In vivo molecular imaging of vascular stress

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Abstract Noninvasive in vivo imaging is an emerging specialty in experimental radiology aiming at developing hardware and appropriate contrast agents to visualize the molecular basis and pathophysiological processes of many pathological conditions, including atherosclerosis. The list of potentially useful tracers and targets for in vivo molecular imaging in the cascade of early atherosclerotic events has been narrowed down to some very promising endothelial factors, i.e., cell adhesion molecules, macrophages, apoptosis, lipoproteins, heat shock proteins, and others. In this review, we will update on the progress of recent developments in the field of noninvasive molecular imaging in experimental atherosclerosis.

Keywords Atherosclerosis · In vivo · Molecular imaging · Radiology · Vascular stress

Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ARMY	Atherosclerosis risk factors in male youngsters
CD	Cluster of differentiation

CEST	Chemical exchange saturation transfer
CT	Computed tomography
CVD	Cardiovascular disease
ELAM	Endothelial lymphocyte adhesion molecule
¹⁸ FDG	¹⁸ Fluorodeoxyglucose
Gd	Gadolinium
GLUT-1	Glucose transporter-1
HDL	High-density lipoprotein
HSP	Heat shock protein
ICAM	Intracellular adhesion molecule
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
Mab	Monoclonal antibody
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
MMP	Matrixmetalloproteinase
MRI	Magnetic resonance imaging
OxLDL	Oxidized low-density lipoprotein
PARACEST	Paramagnetic chemical exchange saturation transfer
PET	Positron emission tomography
RGD	Arginine-glycine-aspartic acid
SMC	Smooth muscle cell
SPECT	Single photon emission tomography
SPIO	Superparamagnetic iron oxide
SRA	Scavenger receptor A
Tc	Technetium
TCR	T-cell receptor
TLR	Toll-like receptor
TNP	Trireporter nanoparticles
US	Ultrasound
USPIO	Ultrasmall superparamagnetic iron oxide
VCAM	Vascular cell adhesion molecule

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Molecular and cellular mechanisms in the autoimmune hypothesis of atherogenesis

Cardiovascular diseases (CVD) cause approximately 40% of deaths in Western industrialized countries (<http://epp.eurostat.cec.eu.int>). Of these, atherosclerosis is considered a paradigmatic age-associated disease, and a preponderance of research in this area has focused on the elderly, as atherosclerosis progresses slowly and manifests clinically significant symptoms primarily in this segment of the population. However, more recent evidence suggests that the atherosclerotic process begins at a much earlier age and thus may be accessible to early diagnostic approaches and therapeutic interventions (Akerblom et al. 1991; Berenson et al. 1998; Knoflach et al. 2003a; Millonig et al. 2002).

It is now common knowledge that inflammatory–immunological processes play a major role during atherogenesis (Hansson 2005), but most of the available data on the in situ situation in atherosclerotic lesions are derived from analyses of advanced stages, i.e., atherosclerotic plaques in surgically excised specimens.

During the past 15 years, a new “autoimmune hypothesis” for atherogenesis has been developed in our laboratory supported by solid experimental animal and clinical data (Wick et al. 2004). This hypothesis is based on observations that early stages of atherosclerosis are of an immunological–inflammatory nature, often still without the presence of foam cells, and represented at young age as arterial intimal infiltration with mononuclear cells at known predilection sites, i.e., primarily at areas of arterial branching (Gerrity 1981; Millonig et al. 2001; Schwartz et al. 1985; Xu et al. 1990).

The exact initiating factors of prodromic atherosclerotic wall changes have not yet been fully elucidated, but have been narrowed down to the effect of well-known classical risk factors. Notwithstanding the proven importance of these factors, the autoimmune hypothesis of atherogenesis postulates that they first act as endothelial stress factors when individuals still appear clinically healthy. Thus, in vitro and in vivo experiments provided evidence that cigarette smoke (Bernhard et al. 2003), proinflammatory cytokines (Amberger et al. 1997), and mechanical stress in vitro or high blood pressure in vivo (Hochleitner et al. 2000) are among the risk factors that lead to the expression of pro-atherogenic stress proteins, called heat shock proteins (HSPs), as well as various pro-inflammatory mediators (Frostegard et al. 1999). HSPs are ubiquitous and structurally highly conserved molecules (Young and Elliott 1989) that have important physiological functions in intracellular protein assembly, folding, and transport as well as the prevention of protein aggregation and misfolding upon stress (chaperone function; Benjamin and McMillan 1998). HSPs also play important roles in a number of diseases,

including cancer and autoimmune diseases (Hightower 1991; Snoeckx et al. 2001; Young and Elliott 1989).

