The role of inflammation in cardiac and vascular remodelling

Jong, R.C.M. de

Chapter 7

Summary and general discussion

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Cardiovascular disease (CVD) is the collective term for all diseases that involve the heart or circulation, like myocardial infarction (MI), stroke, heart failure and restenosis. The major underlying cause of CVD is atherosclerosis, in which a lipid-driven chronic inflammation of the vessel wall leads to formation of atherosclerotic plaque. Clinical manifestations occur when the atherosclerotic plaque ruptures which subsequently leads to formation of a thrombus, that blocks the blood flow to adjacent tissue. Next to total blockage of the blood flow, it is also possible that clinical problems occur when an atherosclerotic plaque grows to a substantial size, leading to severely reduced blood flow. CVDs are not only one of the leading causes of morbidity and mortality worldwide, but they also are a major economic burden. Understanding the pathophysiology and underlying mechanisms of the different CVDs may offer new potential therapeutic approaches to reduce morbidity and mortality as well as to reduce the economic burden of CVDs.

The aim of thesis was to further investigate the role of inflammation in cardiac and vascular remodelling. This thesis focusses on different types of adverse cardiac and vascular remodelling, namely MI, myocardial ischemia-reperfusion (MI-R) injury, restenosis and accelerated atherosclerosis.

Potential immunomodulatory therapies against adverse cardiac remodelling

Phosphorylcholine (PC) is the polar head group of phosphatidylcholine, which is an important cell membrane phospholipid. PC is an endogenous ligand capable of triggering the innate immune system and it is expressed by apoptotic cells and oxidized LDL[1,2]. In **chapter 2** we investigated the effect of an antibody directed against PC on adverse cardiac remodelling following MI. We previously developed a fully humanized IgG antibody against PC (PC-mAb), which we tested in a mouse model for MI. We found that PC-mAb treatment decreased infarct size (IS) and left ventricular (LV) dilatation. To investigate the mechanism behind this cardioprotective effect of PC-mAb we measured systemic (C-C motif) ligand 2 (CCL2) concentrations and local leukocyte recruitment, and we analysed the circulating leukocytes using FACS. We demonstrated that serum CCL2 concentration was decreased upon PC-mAb treatment two days after MI, but not after three weeks. Interestingly, the number of cardiac leukocytes was decreased following PC-mAb treatment three weeks post MI, but not two days after MI. Finally, we observed an decrease in percentage circulating monocytes two days post MI. Taken together, we suggest PC-mAb treatment reduces adverse cardiac remodelling by attenuating both the early and late inflammatory response.

Reperfusion following a MI not only saves a portion of the cardiomyocytes in the ischemic area, it also boosts the inflammatory response leading to MI-R injury[3]. Therefore, adverse

remodelling following MI-R injury may be more susceptible for the anti-inflammatory effects of PC-mAb treatment than adverse cardiac remodelling following MI. The effect of PC-mAb treatment on adverse cardiac remodelling following MI-R injury was described in **chapter 3**. To study the effect of PC-mAb on adverse cardiac remodelling we used our mouse model for MI-R injury, in which the left anterior descending coronary artery was occluded for 45 minutes followed by permanent reperfusion. Furthermore, we investigated the post-reperfusion inflammatory response by examining CCL2 levels, local leukocyte recruitment and circulating leukocytes. We demonstrated that PC-mAb treatment reduces IS, while it preserves LV wall thickness three weeks post MI-R injury. In addition, PC-mAb treatment reduces LV dilatation leading to preservation of cardiac function. The post-reperfusion inflammatory response was decreased following PC-mAb treatment. Two days post MI-R injury systemic CCL2 level was significantly decreased following PC-mAb treatment, however this decrease in CCL2 concentration was not observed after three weeks. Local leukocyte infiltration was not decreased two days post MI-R injury upon PC-mAb treatment, however after three weeks number of infiltrated leukocytes was decreased in the interventricular septum, border zones and infarct area. Finally, we observed a decreased percentage circulating monocytes in the PC-mAb treated mice two days after MI-R injury. Interestingly, this reduction of circulating monocytes was mainly caused by a decrease of pro-inflammatory Ly6C^{high} monocytes, while anti-inflammatory Ly6C^{low} monocytes were not decreased upon PC-mAb treatment. We concluded that PC-mAb treatment limits adverse cardiac remodelling by attenuation of the early systemic inflammatory response and by reduction of the extended local inflammatory response.

