikely that this probe can be used for human applications. Our α_2 -AP-based CA enabled *in vivo* visualization of early thrombus formation with non-invasive MRI. In our MRI

Figure 6.6: In vivo and ex vivo results. In vivo MRI (A-C) and ex vivo TPLSM results (E-G) of early thrombus formation and 24-48 hour old thrombi in murine carotid arteries after injection of the bimodal α_2 -antiplasmin-based CA (Bi- α_2 AP-CA) (A, C, E, G) and the bimodal control CA (Bi-con-CA) (B, F). Contrast-to-noise ratios (CNR) are elevated in the early phases of thrombus formation after Bi- α_2 AP-CA administration (D). Panels E-G: Red: Bimodal CA. Green: Nuclear stain. Lumen is located between dotted lines. Blue arrows indicate leukocytes trapped in the thrombus. Elongated cells are smooth muscle cells (white arrows) and endothelial cells (red arrow). For full color figure see chapter 9.

study a higher dose was used compared to the NIRF studies (23,24). However, the present dose (5.0 $\mu\text{mol/kg}$) is still low for MRI. Biological amplification due to the abundance of fibrin and r_1 relaxivity increase upon binding may explain the observed signal enhancement achieved with this low dose. Other fibrin-targeted imaging studies used comparable CA concentrations (10-18). Fibrin-targeted perfluorocarbon nanoparticles (9), EP-1242 (17), EP-1873 (16), and EP-2104R (10-15,18) proved their ability to visualize thrombi in different animal models with MRI. Recently, initial results showed that EP-2104R allows *in vivo* MR thrombus imaging in humans (18). Additionally, EP-2104R demonstrated a time-dependent thrombus enhancement, showing a slow decrease in CNR over a 8 weeks time period (12). However, since increased CNRs were observed in all thrombi compared with pre-contrast imaging, it is unlikely that this CA could effectively discriminate between the early stages of thrombus formation and organized thrombi. In the present study, we demonstrated that the α_2 -AP-based CA significantly increased CNRs during early thrombus formation compared with 24-48 hours old thrombi. Therefore, our bimodal α_2 -AP-based CA is promising for the differentiation between the early phases of thrombus formation and organized thrombi.

Immunohistochemistry showed presence of FXIII in pulmonary thromboemboli, but cellular FXIII staining was also observed. The cellular form of FXIII consists of a homodimer of A-subunits (19). We used a primary antibody addressed to the α -chain of FXIII, explaining the cellular FXIII staining. The importance of FXIII beyond its function in coagulation is still not well known. An *in vivo* study, in which an ^{111}In -labeled α_2 -AP-based CA was used to visualize FXIII activity in myocardial healing, suggested that FXIII plays a role in wound healing and tissue repair (30). Therefore, the fluorescence observed in the medial layer of some carotid arteries might be explained by tissue repair 24-48 hours after FeCl_3 -treatment. Abdalla et al. (31) suggested that intracellular FXIIIa mediated monocyte adhesion during the atherosclerotic process.

Non-invasive assessment of early thrombus formation versus organized thrombi could be of major clinical relevance since specific detection of early thrombus formation may improve selection of patients eligible for fibrinolytic therapy, thereby reducing serious side-effects. Study limitations: Firstly, there is only a limited time window during which this α_2 -AP-based CA can be used for thrombus detection. Secondly, whether our CA is able to detect thrombi that are sensitive to fibrinolytic therapy remains to be determined. Thirdly, to prove its clinical usefulness, it remains to be established whether this α_2 -AP-based CA inhibits thrombolysis using state-of-the-art thrombolytics. However, this CA only contains the part that is responsible for binding of α_2 -AP, which on its own is insufficient to inhibit plasmin activity. Fourthly, our CA binds covalently to fibrin, indicating that the Gd-complex stability is of major importance since free Gd^{3+} -ions can induce nephrogenic systemic fibrosis (NSF). The ionic-macrocylic DOTA-chelate

is more stable than the used DTPA-chelate, therefore minor chelate adjustment will improve its stability and decrease the risk on NSF. Fifthly, to perform pre- and post-contrast MRI within the same mouse on the exact same position, anticoagulants are needed ensuring that the cannula placed for contrast administration will not become obstructed. Since anticoagulants interfere with thrombus formation, no pre-contrast images were obtained. However, the used control CA validated the increase in CNR of thrombi exposed to the α_2 -AP-based CA. Finally, determination of the optimal dosage is still needed.

Conclusion: Our bimodal α_2 -AP-based CA enabled specific, non-invasive visualization of the early phases of thrombus formation, while no hyperintense signal was observed in 24-48 hours old thrombi. Therefore, this bimodal α_2 -AP-based CA might be able to specifically select patients who are eligible for fibrinolytic therapy.

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References

1. Rosamond W, Flegal K, Friday G, et al. Heart disease and stroke statistics--2007 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2007;115:e69-171.
2. White RH. The epidemiology of venous thromboembolism. *Circulation* 2003;107:I4-8.
3. Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. *Lancet* 1994;343:311-22.
4. Lange RA, Hillis LD. Reperfusion therapy in acute myocardial infarction. *N Engl J Med* 2002;346:954-5.
5. Goldstein LB. Acute ischemic stroke treatment in 2007. *Circulation* 2007;116:1504-14.
6. Miserus RJHM, Heeneman S, Engelshoven JMAv, Kooi ME, Daemen MJAP. Development and validation of novel imaging technologies to assist translational studies in atherosclerosis. *Drug Disc. Today: Techn.* 2006;3:195-204.
7. Wickline SA, Neubauer AM, Winter P, Caruthers S, Lanza G. Applications of nanotechnology to atherosclerosis, thrombosis, and vascular biology. *Arterioscler Thromb Vasc Biol* 2006;26:435-41.
8. Jaffer FA, Weissleder R. Molecular imaging in the clinical arena. *Jama* 2005;293:855-62.
9. Flacke S, Fischer S, Scott MJ, et al. Novel MRI contrast agent for molecular imaging of fibrin: implications for detecting vulnerable plaques. *Circulation* 2001;104:1280-5.
10. Botnar RM, Buecker A, Wiethoff AJ, et al. *In vivo* magnetic resonance imaging of coronary thrombosis using a fibrin-binding molecular magnetic resonance contrast agent. *Circulation* 2004;110:1463-6.
11. Spuentrup E, Buecker A, Katoh M, et al. Molecular magnetic resonance imaging of coronary thrombosis and pulmonary emboli with a novel fibrin-targeted contrast agent. *Circulation* 2005;111:1377-82.
12. Sirol M, Fuster V, Badimon JJ, et al. Chronic thrombus detection with *in vivo* magnetic resonance imaging and a fibrin-targeted contrast agent. *Circulation* 2005;112:1594-600.
13. Spuentrup E, Fausten B, Kinzel S, et al. Molecular magnetic resonance imaging of atrial clots in a swine model. *Circulation* 2005;112:396-9.
14. Spuentrup E, Katoh M, Buecker A, et al. Molecular MR Imaging of Human Thrombi in a Swine Model of Pulmonary Embolism Using a Fibrin-Specific Contrast Agent. *Invest Radiol* 2007;42:586-595.
15. Stracke CP, Katoh M, Wiethoff AJ, Parsons EC, Spangenberg P, Spuentrup E. Molecular MRI of cerebral venous sinus thrombosis using a new fibrin-specific MR contrast agent. *Stroke* 2007;38:1476-81.
16. Botnar RM, Perez AS, Witte S, et al. *In vivo* molecular imaging of acute and subacute thrombosis using a fibrin-binding magnetic resonance imaging contrast agent. *Circulation* 2004;109:2023-9.
17. Sirol M, Aguinaldo JG, Graham PB, et al. Fibrin-targeted contrast agent for improvement of *in vivo* acute thrombus detection with magnetic resonance imaging. *Atherosclerosis* 2005;182:79-85.
18. Spuentrup E, Botnar RM, Wiethoff AJ, et al. MR imaging of thrombi using EP-2104R, a fibrin-specific contrast agent: initial results in patients. *Eur Radiol* 2008; 18: 1995-2005
19. Muszbek L, Yee VC, Hevessy Z. Blood coagulation factor XIII: structure and function. *Thromb Res* 1999;94:271-305.
20. Lee KN, Lee CS, Tae WC, Jackson KW, Christiansen VJ, McKee PA. Crosslinking of alpha 2-antiplasmin to fibrin. *Ann N Y Acad Sci* 2001;936:335-9.
21. Robinson BR, Houng AK, Reed GL. Catalytic life of activated factor XIII in thrombi. Implications for fibrinolytic resistance and thrombus aging. *Circulation* 2000;102:1151-7.
22. Tung CH, Ho NH, Zeng Q, et al. Novel factor XIII probes for blood coagulation imaging. *Chembiochem* 2003;4:897-9.

23. Jaffer FA, Tung CH, Wykrzykowska JJ, et al. Molecular imaging of factor XIIIa activity in thrombosis using a novel, near-infrared fluorescent contrast agent that covalently links to thrombi. *Circulation* 2004;110:170-6.
24. Kim DE, Schellingerhout D, Jaffer FA, Weissleder R, Tung CH. Near-infrared fluorescent imaging of cerebral thrombi and blood-brain barrier disruption in a mouse model of cerebral venous sinus thrombosis. *J Cereb Blood Flow Metab* 2005;25:226-33.
25. Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. In situ neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. *Int J Pept Protein Res* 1992;40:180-93.
26. Dirksen A, Langereis S, de Waal BF, et al. Design and synthesis of a bimodal target-specific contrast agent for angiogenesis. *Org Lett* 2004;6:4857-60.
27. Kulkarni S, Jackson SP. Platelet factor XIII and calpain negatively regulate integrin α IIb β 3 adhesive function and thrombus growth. *J Biol Chem* 2004;279:30697-706.
28. Megens RT, Reitsma S, Schiffers PH, et al. Two-photon microscopy of vital murine elastic and muscular arteries. Combined structural and functional imaging with subcellular resolution. *J Vasc Res* 2007;44:87-98.
29. Rittersma SZ, van der Wal AC, Koch KT, et al. Plaque instability frequently occurs days or weeks before occlusive coronary thrombosis: a pathological thrombectomy study in primary percutaneous coronary intervention. *Circulation* 2005;111:1160-5.
30. Nahrendorf M, Hu K, Frantz S, et al. Factor XIII deficiency causes cardiac rupture, impairs wound healing, and aggravates cardiac remodeling in mice with myocardial infarction. *Circulation* 2006;113:1196-202.
31. AbdAlla S, Lothar H, Langer A, el Faramawy Y, Quitterer U. Factor XIIIa transglutaminase crosslinks AT1 receptor dimers of monocytes at the onset of atherosclerosis. *Cell* 2004;119:343-54.

CHAPTER 7

General discussion

Atherosclerosis is a chronic inflammatory disease affecting arterial vessels, which can eventually result in life-threatening events such as myocardial infarction or stroke. These cardiovascular diseases continue to be a major cause of mortality and morbidity in the Western society (1-4). Therefore, improved detection of atherosclerotic lesions and subsequent thrombus formation is vital. Molecular imaging is an emerging field that combines the knowledge of molecular biology with *in vivo* imaging techniques using imaging agents such as MR contrast agents, PET tracers, or fluorescent probes that are directed at specific molecular targets. Therefore, we hypothesized that molecular imaging can contribute to and further improve the assessment of atherosclerosis and thrombus formation.

In the present thesis, we demonstrated that Gadofosveset, an FDA approved albumin binding CA, could discriminate between the normal and atherosclerotic vessel wall in a rabbit model, suggesting that Gadofosveset-enhanced MR plaque imaging enables the differentiation between atherosclerotic and normal vessel walls (**chapter 3**). In addition, we have demonstrated the possibility of imaging macrophages, apoptotic cells, and activated platelets using novel developed molecular imaging agents directed to CCR5 (**chapter 4**) or phosphatidylserine (**chapter 5**). The obtained *in vitro* and *ex vivo* results with MRI, PET and TPLSM, indicate the potential of these imaging agents for *in vivo* imaging of atherosclerotic lesions in the near future. Finally, we investigated the detection of early thrombus formation using the α_2 -antiplasmin-based CA. We showed that this imaging agent enabled non-invasive *in vivo* MR visualization of early thrombus formation in a mouse model (**chapter 6**).