There is increasing evidence that, in atherosclerosis, as an immune-mediated disease, members of the HSP families may be the main antigens triggering the anti-vascular immune response (Xu et al. 1992; Xu and Wick 1996). In particular, microbial HSP60 is a major antigen recognized by the innate and adaptive immune systems, e.g., during bacterial or parasite infections. As HSP60 is phylogenetically highly conserved, the extensive sequence homology between microbial and human HSP60 may result in a cross-reactive immune response against the latter, when it is produced by arterial endothelial cells stressed by classical atherosclerosis risk factors (Wick et al. 2004; Young and Elliott 1989). HSP60 is a mitochondrial protein with a chaperone function that is also transported to the cell surface, creating a target for an immunological attack. The presence of HSP60 on the endothelial cell surface was demonstrated both by immunohistological (Xu et al. 1994) and metabolic (Soltys and Gupta 1997, 2000) labeling techniques. The immune system recognizes HSP60 as a “danger signal” on stressed endothelial cells resulting in their destruction via preexisting innate and adaptive cellular and humoral immune reactions. HSP60 also circulates in the blood (soluble HSP60–sHSP60), probably derived from necrotic endothelial cells (Xu et al. 2000). It should be emphasized that HSP60 is not only produced by endothelial cells themselves, but sHSP60 can also bind to the endothelial cell surface, e.g., via Toll-like receptors (TLRs), specifically TLR-4 in combination with CD14 as well as TLR-2 in association with TLR-6 (Bulut et al. 2002; Habich et al. 2002; Kol et al. 1999). Both anti-HSP60 antibodies and T-cells may detect HSP60 on the endothelial cell surface (Seitz et al. 1996), the latter in a major histocompatibility complex (MHC)-restricted fashion (α/β T-cell receptor positive cells— α/β TCR⁺ cells) or in a non-MHC restricted fashion (γ/δ TCR⁺ cells). Stressed, but not unstressed, endothelial cells are lysed by anti-HSP60 antibodies via complement activation or antibody-dependent cellular cytotoxicity (ADCC). This is true both for purified antibodies from patients with atherosclerosis and monoclonal murine antibodies to eukaryotic HSP60 (Mayr et al. 1999; Schett et al. 1995).

As the lifelong infectious load has been shown to correlate significantly with the risk of developing atherosclerosis (Mayr et al. 2000; Ridker et al. 2000), bacterial toxins may be taken as surrogate endothelial stressors (Mayr et al. 1999). Injection of bacterial lipopolysaccharide (LPS) has already been shown to be a potent inducer of the simultaneous expression of HSP60 and intracellular adhesion molecule-1 (ICAM-1) in rats (Seitz et al. 1996). As mentioned, it has been demonstrated that classical atherosclerosis risk factors also lead to the simultaneous expres-

sion of adhesion molecules (ICAM-1, endothelial lymphocyte adhesion molecule-1=ELAM-1, vascular cell adhesion molecule-1=VCAM-1; Cybulsky and Gimbrone 1991; Iiyama et al. 1999; Libby 2002) and HSP60 (Amberger et al. 1997; Seitz et al. 1996). This is important because interaction of HSP60-specific T cells with the endothelial target is only possible when appropriate adhesion molecules have also been expressed. The invasion of mononuclear effector cells from the arterial lumen (lymphocytes and macrophages) as well as smooth muscle cells (SMC) from the media into the vascular intima represents the beginning of the early inflammatory stage of atherosclerosis (Xu et al. 1990). The Atherosclerosis Risk-Factors in Male Youngsters (ARMY) Study in 17- to 18-year-old clinically healthy men in Austria showed that sonographically demonstrable early atherosclerotic changes correlated significantly to the presence of HSP60-specific T cells and anti-HSP60 antibodies in the peripheral blood (Knoflach et al. 2003a). A similar analysis in 50- to 60-year-old men within the framework of the prospective Bruneck Study showed a correlation of HSP60 antibodies with the presence and extent of sonographically demonstrable atherosclerotic lesions, but no such correlation emerged with respect to HSP60-specific T cells in the peripheral blood (Knoflach et al. 2007).

