General discussion
INTRODUCTION

Inflammation plays an important role in the pathophysiology of cardiovascular disease. Although an inflammatory response is necessary for healing of the heart, subsequent to an event, f.i. acute myocardial infarction (AMI) or myocarditis, it also induces additional cardiomyocyte damage resulting in expansion of fibrosis of the heart, facilitating heart failure development. Several aspects of this inflammatory response in AMI and myocarditis were subject of this thesis, related to improvement of the diagnostic process of AMI or myocarditis, to advanced insight into their pathophysiology and to putative target detection for further therapy development.

EARLY DETECTION OF ACUTE MYOCARDIAL INFARCTION

Ultrastructural analysis of mitochondria in AMI

Postmortem diagnosis of AMI remains a challenge at autopsies. Macroscopically, nitro blue tetrazolium staining of heart slides is used to detect loss of lactate dehydrogenase (LDH) in cardiomyocytes, indicative for infarctions of at least 3 hours old. Detection of AMI of less than 3 hours, however, is difficult. We have now validated a method quantifying mitochondrial deposits in the heart of AMI patients, in infarctions of 2 - 3 hours old (Chapter 2). From animal models, it namely was known, that mitochondrial deposits are formed, that can be detected at an ultrastructural level as early as 2 hours post-AMI. We now found a significant increase in mitochondrial deposits in the infarcted left ventricle of patients with AMI, as compared to the non-infarcted area within the same patient, but also related to control, non-infarcted hearts, with a predictive positive value of 90%, indicating that in 90% of the patients with AMI, this method absolutely indicates AMI. This method is therefore a useful tool to diagnose AMI in cases of sudden or unexplained death in which LDH decolourisation is not conclusive.

We propose the following schedule to diagnose AMI at autopsies, as depicted in Figure 1. At autopsy, first of all NBT staining is performed on the macroscopical heart slides to detect a putative AMI of at least 3 hours old (1). When LDH decolourisation is found, microscopical analysis of this particular part of the heart is performed to further determine infarct age (2). In case no microscopical changes are found, this indicates an AMI of 3 - 6 hours old. When, however, polymorphonuclear neutrophils (PMNs) accumulate in blood vessels of the heart, this would point to an AMI of 6 - 12 hours old. In case PMNs infiltrate in between affected cardiomyocytes, an AMI of 12 hours - 5 days is diagnosed. When, however, no LDH decolourisation is found in the macroscopical heart slides, heart tissue from the anticipated area at risk should be analyzed at an ultrastructural level (3). The area at risk should be
determined related to the atherosclerotic changes of coronary arteries and/or the area of
the heart with signs of older infarctions (replacement fibrosis), as in the majority of cases
reinfarctions occur to the same coronary artery branch. As a control, heart tissue of the non-
infarcted part of the heart, mostly the right ventricle, should also be analyzed to correct for
putative postmortem changes in mitochondria. In case, at an ultrastructural level, an increase
in the number of mitochondria with deposits is found, that is 1.28 times more positive
compared to the non-infarcted part, an AMI of 2-3 hours is diagnosed2.

Figure 1. A diagnostic schedule to diagnose AMI at autopsy
AMI, acute myocardial infarction; EM, electron microscopy; LDH, lactate dehydrogenase; NBT, nitrotetrazolium blue;
PMNs, polymorphonuclear granulocytes.