Molecular imaging

The success of molecular imaging depends on several factors like target concentration, accessibility of the targets, sensitivity and spatial resolution of the used imaging modality, and pharmacokinetics and toxicity of the imaging agent. For clinical applications, non-invasive imaging modalities are preferable, in which MRI and nuclear imaging modalities like PET and SPECT are the most promising modalities for molecular imaging. The high sensitivity of the nuclear imaging modalities allows administration of relatively low tracer concentrations. This could be important because the risk on side-effects is much lower at low tracer concentrations and moreover, the production of these molecular imaging agents is time-consuming and expensive. On the other hand, MRI allows imaging with far higher spatial resolutions and does not expose the patients to ionizing radiation. As a consequence of the relatively low sensitivity of MRI, larger amounts of contrast agent (in the order of $\mu\text{mol Gd/kg}$ body weight) are required for visualization. Future developments on radiofrequency coils may improve the sensitivity of MRI,

but will not yield comparable sensitivities as of nuclear imaging modalities. Amplification strategies that use contrast agents consisting of nanoparticles loaded with gadolinium can overcome this relatively low sensitivity of MRI. This could especially be important for low target concentrations. Several amplification strategies have already been used for molecular imaging purposes using gadolinium-loaded nanoparticles such as liposomes (5), micelles (5), perfluorocarbon nanoparticles (6), dendrimers (7), and quantum dots (QDs) (8). In this thesis, we focused on the molecular imaging of atherosclerosis and thrombus formation, using MRI and PET as non-invasive imaging modalities, while TPLSM was used to verify the presence of molecular imaging agents at subcellular resolutions.

Molecular imaging of atherosclerosis

Specific processes during the development of atherosclerosis such as neovascularisation, inflammation and apoptosis, can be targeted by molecular imaging agents (table 7.1). This provides a major advantage above non-contrast enhanced imaging and the use of non-targeted contrast agents, as it supplies molecular information of specific atherosclerotic processes, allowing the molecular assessment of atherosclerosis.

Neovascularisation

Neovascularisation of the vessel wall plays an important role in atherosclerotic lesion development and the progression of the disease (9). Therefore, detection of neovasculature will possibly facilitate the assessment of atherosclerotic lesions. Kerwin et al. (10) demonstrated that pharmacokinetic modeling of dynamic contrast-enhanced MRI, using non-targeted CAs enabled quantification of neovasculature in carotid atherosclerotic lesions. Furthermore, since neovascular endothelial cells express $\alpha_v\beta_3$ -integrins within the vasa vasorum and intralesional microvessels (11), $\alpha_v\beta_3$ -integrin may therefore be a suitable target for the molecular imaging of neovascularization. RGD-peptides that bind to $\alpha_v\beta_3$ -integrins, have been used for the characterization of atherosclerotic lesions. Gadolinium-loaded RGD mimetic CA (Gd-DTPA-g-mimRGD) (12) and gadolinium-loaded perfluorocarbon nanoparticles linked to RGD-peptides (13) demonstrated signal enhancement in atherosclerotic lesions compared to the use of non-targeted CAs. The binding specificity of both CAs was confirmed with *in vivo* competition studies. Nevertheless, slight signal enhancement was also obtained when gadolinium-loaded nanoparticles linked to RGD-peptides (13) were administered to healthy animals with no lesions. Combined with the fact that the $\alpha_v\beta_3$ -integrin is expressed on both intralesional microvessels and the vasa vasorum in the adventitia (11), the

potential of $\alpha_v\beta_3$ -integrin-targeted CAs in distinguishing atherosclerotic lesions and the healthy vessel wall could be limited. This might also hamper the discrimination between vulnerable and stable lesions, which is of major clinical relevance.

Another approach on lesion visualization that is indirectly related to neovascularization is using the leaky nature of intraplaque microvessels. In human atherosclerotic lesions, the presence of irregular dysmorphic microvessels that lacked smooth muscle cells were shown (14,15). Additionally, it was demonstrated that endothelial junctions were abnormal or missing in intraplaque microvessels within atherosclerotic lesions of human coronary arteries. Moreover, the endothelial cells lining the microvessels often represented an activated, dysfunctional status and were frequently detached from the basal membrane (15).

Table 7.1: Molecular Imaging of Atherosclerosis.

Target	Imaging agent	Image modality	Study design	Reference
Neovascularisation				
$\alpha_v\beta_3$ -Integrin	Mimetic RGD labeled with Gd-DTPA	MRI	Animal model (mouse)	(12)
$\alpha_v\beta_3$ -Integrin	RGD-peptide linked to paramagnetic nanoparticle	MRI	Animal model (rabbit)	(13)
Albumin	Gadofosveset	MRI	Rabbit model (balloon denudation)	This thesis
Albumin	Gadofosveset	MRI	Human study (carotid lesions)	(20)
Albumin	B-22956/1	MRI	Rabbit model (balloon denudation)	(19)
Inflammation				
Macrophages	^{99m}Tc labeled oxLDL ^a particles	Gamma camera	Human study (carotid lesions)	(25)
Macrophages	USPIO ^b (Sinerem, Guerbet, ferumoxtran-10)	MRI	Human study (carotid lesions)	(26-27)
Macrophages	N1177	CT	Rabbit model (balloon denudation)	(28)
Macrophages	MION (monocrystalline iron oxide nanoparticle) labeled with ^{64}Cu and fluorescent probes	MRI, PET and fluorescent microscopy	Mouse model (ApoE ^{-/-})	(29)
Macrophages	^{18}F fluorodeoxyglucose (^{18}FDG)	PET	Human study (carotid lesions)	(31-34)
Scavenger receptors	Gadolinium carrying micelles with MSR ^c -targeting antibodies	MRI	Mouse model (ApoE ^{-/-})	(35)
Oxidation-specific epitopes	Gadolinium carrying micelles with antibodies targeting oxidation-specific epitopes	MRI	Mouse model (ApoE ^{-/-})	(36)
Matrix metalloproteinases	^{99m}Tc labeled MMP ^d inhibitor	SPECT	Rabbit model (balloon denudation)	(37)
Chemokine receptor 2 (CCR2)	^{99m}Tc labeled MCP-1 ^e	Gamma camera	Rabbit model (balloon denudation)	(38)
Chemokine receptor 5 (CCR5)	^{125}I labeled Met-RANTES	Autoradiography	Mouse model (LDLr ^{-/-})	(39)
Chemokine receptor 5 (CCR5)	^{68}Ga and rhodamine labeled RANTES	PET and TPLSM	Mouse model (ApoE ^{-/-})	This thesis
Apoptosis				
Phosphatidylserine	^{99m}Tc labeled Annexin A5	Gamma camera or SPECT	Rabbit and swine models (balloon denudation)	(45-47)
Phosphatidylserine	^{99m}Tc labeled Annexin A5	SPECT	Human study (carotid lesions)	(48)
Phosphatidylserine	Annexin A5 linked to gadolinium containing QDs	MRI and TPLSM	<i>In vitro</i> and <i>ex vivo</i> (mouse model: wire injury)	This thesis

^aoxLDL: Oxidized low density lipoprotein. ^bUSPIO: Ultrasmall superparamagnetic particle of iron oxide.

^cMSR: macrophage scavenger receptor. ^dMMP: Matrix metalloproteinases. ^eMCP-1: Monocyte chemotactic protein.

These structural abnormalities possibly explain the permeable nature of the intralésional microvessels, allowing proteins such as albumin to leak into the atherosclerotic lesions. It has been shown that albumin content correlated with lesion size and microvessel density (16). Therefore, in this thesis, we used the albumin-binding contrast agent Gadofosveset and compared its feasibility for *in vivo* lesion visualization with the more commonly used Gd-DTPA in a rabbit model (**chapter 3**). Gadofosveset binds reversibly to albumin, resulting in an increase in elimination half-life and r_1 relaxivity (17,18). Our study demonstrated that the signal enhancement was significantly different between atherosclerotic vessel walls and healthy vessel walls of control rabbits using Gadofosveset, while no differences in signal enhancement were found using Gd-DTPA. In addition, microvessel density and the amount of albumin were significantly ($p < 0.001$) higher in the atherosclerotic vessel walls compared to the healthy vessel walls. Since equal amounts of gadolinium atoms were found in the atherosclerotic vessel walls and healthy vessel walls, the increase in r_1 relaxivity upon binding of Gadofosveset to albumin explains the obtained results. These findings suggest that Gadofosveset could distinguish healthy from diseased vessel walls and that microvessels may serve as a port of entry for albumin and Gadofosveset. Cornily et al. (19) obtained similar results using the albumin-binding CA B-22956/1. This CA significantly increased lesion enhancement compared to commonly used Gd-DTPA. In addition, it was shown that atherosclerotic lesion enhancement at early time points after B-22956/1 administration correlated with intralésional microvessel density in the atherosclerotic lesions (19). In contrast to our study, the difference in r_1 relaxivity for the used CAs (B-22956/1 and Gd-DTPA) was not corrected for. In addition, B-22956/1 is not registered for human applications, while Gadofosveset is already FDA approved for human applications.

Recently, it was demonstrated that Gadofosveset administration enhanced signal intensities in human carotid lesions of symptomatic patients whereas limited signal enhancement was found in carotid lesions of asymptomatic patients. These results might be explained by the significantly higher amount of microvessels found in carotid endarterectomy samples of symptomatic compared to asymptomatic patients (20). These initial clinical results together with the animal results obtained during this thesis suggest a promising role for Gadofosveset in the assessment of atherosclerotic lesions. Moreover, neovascularization in human atherosclerotic lesions was associated with features of lesion vulnerability and clinically symptomatic diseases as shown in several pathological studies (21-23). This suggests that Gadofosveset can play an important role in the detection of vulnerable lesions.

Inflammation

Inflammation is involved in every stage of atherosclerosis and is a key process in the atherosclerotic development. In this process, activated cells such as macrophages, T-cells, and endothelial cells play an important role (2,3). These inflammatory cells are predominantly found at sites of fibrous cap rupture, around the lipid core, and around areas of neovascularization (24). Since macrophages are the most prominent inflammatory cells, imaging of these cells should allow the assessment of inflammation within atherosclerotic lesions.

Phagocytic behavior of macrophages facilitates detection of atherosclerosis due to uptake of non-targeted CAs. This behavior allowed visualization of human carotid atherosclerotic lesions with radiolabeled oxidized LDL (oxLDL) particles (25), and ultrasmall superparamagnetic particles of iron oxide (USPIOs) (26,27). Additionally, a CA called N1177 composed of crystalline iodinated nanoparticles, allowed detection of atherosclerosis in a rabbit model using CT (28). Recently, a trimodal non-targeted nanoparticle containing a monocrystalline iron oxide nanoparticle (MION), ^{64}Cu , and a fluorochrome, enabled the detection of atherosclerosis with MRI, PET, and fluorescent microscopy (29), combining the strengths of each imaging modality. However, uptake of these imaging agents depends on the phagocytic ability of the macrophages present in the atherosclerotic lesions. Only the USPIO particles demonstrated the ability to distinguish different lesion stages since only 7% of the stable lesions showed USPIO uptake compared to 75% of the rupture-prone and ruptured lesions (26). It has been shown that the phagocytic ability of macrophages for apoptotic cells is impaired in the atherosclerotic progression (30). This could implicate that phagocytosis of some of these contrast agents is also impaired in advanced atherosclerotic lesions, which suggests that none of these agents will be able to specifically detect vulnerable lesions. Additionally, none of these agents are FDA approved.