The effect of PC-mAb treatment on adverse cardiac remodelling was investigated in two different mouse models, namely permanent MI and MI-R injury. We observed that PC-mAb treatment was capable to preserve cardiac function following MI-R injury, while this was not the case following permanent MI. We believe that there are several reasons for this difference: 1) in the MI-R injury model a portion of the cardiomyocytes in the ischemic area is saved by reperfusion, leading to a better preserved cardiac function, 2) in the MI-R injury model the inflammatory response is boosted and therefore PC-mAb treatment is better suited to target MI-R injury than permanent MI due to its anti-inflammatory properties.

Cardiac remodelling is a carefully orchestrated process, which can be divided to an inflammatory phase and a reparative and proliferative phase. In the inflammatory phase different pro-inflammatory immune cells remove dead cells and matrix debris from the infarct area, thereby promoting the healing process. In the reparative and proliferative phase the pro-inflammatory response is dampened and partly replaced by anti-inflammatory immune cells that boost scar formation[4]. Therefore, we postulate that rather than total abolishment of the post-infarct inflammatory response, timely suppression is beneficial against adverse cardiac remodelling following MI. In chapter

2 and 3 we show an decrease of the local inflammatory response three weeks after MI, but not two days after the ischemic insult. We suggest PC-mAb treatment reduces the adverse long term inflammatory response, while the necessary early inflammatory response in not affected and thereby reduces adverse cardiac remodelling.

Annexins are phospholipid-binding proteins and especially annexin A5 (AnxA5) is known to bind phosphatidylserine (PS)[5]. PS is expressed by apoptotic cells where it functions as an "eat me" signal for phagocytic cells[6,7]. By shielding of this "eat me" signal, AnxA5 exerts anti-apoptotic and anti-inflammatory properties[5]. In **Chapter 4** we investigated the therapeutic potential of (AnxA5) to treat adverse cardiac remodelling following MI-R injury. Using the same mouse model as described in chapter 3, we found that AnxA5 treatment reduced IS, while preserving LV wall thickness in the infarct area. Subsequently, LV dilatation was limited following AnxA5 treatment, which led to preservation of LV function. Interestingly, we found accumulation of AnxA5 in the infarct area of AnxA5 treated mice two days after MI-R injury, suggests AnxA5 is present at the desired location. Furthermore, to unravel the mechanism behind the cardioprotective effect of AnxA5 we analysed local macrophage infiltration. We found that following AnxA5 treatment, local macrophage infiltration was decreased both two days and three weeks post MI-R injury. Interestingly, a reduction of proliferating macrophages was observed in AnxA5 treated mice two days post MI-R injury, but not after three weeks. However, the percentage proliferating macrophages was comparable in both the vehicle and AnxA5 group. The cardiac macrophage population is maintained by both infiltration of monocytes (controlled in part by CCL2) and local macrophage proliferation[8]. Since we did not find any difference in systemic CCL2 levels and percentage proliferating macrophages was unaffected, the mechanism by which AnxA5 treatment reduces the number of cardiac macrophages is subject of future research. Nevertheless, we conclude that AnxA5 is a potential therapeutic agent against adverse cardiac remodelling by suppressing the inflammatory response.

The potential therapeutic effects of PC-mAb and AnxA5 against adverse cardiac remodelling described in chapter 2, 3 and 4 were studied a clinical relevant setting, namely by starting the treatment post-reperfusion and hypercholesterolemia. Hypercholesterolemia is an important risk factor for MI in human[9] and it has been shown that it affect cardiac remodelling in mice[10,11]. To mimic the clinical situation of most cardiovascular patients, we used hypercholesterolemic ApoE*3-Leiden mice, which only develop hypercholesterolemia when fed a high-fat diet[12]. Although plasma cholesterol levels in these mice are higher than in the clinical situation, we believe that this model is the closest resemblance regarding hypercholesterolemia available.
Most studies on potential therapeutic agents used a treatment strategy in which treatment was started before reperfusion was accomplished. In our opinion

this is not mimicking the clinical situation of MI patients. Therefore, at least in the MI-R injury studies, we used a treatment strategy in which we started treatment immediately after reperfusion. The clinical relevance regarding hypercholesterolemia and treatment strategy used in these studies, adds even more value to the already impressive cardioprotective effects of PC-mAb and AnxA5.

Epigenetic manipulation against adverse vascular remodelling

Epigenetic factors are factors that change gene expression, and thereby the phenotype of an organism, without altering the DNA sequence[13]. In this thesis we focus on two epigenetic systems, namely acetylation and microRNAs.

