Targeting glycolysis in endothelial cells to prevent intraplaque neovascularization and atherogenesis in mice

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Chapter 9

General summary and future perspectives

Summary

In recent years, endothelial cells (ECs) metabolism has attracted renewed attention which has unveiled an unexpected complexity of regulatory mechanisms and a plethora of potential novel targets for vascular-related diseases.[1, 2] Despite the fact that ECs have readily available oxygen in the blood, they mainly generate ATP via anaerobic glycolysis rather than Krebs cycle. Thus, by relying on anaerobic rather than aerobic metabolism, ECs are able to sprout in low oxygen conditions such as the tumor environment.[1, 3] Among the different enzymes involved in the control of glycolytic flux, PFKFB3 (6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3) has been shown to play a critical role for the proliferation and migration of ECs. [4, 5] Intraplaque (IP) neovascularization, has been identified as a contributing factor of atherosclerotic plaque vulnerability in human atherosclerosis. In this context, regulation of EC metabolism may represent a novel target to inhibit IP angiogenesis and to promote plaque stability. The experimental work of this thesis focuses on PFKFB3 as a modulator of EC glycolysis and its effect on atherosclerosis progression and intraplaque angiogenesis.

In **chapter 2,** a review of potential pharmacological strategies to inhibit IP angiogenesis is presented. Such strategies include: (1) inhibition of vascular endothelial growth factor signaling, (2) inhibition of glycolytic flux, and (3) inhibition of fatty acid oxidation. Overall targeting IP neovascularization might be suitable therapeutic approach to promote plaque stabilization in combination with lipid-lowering treatment.

In **chapter 3,** a comprehensive review of all animal models of atherosclerosis is presented. The chapter also includes a description of the two main models that have been used in this thesis, namely apolipoprotein E-deficient Fibrillin-1 mutant mice ($ApoE^{-/-}Fbn1^{C1039G+/-}$) and vein grafts in $ApoE^{-/-}$ mice. These two animal models develop unstable atherosclerotic plaques with IP angiogenesis, and thus are suitable to study the role of glycolysis during IP neovascularization and plaque progression.

In **chapter 4,** a pharmacological study with partial glycolysis inhibitor 3PO [3-(3-pyridinyl)-1-(4-pyridinyl)-2-propen-1-one] in the context of advanced atherosclerotic plaques in $ApoE^{-/-}Fbn1^{C1039G+/-}$ mice is described. 3PO treatment restrains IP

angiogenesis and plaque frequency, however it does not affect plaque size and composition. In addition, a 3PO-mediated reduction in plaque formation is observed in regular ApoE$^{-/-}$ mice that develop plaques without IP neovascularization. Overall, these data suggest that vessel wall metabolism may play a role in the early stages of atherosclerosis. In vitro and in vivo data show that 3PO prevent upregulation of VCAM-1 and ICAM-1 in ECs, two key adhesion molecules involved in early-stage plaque development. Mechanistically, 3PO inhibits TNFα-NF-κB signalling pathway in ECs, which led to suppression of VCAM-1 expression both in vitro and in vivo. Furthermore, downregulation of endothelial VCAM-1 (and ICAM-1) expression depends on 3PO-inducted autophagy. Expression of the adhesion molecules is not downregulated by 3PO in TNF-α-treated ECs in which expression of the essential autophagy gene ATG7 was silenced. On the contrary, VCAM-1 (and ICAM-1) expression is upregulated in ATG7-deficient ECs, suggesting that autophagy suppresses expression of these adhesion molecules. These findings support previous data showing that endothelial autophagy is atheroprotective and limits atherosclerotic plaque formation by preventing endothelial apoptosis, senescence and inflammation.[6]