In the past, the major focus of atherosclerosis research was on lipid metabolism alterations (Glass and Witztum 2001; Ross 1993, 1999; Smilde et al. 2001; Steinberg et al. 1989), but recently, inflammatory-immunologic processes during atherogenesis have also received major attention (Hansson 2005; Libby 2002; Wick et al. 2004). Most of this work, however, dealt with the conditions of well-developed lesions with clinically apparent symptoms, such as myocardial infarction, stroke, or peripheral arterial occlusion. In addition, most experimental studies relied on the use of transgenic or knockout mouse models that mimic special hereditary situations in humans, such as functional hypercholesterolemia due to a deficiency of low-density lipoprotein (LDL)-receptor expression (Zhang et al. 1992). In contrast, our interest is focused on the very earliest stages of the disease comprised of mononuclear cell infiltration of the intima in normocholesterolemic humans or experimental animals even in the absence of foam cells (Millonig et al. 2001; Xu et al. 1990).

From these and other data, we concluded that HSP60 expression by endothelial cells is an initiating event that renders them a target for attack by preexisting, in principle protecting, innate and adaptive immunity. Arterial endothelial cells have a lower threshold for HSP60-inducing risk factors as a consequence of lifelong arterial blood pressure compared to venous endothelial cells. Interestingly, endothelial HSP60 expression and mononuclear intima-infiltration are also initial hallmarks of restenosis in coronary and

carotid bypass conduits prone to restenosis (Knoflach et al. 2003b).

Molecular imaging of endothelial stress

As the atherosclerotic process begins at an early age, the prolonged course of disease provides a “window of opportunity” for in vivo diagnosis of disease stages before clinical manifestations, as well as the opportunity for early, specifically targeted, therapeutic interventions.

Novel technological developments in the last decade have advanced beyond “classical” radiological techniques to allow imaging of cardiovascular anatomy and physiology on a macroscopic scale, making it possible to image atherogenesis in vivo on the cellular and sometimes even molecular level (Davies et al. 2005a, 2006; Dobrucki and Sinusas 2005; Jaffer et al. 2006a; Jaffer and Weissleder 2004; Lindner 2004a, b; Strauss et al. 2004; Tsimikas 2002; Tsimikas and Shaw 2002; Wickline and Lanza 2003). Noninvasive in vivo imaging is a fast emerging specialty in experimental radiology aiming at developing imaging modalities and appropriate imaging agents to visualize the molecular basis and pathophysiological processes of many pathological conditions, including cardiovascular diseases.

The main difference between the routine application of clinical imaging techniques, such as computed tomography (CT) or magnetic resonance imaging (MRI) and molecular imaging, is that the former primarily focus on assessing anatomical and structural components of atherosclerosis, while the latter aims to visualize the molecular, functional, and pathophysiological process of cardiovascular diseases before the development of clinically overt symptoms. Several serological markers of atherosclerosis, including pro-inflammatory cytokines and vascular stress proteins, are known predictors for, and/or diagnostic biomarkers of, cardiovascular disease and are accepted for both routine and experimental use to monitor patients at risk or after manifestation of cardiovascular symptoms (Danesh et al. 1997; Ridker et al. 2000).

In a prospective context, the development of noninvasive imaging based on the visualization of biologically active markers could be equally important for diagnostic and therapeutic viewpoints, viz: (a) noninvasive diagnostic in vivo imaging of atherosclerosis in patients at risk before the appearance of clinically apparent peripheral arterial-occlusive disease; (b) noninvasive diagnostic in vivo imaging of atherosclerosis in at risk patients in whom classical radiological investigations were inconclusive; (c) using the immunological antigenic function of endothelial cells to monitor possible future medicinal or molecular biological methods for targeted anti-atherosclerotic therapies; (d) providing reproducible well-tolerated noninvasive imaging for

initial and/or follow-up biomarkers in longitudinal studies or clinical trials of novel anti-atherosclerotic treatments.