Acetylation is the introduction of an acetyl group to a chemical compound, while de-acetylation is the opposite, the removal of an acetyl group from a chemical compound. Gene expression can be controlled by the balance of acetylation and de-acetylation of certain proteins, like histones, the proteins around which the DNA is wrapped, but also many non-histone proteins can be acetylated and de-acetylated resulting in altered gene expression. Acetylation of histone proteins results in more loosely wrapped DNA around histones, thereby making the DNA more accessible for transcription, leading to increased gene expression[14]. In case of non-histone proteins, acetylation can lead to both increased and decreased gene expression, depending on the site of acetylation and the resulting fate of the protein, since acetylation of non-histone proteins affect processes, like protein stabilization and localization15. Proteins are usually acetylated and de-acetylated on specific lysine residues by lysine acetyltransferases and lysine deacetylases.

In **Chapter 5** we investigate the role of lysine acetyltransferase P300/CBP associated factor (PCAF) in adverse vascular remodelling. Using a mouse model for intimal hyperplasia in PCAF deficient mice we showed that PCAF deficiency results in decreases intimal hyperplasia development. It is known that PCAF is involved in acetylation of histone acetylation at the site of nuclear factor kappa-beta (NFκB) regulated genes[16-18]. NFκB is an important transcription factor which regulates expression of different pro-inflammatory genes, like tumor necrosis factor α (TNF-α), that are involved in intimal hyperplasia development[19]. Using *in vitro* experiments we showed that different cell types involved in vascular remodelling, reduce their production of pro-inflammatory cytokines, like TNF-α, interleukin-6 (IL-6), and CCL2, upon PCAF deficiency. However, we were not able to show *in vivo* that PCAF deficiency leads to a decreased inflammatory response, probably because the non-optimal time point to evaluate the *in vivo* inflammatory response. To overcome this problem, we studied the *in vivo* inflammatory response in hypercholesterolemic ApoE*3-Leiden mice, in which the inflammatory response is more explicit, at an earlier time point and using the natural PCAF inhibitor garcinol. Indeed, we found a significant reduction of CCL2 expression, and leukocyte and

macrophage infiltration in the vessel wall of mice treated with garcinol. In agreement, we found that garcinol reduced the TNF-α and CCL2 production *in vitro*.
Intimal hyperplasia is not only the result of inflammation, but vascular smooth muscle cell (VSMC) migration and proliferation play also an important role[20]. We observed that PCAF deficiency leads to a decrease of VSMCs in the intimal layer of the vessel wall. Future research is needed to further unravel if PCAF deficiency directly influences VSMC proliferation/migration or that PCAF deficiency impacts VSMC proliferation/migration indirectly via inflammation.
In conclusion, we show that PCAF is involved intimal hyperplasia development and vascular inflammation. This is caused by a direct or indirect effect of PCAF on the inflammatory response, and VSMC proliferation and migration.

MicroRNAs are short endogenous non-coding RNA molecules, which bind to the 3'UTR of their target genes, which can be up to several hundred for a single microRNA and thereby regulate expression of those target genes[21]. Since microRNAs are capable of fine-tuning gene expression of so many targets genes, they can regulate multifactorial processes like adverse vascular remodelling[22]. In **chapter 6** we focus on the role of several members of the 14q32 microRNA cluster in adverse vascular remodelling. Using Gene Silencing Oligonucleotides (GSOs), a relative new microRNA inhibitor with increased specificity and less adverse side effects than the widely used antagomirs, we inhibited expression of microRNA-329, -494 and -495 in a mouse model for intimal hyperplasia. We found that inhibition of microRNA-495 resulted in a decrease in intimal hyperplasia development, while inhibition of microRNA-329 resulted in a near-significant reduction of intimal hyperplasia. Both inflammation and VSMC proliferation play an important role in intimal hyperplasia development[20]. Interestingly, we found that GSO-495 treatment in reduced both macrophage infiltration and VSMC proliferation *in vivo*.
Since no effect on intimal hyperplasia was observed upon microRNA-494, we decided to only investigate the effect of microRNA-329 and -495 inhibition on accelerated atherosclerosis development. We observed a significant reduction of collar-induced atherosclerotic plaque formation upon microRNA-495 inhibition, while treatment with GSO-329 again resulted in a near-significant effect. Atherosclerotic plaque stability is determined by several parameters, namely necrotic core size, macrophage influx, VSMC content and collagen content[23]. We found that inhibition of microRNA-495 led to a reduction in necrotic core size and macrophage infiltration, while collagen content was increased. Taken together this indicates that GSO-495 treatment not only leads to smaller plaques, but the remaining plaque also showed a more stable phenotype.
Since accelerated atherosclerosis was studied in hypercholesterolemic ApoE$^{-/-}$ mice and microRNA-495 has some putative targets involved in cholesterol metabolism we measured cholesterol levels following GSO-495 treatment. Despite no effects were found on expression of the putative target genes (e.g. Lrp6, Mttp, Ldlr and Abca1),

we found that inhibition of microRNA-495 results in decreased plasma cholesterol levels. Moreover, the observed reduction of plasma cholesterol could be attributed to a reduction of very low-density lipoprotein (VLDL), which is, together with LDL, known as the pro-atherosclerotic lipoprotein. We suggest that the reduction of VLDL plasma cholesterol is, at least partly, responsible for the decreased plaque size and increased plaque stability.