Next to exploiting the phagocytotic characteristics of macrophages, the high metabolic activity of these cells was also used to visualize atherosclerotic lesions. The glucose analogue fluorodeoxyglucose marked with ^{18}F (18F-FDG) enabled the assessment of inflammation severity in human carotid lesions (31) and proved to be highly reproducible (32,33). Furthermore, ^{18}F -FDG uptake demonstrated that lesions from symptomatic patients were more metabolically active compared to lesions from asymptomatic patients. This suggests that the degree of inflammation is more prominent in lesions of symptomatic compared to asymptomatic patients (34). Therefore, the ^{18}F -FDG agent could be able to distinguish vulnerable lesions from stable lesions. However, since uptake of this imaging agent depends on the metabolic activity of the cells, this agent also

strongly accumulates in metabolically active tissues such as the heart. This hampers its use for the detection of atherosclerotic lesions in the coronary arteries.

The use of other ligands that are specifically directed towards inflammatory cells could overcome these limitations. Targets such as macrophage scavenger receptors (35), oxidation-specific epitopes (36), matrix metalloproteinase (MMPs) (37), and chemokine receptors (38) allowed the visualization of atherosclerotic lesions and correlated with macrophage density (table 7.1). In this thesis, another target was used for the imaging of inflammatory cells. We synthesized molecular imaging agents based on RANTES. RANTES is a ligand for CCR5 receptors which are predominantly expressed on activated macrophages. In an initial experiment, we demonstrated the feasibility of (bimodal) RANTES-based molecular imaging agents for the detection of murine atherosclerotic lesions with PET and TPLSM (**chapter4**). Veillard et al. (39) administered radiolabeled Met-RANTES and obtained comparable *ex vivo* imaging results as were obtained with our bimodal RANTES-based imaging agent. In addition, we demonstrated that the RANTES-based agents internalized *in vitro* in THP-1 derived macrophages and in macrophages present in advanced murine atherosclerotic lesions. This finding is of considerable interest since internalization of the molecular imaging agent allows accumulation, thereby enhancing detection of the agent. More importantly, given the fact that CCR5 expression was up-regulated in ruptured human carotid atherosclerotic lesions compared to advanced stable atherosclerotic lesions, RANTES-based molecular imaging agents may possibly distinguish vulnerable and stable atherosclerotic lesions. However, more experiments on the assessment of atherosclerotic lesions using RANTES-based CAS are needed to validate these promising initial results.

Apoptosis

Apoptosis or programmed cell death occurs throughout the atherosclerotic process but the consequences can vary for the different atherosclerotic stages. It is suggested that macrophage apoptosis is associated with decreased lesion progression in early atherosclerosis, whereas in further developed atherosclerotic lesions macrophage apoptosis combined with defective phagocytic clearance of apoptotic macrophages might lead to secondary necrosis, thereby inducing inflammation (40). Next to the influence of macrophage apoptosis, apoptotic smooth muscle cells (SMCs) also play an important role in destabilizing atherosclerotic lesions. Apoptosis of SMCs weakens the fibrous cap by decreasing the number of collagen-synthesizing cells (41). Additionally, it was shown that SMC apoptosis accelerated the atherosclerotic process in already established atherosclerotic lesions in ApoE^{-/-} mice (42).

It has been shown that the frequency of apoptotic cells increased in late stage human atherosclerotic disease (43). Therefore, non-invasive detection of apoptosis can possibly assist in identifying human vulnerable atherosclerotic lesions. Apoptotic cells are known to externalize phosphatidylserine (PS) and Annexin A5 (AnxA5) is a widely used molecular imaging agent that binds to PS with high affinity in the presence of Ca^{2+} ions (44). This agent enabled noninvasive detection of atherosclerotic lesions in the rabbit aorta (45) and in coronary vessels of swine (46). In addition, diet withdrawal and statin therapy decreased the extent of apoptosis in a rabbit model for atherosclerosis (47). A clinical proof of concept study, encompassing four patients who had a recent or remote history transient ischemic attack, suggested that $^{99\text{m}}$ Technetium-labeled AnxA5 enables the assessment of lesion vulnerability (48). A major disadvantage of these studies is the poor resolution due to the use of nuclear imaging modalities. Therefore, in this thesis (**chapter 5**) a bimodal AnxA5 molecular imaging agent was developed, allowing visualization at both anatomical and subcellular level, since our molecular imaging agent consisted of Gd-DTPA complexes and QDs for detection with MRI and TPLSM, respectively. Additionally, a novel amplification strategy was used by linking Gd-wedge structures to these QDs. This bimodal molecular imaging agent allowed *in vitro* imaging of apoptotic Jurkat cells and *ex vivo* imaging of apoptotic cells in a wire-injured murine carotid artery using MRI and TPLSM. However, the feasibility of this molecular imaging agent in detecting atherosclerotic lesions non-invasively still has to be proven. Additionally, QD are potentially toxic, which implicates that this molecular imaging agent in its current form is clinically unusable. However, displacement of QDs by other fluorescent nanoparticles such as gold and silica nanoparticles can improve the clinical applicability since these are potentially nontoxic (49). Nevertheless, the used molecular imaging agent proved to be an ideal imaging agent for *in vitro* and animal research because of its strong fluorescence and the possibility to attach multiple gadolinium particles.

Molecular imaging of thrombus formation

Non-invasive molecular imaging of thrombus formation may enable the detection of different phases of thrombus formation. Several molecular imaging agents specifically directed to activated platelets or other components present in thrombi such as fibrin, demonstrated that detection of thrombus formation is feasible (table 7.2). The possibility to distinguish early phases of thrombus formation and organized thrombi can be of major clinical relevance since it might improve the selection of patients that need to receive fibrinolytic therapy.

Table 7.2: Molecular Imaging of Thrombus Formation.

Target	Imaging agent	Image modality	Study design	Reference
Platelets				
Glycoprotein IIb/IIIa	^{99m} Tc labeled DMP-444	SPECT	Canine model (by flow-limiting stenosis and endothelial electrical stimulation)	(51)
Glycoprotein IIb/IIIa	Abciximab linked to microbubbles	Ultrasound	Rat model (human clot placement in carotid artery)	(52)
Glycoprotein IIb/IIIa	Antibodies linked to MPIOs ^a	MRI	Mouse model (FeCl ₃ -induced thrombus formation)	(55)
Phosphatidylserine	Annexin A5 linked to gadolinium containing QDs	MRI and TPLSM	<i>In vitro</i>	This thesis
Fibrin				
Fibrin	EP-1242	MRI	Pig model (blood stasis and external vessel wall damage)	(56)
Fibrin	EP-1873	MRI	Rabbit model (in-stent thrombosis)	(57)
Fibrin	EP-2104R	MRI	Animal models (swine, pig and rabbit)	(58-63)
Fibrin	EP-2104R	MRI	Human study	(64)
Fibrin	α_2 -antiplasmin based peptide labeled with Alexa fluor 680	NIRF	Mouse model (FeCl ₃ -induced thrombus formation)	(66-67)
Fibrin	α_2 -antiplasmin based peptide bimodal labeled with gadolinium and rhodamine.	MRI and TPLSM	Mouse model (FeCl ₃ -induced thrombus formation)	This thesis

^aMPIO: Microparticles of iron oxide

Platelets

At sites of blood vessel wall injury, firm adherence and aggregation of platelets occurs rapidly. This process plays an important role in thrombus formation (50). Glycoprotein IIb/IIIa is expressed on activated platelets and has been used as a target for molecular imaging. For example, selective glycoprotein IIb/IIIa inhibitors such as DMP-444 (51) and abciximab (52) enabled *in vivo* detection of activated platelets in animal models for thrombus formation, using SPECT and ultrasound, respectively. Recently, single-chain antibodies (directed to glycoprotein IIb/IIIa) attached to microparticles of iron oxide (MPIOs) enabled visualization of *in vitro* generated human thrombi with MRI and enabled binding of these molecular imaging agents to platelets under venous and arterial flow conditions (53). These molecular imaging agents also enabled *ex vivo* detection of activated platelets using MRI in a wire injury mouse model (54). The *in vivo* feasibility of these molecular imaging agents in visualizing activated platelets and monitoring

thrombolytic therapy was demonstrated using a ferric chloride-induced thrombosis model in murine carotid arteries (55). However, a major disadvantage of the MPIO-based imaging agents is that these agents cause a signal void in the MRI image instead of a signal enhancement. Signal voids can easily be misinterpreted, which eventually could lead to an incorrect diagnosis. To avoid this disadvantage, positive MR contrast agents such as gadolinium can be used. Since activated platelets also express PS at the cell surface, this thesis demonstrated *in vitro* thrombus detection using an annexin A5 functionalized molecular imaging agent (**chapter 5**). This agent consisted of a QD and several gadolinium particles. Since QDs are potentially toxic, clinical applicability of this QD-based agent is not to be expected in the near future. However, the advantage of this molecular imaging agent is that it causes MR signal enhancement instead of signal voids and the bimodality of this contrast agent allows visualization at subcellular level with TPLSM as well. Although positive *in vitro* results were obtained, the feasibility of this molecular imaging agent in detecting activated platelets *in vivo* remains to be proven.

Fibrin

As fibrin is abundantly present in thrombi, it is an ideal target for molecular imaging strategies. Thus far, fibrin-specific molecular imaging agents were mainly used for MR imaging. The most widely used MR molecular imaging agents for thrombus formation are EP-1242 (56), EP-1873 (57), and EP-2104R (58-63). These molecular imaging agents consist of a fibrin-specific peptide labeled with 4 Gd-DTPA chelates and enabled MR thrombus visualization in different animal models. Moreover, initial results showed that EP-2104R allows *in vivo* MR imaging of thrombi in humans (64). Nevertheless, none of these contrast agents allowed specific detection of the early phases of thrombus formation, while this specific detection is clinically important since resistance of thrombi to fibrinolytic therapy is achieved within hours (65).

A novel molecular imaging agent based on α_2 -antiplasmin allowed carotid (66) and cerebral (67) thrombus visualization with NIRF microscopy and proved to be specific for the early phases of thrombus formation (66). However, for clinical applications, molecular imaging agents which are detectable with non-invasive imaging modalities are preferable. In this thesis, we synthesized a novel bimodal α_2 -antiplasmin-based molecular imaging agent which allowed non-invasive imaging of early thrombus formation with MRI (**chapter 6**). No amplification strategies were used because fibrin is present in large quantities throughout the thrombus. A non-clinical MR field strength was used since we needed to achieve a relatively high resolution in order to be able to visualize the tiny carotid arteries of mice. We expect that the signal enhancement at clinical field strengths (1.5T) will be larger

since the relaxivity (r_1) of the used molecular imaging agents is higher at 1.5 Tesla ($5.6 \pm 0.4 \text{ mM}^{-1} \text{ s}^{-1}$) compared to 7 Tesla ($3.0 \pm 0.3 \text{ mM}^{-1} \text{ s}^{-1}$).

Specific detection of early thrombus formation could have important clinical implications since the resistance of thrombi to fibrinolytic therapy increases with thrombus age (65), and fibrinolytic agents can induce severe complications such as bleeding (68,69). Since α_2 -antiplasmin is known to inhibit thrombolysis, it still has to be determined whether our agent interferes with the fibrinolytic therapy. However, it seems unlikely as the CA only contains the part responsible for binding. To prove its clinical relevance, it still has to be shown that our molecular imaging agent selectively detects thrombi which are sensitive to thrombolysis.

Another clinically relevant topic next to luminal thrombus visualization is the detection of intralesional hemorrhage, since this is one of the features of a vulnerable lesion. Non-contrast enhanced MRI already proved to be able to detect intralesional hemorrhage in human carotid atherosclerotic lesions (70). However, molecular imaging could assist in the detection of intralesional hemorrhage in smaller arteries like the coronary arteries.