THE PATHOGENIC ROLE OF THE MICROVASCULATURE IN AMI

Thickening of the basement membrane (BM) of the cardiac microvasculature
BM thickening of the cardiac microvasculature has been described in endomyocardial biopsies
taken from patients with diabetes mellitus (DM)4. It was suggested that BM thickening plays
an important pathophysiological role in heart failure development in patients with DM5. We
wondered, whether thickening of the BM would also have a pathogenic role in AMI induction,
independent of DM. We found that the BM was significantly thickened in the infarcted area
of the LV of patients with AMI, independent of changes of their epicardial infarct-related
coronary artery (Chapter 3). Combining the outcome of rat AMI studies with human AMI autopsy studies, this suggests that BM thickening possibly occurs prior to AMI. BM thickening hampers transport of oxygen and nutrients across the vascular wall, facilitating a pro-hypoxic condition. The mechanism of BM thickening, however, is still unclear. It is suggested that local disturbance in the balance of extracellular matrix synthesis and degradation forms a causative factor. Herein, advanced glycation end products (AGEs) could play a role, at least in the heart. In a previous study, we have shown that the AGE product Nε-(carboxymethyl)lysine (CML) forms depositions in the cardiac microvasculature in advance of AMI. AGEs crosslink proteins like collagen, making them more resistance to matrix metalloproteases (MMPs), what could result in accumulation of extracellular matrix components (ECM) and thus thickening the BMs. Indeed in diabetic rats, aminoguanidine, an inhibitor of AGEs, induced a decrease in glomular basement membrane thickening. Next to this, reactive oxygen species (ROS) have also been suggested to play a role in BM thickening, as they increase expression of ECM genes, including transforming growth factor-β and connective tissue growth factor, that both can facilitate ECM synthesis, fibrosis, and BM thickening. Indeed, in diabetic transgenic mice that show overexpression of the antioxidant metallothionein, no BM thickening of myocardial capillaries was found.

These data thus implicate an important role for aberrant cardiac microvasculature in AMI induction, also independent of changes of the epicardial vasculature.

**STRESS-INDUCED HEART FAILURE**

*Stress-induced myocarditis (Figure 2)*

Stress-induced heart failure, including stress-induced myocarditis and stress (Takotsubo)-induced cardiomyopathy, is related to sustained exposure of the heart to elevated levels of catecholamines. This has been described related to patients with burn wounds, sepsis, pheochromocytoma, cerebrovascular accidents, and emotional stress. In these patients, catecholamines, including adrenaline, noradrenaline and dopamine, are elevated both in the plasma as well as locally in the heart. Adrenaline and noradrenaline are known to induce cardiotoxic effects, including microvascular dysfunction, inflammation, fibrosis, cardiac hypertrophy, and cell death, albeit its pathophysiology is not exactly known yet. Recently, it was shown that dopamine infusion in rats did also induce stress-induced cardiomyopathy, indicative for a cardiotoxic effect of dopamine as well. We have now analyzed the pathophysiology of this cardiotoxic effect of dopamine in rat cardiomyoblasts (H9c2 cells; Chapter 4; Figure 2). We then found that dopamine induced intracellular accumulation of lipids coinciding with decreased cell viability, indicative for a so-called lipotoxicity effect. This is in line with 2 other animal studies (in mice and rats), in which isoprenaline, a
synthetic adrenaline-like catecholamine, also induced cardiac lipid accumulation coinciding with necrosis and decreased heart function\(^{24, 25}\). Next to lipotoxicity, the formation of reactive oxygen species (ROS) is another putative pathological factor in catecholamine-induced cardiotoxicity, as it can also induce cell death\(^ {21, 26-28}\). Dopamine indeed induced ROS both in the cytoplasm and the nucleus (4,5). Even more, accumulation of lipids within cardiomyocytes is also associated with increased ROS\(^ {27}\).

Figure 2. The cardiotoxic effects of dopamine
AMI, acute myocardial infarction; ATP, adenosinetriphosphate; GTP, guanosinetriphosphate; PC, phosphatidylcholine; PE, pulmonary embolism; PS, phosphatidylserine; ROS, reactive oxygen species.