Numerous serological factors are known to be involved in the pathophysiology of atherosclerosis, but not all proved useful targets for molecular imaging techniques. The list of potentially useful factors in the cascade of events occurring in atherosclerosis has been narrowed down to some very promising endothelial targets, shown in the context of the immunological hypothesis of atherogenesis in Fig. 1.

Imaging hardware for in vivo visualization of atherosclerosis

The choice of the imaging platform for an experimental imaging study depends on several factors, including sensitivity, spatial and temporal resolution, depth of tissue penetration, radiation, costs, examination time, and availability at a given research facility. As assessing an atherosclerotic lesion for vascular protein expression represents a much smaller target than, for example, a solid

static tumor, vascular in vivo imaging requires highly sensitive and high-resolution strategies that must overcome cardiac and respiratory motion and blood flow. For this purpose, a number of routine clinical radiology hardware platforms can be used, either as stand-alone or fusion technologies.

Ultrasound (US) is widely available, safe, radiation free, and relatively inexpensive, but has insufficient tissue penetration for exact imaging of deep vessels and an overall low spatial resolution and sensitivity.

Single photon emission computed tomography (SPECT) and positron emission tomography (PET) have high sensitivities, but limited spatial resolution, and their data alone do not yield precise anatomic information. Therefore, PET or SPECT examinations need to be combined with, for example, X-ray CT followed by data hybridization of co-registered PET/SPECT and CT image data. Another disadvantage of PET and SPECT is the necessity of radioactive tracers.

CT has a high spatial resolution, but its poor sensitivity to contrast agents has limited its use for molecular imaging to fusion techniques in combination with SPECT or PET.

Fig. 1 Schematic illustration of atherosclerosis in the autoimmune hypothesis of atherosclerosis. Promising imaging targets throughout the process can be found for early and later stages in atherosclerosis. Importantly, as results from our group showed, effector T cells are the

first mononuclear cells to appear in the intima and can then be found through both early and late periods in atherosclerosis. The figure is adapted and modified from Choudhury et al. (2004)

Recently, however, the usefulness of CT for molecular imaging was enhanced by the development of new nanoparticulate contrast agents (Hyafil et al. 2007; Rabin et al. 2006; Winter et al. 2005).

In contrast, MRI has a slightly lower sensitivity than SPECT and PET, but it is considered safe (if individuals do not carry ferromagnetic implants, e.g., a cardiac pacemaker), requires comparable examination times, and provides images with high spatial resolution (Mulder et al. 2005, 2006). A downside of MRI in the context of molecular imaging of atherosclerosis is that direct visualization often needs large amounts of nanoparticles (14 to 56 mg Fe/kg) (Ruehm et al. 2001).

Optical imaging is another frequently used method for molecular imaging, and a variety of different approaches have been described (Ntziachristos et al. 2002; Weissleder and Mahmood 2001). As depth penetration (1–2 mm) is a major limiting factor in *in vivo* optical imaging, this technology is mainly applied to surface structures only.

Examples of contrast agents for *in vivo* molecular imaging of atherosclerosis

Molecular imaging of atherosclerosis relies on the use of contrast agents that target specific cells or molecules that are relevant to its pathogenesis (Choudhury et al. 2004). Although most of the available probes for molecular imaging are limited to experimental use, such agents are typically a combination of two major components: (a) a radiologically detectable compound, such as a radioisotope, magnetic particle, fluorochrome, or sonic signal enhancer, and (b) a highly target-specific affinity-ligand moiety, such as an antibody for a given adhesion molecule. In addition, a specific-target cell can take up the probe.

For this reason, not all serologically useful atherosclerosis biomarkers can function as molecular imaging agents,

such as secreted proteins or stationary proteins expressed on endothelial cells only in low densities. In contrast, internalized receptors, abundant extracellular markers, and low-background enzyme-sensing quenched substrates provide high-yield imaging targets. A list of currently favored markers under clinical evaluation as targets for noninvasive imaging in experimental atherosclerosis is given in Table 1.

