Targeting the unstable atherosclerotic plaque : diagnostic and therapeutic implications

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Chapter 7

General Discussion and Future Perspectives

Introduction

In the past few decades, increasing evidence has led to the concept that atherosclerosis is a lipid driven inflammatory disorder of the arterial tree. Disease onset is in early adolescence and it gradually develops during life; and thus is often considered as a chronic inflammatory disease[1-3]. The presence of atherosclerosis remains largely undetected and non-symptomatic until plaques have advanced to a vulnerable, thin cap fibroatheroma that will give rise to clinical events. Due to the long time span before clinical manifestations occur, overt opportunities for primary prevention through change in lifestyle or targeted drug therapy in individuals with subclinical disease are missed. The early detection of vulnerable plaques thus remains an important challenge in cardiovascular research. Recently, it has become clear that the pathological consequences of atherosclerotic plaques (stable versus unstable) are not primarily determined by its size, but rather by its inflammatory status and cellular and extracellular composition[4-6]. Conventional atherosclerosis imaging techniques, such as X-ray angiography, fall short in this regard as they are able to visualize vessel lumen and thus only highly stenotic atherosclerotic plaques. However, recent studies indicate that atherosclerotic lesions, including vulnerable, rupture-prone plaques are often located at sites that are outward remodelled[7]. This notion has led to extensive focus on direct plaque imaging or molecular imaging approaches that detect plaque features rather than stenosis[8-12].

Magnetic Resonance Imaging (MRI) is a powerful non-invasive imaging modality for the early assessment of subclinical atherosclerotic disease symptoms[13] or even for monitoring the efficacy of anti-atherosclerotic treatments[14]. Above all, it has been shown that MRI is able to distinguish between compositional/structural features of advanced atherosclerotic plaques (i.e. fibrous cap, lipid core, and hemorrhage)[15]. Despite its promise for cardiovascular indications, several issues regarding image resolution (signal-to-noise ratio (SNR)), partial volume effect and motion disturbance limit widespread clinical use of MRI for plaque characterization[16]. As a result, a large body of research focuses on improving imaging strategies and/or targeted molecular imaging contrast agents to deliver high levels of MR signal to plaque or plaque components in order to improve detection of vulnerable, unstable atherosclerotic plaques.

Considerable progress has been made in targeted molecular imaging and numerous reviews describe in detail the latest discoveries and newest targeted contrast agents[17-21]. In this thesis we described the developments of a targeted delivery of iron oxide based contrast agents for magnetic resonance imaging.

Targeting the unstable atherosclerotic plaque

As inflammatory processes are manifest at all stages of atherosclerotic lesion development and as the inflammatory status of the plaque is closely related with plaque vulnerability, inflammatory cells are considered primary targets for unstable plaque imaging[22]. A first and obvious candidate cell type is the plaque macrophage. First they are the most abundant inflammatory cell type within the atherosclerotic lesion. Second infiltration of monocytes in the subendothelial space, their differentiation into macrophages and functional activation are considered crucial in atherogenesis, and intimal macrophage accumulation is thus regarded a hallmark of atherosclerosis at all stages of disease development. Indeed, recent studies have already shown that macrophages have potential as marker of disease activity and plaque stability[23]. To target plaque macrophages we focused on scavenger receptor class A (SR-A). This receptor is abundantly present on this cell type and is involved in the uptake of modified lipoproteins, resulting in foam cell formation and lipid accumulation in the plaque.
In **chapter 2** we have used the phage display technique to identify a small 15 amino acid peptide (PP1) which acted as a high affinity and specificity ligand for SR-AI. A synthetic peptide sequence encoded by the major SR-AI binding phage clone was able to displace ligand binding to SR-AI at an IC_{50} value of 29μM. Furthermore we defined the minimal essential binding motif for SR-AI interaction. Docking biotinylated PP1 peptide to a streptavidin scaffold further increased its affinity for SR-AI on RAW macrophages by 7-fold and PP1 equipped streptavidin was shown to be internalized in a SR-AI dependent manner. The enriched phage pool and streptavidin immobilized PP1 exhibited a similar in vivo biodistribution profile in mice with marked accumulation in macrophage-rich organs, such as liver and spleen. Furthermore, co-localization studies in both organs clearly showed PP1-mediated uptake in F4/80 positive macrophages. But more importantly, PP1 docking led to a significant, 2-fold increase of [^{125}I]-streptavidin uptake by advanced atherosclerotic lesions in the aorta of ApoE$^{-/-}$ mice as compared to the control. Given the complex chemical nature of its natural macromolecular substrates, not many reports exist on the synthesis of a high specific ligand for the scavenger receptor. Only Lysko *et al.*[24] reported for the first time the design of a rather unselective SR-A antagonist. Our results described the design and validation of a specific and high affinity peptide ligand, with promising SR-AI targeting capacities.