Interestingly, in a study analyzing the cardiotoxic effects of noradrenaline in fetal rat hearts and H9C2 cells, a role of NOX1 was suggested\(^ {29}\). In this particular study, NOX4 was unchanged and remarkably, NOX2 was not detected. We found that dopamine induced increased (peri)nuclear and cytoplasmic expression of not only NOX1 (6), but also NOX4 (7), NOX2, and p47\(^ {phox}\) (an important co-factor for NOX2 activation)(8). We have previously shown that ischemia induced NOX2 activation in H9c2 cells, coinciding with ROS production, resulting in cell death, partly via caspase-3 activation and thus apoptosis\(^ {30, 31}\). However, in dopamine stimulated cells, we did not find caspase-3 activation. This is in line with studies in patients with stress-induced cardiomyopathy, were neither caspase-3 activity were found \(^ {27, 37}\). We,
however, did find that dopamine induced the so-called flip-flop of the plasma membrane (9), namely, a translocation of phosphatidylinerse (PS) from the inner to the outer plasma membrane leaflet. This can be induced in apoptosis, but also occurs in non-lethal stressed cardiomyocytes after an ischemic event32, 33. Such loss of plasma asymmetry constitutes a pro-inflammatory status of the plasma membrane and a risk for non-lethal cells, as it facilitates the binding of inflammatory mediators, such as sPLA2-IIA, CRP and subsequently inflammatory cells, resulting in cell death34, 35.

The phospholipid transporter flippase plays an important role in preventing membrane flip-flop in general, as it translocates PS from the outer back to the inner plasma membrane leaflet. Importantly, we now found that dopamine inhibited flippase (10). Interestingly, reduced ATP levels were found in an in vivo model of catecholamine-induced cardiotoxicity36. Because flippase is an ATP dependent protein37, we wondered whether the effect of dopamine on flippase could be explained by an effect on ATP levels. We, however, found that dopamine induced only a minor reduction in ATP (11). It is therefore doubtful whether this would have an effect on flippase activity in dopamine stimulated cells, because from studies in erythrocytes it is known that flippase activity was not reduced until ATP levels were almost depleted, at least in these cells38-40.

Taken together, our data point to the induction of a pro-inflammatory status of the plasma membrane by dopamine, that might be an important reason for the increased presence of inflammatory cells in the heart, as found in both animal models36, 41, 42 and in patients with stress-induced heart disease17, 28, 43, 44.

Pulmonary embolism (Figure 3)

Thrombi in the pulmonary vasculature, also known as pulmonary embolism (PE), can be lethal in the acute phase, but can also result in clinical complications on the long term45. PE, namely, can induce dilation of the right ventricle (RV) due to a sudden increase in pulmonary vascular resistance45 (1). This then reduces RV output and left ventricular (LV) pre-load. In combination with an increased oxygen demand a state of hypoxia can arise, that theoretically can result in ischemia and/or infarction of the RV, facilitating failure of the RV (2), but eventually also the LV (3). Although in PE patients increased numbers of macrophages have been described in the RV, ischemic changes were not proven in these studies46, 47. Therefore, we wondered whether this inflammation might be caused by a PE-induced (stress) myocarditis43 (Chapter 5). In patients with acute PE, we indeed found an increase of inflammatory cells, namely neutrophilic granulocytes, lymphocytes, and macrophages, in both the RV and LV coinciding with myocytolyses, indicative for a stress-induced myocarditis43 (4). This is in line with the excessive activation of the neurohormonal system, as described in patients with PE48. This stress-induced myocarditis of the LV and RV, could, next to infarction of the RV, also explain RV but especially LV failure in patients with PE (5).
It has been suggested that PE also affects the atria of the heart, as the sudden increase in RV afterload induces dilatation of both the RV and right atrium (RA), that in the end, impairs left atrial (LA) diastolic filling. In Chapter 8 we describe, in patients without PE, that stress-induced myocarditis of the ventricles of the heart coincided with inflammation of the atria. Interestingly, atrial inflammation coincides with atrial fibrillation (AF). A recent study, shows that in 24% of patients with PE with right-sided atrial and ventricular endocardial thrombi, AF was detected. Although we found in our study endocarditis of the LV and RV, partly coinciding with endocardial thrombi, it is unknown whether these patients had AF. So, whether inflammation of the atria in patients with PE plays a role in AF induction is still unknown. These cardiac thrombi, theoretically, can embolize causing peripheral or cerebral ischemia and/or recurrent pulmonary embolism turning this disease into a vicious circle.

In conclusion, in patients with PE, we found a stress-induced myocarditis in ventricles of the heart that, in part, can explain their morbidity and mortality.