MRI contrast agents

As mentioned, MRI with clinical magnetic field strengths, e.g., at 1.5 T, has a considerably higher sensitivity to contrast agents than CT or US, but the sensitivity is still significantly lower compared to SPETC or PET, which limits its use for *in vivo* imaging on cellular or molecular levels. Nevertheless, in contrast to SPECT and PET, it can be used without potentially harmful radioactive agents and, in contrast to CT, without X-ray load, which makes a possible future application in humans more likely than other modalities. In addition, recent improvements in the development of appropriate MRI contrast agents and optimized hardware with higher magnetic fields (3 and 4.7 T and higher) have now almost overcome the limited specificity of MRI (Aime et al. 2002a, b).

Other than for SPECT/PET, contrast agents for MRI are not in themselves active signal emitters. They also do not directly modify the MRI signal, as do X-ray contrast agents. Instead, MRI contrast agents lead to an indirect effect on the MRI signal by modifying tissue relaxation properties, which are described by the two time constants T_1 and T_2 . The T_1 - and T_2 -relaxation times, together with MRI measurement parameters, determine the contrast in magnetic resonance images. Any MRI contrast agent leads to a reduction of both T_1 and T_2 and thus to a detectable change in image contrast. The effectiveness of a contrast agent in modifying T_1 or T_2 is described by the so-called relaxivity of the contrast agent, and the ratio of T_2 to T_1 relaxivity determines if the contrast agent will produce a positive

Table 1 Currently favored potential markers as targets for noninvasive molecular imaging of atherosclerosis; adapted from Jaffer et al. (2006b)

GLUT-1 glucose transporter-1, *HSP* heat shock protein, *MMP* matrixmetalloproteinase, *MPO* myeloperoxidase, *oxLDL* oxidized low-density lipoprotein, *SRA* scavenger receptor A, *Tc* technetium, *VCAM-1* vascular cell adhesion molecule-1

^a Agents under clinical evaluation

Biological Process	Class	Specific molecular target
Angiogenesis	Increased vascularity	Perfusion markers
	Endothelium	$\alpha_v\beta_3$ ^a , E-selectin
Apoptosis	Cell membrane	Phosphatidylserine, Annexin A5, ^{99m} Tc-annexin
Cell adhesion	Surface receptors	VCAM-1
Lipoproteins	Modified lipoproteins	oxLDL, HDL, others
Macrophage activity	Surface receptors	SRA, CD36, dextran receptor, others
	Metabolism	Hexokinase, GLUT-1
	Proteases	MMP (1, 8, 9, 13), cathepsins-NIRF ^a (B, S, K)
	Peroxidases	MPO
Vascular stress	Stress proteins	HSP-60
Thrombosis	Platelet glycoprotein	^{99m} Tc-apcitide
	IIb/IIa receptor	
	Fibrin	Fibrin, EP-2104R

contrast, i.e., signal increase on T_1 weighted images, or a negative contrast, i.e., signal decrease on T_2 weighted images. In general, only paramagnetic substances are suitable as contrast agents for MRI. At present, two basic classes of MRI contrast agents are available: (1) contrast agents based on gadolinium [Gd (III)] complexes and (2) contrast agents based on iron oxide. For Gd(III)-based complexes, the ratio of T_2 to T_1 relaxivity is the order of 1 leading to a positive MRI contrast (Weinmann et al. 1984). In molecular form, iron oxide has considerably less influence on relaxation times than Gd(III) and was originally not considered a contrast agent for MRI. As a nanoparticle, iron oxide becomes superparamagnetic with strongly enhanced relaxivities (Bjornerud et al. 2002; Weissleder et al. 1987). For such superparamagnetic iron oxide preparations, the ratio of T_2 to T_1 relaxivity is significantly larger than 1, resulting in negative contrast on T_2 weighted images.

Targeting molecular imaging applications requires consideration of the potentially low concentrations of the molecular targets of interest (nanomolar to picomolar) that have to be detected within voxels sized in the order of 1 mm^3 . For basic Gd(III) complexes, it is known that millimolar concentrations of Gd are required at the target site to produce an adequate signal; thus, molecular imaging is difficult to achieve with these contrast agents (Gillies 2002).

