Crosstalk between apoptosis and inflammation in atherosclerosis

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Chapter 7

Summary and Discussion

Despite available treatments, be it lipid lowering or blood pressure lowering therapy, surgical intervention or life style changes, cardiovascular diseases (CVD) continue to be one of the main causes of death in the world[1]. Most clinical complications of CVD can be attributed to atherosclerotic plaque disruption and subsequent thrombus formation[2,3]. Atherosclerosis is a progressive disease of medium and large sized arteries characterized by accumulation of lipids in the artery wall[4]. It is a multifactorial process featuring in addition to subendothelial lipid deposition, excessive tissue remodeling, inflammation, and oxidative stress[5].

An important process generally considered to contribute to plaque progression and instability is apoptotic cell death. Apoptosis, programmed cell death characterized by morphological changes like cell shrinkage, DNA fragmentation, condensation of chromatin and membrane blebbing[6], occurs in atherosclerotic lesions and affects all major cell types relevant to the disease process such as endothelial cells, macrophages, T cells and vSMC[7]. Apoptosis increases with plaque progression, being virtually absent in initial lesions and overtly present in advanced lesions[8], as a result of enhanced apoptosis rates or impaired phagocytosis. Although originally considered anti-inflammatory or inflammation-neutral, recent insights support the notion that apoptosis may under conditions present in atheromathous tissue, promote inflammation[9].The consequences of apoptosis also depend on the cell type. Apoptosis of vSMC was seen to promote plaque vulnerability[10] whereas the impact of macrophage apoptosis is less well defined. At early stages macrophage apoptosis has been reported to be beneficial[11,12], whereas in advanced plaques phagocytosis is impaired[13] and apoptotic macrophages may undergo secondary necrosis, potentially leading to lipid core expansion, plaque inflammation and thus progression. On the contrary, others did not observe any effects of macrophage apoptosis on plaque size or inflammation in advanced stages of atherosclerosis[14].

In chapter 2 we review the inflammatory consequences of altered apoptosis and effects of plaque inflammation on cell death. Several pro-inflammatory mediators that contribute to atherogenesis can have pro- or anti-apoptotic effects. For example, proapoptotic members of the TNF super family, Fas, CD40 and Fn14, were also seen to promote secretion of inflammatory cytokines like MCP-1 and IL8 and/or adhesion molecules[15-17]. Apoptotic cells modulate inflammatory responses either on their own account by inducing amongst others cytokines[18] or after uptake and processing by phagocytes[19]. Furthermore, impaired and altered phagocytosis, as apparent during progressive atherosclerosis[20], elicits a proinflammatory response as a result of secondary necrosis thereby further aggravating inflammation leading to lesion progression and instability[9]. Reactive oxygen species and Ox-LDL, accumulating in the vessel wall during plaque progression, are able to induce cell death in endothelial cells, vSMC and macrophages[21-23] by upregulating several apoptotic proteins including Fas, TNFα, p53 and Bcl-2 family proteins[24-26]. In addition, several cytokines present in atherosclerotic lesions, including IFNγ[27,28], TNFα[29,30], IL1β[31], IL2[32], IL4[33] and MIF[34,35], are able to induce apoptosis themselves.

Since most clinical complications of cardiovascular disease are caused by plaque rupture in this thesis we first aimed to identify genes or pathways promoting thin cap fibroatheroma formation. Thin cap fibroatheroma (ThCFA) can be defined as plaques containing a large lipid core with an overlying thin fibrous cap, as opposed to thick cap fibroatheroma (TkCFA) which are more fibrous in nature and have as indicated by their name a thick cap. ThCFA and TCFA are categorized as type IV and V lesions, respectively, according to the classification system of the American Heart Association (AHA). ThCFA are considered as the most vulnerable to rupture[36]. In chapter 3 of this thesis we describe a microarray analysis on RNA obtained from mouse TkCFA and ThCFA early on in the plaque destabilization process in two different models of thin cap fibroatheroma formation. As a first model we chose spontaneous vulnerable plaque development in the brachiocephalic artery of ApoE$^{-/-}$ mice, after 9 weeks of Western type diet feeding, when plaques were reported to display the first features of enhanced vulnerability[37]. As a second model we considered low shear stress induced plaque formation in the carotid artery of ApoE$^{-/-}$ mice that were further destabilized by cap overexpression of the tumor suppressor protein p53[38]. We were able to identify several functional clusters consisting of differentially regulated genes that were overrepresented in both models of thin cap fibroatheroma. These functional gene clusters include lipid metabolism, small molecule biochemistry, metabolic disease, cellular growth and proliferation and cell-to-cell signaling and interaction. Genes regulating cell death were most significantly dysregulated in thin cap fibroatheroma versus thick cap fibroatheroma. Within this functional cluster, five upregulated genes were shared between both thin cap fibroatheroma models (Cd5l, Plagl1, Bim and two genes of unknown function). This indicates that CD5l, Plagl1 and Bim, the differential expression of which was confirmed by realtime PCR, may play a role in the transition of thick cap fibroatheroma into a more vulnerable