In **chapter 5,** the mechanism behind glycolysis inhibition by 3PO is investigated. 3PO is considered a PFKFB3 competitive inhibitor however conclusive affinity assays are still lacking**.** The study presented in chapter 5 shows data from isothermal titration calorimetry indicating that 3PO does not bind to PFKFB3, up to 750 μM. Conversely, AZ PFKFB3 67 at 3 μM concentration shows strong and potent PFKFB3 inhibition. This study also confirms that 3PO inhibits glycolysis in ECs and demonstrates that the inhibitory effect of 3PO on glycolysis relies on its capacity to cause an imbalance in intracellular pH through the accumulation of lactic acid inside the cell. This finding is not surprising as other glycolytic enzymes such as lactate dehydrogenase and phosphofructokinase-1 (PFK-1) are also extremely sensitive to pH. A change of less than one pH unit, even a few tenths, reduces the activity of PFK-1 by more than 10-fold. Moreover, PFKFB3, also known as phosphofructokinase-2 (PFK-2), has been shown to be allosterically regulated by hydrogen ion concentrations. On the other hand, lactate is a modulator of intracellular pH, hence the accumulation of this metabolite leads to intracellular milieu acidification and affect indirectly the glycolysis rate

In **Chapter 6,** a genetic approach to study the role of endothelial PFKFB3 in IP angiogenesis and plaque development is described. ApoE$^{-/-}$ mice were crossbreed with PFKFB3$^{fl/fl}$ Cdh5^{iCre} mice (containing a tamoxifen-inducible EC-specific Cre) to generate an ApoE$^{-/-}$ PFKFB3^{ECKO} mouse strain. A vein graft model is then used to study the effect of an EC-specific deletion of PFKFB3 in advanced plaques with IP neovascularization. Analysis of IP neovascularization in vein graft lesions of ApoE$^{-/-}$ PFKFB3^{ECKO} mice show a significant decrease in the amount of microvessels, which is in line with the effects obtained with glycolysis inhibitor 3PO. However, in contrast with 3PO, a decrease in vein graft lesion area and percentage of stenosis in ApoE$^{-/-}$ PFKFB3^{ECKO} mice is observed. One possible explanation for such differences is the use of 3PO as therapeutic agent (administration after 4 or 16 weeks of western diet), while PFKFB3 is deleted ahead of the western diet regimen. Furthermore, 3PO is not an EC-specific agent and may also interfere with the metabolism of other cell types involved in plaque development. The reduction in IP angiogenesis observed in vein graft lesions was accompanied by a reduction in leakage of these microvessels. These findings are in line with in vitro and in vivo observations showing that PFKFB3 inhibition in ECs reduces VE-cadherin endocytosis and promotes normalization of the endothelial barrier in the settings of cancer biology.[7] Together with a decreased number of microvessels, reduction of macrophage infiltration in lesions of ApoE$^{-/-}$ PFKFB3^{ECKO} mice is also observed, suggesting a link between EC metabolism and macrophage infiltration. In agreement with previous findings, it is possible that such reduced lesion infiltration *in vivo*, is in part due to an improved restoration of EC cell-cell junction after PFKFB3 deletion.[7] Interestingly, EC-specific PFKFB3 deletion inhibits plaque development in ApoE$^{-/-}$ mice, a native atherosclerosis animal model that does not develop features of advanced lesions such as IP microvessels. Altogether, these findings show the potential value of targeting EC glycolysis and in particular PFKFB3 as a therapeutic strategy to counteract plaque development in vein grafts and native atherosclerosis.

In **chapter 7,** a study performed in collaboration with the University of Aberdeen is presented. Here the development of a PFKFB3-targeted PET radiotracer, [^{18}F]ZCDD083 (^{18}F-radiolabelled phenoxindazole compound, ZCDD083), for imaging the atherosclerotic plaque in vivo is described. ZCDD083 is a close structural mimic of the potent PFKFB3 inhibitor AZ68 (IC50 = 4 nM), whose

radiofluorination is deemed to be chemically viable and unlikely to affect the binding to PFKFB3. The specificity of the tracer for atherosclerotic plaques is demonstrated by the combination of ex vivo autoradiography with en face Oil Red O staining of the same aortic specimens. Indeed, co-localisation of the $^{[18}$F]ZCDD083 signal with plaque distribution along the aorta is observed. These cross studies in C57BL/6J, ApoE$^{-/-}$ and ApoE$^{-/-}$Fbn1$^{C1039G+/-}$ mice demonstrates high sensitivity of $^{[18F]}$ZCDD083 to detect atherosclerotic plaques, whereas little signal is detected in normal vessels or outside atherosclerotic lesions. This tracer is a promising non-invasive diagnostic tool to detect rupture-prone atherosclerotic plaques, which in turn could help improving risk stratification and evaluation of the efficacy of anti-atherosclerotic therapies.