Outlook and conclusion

During the last years, promising molecular imaging results have been obtained for the assessment of atherosclerosis and thrombus formation using various molecular imaging agents and targets. This thesis demonstrated that the FDA-approved CA Gadofosveset and newly developed molecular imaging agents enabled the detection of atherosclerotic processes and thrombus formation. Future studies need to evaluate the value of the FDA approved Gadofosveset in the assessment of atherosclerotic lesions using large clinical trials. In addition, the initial results obtained on the imaging of atherosclerosis using the novel QD-based and RANTES-based CAs need to be validated. A disadvantage of the QDs used in this thesis, is their potential toxicity. This will hamper its use in clinical studies. In contrast, the prospective for the α_2 -antiplasmin-based CA is promising, although the influence on fibrinolytic therapy remains to be determined.

The next step in the assessment of atherosclerosis is the specific detection of vulnerable lesions, which is of major clinical relevance. To achieve this, specific targets that are up-regulated in vulnerable compared to stable lesions are needed. Ideally, the molecular imaging target should not be expressed in stable lesions and be strongly up-regulated in vulnerable lesions. Analysis of differential gene and protein expression of atherosclerotic tissue samples could provide new targets for novel molecular imaging agents. In this thesis, micro array analysis demonstrated that CCR5 was up-regulated in ruptured atherosclerotic lesions and a preliminary validation study suggested that it could be a promising target for the assessment of

atherosclerosis. These newly developed molecular imaging agents still face a long validation track such as determination of pharmacokinetics, toxicity and (for thrombus detection) the influence on thrombus formation and fibrinolytic therapy before they are suitable for human studies. Next to these challenges, factors such as target concentration, accessibility of the targets, specificity, sensitivity and spatial resolution of the used imaging modality, and the costs for producing the molecular imaging agents will determine the success of these agents. Additionally, the stability of the gadolinium complexes used in the molecular imaging agents is of major importance, since free Gd^{3+} -ions can induce nephrogenic systemic fibrosis (NSF) (71).

In addition to the optimization of molecular imaging agents, technical developments like the introduction of the combined MRI-PET system could be beneficial for molecular imaging, since this allows a combination of high resolution and high sensitivity. Additionally, it could be useful in the detection of MRI and PET-labeled molecular imaging agents, allowing the detection of two different targets by administering two different molecular imaging agents. This will eventually further improve the assessment of atherosclerosis and thrombus formation.

In conclusion, we demonstrated the feasibility of an already approved CA (Gadofosveset) and novel molecular imaging agents (CCR5 and PS-targeted) for the non-invasive assessment of atherosclerosis and thrombus formation (α_2 -antiplasmin-based CA) in animal models. Although only a few clinical molecular imaging studies have been performed in atherosclerotic patients, we expect molecular imaging to be encompassed in the clinical arena in a few decades. It might assist in distinguishing stable and vulnerable lesions and further improve the detection of thrombus formation. Eventually, this will have important clinical implications such as improved patient selection and individualized treatment.

References

1. Weintraub HS. Identifying the vulnerable patient with rupture-prone plaque. *Am J Cardiol* 2008;101:3F-10F.
2. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105:1135-43.
3. Lusis AJ. Atherosclerosis. *Nature* 2000;407:233-41.
4. Lloyd-Jones D, Adams R, Carnethon M, et al. Heart disease and stroke statistics--2009 update: a report from the American Heart Association Statistics Committee and Stroke Statistics Subcommittee. *Circulation* 2009;119:e21-181.
5. Mulder WJ, Strijkers GJ, van Tilborg GA, Griffioen AW, Nicolay K. Lipid-based nanoparticles for contrast-enhanced MRI and molecular imaging. *NMR Biomed* 2006;19:142-64.
6. Morawski AM, Winter PM, Crowder KC, et al. Targeted nanoparticles for quantitative imaging of sparse molecular epitopes with MRI. *Magn Reson Med* 2004;51:480-6.
7. Langereis S, de Lussanet QG, van Genderen MH, et al. Evaluation of Gd(III)DTPA-terminated poly(propylene imine) dendrimers as contrast agents for MR imaging. *NMR Biomed* 2006;19:133-41.
8. Mulder WJ, Koole R, Brandwijk RJ, et al. Quantum dots with a paramagnetic coating as a bimodal molecular imaging probe. *Nano Lett* 2006;6:1-6.
9. Doyle B, Caplice N. Plaque neovascularization and antiangiogenic therapy for atherosclerosis. *J Am Coll Cardiol* 2007;49:2073-80.
10. Kerwin W, Hooker A, Spilker M, et al. Quantitative magnetic resonance imaging analysis of neovascularity volume in carotid atherosclerotic plaque. *Circulation* 2003;107:851-6.
11. Hoshiga M, Alpers CE, Smith LL, Giachelli CM, Schwartz SM. Alpha-v beta-3 integrin expression in normal and atherosclerotic artery. *Circ Res* 1995;77:1129-35.
12. Burtea C, Laurent S, Murariu O, et al. Molecular imaging of alpha v beta3 integrin expression in atherosclerotic plaques with a mimetic of RGD peptide grafted to Gd-DTPA. *Cardiovasc Res* 2008;78:148-57.
13. Winter PM, Morawski AM, Caruthers SD, et al. Molecular imaging of angiogenesis in early-stage atherosclerosis with alpha(v)beta3-integrin-targeted nanoparticles. *Circulation* 2003;108:2270-4.
14. Dunmore BJ, McCarthy MJ, Naylor AR, Brindle NP. Carotid plaque instability and ischemic symptoms are linked to immaturity of microvessels within plaques. *J Vasc Surg* 2007;45:155-9.
15. Sluimer JC, Kolodgie FD, Bijnens AP, et al. Thin-walled microvessels in human coronary atherosclerotic plaques show incomplete endothelial junctions relevance of compromised structural integrity for intraplaque microvascular leakage. *J Am Coll Cardiol* 2009;53:1517-27.
16. Zhang Y, Cliff WJ, Schoefl GI, Higgins G. Immunohistochemical study of intimal microvessels in coronary atherosclerosis. *Am J Pathol* 1993;143:164-72.
17. Parmelee DJ, Walovitch RC, Ouellet HS, Lauffer RB. Preclinical evaluation of the pharmacokinetics, biodistribution, and elimination of MS-325, a blood pool agent for magnetic resonance imaging. *Invest Radiol* 1997;32:741-7.
18. Lauffer RB, Parmelee DJ, Dunham SU, et al. MS-325: albumin-targeted contrast agent for MR angiography. *Radiology* 1998;207:529-38.
19. Cornily JC, Hyafil F, Calcagno C, et al. Evaluation of neovessels in atherosclerotic plaques of rabbits using an albumin-binding intravascular contrast agent and MRI. *J Magn Reson Imaging* 2008;27:1406-11.
20. Lobbes MBI, Heeneman S, Lima Passos V, et al. Gadofosveset-enhanced magnetic resonance imaging of human carotid atherosclerotic plaques: initial experience. Submitted 2009.
21. Moreno PR, Purushothaman KR, Fuster V, et al. Plaque neovascularization is increased in ruptured atherosclerotic lesions of human aorta: implications for plaque vulnerability. *Circulation* 2004;110:2032-8.

22. Fleiner M, Kummer M, Mirlacher M, et al. Arterial neovascularization and inflammation in vulnerable patients: early and late signs of symptomatic atherosclerosis. *Circulation* 2004;110:2843-50.
23. McCarthy MJ, Loftus IM, Thompson MM, et al. Angiogenesis and the atherosclerotic carotid plaque: an association between symptomatology and plaque morphology. *J Vasc Surg* 1999;30:261-8.
24. Shah PK. Inflammation and plaque vulnerability. *Cardiovasc Drugs Ther* 2009;23:31-40.
25. Iuliano L, Signore A, Vallabajosula S, et al. Preparation and biodistribution of 99m technetium labelled oxidized LDL in man. *Atherosclerosis* 1996;126:131-41.
26. Kooi ME, Cappendijk VC, Cleutjens KB, et al. Accumulation of ultrasmall superparamagnetic particles of iron oxide in human atherosclerotic plaques can be detected by *in vivo* magnetic resonance imaging. *Circulation* 2003;107:2453-8.
27. Trivedi RA, JM UK-I, Graves MJ, et al. *In vivo* detection of macrophages in human carotid atheroma: temporal dependence of ultrasmall superparamagnetic particles of iron oxide-enhanced MRI. *Stroke* 2004;35:1631-5.
28. Hyafil F, Cornily JC, Feig JE, et al. Noninvasive detection of macrophages using a nanoparticulate contrast agent for computed tomography. *Nat Med* 2007;13:636-41.
29. Nahrendorf M, Zhang H, Hembrador S, et al. Nanoparticle PET-CT imaging of macrophages in inflammatory atherosclerosis. *Circulation* 2008;117:379-87.
30. Schrijvers DM, De Meyer GR, Kockx MM, Herman AG, Martinet W. Phagocytosis of apoptotic cells by macrophages is impaired in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2005;25:1256-61.
31. Tawakol A, Migrino RQ, Bashian GG, et al. *In vivo* 18F-fluorodeoxyglucose positron emission tomography imaging provides a noninvasive measure of carotid plaque inflammation in patients. *J Am Coll Cardiol* 2006;48:1818-24.
32. Rudd JH, Myers KS, Bansilal S, et al. (18)Fluorodeoxyglucose positron emission tomography imaging of atherosclerotic plaque inflammation is highly reproducible: implications for atherosclerosis therapy trials. *J Am Coll Cardiol* 2007;50:892-6.
33. Rudd JH, Myers KS, Bansilal S, et al. Atherosclerosis inflammation imaging with 18F-FDG PET: carotid, iliac, and femoral uptake reproducibility, quantification methods, and recommendations. *J Nucl Med* 2008;49:871-8.
34. Rudd JH, Warburton EA, Fryer TD, et al. Imaging atherosclerotic plaque inflammation with [18F]-fluorodeoxyglucose positron emission tomography. *Circulation* 2002;105:2708-11.
35. Amirbekian V, Lipinski MJ, Briley-Saebo KC, et al. Detecting and assessing macrophages *in vivo* to evaluate atherosclerosis noninvasively using molecular MRI. *Proc Natl Acad Sci U S A* 2007;104:961-6.
36. Briley-Saebo KC, Shaw PX, Mulder WJ, et al. Targeted molecular probes for imaging atherosclerotic lesions with magnetic resonance using antibodies that recognize oxidation-specific epitopes. *Circulation* 2008;117:3206-15.
37. Fujimoto S, Hartung D, Ohshima S, et al. Molecular imaging of matrix metalloproteinase in atherosclerotic lesions: resolution with dietary modification and statin therapy. *J Am Coll Cardiol* 2008;52:1847-57.
38. Hartung D, Petrov A, Haider N, et al. Radiolabeled Monocyte Chemoattractant Protein 1 for the detection of inflammation in experimental atherosclerosis. *J Nucl Med* 2007;48:1816-21.
39. Veillard NR, Kwak B, Pelli G, et al. Antagonism of RANTES receptors reduces atherosclerotic plaque formation in mice. *Circ Res* 2004;94:253-61.
40. Tabas I. Consequences and therapeutic implications of macrophage apoptosis in atherosclerosis: the importance of lesion stage and phagocytic efficiency. *Arterioscler Thromb Vasc Biol* 2005;25:2255-64.
41. Halvorsen B, Otterdal K, Dahl TB, et al. Atherosclerotic plaque stability--what determines the fate of a plaque? *Prog Cardiovasc Dis* 2008;51:183-94.