plaque phenotype.
In addition to the identification of cell death regulating genes possibly involved in thin cap fibroatheroma development, one particular gene, Npy, a neurotransmitter with both pro- and anti-atherogenic properties[39-41], was evident. Apart from an upregulation in thin cap fibroatheroma in the brachiocephalic artery compared to thick cap fibroatheroma in the carotid artery, Npy expression was shown to increase with disease progression not only in murine but also in human plaques.

Having identified cell death associated genes and gene clusters which were differentially regulated in thin cap fibroatheroma versus thick cap fibroatheroma, we set out to assess the role of several apoptosis regulating proteins in atherosclerotic plaque development and stability in different mouse models. One of the first genes of interest was Bim, which as microarray analysis indicated is one the cell death regulating factors differentially expressed in thin cap fibroatheroma compared to thick cap fibroatheroma. In chapter 4 we have investigated effects of leukocyte Bim deficiency on plaque development in LDLr$^{-/-}$ mice. Previous studies in Bim deficient mice have already revealed that Bim is a key regulator of leukocyte apoptosis[42,43], and particularly important in the deletion of autoreactive and activated T and B cells[42,44-46]. T and B cell levels, including autoreactive T and B cell numbers, are markedly increased in circulation, spleen and thymus of Bim deficient mice[42] facilitating the development of auto-immunity and lymphadenopathy[42,44,45]. As lymphocytes are important regulators of immune responses in atherogenesis[47,48] and atherosclerosis is considered to have features of an autoimmune disease[49,50] Bim may be of particular relevance in atherosclerosis.
Deletion of leukocyte Bim in Western type diet fed LDLr$^{-/-}$ mice resulted in an increased pro-inflammatory status as demonstrated by marked splenomegaly and enhanced T cell activation and proliferation resulting in increased circulating and mediastinal lymph node T cell levels. Leukocyte Bim deficiency led to increased lesion T cells as well. Leukocyte Bim deficient LDLr$^{-/-}$ mice had increased Ox-LDL antibody levels of the IgG1 but not IgG2a isotype in serum and in addition we found dramatically increased total immunoglobulin levels in atherosclerotic lesions of Bim$^{-/-}$ BM transplanted mice. In atherosclerotic lesions of ApoE$^{-/-}$ mice, human and rabbit as well as in serum Ox-LDL specific antibodies have been detected[24-26]. Studies in which mice were immunized with modified LDL resulted in decreased lesion formation[54-57], suggesting that the Bim deficiency related increase in Ox-LDL specific auto-antibodies are atheroprotective. Although Bim$^{-/-}$ bone marrow derived macrophages were less sensitive to cell death after culturing and growth factor withdrawal as well as after stimulation with oxidized LDL, lesion apoptotic cell content was not altered in leukocyte Bim deficient LDLr$^{-/-}$ mice. Since apoptotic cells in spleen were clearly elevated, the observed lack of effect on apoptosis in lesions may be a result of increased resistance of plaque macrophages against Bim deficiency associated cell death or of the low T cell content in atherosclerotic lesions. Despite these profound effects on lymphocyte homeostasis, inflammatory

status and unexpectedly, the reduction in lipid serum levels, atherosclerotic lesion burden and stability in both aortic root and descending aorta was unchanged in Bim$^{-/-}$ BM transplanted LDLr$^{-/-}$ mice. We hypothesize that the observed pro- and anti-atherogenic effects of Bim deletion on inflammatory response, together with decreased lipid levels counterbalance, resulting in unaltered atherogenesis. Possibly cell-type specific modulation of Bim expression will clarify the contribution of Bim functioning in specific cells to atherogenesis.