Another important cell type with a specific role in plaque inflammation and destabilization is the T lymphocyte. Both CD4$^+$ (helper) and CD8$^+$ (cytotoxic) T cell subsets were seen to be associated with atherosclerotic lesion development. Their functional presence in the plaque also implies that atherosclerosis can be regarded as a chronic adaptive immune response [25]. An important receptor in the T-cell associated pathogenesis of atherosclerosis is CD40[26]. This TNF receptor

family member is an important co-stimulatory molecule in adaptive immune responses. The prominent role of CD40 in atherosclerosis and the beneficial effects of interruption of the CD40 pathway on disease progression[26, 27] makes the CD40/CD40L dyad an interesting target for imaging and intervention purposes. In **chapter 3**, we show that affinity selection of phage displayed peptide libraries led to the successful identification of a specific CD40 binding peptide, NP31. The essential binding motif for the human CD40 receptor was identified by ELISA based peptide competition studies. Tetramerization on a streptavidin scaffold increased avidity and biological potency, similar to PP1 peptide (chapter 2) and concordant with the presumed trimeric configuration of CD40 on T-cells. NP31 was also able to partially inhibit CD40 signalling as shown by decreased VEGF activation and IL-6 production. Biodistribution profiles of NP31 in $ApoE^{-/-}$ mice were identical to biodistribution data obtained with CD40-directed antibodies. NP31 peptide exposing phage strongly accumulated in the inflamed joint in a mouse model of rheumatoid arthritis, whereas NP31 peptide itself was able to induce accumulation of streptavidin in advanced atherosclerotic plaques in the aortic root and the abdominal aorta of $ApoE^{-/-}$ mice. Furthermore, NP31 uptake colocalized with CD40 in atherosclerotic lesions in the aortic root. These data demonstrate that NP31 holds promise as a targeting device for molecular imaging and as delivery tool for therapeutic agents in the inflammatory active vulnerable plaque.
Furthermore, interruption of the CD40/CD40L pathway has been shown to inhibit immune responses and blockade of this dyad was reported to be beneficial in the prevention of transplantation rejection, and in the treatment of various autoimmune disorders and atherosclerosis[26]. The use of specific antibody treatment to interrupt CD40/CD40L pathway has also unveiled severe side-effects such as thrombo-embolic symptoms, which precluded further clinical studies on CD40L intervention[28]. Therefore peptide inhibitors as partial antagonists may provide a better and safer alternative in diagnostic and therapeutic approaches for the treatment of autoimmune diseased and atherosclerosis. Given the partial antagonistic capacity of NP31, this peptide may be a valuable lead for further optimization and development of a more refined intervention in atherosclerosis related disorders that does not affect thrombosis.

Finally, another key mediator in inflammatory reactions during initiation and progression of atherosclerosis is a specific subclass of small chemotactic cytokines, i.e. chemokines[29]. These chemokines act via chemokine receptors and exhibit strong leukocyte homing capacities which causes inflammatory cells to accumulate in atherosclerotic lesions. Over 20 of chemokine receptors have already been documented. Moreover, it is widely accepted that many of these chemokine receptors can recognize multiple high affinity ligands, while conversely chemokines can bind various receptors. This illustrates the complex redundancy of chemokine interaction and function. Given the druggable nature of G-protein