42. Clarke MC, Littlewood TD, Figg N, et al. Chronic apoptosis of vascular smooth muscle cells accelerates atherosclerosis and promotes calcification and medial degeneration. *Circ Res* 2008;102:1529-38.
43. Lutgens E, de Muinck ED, Kitslaar PJ, Tordoir JH, Wellens HJ, Daemen MJ. Biphasic pattern of cell turnover characterizes the progression from fatty streaks to ruptured human atherosclerotic plaques. *Cardiovasc Res* 1999;41:473-9.
44. Reutelingsperger CP, van Heerde WL. Annexin V, the regulator of phosphatidylserine-catalyzed inflammation and coagulation during apoptosis. *Cell Mol Life Sci* 1997;53:527-32.
45. Kolodgie FD, Petrov A, Virmani R, et al. Targeting of apoptotic macrophages and experimental atheroma with radiolabeled annexin V: a technique with potential for noninvasive imaging of vulnerable plaque. *Circulation* 2003;108:3134-9.
46. Johnson LL, Schofield L, Donahay T, Narula N, Narula J. 99mTc-annexin V imaging for *in vivo* detection of atherosclerotic lesions in porcine coronary arteries. *J Nucl Med* 2005;46:1186-93.
47. Hartung D, Sarai M, Petrov A, et al. Resolution of apoptosis in atherosclerotic plaque by dietary modification and statin therapy. *J Nucl Med* 2005;46:2051-6.
48. Kietselaer BL, Reutelingsperger CP, Heidendal GA, et al. Noninvasive detection of plaque instability with use of radiolabeled annexin A5 in patients with carotid-artery atherosclerosis. *N Engl J Med* 2004;350:1472-3.
49. Douma K, Prinzen L, Slaaf DW, et al. Nanoparticles for Optical Molecular Imaging of Atherosclerosis. *Small* 2009; 5: 544-557.
50. Wohner N. Role of cellular elements in thrombus formation and dissolution. *Cardiovasc Hematol Agents Med Chem* 2008;6:224-8.
51. Mitchel J, Waters D, Lai T, et al. Identification of coronary thrombus with a IIb/IIIa platelet inhibitor radiopharmaceutical, technetium-99m DMP-444: A canine model. *Circulation* 2000;101:1643-6.
52. Alonso A, Della Martina A, Stroick M, et al. Molecular imaging of human thrombus with novel abcximab immunobubbles and ultrasound. *Stroke* 2007;38:1508-14.
53. von Zur Muhlen C, von Elverfeldt D, Choudhury RP, et al. Functionalized magnetic resonance contrast agent selectively binds to glycoprotein IIb/IIIa on activated human platelets under flow conditions and is detectable at clinically relevant field strengths. *Mol Imaging* 2008;7:59-67.
54. von zur Muhlen C, Peter K, Ali ZA, et al. Visualization of activated platelets by targeted magnetic resonance imaging utilizing conformation-specific antibodies against glycoprotein IIb/IIIa. *J Vasc Res* 2009;46:6-14.
55. von zur Muhlen C, von Elverfeldt D, Moeller JA, et al. Magnetic resonance imaging contrast agent targeted toward activated platelets allows *in vivo* detection of thrombosis and monitoring of thrombolysis. *Circulation* 2008;118:258-67.
56. Sirol M, Aguinaldo JG, Graham PB, et al. Fibrin-targeted contrast agent for improvement of *in vivo* acute thrombus detection with magnetic resonance imaging. *Atherosclerosis* 2005;182:79-85.
57. Botnar RM, Perez AS, Witte S, et al. *In vivo* molecular imaging of acute and subacute thrombosis using a fibrin-binding magnetic resonance imaging contrast agent. *Circulation* 2004;109:2023-9.
58. Botnar RM, Buecker A, Wiethoff AJ, et al. *In vivo* magnetic resonance imaging of coronary thrombosis using a fibrin-binding molecular magnetic resonance contrast agent. *Circulation* 2004;110:1463-6.
59. Spuentrup E, Buecker A, Katoh M, et al. Molecular magnetic resonance imaging of coronary thrombosis and pulmonary emboli with a novel fibrin-targeted contrast agent. *Circulation* 2005;111:1377-82.
60. Sirol M, Fuster V, Badimon JJ, et al. Chronic thrombus detection with *in vivo* magnetic resonance imaging and a fibrin-targeted contrast agent. *Circulation* 2005;112:1594-600.
61. Spuentrup E, Fausten B, Kinzel S, et al. Molecular magnetic resonance imaging of atrial clots in a swine model. *Circulation* 2005;112:396-9.
62. Spuentrup E, Katoh M, Buecker A, et al. Molecular MR Imaging of Human Thrombi in a Swine Model of Pulmonary Embolism Using a Fibrin-Specific Contrast Agent. *Invest Radiol* 2007;42:586-595.

63. Stracke CP, Katoh M, Wiethoff AJ, Parsons EC, Spangenberg P, Spuntrup E. Molecular MRI of cerebral venous sinus thrombosis using a new fibrin-specific MR contrast agent. *Stroke* 2007;38:1476-81.
64. Spuntrup E, Botnar RM, Wiethoff AJ, et al. MR imaging of thrombi using EP-2104R, a fibrin-specific contrast agent: initial results in patients. *Eur Radiol* 2008; 18:1995-2005
65. Fibrinolytic Therapy Trialists' (FTT) Collaborative Group. Indications for fibrinolytic therapy in suspected acute myocardial infarction: collaborative overview of early mortality and major morbidity results from all randomised trials of more than 1000 patients. *Lancet* 1994;343:311-22.
66. Jaffer FA, Tung CH, Wykrzykowska JJ, et al. Molecular imaging of factor XIIIa activity in thrombosis using a novel, near-infrared fluorescent contrast agent that covalently links to thrombi. *Circulation* 2004;110:170-6.
67. Kim DE, Schellingerhout D, Jaffer FA, Weissleder R, Tung CH. Near-infrared fluorescent imaging of cerebral thrombi and blood-brain barrier disruption in a mouse model of cerebral venous sinus thrombosis. *J Cereb Blood Flow Metab* 2005;25:226-33.
68. Lange RA, Hillis LD. Reperfusion therapy in acute myocardial infarction. *N Engl J Med* 2002;346:954-5.
69. Goldstein LB. Acute ischemic stroke treatment in 2007. *Circulation* 2007;116:1504-14.
70. Cappendijk VC, Cleutjens KB, Heeneman S, et al. *In vivo* detection of hemorrhage in human atherosclerotic plaques with magnetic resonance imaging. *J Magn Reson Imaging* 2004;20:105-10.
71. Morcos SK, Thomsen HS. Nephrogenic systemic fibrosis: more questions and some answers. *Nephron Clin Pract* 2008;110:c24-31.

CHAPTER 8

Summary

Samenvatting

Dankwoord

Curriculum Vitae

List of publications

Summary

Atherosclerosis is a chronic inflammatory disease of the arterial vessel wall which starts already at young age and progresses during ageing. Eventually, thrombus formation can occur due to rupture or erosion of atherosclerotic lesions, which can lead to life-threatening events such as myocardial infarction and stroke. This indicates the importance of proper detection and assessment of atherosclerotic lesions and thrombus formation. We hypothesized that molecular imaging can further improve the assessment of the phenotype of the atherosclerotic plaque and thrombus formation. Molecular imaging is a relatively young and rapidly developing research field that uses imaging agents to detect specific disease-related targets. In the present thesis, the potential of a FDA approved MRI contrast agent and several newly developed imaging agents for the assessment of atherosclerosis and thrombus formation were investigated. Magnetic resonance imaging (MRI) and positron emission tomography (PET) were used as imaging modalities. In addition, two-photon laser-scanning microscopy (TPLSM) was used for the validation on sub-cellular level.

Development and validation of novel imaging technologies to assist translational studies in atherosclerosis

Chapter 2 presents a literature review, summarizing different imaging technologies that can be used for invasive and non-invasive detection of atherosclerotic lesions such as: ultrasound, computed tomography (CT), positron emission tomography (PET), single photon emission tomography (SPECT), magnetic resonance imaging (MRI), angiography, optical computed tomography, and thermography. Additionally, it provides an overview of molecular imaging agents that have been used to detect specific features of plaque destabilization, such as inflammation, macrophage activation, angiogenesis, lipid accumulation, and thrombus formation. For non-invasive molecular imaging, MRI and PET are very promising modalities since they allow imaging with high spatial resolution and high sensitivity, respectively.

Contrast-enhanced magnetic resonance vessel wall imaging in a rabbit model of atherosclerosis: comparison of Gadofosveset and Gd-DTPA

Since albumin is abundant in atherosclerotic lesions, the potential of the albumin-binding contrast agent Gadofosveset (also published under the name Vasovist®) for contrast-enhanced magnetic resonance imaging of atherosclerotic lesions was examined in **chapter 3**. For this study, Gadofosveset (0.05 mmol/kg) was administered to normal rabbits and to rabbits with atherosclerotic lesions in the abdominal aorta. Gadofosveset was compared with the clinically available Gd-DTPA

(0.5 mmol/kg). A different dose was used to correct for the higher r_1 relaxivity of Gadofosveset when bound to albumin. After pre- and postcontrast MRI, the animals were sacrificed and presence of microvessels and albumin content were determined in the abdominal aorta. Finally, proton induced x-ray emission (PIXE)-analysis was performed on a few specimens to determine the amount and distribution of Gadofosveset and Gd-DTPA within the vessel walls.

It was demonstrated that the use of Gadofosveset resulted in increased MRI signal intensities of atherosclerotic versus normal vessel walls, while Gd-DTPA did not discriminate between rabbits with atherosclerotic lesions and normal rabbits. Additionally, albumin content and microvessel density increased in the vascular wall of atherosclerotic rabbits. PIXE analysis demonstrated that the atherosclerotic and normal vessel walls contained equal amounts of Gadofosveset and Gd-DTPA. When corrected for dose and clearance, the gadolinium content was similar in the different study groups. We hypothesized that binding of Gadofosveset to intraplaque albumin resulted in a drastic relaxivity increase, leading to signal amplification. The data suggest that Gadofosveset has potential for imaging of atherosclerosis.

Molecular imaging of murine atherosclerosis using RANTES-based imaging agents: a feasibility study

Chapter 4 presents a feasibility study in which a novel molecular imaging agent was designed and validated. In this study, mRNA analysis and immunohistochemistry demonstrated strong upregulation of CCR5 mRNA and protein expression levels in ruptured compared to stable human carotid lesions. Therefore, newly developed CCR5-targeting imaging agents were investigated for their potential in the assessment of atherosclerotic lesions. Three RANTES-based (CCR5 ligand) imaging agents were synthesized: RANTES-rhodamine (for TPLSM), RANTES-DTPA for capturing gallium-68 (for PET imaging), and bimodal-RANTES containing rhodamine and the DTPA-chelate (for TPLSM and PET). Uptake of RANTES-rhodamine and RANTES-DTPA was evaluated *in vitro* using murine bone marrow-derived macrophages (BMMs) and CCR5 negative control cells (3T3 cells). Additionally, RANTES-based agents were intravenously administered to ApoE^{-/-} mice to determine the *in vivo* uptake of RANTES-rhodamine and bimodal-RANTES in atherosclerotic lesions. After sacrificing the animals, the excised atherosclerotic lesions were imaged with TPLSM and microPET.

TPLSM demonstrated internalization of RANTES-rhodamine in BMMs. A significant increase of RANTES-DTPA-⁶⁸Ga uptake was found in BMMs compared to control 3T3 cells. Intravenously administered RANTES-rhodamine and bimodal-RANTES in ApoE^{-/-} mice and subsequent visualization with TPLSM demonstrated that both imaging agents internalized in macrophages present in the

atherosclerotic lesions. Additionally, microPET using the bimodal-RANTES agent showed increased activity in atherosclerotic murine lesions compared to vessels of non-atherosclerotic C57BL6 mice. These findings indicate the potential of RANTES-based imaging agents for the assessment of atherosclerotic lesions. However, additional experiments are warranted to validate these initial results.