**Figure 3. The hypothetical pulmonary embolism cycle**

AF, atrial fibrillation; LV, left ventricle; RV, right ventricle.
**Burn wounds (Figure 4)**

A burn wound induces a massive inflammatory response, not only locally in the burn wound, but also systemically, affecting different organs, including the heart. An important inflammatory mediator herein, is complement. Complement is upregulated locally and in the circulation up to months after burn injury\textsuperscript{52, 53}. Indeed, inhibition of complement using C1-esterase inhibitor (C1inh) had beneficial effects in burn wound healing\textsuperscript{54, 55}. In addition, complement inhibition through C1inh or with C5a blockade, also reduced cardiac dysfunction, as it attenuated the reduction in ventricular function, sarcomere shortening, and impaired contractility in animals with burn wounds\textsuperscript{55, 56}. It has to be noticed that in these models, the effect of complement inhibition was measured up to a maximum of 4 days post-burn only, while it is known that the complement system is much longer activated in burn wounds. Therefore, we have studied the effects of a prolonged period of complement inhibition, namely up to 14 days post-burn, using C1inh. This was analyzed in a rat model in which the burn wound was induced using a copper stamp heated to 100°C that was pressed to the shaved part of the skin for 15 seconds\textsuperscript{41} (Chapter 6), inducing a full thickness, third degree burn wound (FTB)(1). We then found that C1inh resulted in improved re-epithelialization (RE) and less dermal granulation tissue formation (GT) (2), although, then only a minor decrease in complement C3 and C4 levels in the burn wound was found (3), what could indicate that the concentration and/or the application scheme of C1inh we used, was not optimal yet. Albeit, the minor decrease in complement levels in the burn wound could explain the lack of effect of C1inh on the number of neutrophils in the wound (4). C1inh, however, significantly decreased the total number of macrophages and increased the number of anti-inflammatory macrophages (ED2) (5). Besides complement inhibition, C1inh has also been shown to have inhibitory effects on both the coagulation and the contact system\textsuperscript{57} and reduced capillary leakage in an in vivo burn wound model\textsuperscript{55}. Because burn wounds are associated with a hypercoagulable state and severe edema\textsuperscript{58}, that adversely affect the healing process, these inhibitory effects of C1inh may also beneficially affect burn wound healing.

In the heart (6), the burn wound induced an increase of macrophages, predominantly of the pro-inflammatory phenotype, in both the ventricles and atria (7). This can be related to the massive increase in catecholamines in burn wounds\textsuperscript{13, 59}, inducing stress-related heart failure\textsuperscript{60}. C1inh then significantly reduced the number of infiltrating macrophages, that predominantly were of an anti-inflammatory phenotype (8).

These results thus show that systemic treatment with C1inh, improves wound healing of the skin and reduced inflammation of the heart, and thus might form a promising therapeutic intervention in burn wound patients.
Figure 4. Local and systemic response of burn wounds
C, capillary; C1inh, C1 esterase inhibitor; C3, complement factor C3; C4, complement factor C4; CM, cardiomyocyte; ED1, pro-inflammatory macrophage; ED2, anti-inflammatory macrophage; FTB, full thickness burnwound; GT, granulation tissue; PMNs, neutrophilic granulocytes; RE, re-epithelialization.