Bim activity is in part regulated by the Bcl-2 family member myeloid cell leukemia 1 (Mcl-1) which directly interacts with Bim, Bid, another pro-apoptotic BH3-only protein, and multidomain pro-apoptotic Bak[39-41], to inhibit apoptosis. Mcl-1 plays an important role in promoting survival and differentiation of leukocyte subsets[58,59] being essential for neutrophil survival[60]. In chapter 5 we investigated the consequences of modulating macrophage apoptosis on atherosclerotic plaque development and stability by deleting Mcl-1 in lysozyme M (lysM) expressing, myeloid cells. Lethally irradiated LDLr$^{-/-}$ mice were transplanted with Mcl-1$^{fl/fl}$ LysMcre or wildtype bone marrow, followed by Western-type diet feeding to induce atherosclerotic lesion development.
We show that Mcl-1 participates in Ox-LDL induced cell death, and regulates gene expression of other apoptosis regulating Bcl-2 family members Bim, PUMA and NOXA after Ox-LDL exposure. In atherosclerotic aortic root lesions of Mcl-1$^{-/-}$ bone marrow transplanted mice apoptotic cell content was elevated by 77% compared to WT controls. However, atherosclerotic plaque size did not differ between Mcl-1$^{-/-}$ and WT bone marrow recipients as a result of additional effects of myeloid Mcl-1 deficiency. Mcl-1$^{-/-}$ macrophages exhibited increased lipid uptake and increased peritoneal foam cell counts, marked neutropenia and altered neutrophil phenotype in Mcl-1$^{-/-}$ bone marrow transplanted mice. Mcl-1$^{fl/fl}$ LysMcre transplanted mice not only carry an Mcl-1 deletion in macrophages but also in neutrophils[61]. In keeping with the notion that Mcl-1 is essential for neutrophil survival, neutrophil levels in circulation and lesions were sharply decreased in Mcl-1$^{-/-}$ bone marrow transplanted mice. The remaining neutrophils, that escaped Cre mediated deletion, displayed a decreased response to chemoattractant KC, partly due to reduced responsiveness and partly a result of the substantially reduced neutrophil pool Furthermore, CXCR4 expressing neutrophil levels in Mcl-1$^{-/-}$ BM transplanted mice were increased compared to WT BM recipients, indicative of increased release of neutrophils from stroma.
Finally, myeloid Mcl-1 deficiency resulted in alteration of the inflammatory properties of LDLr$^{-/-}$ mice. Isolated peritoneal macrophages from Mcl-1$^{-/-}$ BM recipients contained a high amount of multinucleated giant cells as compared to WT macrophages, suggestive of an altered apoptotic response and macrophage polarization. Another indication for an elevated inflammatory status of Mcl-1$^{-/-}$ BM transplanted mice was found in the apparent shift towards a pro-inflammatory M1 macrophage phenotype as judged from their cytokine expression patterns and reduced capacity for apoptotic

cell clearance. Thus analogous to leukocyte Bim deletion, myeloid Mcl-1 deletion in LDLr$^{-/-}$ mice results in several cell death and inflammation related effects but, surprisingly, without affecting atherosclerotic plaque size or stability, emphasizing the importance of pro-atherosclerotic neutrophil recruitment into plaques.