Optical and magnetic resonance imaging of cell death and platelet activation using Annexin A5-functionalized quantum dots

In **chapter 5**, annexin A5-functionalized quantum dot-based nanoparticles were used for the detection of phosphatidylserine (PS) with MRI and TPLSM. PS is expressed on apoptotic cells. Since apoptotic cells contribute to the formation of the necrotic core and weakening of the overlying fibrous cap, this annexin A5-functionalized nanoparticle could aid in the assessment of atherosclerosis. In addition, this nanoparticle might be useful for the detection of thrombus formation as PS is also expressed on activated platelets. To validate the annexin A5-functionalized nanoparticles, apoptotic Jurkat cells and *in vitro* generated thrombi were incubated with the nanoparticles and subsequently visualized with MRI and TPLSM. To demonstrate its feasibility as a target specific contrast agent for the detection of cell death in the vascular wall, a murine carotid artery was damaged using a metal wire, inducing rapid PS exposure. After sacrificing the mouse, the damaged and undamaged carotid arteries were excised and exposed to the annexin A5-functionalized quantum dot-based nanoparticles and imaged with TPLSM and MRI.

Increased MR signal intensities were found in the apoptotic Jurkat cells and the *in vitro* generated thrombi exposed to annexin A5-functionalized nanoparticles. TPLSM demonstrated that the nanoparticles bound to the apoptotic Jurkat cells and the activated platelets. In addition, TPLSM demonstrated that the nanoparticles also bound to PS-expressing cells in the wire-injured carotid artery. Slightly higher MR signal intensities were found in the wire-injured carotid artery compared to the undamaged control artery. These nanoparticles demonstrated their potential for the detection of apoptotic cells and activated platelets using MRI and TPLSM. Therefore, this nanoparticle is a promising bimodal contrast agent for future *in vivo* imaging studies.

Molecular MRI of early thrombus formation using a bimodal α_2 -antiplasmin-based contrast agent

In **chapter 6**, the potential of a novel fibrin targeted contrast agent for the detection of early thrombus formation was investigated. Specific detection of early thrombus formation is still challenging in clinical practice and might aid in the

selection of patients that are eligible for fibrinolytic therapy as the resistance of thrombi against fibrinolysis increases with time. Since activated factor XIII crosslinks α_2 -antiplasmin to fibrin during the early phases of thrombus formation, a bimodal α_2 -antiplasmin-based contrast agent and control agent (1 amino acid substitution) were synthesized. *In vitro* generated thrombi were incubated with the contrast agents and subsequently imaged with TPLSM and MRI. To check the presence of factor XIII and α_2 -antiplasmin in different stages of thrombus formation, immunohistochemical stainings were performed on sections of human pulmonary thrombo-emboli. In addition, the bimodal α_2 -antiplasmin-based contrast agents were administered (5.0 $\mu\text{mol/kg}$) to mice containing fresh or 24-48 hours old (iron chloride-induced) thrombi in the carotid artery. After non-invasive MRI, mice were sacrificed and the excised carotid arteries were imaged with TPLSM.

In the *in vitro* generated thrombi that were exposed to the α_2 -antiplasmin-based contrast agent, hyperintense MR signal intensities were found at the edge of the thrombus while no hyperintense signal was observed using the control contrast agent. TPLSM demonstrated that the α_2 -antiplasmin-based contrast agent bound to fibrin. In the human pulmonary thrombo-emboli sections, strong α_2 -antiplasmin immunohistochemical staining was found in fresh compared to lytic and organized thrombi. *In vivo* contrast agent administration to mice containing fresh thrombi resulted in increased contrast-to-noise ratios at the site of thrombus formation compared to the use of the control contrast agent and the α_2 -antiplasmin-based contrast agent administration 24-48 hours after thrombus formation. These findings demonstrate that non-invasive MR visualization of early thrombus formation is feasible using the α_2 -antiplasmin-based contrast agent.

Discussion and conclusion

In **chapter 7**, the findings and implications of this thesis were discussed in relation to the available literature about molecular imaging of atherosclerosis and thrombus formation.

In conclusion, the potential of the FDA-approved contrast agent Gadofosveset and several newly developed molecular imaging agents for the assessment of atherosclerosis and thrombus formation were investigated and validated in this thesis.

Nederlandse samenvatting

Atherosclerose, in de volksmond beter bekend als aderverkalking, is een chronische aandoening van de vaatwand van slagaders die al op jonge leeftijd kan beginnen en zich in de jaren daarna verder ontwikkelt. In een bepaald stadium van plaque ontwikkeling, kan de plaque scheuren (ruptureren), waardoor er bloedstolsels (trombi) gevormd kunnen worden. Plaques met een hoog risico op ruptureren worden ook wel instabiele plaques genoemd. De gevormde trombi kunnen levensgevaarlijk zijn, aangezien ze kunnen leiden tot een hart- of herseninfarct (beroerte). Om deze reden is het van groot belang om atherosclerotische plaques en trombusvorming zo goed mogelijk in beeld te brengen. Contrastmiddelen waarmee specifieke moleculen of processen ("molecular imaging") in beeld gebracht kunnen worden, kunnen de beoordeling van atherosclerose en trombusvorming mogelijkwijs verbeteren. Molecular imaging is een relatief jong onderzoeksveld dat zich snel ontwikkelt. In dit proefschrift zal de mogelijkheid voor het in beeld brengen en beoordelen van atherosclerotische plaques en trombusvorming van een reeds door de "Food and Drug Administration" (FDA) goedgekeurd Magnetische Resonantie Imaging (MRI) contrastmiddel en een aantal nieuw ontwikkelde moleculaire contrastmiddelen worden onderzocht. MRI en Positron Emissie Tomografie (PET) zijn hierbij gebruikt als afbeeldingstechnieken. Tevens is fluorescentie microscopie (twee-foton laser-scanning microscopie; TPLSM) gebruikt om de locatie van de gebruikte contrastmiddelen te controleren op microscopisch niveau.

Ontwikkeling en validatie van nieuwe technieken voor het afbeelden van atherosclerose

Hoofdstuk 2 is een literatuuroverzicht over de verschillende afbeeldingstechnieken die gebruikt kunnen worden voor het afbeelden van atherosclerotische plaques zoals: "ultrasound", "computed tomography", PET, "single photon emission computed tomography" (SPECT), MRI, angioscopie, "optical computed tomography", en thermografie. Tevens bevat dit hoofdstuk een overzicht van moleculaire contrastmiddelen die gebruikt zijn voor het detecteren van processen die een rol spelen in de vorming en ruptuur van risicovolle plaques zoals ontsteking, activatie van ontstekingscellen, de vorming van nieuwe vaatjes, stapeling van vetten en trombusvorming. Voor het afbeelden van atherosclerotische plaques zijn MRI en PET veelbelovende afbeeldingstechnieken vanwege respectievelijk hun vermogen om kleine details af te beelden en hun gevoeligheid.

Contrast versterkte MRI van de atherosclerotische vaatwand in een konijnenmodel: vergelijking tussen Gadofosveset en Gd-DTPA

Aangezien het plasma-eiwit albumine veel voorkomt in atherosclerotische plaques is in **hoofdstuk 3** de toegevoegde waarde van het aan albumine bindende contrastmiddel Gadofosveset (ook gepubliceerd onder de naam Vasovist®) voor het afbeelden van atherosclerotische plaques met MRI onderzocht. Voor deze studie is Gadofosveset (0.05 mmol/kg) toegediend aan gezonde konijnen en konijnen met atherosclerotische plaques in de buikslagader. Gadofosveset is vergeleken met het in de kliniek gebruikte contrastmiddel Gd-DTPA (0.5 mmol/kg). Nadat er zowel vóór als ná contrasttoediening MRI opnames waren gemaakt werd de buikslagader van de konijnen gebruikt voor verdere analyse. Er is onderzocht of er albumine en kleine bloedvaatjes aanwezig waren in de vaatwand. Tevens zijn er analyses verricht op de uitgenomen buikslagaders met behulp van de zogenaamde “proton induced x-ray emission” (PIXE) techniek om de hoeveelheid en verdeling van Gadofosveset en Gd-DTPA in de vaatwand te bepalen. Deze studie liet zien dat het contrastmiddel Gadofosveset een verhoogd MRI-signaal veroorzaakte in de atherosclerotische vaatwand vergeleken met de normale gezonde vaatwand, terwijl dit met Gd-DTPA niet mogelijk was. Het bleek dat in de vaatwand van de atherosclerotische konijnen de hoeveelheid albumine groter was dan in de vaatwand van de gezonde konijnen. Bovendien waren er meer kleine bloedvaatjes aanwezig in de vaatwand van de atherosclerotische konijnen. Tevens is aangetoond dat de atherosclerotische en normale vaatwanden gelijke hoeveelheden Gadofosveset bevatten. Ook werden er gelijke hoeveelheden Gd-DTPA teruggevonden in de atherosclerotische en normale vaten. Wanneer de gevonden hoeveelheden gecorrigeerd worden voor de toegediende dosis contrastmiddel en de klaring hiervan, bleken de gadolinium hoeveelheden in de verschillende studiegroepen vergelijkbaar te zijn. Het verhoogde MRI-signaal na Gadofosveset, maar niet na Gd-DTPA toediening kan meest waarschijnlijk verklaard worden door de aanwezigheid van albumine in de atherosclerotische plaques. Binding van Gadofosveset aan albumine leidt namelijk tot een sterk verhoogd MRI-signaal. Samenvattend betekenen deze resultaten dat het contrastmiddel Gadofosveset een veelbelovend contrastmiddel is voor de afbeelding van atherosclerotische plaques.

Molecular imaging van atherosclerose waarbij gebruik gemaakt wordt van contrastmiddelen die zijn gebaseerd op RANTES

Hoofdstuk 4 is een studie waarin een aantal belangrijke stappen staan beschreven die nodig zijn voor de ontwikkeling en validatie van een nieuwe moleculaire tracer/probe. In dit hoofdstuk is aangetoond dat de chemokine receptor 5 (CCR5)

meer aanwezig is in geruptureerde atherosclerotische plaques vergeleken met niet geruptureerde atherosclerotische plaques. Om deze reden hebben we een moleculaire tracer/probe gemaakt die gebaseerd is op een eiwit dat aan CCR5 bindt. Dit eiwit heet RANTES. De mogelijkheden van dit contrastmiddel om atherosclerotische plaques aan te tonen zijn in dit hoofdstuk onderzocht. In totaal zijn drie op RANTES gebaseerde deeltjes gemaakt: 1) RANTES-rhodamine, rhodamine is een fluorescerend deeltje waardoor visualisatie met fluorescentie microscopie (TPLSM) mogelijk is. 2) RANTES-DTPA-Gallium, als DTPA aan RANTES wordt toegevoegd is het vervolgens mogelijk om een isotoop die zichtbaar is met PET, namelijk gallium-68, te koppelen aan het RANTES. 3) Bimodaal-RANTES, doordat dit middel zowel rhodamine als het DTPA bevat (voor de koppeling van gallium-68), kan dit deeltje zichtbaar gemaakt worden met zowel TPLSM als PET (dit wordt bimodaliteit genoemd). De opname van RANTES-rhodamine en RANTES-DTPA in cellen buiten het lichaam is onderzocht door gebruik te maken van cellen die CCR5 positief zijn en controlecellen die CCR5 negatief zijn. Tevens zijn RANTES-rhodamine en het bimodale RANTES via de bloedstroom toegediend aan muizen met atherosclerose om de opname van deze contrastmiddelen in levende wezens te onderzoeken. Vervolgens werden de uitgenomen atherosclerotische plaques afgebeeld met TPLSM en PET. TPLSM liet zien dat RANTES-rhodamine werd opgenomen in de CCR5-positieve cellen. Ook bleek dat RANTES-DTPA met gallium-68 in hogere mate werd opgenomen in de CCR5 positieve cellen in vergelijking met de CCR5-negatieve cellen. Toediening van RANTES-rhodamine en het bimodale RANTES in muizen liet met behulp van TPLSM zien dat beide contrastmiddelen werden opgenomen in de macrofagen die aanwezig waren in de atherosclerotische plaque. Tevens werd er met behulp van PET een hogere activiteit in de atherosclerotische plaque gevonden dan in de vaten van controlemuizen zonder atherosclerose. Deze resultaten laten zien dat het mogelijk is de op RANTES gebaseerde contrastmiddelen te gebruiken voor het afbeelden van atherosclerotische plaques. Echter, er moeten nog vervollexperimenten worden uitgevoerd om deze bevindingen te bevestigen.