With iron oxide nanoparticles, it has been shown that the necessary sensitivity can be obtained (Bulte and Kraitman 2004), but the detection of negative contrast is frequently considered difficult, and a great deal of effort has been directed into finding methods that provide clear positive contrast mechanisms. For iron oxide nanoparticles, this could be achieved by employing special measurement sequences that give rise to a bright signal at iron accumulation sites (Stuber et al. 2007). For Gd(III)-based contrast agents, the quest for high detection sensitivity has led to the development of a variety of different Gd-loaded nanoparticles that can deliver up to 50,000 to 90,000 Gd ions per particle (Lanza et al. 2004). A wide variety of Gd-loaded nanoparticles have been described (Mulder et al. 2007) that are based on polymers or polysaccharides (Armitage et al. 1990; Faranesh et al. 2004), proteins (Aime et al. 2002c), dendrimers (Winalski et al. 2002), micelles (Torchilin 2002), liposomes (Mulder et al. 2006; Winter et al. 2003a), and even on viruses (Allen et al. 2005; Manchester and Singh 2006).

As nanoparticulate contrast agents seem mandatory to sufficient sensitivity for targeted molecular MRI, it is important to be aware of the biodistribution of these agents after intravenous administration, when clearance from the blood follows a two-step process based on opsonization and phagocytosis by monocytes or macrophages. Due to these processes, colloidal drug carriers are usually cleared from

the blood within minutes and accumulate in liver, spleen, and bone marrow. For targeted contrast agents, it is therefore important to slow their clearance from the blood stream, i.e., to extend the circulation time as much as possible by controlling different physico-chemical factors such as size, charge, hydrophilicity/hydrophobicity (Moghimi et al. 2001).

The MRI contrast agents discussed thus far have been based on chelated metal ions or metal particles and had a direct influence on tissue relaxation times. Recently, contrast agents based on magnetization transfer, called chemical exchange saturation transfer (CEST) agents, have been introduced, which in principle, can be totally metal free. By selective excitation of exchangeable protons on these agents, magnetization will be transferred to the free water pool, leading to a drop in signal intensity (Ward et al. 2000). Many small diamagnetic organic compounds, such as sugars or amino acids, have pools of exchangeable protons and may therefore serve as CEST agents. Paramagnetic lanthanide (Eu, Dy, Ho, Er, Tm, Yb) chelates (PARACEST agents) can result in significant detectable saturation transfer effects down to picomolar concentrations (Aime et al. 2005).

PET contrast agents

The most common radiotracer used for molecular imaging of atherosclerosis with PET is the ^{18}F -fluoro-2-deoxyglucose (^{18}FDG ; Davies et al. 2005b; Rudd et al. 2002; Tahara et al. 2007), which competes with glucose for uptake into metabolically active cells, such as macrophages in atheromas. This has been clearly shown in vivo in humans with symptomatic carotid atherosclerosis, where focal ^{18}FDG uptake 3 h after intravenous (i.v.) injection could be correlated with stenosis on co-registered CT images, with heavy macrophage infiltration in histology and with micro-autoradiography after surgical resection of the respective carotid plaques (Rudd et al. 2002). However, the concept of ^{18}FDG accumulation in inflammatory cells in atherosclerosis was not unequivocally determined (Laurberg et al. 2007), until the recent successful visualization of Nahrendorf et al. (2008) using PET-CT, of macrophages in atherosclerotic lesions in apolipoprotein E-deficient mice using both radioactive ^{64}Cu -trireporter labeled nanoparticles (^{64}Cu -TNP) and ^{18}FDG .

Examples of in vivo molecular imaging in atherogenesis

Imaging heat shock proteins

From our own and other data, it has been shown that HSP60 expression by endothelial cells is an initiating event that renders them a target for attack by preexisting,

presumably protective, innate and adaptive immunity, even under normocholesterolemic conditions.