In **chapter 8,** a novel imaging method for a three dimensional reconstruction of the IP vessel network in a mouse model of advanced atherosclerosis that spontaneously develops IP angiogenesis is presented. This method based on immunolabeling-enabled 3D Imaging of Solvent Cleared Organs (iDISCO) and confocal microscopy, may represent a useful tool for studies aimed at determining whether there is a causal relationship between the presence of IP neovessel structures and atherogenesis or between angiogenic stimuli and plaque angiogenesis.

General discussion and future perspective

Glycolytic flux in ECs is 200-fold higher than glucose oxidation, fatty acid oxidation, and glutamine oxidation, resulting in the generation of >85% of the total cellular ATP content.[1] The glycolytic activity of ECs is often as high as in cancer cells. Recent studies have shown that the proangiogenic response to growth factors such as VEGF (vascular endothelial growth factor) or FGF (fibroblast endothelial growth factor) relies on metabolic changes such as increased glycolytic flux and fatty acid oxidation (FAO) in endothelial cells (ECs).[3, 5, 9]

In the context of atherosclerosis, there has been growing interest on the role of EC metabolism and how it affects both plaque formation and intraplaque (IP) angiogenesis.[2] For example, recent results have shown that laminar shear stress *in vitro* reduces glucose uptake by ECs, via an upregulation of the transcription factor

Krüppel-like factor 2 (KLF2). This mechanism allows to maintain a quiescent metabolic phenotype in ECs and may promote an atheroprotective effect.[10] Another link between ECs metabolism and atherosclerosis has been identified in the glycolytic enzyme PFKFB3, which is upregulated in atheroprone regions of arterial vessels (usually exposed to turbulent blood flow) and in carotid plaques of patients with elevated levels of lipoprotein(a).[8, 10] However, to date there are no published studies on the role of EC metabolism in animal model of atherosclerosis.

ECs are also capable of switching from a quiescent state to a highly proliferative and migratory state when angiogenesis needs to take place. Studies on the metabolic requirements for ECs to undergo an angiogenic switch are slowly emerging. Our increasing understanding of EC-metabolism regulation in the context of IP angiogenesis may unveil novel pharmacological targets to promote plaque stability. Interestingly, neovascular networks are often found inside advanced or vulnerable atherosclerotic plaques and recent evidence suggests that 50% of patients with acute coronary syndrome present one or more lesions with IP angiogenesis. This is five times more frequently than in lesions from patients with stable coronary artery disease.[11] The experimental data generated in this thesis with the use of animal models that recapitulate advanced human atherosclerosis, strongly suggest a critical role of EC metabolism in the formation and progression of atherosclerosis in addition to IP angiogenesis.

This thesis includes a number of pharmacological and genetic preclinical studies that describe the effect of glycolysis inhibition in the activated vessel wall and endothelium in the context of atherosclerosis. In particular, the work of this thesis focuses on PFKFB3, nevertheless, other metabolic enzymes have also been recently identified in the control of EC glycolytic flux and angiogenesis. Indeed, a recent study has shown that deletion of hexokinase 2 (HK2) in ECs leads to decreased glycolysis, which impaired EC proliferation, migration and angiogenesis in vivo.[9] Finally, endothelial fatty acid oxidation (FAO) has been shown to play a rather unexpected role in vessel sprouting. A recent study proved that EC-specific deletion of carnitine palmitoyltransferase 1a (CPT1a), which imports fatty acids (FAs) into mitochondria and thereby rate-limits FAO flux, decreased EC proliferation and caused sprouting reduction both in vitro and in vivo.[12] The role of these EC metabolic

pathways in the context of atherosclerotic plaque formation and progression has not been investigate thus far.