Optische afbeelding en MRI van celdood en geactiveerde plaatjes met behulp van Annexine A5 gefunctionaliseerde quantum dots

In **hoofdstuk 5** is een ander bimodaal deeltje gebruikt dat bestaat uit een quantum dot (fluorescerend deeltje) waaraan gadolinium (contrastmiddel voor MRI) is gekoppeld. Hierdoor is het deeltje zichtbaar met TPLSM en MRI. Ook is Annexine A5 aan dit deeltje gekoppeld, waardoor het specifiek gericht is op cellen die geprogrammeerde celdood (apoptose) ondergaan. Tijdens het proces van apoptose wordt een specifiek molecuul, fosfatidylserine (PS), van de binnenkant naar de buitenkant van de cel verplaatst. Annexine A5 kan aan PS hechten. PS is ook

aanwezig aan de buitenkant van geactiveerde bloedplaatjes, die aanwezig zijn in trombi. Hierdoor zouden deze deeltjes gebruikt kunnen worden om zowel apoptotische cellen als trombi in beeld te brengen. Om dit te onderzoeken werden apoptotische cellen en buiten het lichaam gemaakte trombi blootgesteld aan dit contrastmiddel, waarna ze afgebeeld zijn met MRI en TPLSM. Tevens werd de mogelijkheid van dit contrastmiddel voor het detecteren van apoptotische cellen in de vaatwand gedemonstreerd door schade te veroorzaken aan de halsslagader van een muis, waarna de halsslagader werd blootgesteld aan het contrastmiddel. Met MRI werden verhoogde signaal intensiteiten gevonden in de cellen en trombi die blootgesteld waren aan het contrastmiddel. TPLSM liet zien dat het bimodaal deeltje bond aan de apoptotische cellen en de geactiveerde bloedplaatjes in de trombi. Tevens was er een positief signaal te zien in de beschadigde vaatwand met behulp van MRI en TPLSM. In toekomstige studies zal dit contrastmiddel in levende proefdieren moeten worden gevalideerd.

Moleculaire MRI van vroege trombusvorming met behulp van een op α_2 -antiplasmin gebaseerd bimodaal gelabeld contrastmiddel

In **hoofdstuk 6** is een nieuw contrastmiddel voor de detectie van vers gevormde trombi onderzocht. Tot op heden is de detectie van verse trombi in de klinische praktijk moeilijk uitvoerbaar. Als dit wel mogelijk zou zijn, zou het de selectie van patiënten die nog in aanmerking komen voor een therapie waarbij de gevormde trombus kan worden opgelost (fibrinolytische therapie; fibrinolyse) verbeteren. De mate waarin trombi bestand zijn tegen fibrinolyse wordt namelijk groter naarmate de trombi ouder worden. Aangezien α_2 -antiplasmin bindt aan fibrine (een bestanddeel van trombi) gedurende de vroege fase van de trombusvorming, werd een bimodaal contrastmiddel gemaakt gebaseerd op α_2 -antiplasmin. Het contrastmiddel werd bimodaal gemaakt door een fluorescerend deeltje (rhodamine) en een contrastmiddel voor MRI (gadolinium) hieraan te binden. Tevens werd een controle contrastmiddel gemaakt dat geen binding kon aangaan met fibrine. Om dit contrastmiddel te testen werden buiten het lichaam gevormde trombi blootgesteld aan beide contrastmiddelen en vervolgens afgebeeld met MRI en TPLSM. De aanwezigheid van α_2 -antiplasmin in de verschillende stadia van de trombusvorming werd gecontroleerd aan de hand van kleuringen van trombi afkomstig uit mensen. Bovendien werd trombusvorming opgewekt in de halsslagaders van een aantal muizen, waarna het op α_2 -antiplasmin gebaseerde contrastmiddel via de bloedstroom werd toegediend (5.0 $\mu\text{mol/kg}$) direct na (verse trombus) en 24-48 uur (oude trombus) na het opwekken van trombusvorming. Na afbeelding met MRI werd de halsslagader van de muis bekeken met TPLSM. Een verhoogd MRI-sigitaal werd teruggevonden aan de randen van buiten het lichaam gevormde trombi die blootgesteld waren aan het op α_2 -antiplasmin gebaseerde

contrastmiddel. TPLSM van deze trombi toonde aan dat het op α_2 -antiplasmin gebaseerde contrastmiddel aan het in de trombi aanwezige fibrine bond. De kleuringen van trombi afkomstig van mensen toonde aan dat α_2 -antiplasmin voornamelijk aanwezig was in verse trombi vergeleken met oudere trombi. Toediening van het op α_2 -antiplasmin gebaseerde contrastmiddel aan muizen met een verse trombus resulteerde in een verhoogd MRI-sigitaal op de plaats waar de trombusvorming was opgewekt in vergelijking met de 24-48 uur oude trombi. De behaalde resultaten tonen aan dat de detectie van vroege trombusvorming mogelijk is met behulp van het op α_2 -antiplasmin gebaseerde contrastmiddel.

Discussie en conclusie

In **hoofdstuk 7** zijn de resultaten van dit proefschrift bediscussieerd en vergeleken met de aanwezige literatuur over moleculaire afbeeldingstechnieken van atherosclerose en trombusvorming.

Geconcludeerd kan worden dat het contrastmiddel Gadofosveset en de verschillende nieuwe moleculaire contrastmiddelen perspectieven bieden voor het in beeld brengen en beoordelen van atherosclerotische plaques en trombusvorming.

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List of publications

Miserus R.J.J.H.M, Heeneman S, van Engelshoven J.M.A, Kooi M.E, Daemen M.J.A.P. Development and Validation of Novel Imaging Technologies to Assist Translational Studies in Atherosclerosis. *Drug Discovery Today: Technologies* (2006);3:195-204.

Prinzen L, **Miserus R.J.J.H.M**, Dirksen A, Hackeng T.M, Deckers N, Bitsch N, Megens R.T.A, Douma K, Heemskerk J.W, Kooi M.E, Frederik P.M, Slaaf D.W, van Zandvoort M.A.M.J, Reutelingsperger C.P.M. Optical and Magnetic Resonance Imaging of Cell Death and Platelet Activation using Annexin A5-functionalized Quantum Dots. *Nanoletters* (2007);7:93-100.

Lobbes M.B.I, **Miserus R.J.J.H.M**, Heeneman S, Lima Passos V, Mutsaers P.H.A, Debernardi N, Misselwitz, Post M, Daemen M.J.A.P, Van Engelshoven J.M.A, Leiner T, Kooi M.E. Atherosclerosis: Contrast-enhanced MR imaging of vessel wall in rabbit model: comparison of Gadofosveset and Gadopentetate Dimeglumine. *Radiology* (2009);250:682-691.

Miserus R.J.J.H.M, Herías M.V*, Prinzen L*, Lobbes M.B.I, van Suylen R.J, Dirksen A, Hackeng T.M, Heemskerk J.W.M, van Engelshoven J.M.A, Daemen M.J.A.P, van Zandvoort M.A.M.J, Heeneman S, Kooi M.E. Molecular MRI of early thrombus formation using a bimodal α_2 -antiplasmin-based contrast agent. *J Am Coll Cardiol Img* (2009);2:987-996.

Miserus R.J.J.H.M, Suylen D, Herías M.V, Prinzen L, Dirksen A, van Zandvoort M.A.M.J, Biessen E.A.L, Vermaelen P, van Engelshoven J.M.A, Daemen M.J.A.P, Dawson P.E, Hackeng T.M, Kooi M.E*, Heeneman S*. Molecular imaging of murine atherosclerosis using RANTES-based imaging agents: A feasibility study. *In preparation*.

* Authors contributed equally.

CHAPTER 9

Full color figures

Chapter 2: full color figures

Figure 2.1: Transverse MR images and corresponding histologic slice obtained at the same carotid artery level. Weightings included T_1 -weighted turbo field echo (TFE) and intermediate-weighted, T_2 -weighted, T_1 -weighted double-inversion-recovery black-blood technique, and partial T_2 -weighted turbo spin echo (TSE). Hemorrhage (h) was well differentiated from fibrous tissue (f) on the T_1 -weighted TFE image. On the T_2 -weighted TSE image, fibrous tissue had high signal intensities whereas calcification (c, arrows) had very low signal intensities. Calcification also had very low relative signal intensities on intermediate-weighted, T_1 -weighted, and partial T_2 -weighted TSE images (copyright ©Radiological Society of North America, 2005).

Figure 2.2: Schematic representation of the atherosclerotic process indicating several molecular targets. Abbreviations: ICAM, intercellular adhesion molecule; LDL, low-density lipoprotein; LRNC, lipid-rich necrotic core; MMPs, matrix metalloproteinases; oxLDL, oxidized low density lipoprotein; PS, phosphatidylserine; VCAM, vascular cell adhesion molecule.

Chapter 3: full color figures

Figure 3.2. Box plot of precontrast rSI values (A) and postcontrast signal enhancement (B) for each rabbit, clustered by groups. Abbreviation: Gadofosveset (GFV).

Figure 3.3. Observed postcontrast rSI as a function of precontrast rSI for individual rabbits according to study group. The regression lines are based on the predicted postcontrast rSI values for fixed effects only. Abbreviation: Gadofosveset (GFV).

Figure 3.4. Histological examples of the aortic vessel wall of atherosclerotic and control rabbits. (A) HE stained anatomical overview. (B) HE stained detail of vessel wall. (C) CD31 immunoreactivity of adventitial microvessels visualized with Vector Red. (D) Positive CD31 red staining of endothelium. Abbreviations: intima (int), media (m), adventitia (adv).

Figure 3.5. Immunohistochemical staining for albumin in vessel walls. Albumin is present in the atherosclerotic vessel wall lesions (A) and not in control animals (B). CD31 immunoreactive neointimal microvessels (arrows) in atherosclerotic animals might serve as a port of entry (C). Intraluminal staining is caused by albumin-containing blood remnants and demonstrates positive staining for albumin in A and B.

Figure 3.6. PIXE analysis. Hematoxylin-Eosin stained sections served as orientation point (square) for the analysis of the distribution of gadolinium. Colors are indicative of the amount of gadolinium present. Scaling of bars is in micrograms per gram dry tissue. (*) Inferior vena cava.

Chapter 4: full color figures

Figure 4.1: Immunohistochemistry stainings for CCR5 (A, B, D, and E) and macrophages (CD68) (C and F) on human atherosclerotic lesions. Stable lesions (A-C) show no or hardly any CCR5 staining while ruptured lesions (D-F) strongly express CCR5. Mean CCR5 score was significantly higher in ruptured lesions (1.4 ± 0.1) compared to stable lesions (0.7 ± 0.2) (G). Blue: haematoxylin staining. Red: CCR5 staining. Brown: macrophage staining. Bars: 100 μ m (A and D) or 50 μ m (B, C, E, and F)

Figure 4.2: Murine and human RANTES. A: Human RANTES solution NMR structure showing antiparallel β -sheet and α -helical secondary structure, and Cys¹⁰-Cys³⁴ and Cys¹¹-Cys⁵⁰ disulfide bonds. B: Murine (P30882) and human (P13501) RANTES sequence with differences indicated in red. The underlined Lys-Cys sequence represents the native chemical ligation site. A C-terminal Lys(Fmoc)⁶⁹ handle is attached as a linker to attach (multimodal) imaging probes.

Figure 4.3: Total chemical synthesis of RANTES-rhodamine. A: Strategy: deprotection of Lys(Fmoc) yielded a single amine moiety for attachment of rhodamine(label)-succinimidyl ester. B: Native Chemical Ligation between RANTES fragments proceeded through an initial thioester intermediate that spontaneously rearranged into a full peptide backbone. C: ESI-MS spectra of folded RANTES-rhodamine yielding a molecular mass of 8410.5 Da. D: Final RANTES product with a rhodamine dye attached to the C-terminus.