INFLAMMATION OF THE ATRIA

Acute myocardial infarction (Figure 5) and atrial inflammation (Figure 6)
AMI, is in majority, induced in the ventricles of the heart, mainly the LV, resulting in an inflammatory response especially in this particular area of the heart. Much less is known about its effect on the non-infarcted part of the heart. We have now found (figure 5), in patients with AMI and in a rat AMI model, that AMI induces infiltration of neutrophilic granulocytes, lymphocytes, and macrophages, not only in the infarcted LV, but also in the non-infarcted RV (1) and both atria (2) (Chapter 7). Even more, in rats with AMI, the AGE product Nε-(carboxymethyl)lysine (CML), an important pro-inflammatory mediator that coincides with endothelial activation, was also increased in the cardiac microvasculature of the infarcted LV, the non-infarcted RV and both atria of the heart (3) (Chapter 7). Interestingly, we did find in the rat AMI model, that application of C1inh not only reduced the number of lymphocytes and macrophages, but also CML depositions of the cardiac microvasculature in both ventricles and atria (4).
Interestingly, in 7 up to 21% of patients with AMI, atrial fibrillation (AF) is described. It was also found that patients with AF, not only have increased numbers of macrophages and lymphocytes in the atria\textsuperscript{61-65}, but also have increased CML levels in the plasma\textsuperscript{66}. Unknown, however, is the role of AGEs in the atria itself in AF. We have now found a significantly increase of CML accumulation in the cardiac microvasculature in the left atrial appendage of patients with AF (Chapter 9 / figure 6)\textsuperscript{1}, coinciding with endothelial activation, as visualized by VCAM-1 expression\textsuperscript{2} and increased infiltration of, not only lymphocytes and macrophages, but also neutrophilic granulocytes\textsuperscript{3}(Chapter 9). Inflammation of the atria has been shown to play role in AF induction\textsuperscript{67}. This because atrial inflammation can induce cell death of cardiomyocytes, facilitating fibrosis\textsuperscript{67} (4), that is directly associated with AF\textsuperscript{50}. Remarkably, in the patients with AF, we analyzed, we did not find increased fibrosis (chapter 9). However, the majority of patients with AF that we analysed, had paroxysmal AF (chapter 9) and fibrosis is often associated with the more persistent forms of AF\textsuperscript{68}. Even more, other studies have shown that AF may also occur in the absence of fibrosis, through direct effects of adipokines / cytokines on cardiomyocytes and ganglion plexi (GPs)\textsuperscript{69-73} (5). AF, in turn, can also induce inflammation\textsuperscript{50} (6), and in this way, could sustain itself.
Interestingly, we did find that inflammation in the atria due to AMI, was reduced with C1inh in rats. Because it is known that AMI can induce AF, this could point to a role of C1inh as a new potential therapeutic to prevent AF in AMI (7). Indeed, studies have been performed analyzing the effects of C1inh in patients with AMI74-77; however, the effects of C1inh on preventing AF in these patients was not studied herein and further research is thus required to establish this. Additionally, as C1inh has beneficial effects on reducing atrial inflammation and endothelial AGE formation, it may also be a potential therapeutic in preventing AF, independently of AMI.

Figure 6. Relation between atrial inflammation and atrial fibrillation
AF, atrial fibrillation; AGEs, advanced glycation end products; AMI, acute myocardial infarction; C1inh, C1 esterase inhibitor; GPs, ganglion plexi; Itis, myocarditis; L, lymphocytes; Mϕs, macrophages; PMNs, polymorphonuclear granulocytes; ROS, reactive oxygen species.
CONCLUSIONS

In this thesis, we have studied several aspects of the pathophysiology of AMI and stress-induced myocarditis, with diagnostic and therapeutical implications.

At an ultrastructural level, we have validated in deceased patients that AMI induces depositions in mitochondria 2 hours post-AMI, that can be used to diagnose early AMI at autopsies. In addition, changes in the cardiac microvasculature, namely basement membrane thickening and increased depositions of AGEs, were detected in AMI that both could play a role in the induction of AMI, but also in the post-AMI inflammatory response.

We have also found that a so-called stress-related myocarditis is induced in patients with pulmonary embolism or in burn wounds. Although it has been known for decades that the catecholamines adrenaline and noradrenaline induce cardiotoxicity, we now describe that the catecholamine dopamine induced lipotoxicity, oxidative stress, and a pro-inflammatory status of the plasma membrane of cardiomyocytes, that in part can explain inflammation and adverse cardiac effects found in patients with stress-induced myocarditis.

In both AMI and stress-induced myocarditis, we found that inflammation was not only limited to the ventricles, but that it also extended in the atria. This might play role in AF induction, in which the AGE product CML can also play an important role. Moreover, both inflammation and CML accumulation in the atria, induced by AMI, can be prevented by the complement inhibitor C1inh, indicating that C1inh might form a new potential therapeutic drug to prevent AF in AMI.
REFERENCES