Overall, the results presented in this thesis strongly argues that glycolysis inhibition in ECs may promotes beneficial effects such as a reduction in plaque formation, plaque progression and IP angiogenesis. Given the strong evidence that decreased IP vascularization makes plaques less vulnerable, glycolysis inhibition may represent a novel strategy to reduce the risk of plaque rupture and frequency of adverse cardiovascular events.[13-15]

The work of this thesis also presents new compelling data that will be expanded in future studies. For example, in vein graft lesions of ApoE$^{-/-}$ PFKFB3^{ECKO} mice, a marked reduction in IP neovascularization is associated with reduced plaque growth (See Chapter 6), however whether these events are causally linked require additional investigation. Interestingly, mice treated with glycolysis inhibitor 3PO do not display changes in progression of established plaques however the number of plaques is reduced. Histological data from human plaques indicate that in early lesions VCAM-1 and ICAM-1 are predominantly expressed by the endothelium, whereas in more advanced lesions, the majority of VCAM-1 expression is found in subsets of intimal VSMCs and macrophages. These findings may explain why downregulation of adhesion molecules by 3PO in ECs mainly affects plaque formation, but not further steps of plaque progression. Interestingly, the metabolic stress caused by 3PO stimulated autophagosome formation in ECs and led to induction of autophagy. Similar observations were previously reported in cancer cells.[16]

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the enzyme that catalyses the rate limiting step in cholesterol synthesis and they are one the most widely prescribed drugs. Statins are used to slow down atherosclerosis progression and to reduce cardiovascular risk. Apart from their cholesterol lowering action, they also have anti-inflammatory properties and improve endothelial function by increasing the expression of endothelial progenitor cells involved in vascular repair as well as the expression of endothelial nitric oxide synthase.[17] More recently, the class of PCSK9 Inhibitors (e.g. alirocumab, evolocumab) that inactivate liver *proprotein convertase subtilisin kexin* 9 achieve

even lower circulating LDL-C levels, and have been approved as first-line drugs to reduce the risk of cardiovascular events in patients that cannot tolerate statins.[18] Statins and PCSK9 inhibitors act on hyperlipidaemia, which is a well-established risk factor for plaque progression, rupture and adverse cardiovascular events. Drugs that specifically target EC metabolism may offer a novel approach to inhibit the development of atherosclerosis lesions by targeting the underlying mechanisms of endothelial disfunction. Furthermore, a combination therapy for both ECs and lipid metabolism in established atherosclerosis may result in reduced cardiovascular complications due to vulnerable plaques and leads to an overall improved survival.

Development of new diagnostic and imaging tools in atherosclerosis has been active area of research in the last few decades.[19] Indeed, studying the complex mechanisms behind the progression of atherosclerosis has been made hard by the difficulty of following the morphological changes in a patient over time. Moreover, there are a limited number of non-invasive image modalities that allow to collect information on the composition of atherosclerotic plaques. For example, intravascular ultrasound (IVUS), optical coherence tomography (OCT) and near-infrared spectroscopy (NIRS), can provide a limited plaque characterization but they are invasive and thus not ideal in early-diagnosis or follow-up.[20]

To date Positron emission tomography (PET) is a non-invasive nuclear imaging method which can detect and quantify the pathophysiological processes associated with atherogenesis and subsequent risk of plaque destabilization. However, there is a need for a better PET tracer for routine clinical imaging. Numerous pathways and targets have been studied but currently there is only one approved tracer for clinical use (18F-FDG) .[21] However, carotid artery imaging using 18F-FDG PET often shows unspecific myocardial uptake.[22, 23] A new PFKFB3-targeted PET radiotracer ([^{18}F]ZCDD083) has been presented in this thesis with several advantages which include a higher in vivo metabolic stability and specific uptake in atherosclerotic plaques. This new radiotracer may represent a better tool for the evaluation of plaque morphology and identification of vulnerable plaques.

The majority of the studies aimed at identifying pathological pathways of human atherosclerosis plaques have been done using two dimensional cross-sections of autoptic or surgical samples. However, a more detailed study of atherosclerotic lesions with particular regard to the morphological features that make a plaque

vulnerable such as the presence of intraplaque neovessels, could be obtained through the three-dimensional reconstruction of human atherosclerotic plaques. For the first time in this thesis, is presented a preclinical study in which using innovative clearing techniques it is possible to reconstruct the small vessels inside the atherosclerotic plaque three-dimensionally. Additional studies with human samples are still required but this method may provide the missing tool to characterize and understand the complexity of intraplaque neovessel network in human atherosclerotic plaque.

The studies and experimental results described in this thesis contribute to a better understanding of EC metabolism in the context of atherosclerosis and intraplaque angiogenesis. This work also suggests a strong link between EC metabolism, intraplaque angiogenesis and plaque stability which potentially unveil a completely, novel therapeutic approach to enhance plaque stability.

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