coupled chemokine receptors, the distinct roles they play at different stages in the pathogenesis of atherosclerosis and their inflammatory function, chemokines receptors and their ligands are becoming more and more interesting targets for diagnostic and therapeutic purposes. In **chapter 4**, we mapped the vascular expression levels of chemokines and their receptors during atherosclerotic lesion initiation and progression in an animal model for atherosclerosis. Genome wide micro-array analysis revealed upregulation of 'traditional' genes of proteins/ receptors known be involved in atherosclerotic lesion development. Among these genes, encoding for cell type-specific markers, we observed an upregulation of macrophages and foam cell markers (CD68, MSR2, Arginin-1 and MMP-14), whereas a decrease in relative expression of vascular smooth muscle cell markers could be observed. This suggests a substantial influx of macrophages leading to macrophage rich fatty streak lesions. On the other hand cellular markers for T lymphocytes (CD3, CD4 and CD8) and endothelial cells (CD31, Cadherin, von Willebrand Factor and Smoothelin) were not significantly altered. As to chemokine and chemokine receptors, progressively increased gene expression patterns were observed for chemokine receptor 5 (CCR5) and CXCR4, whereas CCR1, 2, 3 and 4 expression was not altered during atherosclerotic lesion development. The chemokine ligand CCL5 shows a progressive increase in expression throughout lesion development, and CCL3 and CXCL12 show an initial peak in expression level at lesion initiation and gradually start increasing 4-6 weeks after lesion induction. Furthermore, an attempt to target both upregulated chemokine receptors (CCR5 and CXCR4) by *in vivo* administration of radiolabeled chemokines (CCL3 and CCL5) in $ApoE^{-/-}$ mice with advanced atherosclerotic lesions resulted in an accumulation of both chemokines in the plaque which could be largely attributed to increased CCR5 expression. These observations not only indicate that CCR5 is upregulated during atherosclerotic lesion formation but also that it can be used as target for inflammatory plaque detection. This chemokine receptor has already been shown to play an important role in leukocyte attraction to sites of inflammation and in plaque development[30] and CCR5 deficiency led to increased plaque stability, decreased mononuclear cell infiltration and Th1 immune response[31]. Of note, studies by van Wanrooij et al. showed that pharmacological inhibition attenuated atherosclerotic lesion formation[32], once again emphasizing the importance of CCR5 as a target in atherosclerotic plaque formation. Thus the rather small, synthetically accessible CCR5 receptor, which has been shown to be associated with leukocyte influx, reflecting ongoing and future plaque inflammation, could be seen as a very interesting target in diagnosing (plaque) inflammation and could be helpful in monitoring therapy efficacy.

Diagnostic (and therapeutic) implications

After identification and validation of interesting candidate ligands for receptors/pathways involved in inflammatory processes in the pathogenesis of atherosclerosis, SR-AI binding peptide PP1 was selected for further verification of its potential in molecular plaque imaging. In collaboration with Guerbet, worldwide leader in contrast agents, a SR-AI targeted contrast agent (T-USPIO), coated with PP1 peptides, was developed for non-invasive magnetic resonance imaging. Until now most imaging studies on iron-based contrast agents (USPIO) reported increased signal due to passive, thus non-aided, targeting of active macrophages, either within the plaque or indirectly after uptake by macrophages that have subsequently migrated into the atherosclerotic plaque. However the high doses of contrast agents required for passive targeting combined with the long half-life of untargeted USPIO in blood, lead to a high background signal for an extended period. Moreover untargeted USPIO will also be taken up in a nonspecific manner and extravasate passively from leaky vessels that are associated with inflammation, thereby potentially compromising the specificity of molecular targeting[33]. Therefore, we investigated in **chapter 5** whether targeted contrast agents could overcome these hurdles and increase vascular signal-to-noise ratio during MRI analysis. Our data show that the SR-A targeted contrast agent (T-USPIO) is, like its target peptide PP1, effectively taken up by both murine and human macrophages in a SR-A mediated manner. In a pilot study in hyperlipidemic ApoE$^{-/-}$ mice with advanced atherosclerosis the SR-AI targeted USPIO showed increased serum clearance, and accumulation in macrophage-rich organs compared to its non-targeted counterpart. The biodistribution profile of targeted USPIO was comparable to that of PP1 docked streptavidin (see chapter 2). Due to its high affinity for SR-AI, T-USPIO showed effective, scavenger receptor-AI mediated uptake by atherosclerotic plaque-associated macrophages in ApoE$^{-/-}$ and a favorable signal-to-noise ratio in MRI detection of atherosclerotic plaques in a humanized SR-AI model of atherosclerosis. With these features this targeted contrast agent constitutes a promising new platform for the non-invasive detection of macrophage-rich foci in chronic inflammatory diseases such as the atherosclerotic plaque and might even allow selective discrimination of unstable plaques.

Non-targeted USPIO are nowadays commonly used contrast agents for clinical detection by MRI of metastases, multiple sclerosis lesions, inflammatory foci in the central nervous system and atherosclerotic plaques[16]. As mentioned before, high doses of untargeted USPIO need to be administered, which slowly accumulate in macrophages by means of passive (receptor-mediated) endocytosis and phagocytosis. Upon macrophage uptake USPIO are retained in the lysosomal compartment for up to several days[34]. This lysosomal retention is not accompanied by cytotoxicity, by inflammatory or chemotactic responses nor by respiratory burst/superoxide release and does not interfere with Fc-receptor-mediated