In our radiology studies for *in vivo* molecular imaging of HSP60 expression, we radiolabeled the murine monoclonal antibody (Mab) II-13, with exquisite specificity for eukaryotic HSP60, and for which *in vitro* and *in vivo* reactivity with stressed endothelial cells has been previously demonstrated (Soltys and Gupta 1997; Xu et al. 1994). Endothelial stress was induced in normocholesterolemic New Zealand white rabbits by *i.v.* injection of bacterial endotoxin (LPS at 10 µg/kg). *In vivo* molecular imaging was performed using co-registered CT and PET after *i.v.* injection of ^{124}I -labeled monoclonal anti-HSP60 or ^{124}I -radiolabeled isotype control antibodies. *In vitro* correlation of *in vivo* imaging was achieved by *en face* immunohistochemistry and autoradiography of the aortae. For radiolabeling, ^{124}I iodine was selected because of its proven usefulness in the applied imaging systems and its suitable half-life (Davies et al. 2005a, 2006; Rudd et al. 2005). Post-*in vivo* imaging autoradiography and *en face* immunohistochemistry convincingly corroborated the data obtained by CT/PET analysis. Importantly, the *en face* immunohistochemical data also provided additional proof for the concept that HSP60 expression is most intense at arterial branching points, especially at areas subjected to turbulent rather than laminar flow shear stress as shown in several previous studies by immunohistological methods (Fig. 2).

Imaging cell adhesion molecules

Endothelial cell stressors lead to the simultaneous expression of HSP60 and adhesion molecules, e.g., ICAM-1, ELAM-1, and VCAM-1 (Amberger et al. 1997), which is a prerequisite for the interaction not only of potentially bacterial/human HSP60 cross-reactive antibodies, as men-

Fig. 2 Fatty streak in a surgically removed specimen from the aorta of a 50-year-old male patient. Immunohistochemical demonstration of HSP60 (black) by endothelial cells (arrowhead) and cells infiltrating the intima, including foam cells (arrow), and HLA-DR (red) by macrophages transformed into foam cells. Original magnification $\times 400$

tioned, but also of T cells with endothelial targets. However, this does not confer immunologic specificity, and adhesion alone does not lead to a cellular immune attack. Cell adhesion molecules are expressed in an early stage of atherosclerosis development and, hence, might serve as diagnostic targets. VCAM-1-internalizing targeted nanoparticles, injected into apoE-knockout mice, have been successfully determined in MRI in experimental atherosclerosis and revealed good correlation with *ex vivo* histological VCAM-1 staining (Nahrendorf et al. 2006). Using PET, radiolabeled B2702-p, which is a ligand that specifically binds to VCAM-1, also proved to be a promising tracer for noninvasive imaging of adhesion molecules (Broisat et al. 2007). *In vivo* imaging studies of ICAM-1- or selectin expression in atherosclerosis have not yet been published.

Imaging macrophages

Macrophages can be best imaged with the use of MRI. They phagocyte *i.v.* injected dextran-coated ultrasmall superparamagnetic ironoxide nanoparticles (USPIOs), which accumulate in atherosclerotic plaques over time, via dextran receptors or scavenger receptors (Kooi et al. 2003; Ruehm et al. 2001). Due to their superparamagnetic properties, USPIOs generate a negative contrast in T_2 MRI sequences based on local signal quenching. Amirbekian et al. (2007) targeted macrophages with paramagnetic and fluorescent micelles that specifically bind the macrophage scavenger receptor. Their *in vivo* images correlated with *ex vivo* fluorescence microscopy-verified macrophage-rich plaques.

Very recently, Nahrendorf et al. (2008) successfully utilized PET/CT technology for *in vivo* imaging of macrophages in inflammatory atherosclerosis. Dextranated DTPA-modified magnetofluorescent 20 nm nanoparticles were radiolabeled with ^{64}Cu and its *in vivo* accumulation in apolipoprotein E-deficient mice correlated with atherosclerotic plaques in specimen autoradiographies and with infiltrating macrophages in *ex vivo* fluorescence microscopy.

Imaging angiogenesis

Angiogenesis derived from the *vasa vasorum* is a typical part of the atherosclerotic pathology that occurs predominantly in more advanced stages of the development of an atherosclerotic lesion (Moreno et al. 2006). Contrast agents can detect angiogenic areas by either (a) determination of the permeability of the newly formed vessels with dynamic contrast-enhanced MRI or, more advanced, (b) targeted contrast agents that specifically mark the surface of angiogenically activated endothelial cells (Choudhury et al. 2004). In particular, the vitronectin receptor ($\alpha_v\beta_3$),

which is a member of the integrin superfamily, mediates cell attachment on arginine–glycine–aspartic acid (RGD)-containing adhesive proteins and is a heterodimeric protein demonstrable in human plaques. The $\alpha_v\beta_3$ -integrin has been shown to be a promising and useful target that can be visualized via $\alpha_v\beta_3$ -specific antibodies or the $\alpha_v\beta_3$ -specific RGD peptide (Mulder et al. 2005; Sipkins et al. 1998). Using the first above-mentioned approach, the application of the macromolecular agent gadofluorine, which accumulates in plaques over time, has effectively been detected in lipid-rich experimental plaques using the MRI technique (Sirol et al. 2004).