In chapter 6 we studied the atherogenic role of focal adhesion kinase (FAK), a kinase not only involved in cell death and proliferation, but also in adhesion and migration of various cell types[62]. Plaque initiation starts with adhesion of leukocytes to the luminal endothelial cell layer followed by migration into the subendothelial space[63]. After initial tethering and rolling of circulating leukocytes, mediated by selectins, adhesion involves the engagement of β-integrins which interact with ICAM-1 and VCAM-1[64,65]. FAK is activated by (auto)phosphorylation upon integrin binding, resulting in increased turnover of focal adhesions thereby stimulating migration[66]. We generated ApoE$^{-/-}$ mice with heterozygous FAK expression, since FAK deletion is embryonically lethal[67]. Atherosclerosis was induced by Western type diet feeding.
Reduced FAK levels resulted in an increased inflammatory status in ApoE$^{-/-}$ mice as witnessed by splenomegaly and enlarged splenic germinal centers. Furthermore, leukocyte composition in spleen, peritoneal cavity and circulation was affected by FAK reduction. Macrophage content in spleen was found to be decreased, whereas that of lymphocytes was increased. In contrast, peritoneal cavity and circulation showed increased monocyte numbers accompanied by reduced CD4^{+} and CD8^{+} T cell content. These differential leukocyte composition was likely caused by impaired monocyte ingress from circulation to spleen even despite enhanced blood monocyte counts and enhanced retention of lymphocytes in spleen. FAK$^{+/-}$ApoE$^{-/-}$ mice had significantly lower plasma total cholesterol levels, which was shown to be specifically evident upon a Western-type diet and attributable to a decreased hepatic VLDL production rather than to an altered intestinal cholesterol absorption. Hepatic lipid content was unchanged in FAK$^{+/-}$ApoE$^{-/-}$ mice. However, hepatic expression of critical genes in lipogenesis, SREBP1 and 2, SCD1 and FAS, was increased in FAK$^{+/-}$ApoE$^{-/-}$ mice in addition to a slight increase in CD36 expression. OxLDL mediated CD36 signaling was shown to inhibit macrophage migration and stimulate cell spreading by continued FAK phosphorylation and activation[68], suggesting a role for FAK in trapping macrophages in the arterial wall and promoting atherogenesis.
Surprisingly, despite these marked effects on immune status, impaired FAK expression neither affected size and composition of semi-constrictive perivascular collar induced carotid artery plaques nor that of spontaneous brachiocephalic artery and aortic root plaques. We therefore assessed atherosclerotic lesion size in chow fed ApoE$^{-/-}$ mice with reduced FAK expression in order to eliminate the impact of reduced lipid levels on atherosclerosis development. However, also here atherosclerotic lesion size of FAK$^{+/+}$ and FAK$^{+/-}$ mice was essentially comparable. Thus, FAK reduction beneficially affects lipid metabolism in Western type diet fed ApoE$^{-/-}$ mice and alters their inflammatory status without resulting in an altered atherogenic response.

In chapter 6 we show that systemic reduction of FAK activity alters various inflammatory parameters. In addition we are the first to show an unexpected effect of FAK on hepatic lipid metabolism in mice. The results obtained from the studies described in chapter 4 and 5 provide new insights on the role of apoptosis regulating Bcl-2 family members in atherosclerosis. We show that both pro-apoptotic Bim and anti-apoptotic Mcl-1 have several functions during atherogenesis including intraplaque cell death, foam cell formation and lipid metabolism. Moreover, modulation of Bim and Mcl-1 expression resulted in major effects on inflammatory processes. Apoptosis, and in particular that mediated by Bcl-2 family members, is essential in homeostasis and functioning of the immune system[69]. Deletion of autoreactive T and B cells, termination of the immune response and survival of activated T and B cells are all regulated by Bcl-2 family member and death receptor dependent apoptosis. Dysregulated apoptosis of immune cells may result in various pathological conditions including immunodeficiency, autoimmunity, tumor growth and infection[69]. The Bim and Mcl-1 associated modulation of inflammatory parameters observed in our studies correspond with the previously reported role of these proteins in immune system homeostasis. We further show that both Bim and Mcl-1 regulate specific cell death and inflammatory processes relevant to atherosclerosis. However, the key role in systemic immunity and the multifaceted mode of action of these proteins involving apoptotic, phagocytic and inflammatory processes and lipid metabolism should be taken into account when considering therapeutic approaches targeting these apoptosis regulating proteins. In the studies described in chapter 4 and 5 we specifically deleted Bcl-2 family proteins in total hematopoietic (Bim) or myeloid cells (Mcl-1), whereas in the study described in chapter 6 FAK expression was reduced at a systemic level. Local modulation of gene expression, for example by targeted gene or drug delivery, or in the case of FAK, cell type specific modulation, might be a valuable approach to restrict therapeutic effects to the atherosclerotic lesions without affecting the systemic immune response. In particular, local intervention in Mcl-1 signaling, to prevent pathological consequences of altered systemic immunity, to modify macrophage lipid accumulation and plaque apoptosis could potentially be a valuable approach to modulate atherogenesis. Further studies are necessary to confirm this hypothesis.

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