phagocytosis[35]. Furthermore, extensive toxicology and safety assessments in rats, rabbits and monkeys showed a safety profile of USPIO that is satisfactory for its proposed use as single-dose diagnostic agent in humans. However, in **chapter 6** we are the first to describe adverse effects of USPIO administration on macrophage derived foam cells. We show that (U)SPIO can induce cell death of lipid-laden macrophages both *in vitro* and *in vivo*. Single, as well as multiple administrations, of USPIO significantly increased lesional macrophage cell death in various hyperlipidemic mouse models, which at early stages of plaque development was accompanied by plaque regression, while at later stages could potentially lead to necrotic core expansion and plaque destabilization. USPIO induced foam cell apoptosis was attributable to increased oxidative stress and could be prevented both *in vitro* and *in vivo* by prior anti-oxidant treatment. These potentially harmful effects of USPIO on foam cells have thus far only be studied in a mouse model of atherosclerosis and await confirmation in a human setting. Though remarkable, our data leave unaddressed the overall effect of increased foam cell apoptosis on plaque progression and stability in long term studies.

The use of targeted delivery of contrast enhancing agents for molecular imaging dramatically reduces the dose of the contrast agent injected, and will furthermore lead to faster clearance from the circulation. This reduction in exposure time and the delivery of the contrast agents at aforementioned disease/inflammation sites will largely reduce cytotoxic implications and possible adverse side-effects at other locations throughout the body.

Future Perspectives

In conclusion, we have addressed the potential of interesting candidates for targeted imaging of inflammatory plaques. We have designed (oligo)peptide based plaque homing devices and shown their aptness as diagnostic tool for contrast enhancing imaging protocols.

Of the various options explored in this thesis for molecular imaging of the unstable atherosclerotic plaque, the PP1 targeted contrast agents for MR imaging of plaque macrophages and thus inflammation probably holds the most promise for clinical use. The fact that our data demonstrate both high affinity and specificity of PP1 for both murine and human macrophage SR-A combined with the fact that unstable, vulnerable lesions are highly enriched in macrophages, in particular the rupture prone shoulder, that abundantly express SR-A, makes PP1 a powerful homing tool for diagnostic purposes as already shown in our study in a humanized mouse model (chapter 5). Though promising, PP1 targeted contrast agents need to be tested in larger animal models and also tested for safety. Possibly a further evaluation in larger animal models will be needed before application in clinical settings becomes reality. Next to its use in molecular imaging, the PP1 peptide could also be of added value in a therapeutic setting by

faciltating targeted delivery of nanoparticle-sized pharmacological drug(carrier) s. In this way, more specific accumulation of the pharmaceutical compound could be accomplished, directly at the site of inflammation, which will help to minimize both dose and toxicity. As to the use of PP1 itself as antagonist of scavenger receptor AI for the treatment of atherosclerosis I am less optimistic given the considerable redundancy in scavenger receptor function and the critical role of scavenger receptors in clearance of a wide variety of ligands.

The peptide ligand for CD40/CD40L, NP31 and chemokines CCL5/CCL3 may have better perspectives for monitoring of specific inflammatory processes in the plaque and to a lesser extent identify vulnerable atherosclerotic plaques. NP31 in contrast with PP1, targets mainly T-lymphocytes which are not as abundant as macrophages throughout atherosclerotic lesion formation leading to less signal density compared to PP1 Given the prominent role of CD40 as well as CCR5 in Th1 dominated immune responses and disorders like multiple sclerosis, rheumatoid arthritis and others, they might be useful in these ailments as well for both diagnostic and therapeutic implications. Next to its potential as diagnostic tool, NP31 could also be a promising tool in monitoring therapy efficacy as its target CD40 has been seen to be causally involved in plaque stabilization in various studies. Peptide ligands as a diagnostic tool for CD40 imaging might lower the risk of severe side-effects that are generally encountered after complete administration[28]. These considerations make NP31 peptide a valuable lead for further optimization.

Like PP1, both NP31 and CCR5 receptor antagonists could be used for therapeutic application atherosclerosis and inflammation-driven diseases. CCR5 receptor antagonists have already been designed and are currently being tested in clinical trials for HIV positive patients. However these antagonists could prove their beneficial effects during atherosclerotic lesion formation as CCR5 deficiency has been showed to correlate with increased plaque stability, decreased mononuclear cell infiltration and Th1 immune response.

Finally, I envision that a combination of the here described targeted imaging approaches as each will allow detection of specific functional or compositional features of the unstable plaque. For instance a combined accumulation of macrophage targeting peptide such as PP1 and of contrast agents that are directed against t-lymphocytes with a low abundancy of vascular smooth muscle cell or collagen targeting agents could more accurately identify a vulnerable rupture prone lesion. Such multi-target imaging approaches to capture the full complexity of the vulnerable plaque are still remote future to date, but deserve certainly serious consideration.

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