Based on the second above-mentioned approach for imaging of angiogenesis in atherosclerosis, Lanza et al. (2006) developed nanoparticles with a perfluorocarbon core and a lipid monolayer into which they incorporated Gd-DTPA-*bis*-oleate, a paramagnetic lipid with high detectability in MRI. Winter et al. (2003b, 2006) used these nanoparticles and specifically targeted them against $\alpha_v\beta_3$ -integrin, which is specifically upregulated on endothelial cells during angiogenesis. Consequently, they were able to specifically detect angiogenesis in cholesterol-fed rabbits with intimal hyperplasia. In another study, they even used this nanoparticle as a vehicle for an anti-angiogenic drug. Other $\alpha_v\beta_3$ -targeted imaging agents have also been designed for PET, SPECT, MRI, and US (Haubner 2006; Haubner et al. 2005; Hua et al. 2005; Meoli et al. 2004; Sipkins et al. 1998).

Imaging apoptosis

Apoptosis, or programmed cell death, is essential for tissue development and homeostasis. Because apoptosis has been found to play a critical role in the etiology of atherosclerosis, *in vivo* early detection of apoptosis could be of great importance in determining the disease stage (Cederholm and Frostegard 2007). Annexin V targets the phosphatidylserine, which is expressed on the outer layer of the apoptotic cell membrane (Kolodgie et al. 2003; Li et al. 2004; Shiomi et al. 2003). *In vivo*, apoptosis in atherosclerotic lesions has been detected via radiolabeled and superparamagnetic-iron oxide particle-labeled annexin V (Schellenberger et al. 2002, 2004; Smith et al. 2007).

Imaging lipoproteins

Lipoproteins, particularly LDL and high-density lipoprotein (HDL), play significant roles in the transport of cholesterol and atherosclerotic plaque composition (Ross 1999). While LDL is involved in progressive plaque formation, HDL has the capacity to remove lipids from an atherosclerotic plaque and thus may even serve as a possible therapeutic agent for plaque regression. For molecular imaging purposes,

lipoproteins may be labeled with contrast-enhancing agents such as radiotracers, fluorescent dyes, or paramagnetic nanoparticles. Frias et al. (2006, 2004) successfully synthesized a HDL-based paramagnetic contrast agent for MRI and applied it in a mouse model of experimental atherosclerosis wherein *in vivo* and *ex vivo* imaging studies provided specific signal enhancement in atherosclerotic lesions in the abdominal aorta.

Imaging atherosclerotic thrombus

Thrombi can be found in late stage atherogenesis, especially on plaques at the verge of rupture. Because thrombi mainly consist of highly abundant fibrin, specifically anti-fibrin targeted probes can be used. For that purpose, paramagnetic perfluorocarbon nanoparticles that contain Gd and that are conjugated with fibrin-specific antibodies or fibrin-binding Gd-labeled peptides have been used successfully for effective *in vivo* imaging of thrombi in experimental atherosclerosis after carotid endothelium denudation (Sirol et al. 2005a, b; Yu et al. 2000).

In conclusion, the development of increasingly powerful single platform imaging hardware and rapid progress on the design and successful utilization of highly atherosclerosis-specific targeted contrast agents that are noninvasive and detectable at different stages of atherosclerosis development highlight the crucial role of *in vivo* molecular imaging of atherosclerosis in future experimental and clinical radiology of cardiovascular disease. Promising targets for detecting the earliest endothelial response to atherosclerosis risk factors (e.g., HSP60 and VCAM-1) as well as intermediate and later stages (morphologically changed vessel walls, e.g., $\alpha_v\beta_3$ -integrin, annexin V, or fibrin) cannot only provide diagnostic indicators of patients at risk for arterial stenosis but, upon their noninvasive-specific radiological detectability, also function as biomarkers to monitor possible future methods for targeted anti-atherosclerotic therapies.

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