Figure 4.4: Immunohistochemistry (IHC) stainings for CCR5 of 3T3 fibroblast cells and bone marrow-derived macrophages (BMMs). 3T3 cells show no or hardly any CCR5 staining while BMMs with and without 4 hours of LPS stimulation, strongly express CCR5 (red). Bars: 40 μ m

Figure 4.5: Two-photon laser-scanning microscopy (TPLSM) of 3T3 control cells and bone marrow-derived macrophages (BMMs) exposed to 250 nM RANTES-rhodamine. Left: No RANTES-rhodamine uptake was detected in the 3T3 control cells. Right: Clear RANTES-Rhodamine internalization is shown in the BMMs exposed to RANTES-rhodamine (white arrows). Red: RANTES-rhodamine. Blue: nucleic acid stain using Syto 44. Green: macrophage staining using CD11b-Alexa 488.

Figure 4.6: Top left panel: Clearance of RANTES-rhodamine as measured with two-photon laser-scanning microscopy (TPLSM) on different time points after RANTES-rhodamine administration (1, 5, 10, 15, 30, 60, and 90 minutes, $n=3$). Top right panel: TPLSM examination of kidney, spleen, liver, fat, lung and intestine demonstrated that the RANTES-rhodamine predominantly accumulated in the kidney, spleen, and liver. Red: RANTES-rhodamine. Blue / white: autofluorescence. Bottom panels: Ex vivo TPLSM of a murine aortic atherosclerotic lesion demonstrating in vivo RANTES-rhodamine uptake in macrophages (white arrows) and a non-atherosclerotic part (showing mainly smooth muscle cells) of the same mouse without any RANTES-rhodamine uptake. Red: RANTES-rhodamine. Blue: nucleic acid stain using Syto 44. Green: macrophage staining using CD11b-Alexa 488.

Figure 4.7: Total chemical synthesis of RANTES-DTPA. **A:** Strategy: deprotection of Lys(Fmoc) yielded a single amine moiety for attachment of a thiaproline residue. After oxidative folding of RANTES thiaproline was converted into cysteine, and DTPA maleimide was attached. **B:** Final RANTES product with a DTPA-moiety on the C-terminus. **C:** Analytical RP-HPLC of RANTES-DTPA. RANTES was eluted with a linear gradient of 0-60% acetonitrile in water/0.1% TFA in 30 minutes. **D:** MALDI-MS spectrum of folded RANTES-DTPA yielding a molecular mass of 8764.5 Da (8100.7 Da represents unlabeled RANTES). Chelation of ^{68}Ga to RANTES-DTPA yielded a contrast agent for positron emission tomography (PET) imaging. Note that DTPA conjugate structure is enlarged in respect to RANTES structure for reasons of clarity. Label represents 7.5% of the total contrast agents' mass.

Figure 4.9: Total chemical synthesis of bimodal-RANTES **A:** Strategy: deprotection of Lys (Fmoc) yielded a single amine moiety for attachment of a levulinic acid. After oxidative folding RANTES was reacted with aminooxyacetyl-bimodal label to yield an oxime bond. **B:** RANTES with a levulinic acid on the C-terminus. **C:** MALDI-MS spectrum of folded RANTES-levulinic acid yielding a molecular mass of 8093.7 Da. **D:** Bimodal rhodamine-DTPA synthon on Lys-Cys backbone functionalized with an aminooxyacetyl group. **E:** MALDI-MS spectrum of bimodal rhodamine-DTPA synthon yielding a molecular mass of 1396.6 Da. **F:** Final bimodal RANTES imaging agent with an oxime bond between RANTES and bimodal label. **G:** MALDI-MS spectrum of bimodal RANTES-rhodamine-DTPA yielding a molecular mass of 9474.1 Da.

Figure 4.11: Bimodal-RANTES results in aged $ApoE^{-/-}$ mice ($n=2$) and wild type control mice ($n=2$). Top left: Ex vivo microPET image and photograph of the arterial tree. Top right: Hematoxylin/eosine (HE) staining and CCR5 immunohistochemistry (IHC), indicating the presence of CCR5 in the murine atherosclerotic lesions. Bars represent 100 μm (HE staining) and 300 μm (CCR5 IHC). Middle: HE staining of a thoracic part of the arterial tree without atherosclerotic lesions. This part corresponds with limited activity detected with PET. Bar represent 300 μm . Bottom left: Mean standard uptake value (SUV) increase measured in the different parts of the arterial tree. SUV values measured in 58-59 weeks aged $ApoE^{-/-}$ mice were strongly increased in the aortic arch (236% $\pm$ 95%), right carotid artery (125% $\pm$ 56%) and left carotid artery (205% $\pm$ 25%), while only a minor SUV increase was measured in the abdominal aorta (85% $\pm$ 48%). Bottom right: TPLSM confirmed the uptake of the bimodal-RANTES agent in macrophages that were present in the atherosclerotic lesions. Red: bimodal-RANTES agent. Green: macrophage staining using CD11b-Alexa 488.

Chapter 5: full color figures

Figure 5.1: Molecular structure of the biotinylated Gd-DTPA (A) and biotinylated Gd-wedge (B) structures with biotin (red) and Gd-DTPA (blue).

Figure 5.2: Schematic representation of the two nanoparticles. QDs contain approximately 1 AnxA5 and 10 streptavidin molecules. A: the nanoparticle with single biotinylated Gd-DTPA (AnxA5-QD-Gd); B: the nanoparticle with the biotinylated Gd-wedge, containing eight Gd-DTPA complexes each (AnxA5-QD-Gd-wedge). Green: QD, yellow: streptavidin, red dot: Gd-DTPA, red star: lysine-wedge, blue: AnxA5.

Figure 5.4: TPLSM images showing cellular AnxA5-QD525 (green) distribution. All cells were counterstained with PI (red) to determine membrane leakage, i.e., determine the stage of apoptosis. Top-row: early apoptotic Jurkat cells labeled with A; AnxA5-QD-Gd and B; AnxA5-QD-Gd-wedge. Bottom-row: late apoptotic cells labeled with C; AnxA5-QD-Gd and D; AnxA5-QD-Gd-wedge. Bar = 10 μ m.

Figure 5.5: TPLSM image of a Jurkat cell, incubated with AnxA5-QD525-Gd (green), while simultaneously inducing apoptosis by anti-Fas antibodies. After 5.5 hours cells were washed in EDTA buffer to remove AnxA5 present on the outside of the cells, and subsequently stained with AnxA5-QD585-Gd (red). Yellow indicates co-localization of QD525 and QD585 (probably caused by QD525 on the cell exterior that had not completely been removed). Cells were counterstained with Syto41 (blue), a nucleic acid stain. Bar = 10 μ m.

Figure 5.6: TPLSM images showing the cellular distributions of QD585 (red) coupled to AnxA5 or a mutant of AnxA5, unable to bind to PS (M1234), on apoptotic Jurkat cells. All cells were counterstained with Syto41 (blue), a nucleic acid stain. A: AnxA5-QD-Gd. B: AnxA5-QD-Gd-wedge. C: M1234-QD-Gd-wedge. Bar = 50 μ m.

Figure 5.9: TPLSM images of whole-blood clots incubated with AnxA5 after clot-formation. A; green: AnxA5-FITC, blue: syto41, a nucleic acid stain; B; green: AnxA5-QD525-Gd, red: acridine red, a cytoplasmic stain. C; red: AnxA5-QD585-Gd, green: anti-fibrinogen-FITC. Platelets show a bright cellular membrane, similar to apoptotic cells (compare to figures 5.4/5.5/5.6). Bar = 50 μm .

Figure 5.10: A; TPLSM image of a wire-injured murine carotid artery, showing the various vascular wall layers. The tunica intima consists of endothelial cells (ECs); the tunica media mainly consists of smooth muscle cells (SMCs). Uptake of nanoparticles is visible in ECs and SMCs. Green: AnxA5-QD-Gd-wedge; red: eosin, labeling elastin laminae; blue, syto41, labeling cell nuclei. L: lumen; IEL: internal elastic lamina; EEL: external elastic lamina. Bar = 10 μm . The imaging plane was slightly oblique, as opposed to a longitudinal direction of the artery. B; transversal MR image of two murine carotid arteries: undamaged (control, left) and damaged (right, same artery as shown in A). Bar = 0.5 mm.

Chapter 6: full color figures

Figure 6.1: Structure of the bimodal α_2 -antiplasmin-based contrast agent. Schematic representation of the α_2 -AP-based peptide that is bimodally labeled with rhodamine and a DTPA-chelate ($\text{Bi-}\alpha_2\text{AP-CA}$) and the corresponding mass spectra. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) shows a molecular mass of 2832.92 g/mol for the $\text{Bi-}\alpha_2\text{AP-CA}$. Substitution of only one amino acid ($\text{Q}^3 \rightarrow \text{A}^3$) results in a bimodal control CA (Bi-con-CA).

Figure 6.2: In vitro results. TPLSM and MRI results for human (A-F) and murine (G-J) thrombi incubated with the bimodal α_2 -AP-based contrast agent ($\text{Bi-}\alpha_2\text{AP-CA}$) (A-B, E-H) and the bimodal control CA (Bi-con-CA) (C-D, I-J). For TPLSM, red color originated from the rhodamine-labeled CAs. Green color indicated the fibrin network due to use of fibrinogen-Oregon green 488. Yellow color illustrated co-localization of red and green. Hyperintense MR signals were found at the edge of thrombi incubated with $\text{Bi-}\alpha_2\text{AP-CA}$ (A, G). Lower MR signal intensities were found at the edge of a thrombus exposed to dansylcadaverine and $\text{Bi-}\alpha_2\text{AP-CA}$ (E).

Figure 6.3: Immunohistochemical stainings of thrombi. A) Immunohistochemical staining for factor XIII. B) Immunohistochemical staining for α_2 -antiplasmin. Positive immunohistochemical staining is depicted in red/pink color. The immunohistochemical staining for factor XIII (scanty spots) was present throughout the whole thrombus while the immunohistochemical α_2 -antiplasmin staining was mainly depicted at the edge of the thrombus (arrows). Bars: 50 μ m.

Figure 6.4: Pulmonary thrombo-emboli sections. Middle panels: overview of thrombo-emboli, from top to bottom: Hemathoxyline/Eosin stained, immunohistochemically stained for fibrin, factor XIII, and α_2 -antiplasmin. Bars: 300 μ m. Left panels: enlargements of boxed areas indicating an organized part of the thrombo-emboli. In the enlarged FXIII stained area clear cellular FXIII staining is visible. Bars: 50 μ m. Right panels: enlargements of boxed areas indicating a fresh segment (arrows). Bars: 50 μ m.

Figure 6.5: Clearance and kidney uptake of the contrast agents. Relative fluorescence measured in blood samples decreases with time after contrast administration (A). Half-life of the CAs are 14.1 ± 8.4 minutes for the bimodal α_2 -antiplasmin-based CA (Bi- α_2 AP-CA) and 16.3 ± 4.0 minutes for the bimodal control CA (Bi-con-CA). Both CAs are mainly cleared through the kidney (B-C). Panels B-C: Red: Bimodal CA in kidney tubuli.

Figure 6.6: In vivo and ex vivo results. In vivo MRI (A-C) and ex vivo TPLSM results (E-G) of early thrombus formation and 24-48 hour old thrombi in murine carotid arteries after injection of the bimodal α_2 -antiplasmin-based CA (Bi- α_2 AP-CA) (A, C, E, G) and the bimodal control CA (Bi-con-CA) (B, F). Contrast-to-noise ratios (CNR) are elevated in the early phases of thrombus formation after Bi- α_2 AP-CA administration (D). Panels E-G: Red: Bimodal CA. Green: Nuclear stain. Lumen is located between dotted lines. Blue arrows indicate leukocytes trapped in the thrombus. Elongated cells are smooth muscle cells (white arrows) and endothelial cells (red arrow).