Next to the targets mentioned in the paragraph above, we investigated the effect of microRNA-495 inhibition on the expression of several targets putative targets. We found that treatment with GSO-495 led to significant upregulation of Tgfβ2 and Il13ra1, while a modest upregulation was shown for Acvr1 and Il10. Using reversed target prediction a total of 37 putative murine atherosclerosis-related targets genes for microRNA-495 were identified[24]. In our study we were able to show upregulation of only two of the putative targets, which seem unable to result in the observed effect on adverse vascular remodelling. However, in agreement with Van Rooij *et al*.[21], we believe that rather than upregulation of one or two targets genes, it is the sum of many modest upregulated target genes that are responsible for the observed effect on adverse vascular remodelling.

Previously it was shown that microRNA-495 inhibition results in increased therapeutic neovascularization following ischemia[25]. Both therapeutic neovascularization and atherogenesis are influenced by similar cellular mechanisms, like cytokine and adhesion molecule expression. However, factors that increase therapeutic neovascularization usually also increase atherogenesis, this trade-off is also called the Janus phenomenon[26]. The fact that microRNA-495 positively influences both therapeutic neovascularization and adverse vascular remodelling, and thus breaks with the Janus phenomenon, makes it a very interesting potential therapeutic target.

Future perspective

The aim of this thesis was to investigate the role of inflammation in adverse vascular and cardiac remodelling and thereby find new potential therapeutic targets and agents. Potential immunomodulatory therapies are subject of research for decades and in 2017 Ridker *et. al.* showed for the first time that an anti-inflammatory agent reduces CVD[27]. However, the results in most clinical trials are, despite promising pre-clinical studies, disappointing. One of the reasons for the disappointing results is that the immunomodulatory therapies were focussed on single factors and/or factors that take place relatively late in the inflammatory pathway. To overcome this problem we investigated potential targets that influence the inflammatory process earlier in the pathway or factors that are capable to influence multiple processes. In chapter 2, 3 and 4 we investigated PC-mAb and AnxA5 treatment against adverse cardiac remodelling, both potential therapeutic agents capable of binding to endogenous ligands that can trigger the innate immune system. By shielding of endogenous ligands before they

are recognized by the immune system, the inflammatory response is prevented at the earliest time point. In chapter 5 and 6 we described the role of PCAF and microRNA-495 in adverse vascular remodelling, two factors that are capable to influence multiple processes. In the next paragraph the current status of the potential therapeutic agents will be discussed.

Phase 1 clinical trials showed PC-mAb is safe to use in healthy volunteers and peripheral artery disease patients. Therefore, PC-mAb is currently investigated in a phase 2 clinical trial to study efficiency in a relevant patients population. AnxA5 treatment has been shown to be very effective in different mouse models, but AnxA5 therapy is not subject of a clinical trial yet. However, radiolabeled AnxA5 is widely used as a diagnostic tool. Thus, one can imagine that AnxA5 therapy alone likely will not possess severe side effects. In chapter 5 we investigated the role of PCAF in adverse vascular remodelling, in which we used the natural PCAF inhibitor garcinol. Due to its anti-inflammatory and anti–proliferative properties, garcinol treatment is investigated as potential therapy against several diseases, including cancer and bacterial infection, but also against Alzheimer disease. However, clinical data is lacking, therefore, further research regarding pharmacokinetics is required before garcinol can enter a clinical trial. Next to garcinol, many other PCAF inhibitors are under investigation for their therapeutic potential against different diseases, increasing the possibility that a PCAF specific therapy will eventually be found. Miravirsen, a microRNA-122 inhibitor which reduce the replication of hepatitis C virus, is currently the only one microRNA inhibitor that entered a clinical trial[28]. The phase 1 trial showed it is safe to use a microRNA-based treatment in human. However, Miravirsen is a so-called locked nucleic acid, which is chemically different compared to our GSOs. Therefore, further research regarding pharmacokinetics and dosing are necessary should be performed regarding GSO therapy before it can enter clinical trials.

In conclusion, this thesis presents further understanding in the role of inflammation in adverse cardiac and vascular remodelling. Furthermore, the studies included in this thesis identified potential immunomodulatory therapeutic agents against adverse vascular and cardiac remodelling. Future research will show if the potential therapeutic agents can successfully be used in patients suffering from CVD.

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