Cell cycle and apoptosis genes in atherosclerosis

Boesten, Lianne Simone Mirjam

Summary

The work described in this thesis was aimed at identifying the role of cell cycle and apoptosis genes in atherosclerosis. Atherosclerosis is the primary cause of cardiovascular disease, a multi-factorial disorder occurring in the large and medium-sized arteries of the body. Although in the beginning 90s promising lipid lowering therapies predicted a strong reduction in cardiovascular deaths for the upcoming years, in westernized societies it is still the underlying cause of about 40% of all deaths, indicating that treatment of atherosclerosis goes beyond lipid lowering solely. In addition to lipids, continuous cell growth (cell cycle), cell death (i.e. apoptosis and necrosis) and inflammatory processes play a central role in the development and maintenance of atherosclerotic lesions. To investigate in detail the role of several cell cycle and apoptosis genes in atherosclerosis we generated and characterized several mouse models as described in this thesis.

Based on the findings that germline null alleles of the cell cycle genes of our interest (i.e. p53, Rb and Mdm2) lead to either the formation of tumors after the age of 6 months (p53) or embryonic lethality (Rb and Mdm2) we chose to use site-specific recombinase (SSR) technology. To obtain cell type specificity we used either the LysMcre or the SM-CreER^{T2}(ki) mouse model for targeting macrophages or SMCs, respectively.

The tumor suppressor gene p53 has been shown to inhibit cell proliferation and stimulate apoptosis in many cell types. To study the role of macrophage p53 in the development of atherosclerosis, we generated apoE-deficient mice with a macrophage-restricted deletion of p53 and control littermates and analyzed early and advanced atherosclerosis development (**chapter 2**). Absence of macrophage p53 did not affect lesion area in both early and advanced atherosclerosis, neither in the aortic root nor in the aortic arch and thoracic aorta. In early atherosclerosis, absence of macrophage p53 resulted in reduced apoptosis, though without changes in lesion composition. In contrast, in advanced atherosclerosis, reduced apoptosis upon absence of macrophage p53 coincided with increased necrotic death, increased foam cell content, and reduced lipid core formation. Proliferation was not affected by the absence of macrophage p53 in both early and advanced atherosclerosis. Hence, these studies demonstrate that macrophage p53 is a major mediator of foam cell apoptosis and inhibition of this pathway results in a shift of cell death towards necrotic death of lesional macrophages, thereby affecting lesion composition.

To expand the knowledge on the role of cell cycle genes in vascular disease *in vivo* we investigated the role of macrophage Retinoblastoma (Rb) in atherosclerosis development. The tumor suppressor gene Rb has been shown to regulate both cell proliferation and cell death in many cell types. In **chapter 3** we describe the role of macrophage Rb in atherosclerosis development in apoE-deficient mice. To this end, we fed a cholesterol-rich diet for 12 weeks to apoE-deficient mice with a macrophage-restricted deletion of Rb and control littermates. Macrophage-restricted Rb deletion resulted in a strong increase in atherosclerotic lesion area. In addition, the increase in atherosclerosis was characterized by the presence of more advanced lesions that were rich in smooth muscle cells and poor in macrophages. Additional analyses showed that the increase in atherosclerosis was independent of *in vitro* macrophage modified lipoprotein uptake or cytokine production. Immunohistochemical analysis showed that macrophage-restricted Rb deletion did not affect

lesional macrophage apoptosis, but lesional macrophage proliferation was strongly increased. These studies clearly demonstrate that macrophage Rb is a suppressing factor in the progression of atherosclerosis via reduction of macrophage proliferation.

The Mdm2 oncoprotein inhibits p53 activity during embryonic development and in adult homeostatic tissues. Overexpression of p53 can be achieved by specific inactivation of its inhibitor Mdm2 (**chapter 4**). To this end, conditional allelic inactivation of Mdm2 was carried out in mice harboring Mdm2 floxed alleles and a tamoxifen-inducible Cre-recombinase under control of the SM22 promoter (SM-CreER^{T2}(ki) mice), resulting in mice that inducibly lack Mdm2 in their smooth muscle cells. This mouse model would allow us to study p53 overexpression in the SMC-rich cap of atherosclerotic lesions. However, upon SMC-specific Mdm2 deletion mice became rapidly ill and died, hampering studies on the role of SMC-p53 in atherosclerosis. Unexpectedly, the mouse model showed that Mdm2 prevents accumulation of active p53 in quiescent SMCs and thereby the induction of p53-mediated necrotic cell death *in vivo*.

Chapter 5 describes a means to conditionally and locally modify genes of the vasculature using a perivascular drug delivery device (PDD). A 4-hydroxytamoxifen (4-OHT)-eluting PDD was applied around the carotid or femoral artery of a mouse strain, carrying both the tamoxifen-inducible and smooth muscle cell (SMC)-specific Cre-recombinase (SM-Cre-ER^{T2}(ki)) transgene and a stop-floxed β-galactosidase gene in the Rosa26 locus. A dose and time curve of 0-10% (w/w) 4-OHT and 0-14 days application of the PDD showed optimal gene recombination at 1% (w/w) 4-OHT loading at 7 days post application. Recombination was similar to the level achieved by systemic tamoxifen administration and was completely confined to the PDD-treated vessel wall segment. Thus, local application of a 4-OHT-eluting PDD results in vascular SMC-specific Cre-mediated recombination without affecting additional SMCs.

In addition to cell proliferation and cell death, inflammation plays a key role in the development of atherosclerosis. Immune cells are of paramount importance in early atherosclerosis development and their effector molecules accelerate progression of atherosclerosis. Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine exerting both cell death and inflammatory activity. Although TNFα and its receptors are thought to be considerably important in a number of biological activities relevant to atherosclerosis, its complete function in atherogenesis remains unclear. Earlier studies in mice indicated that TNFα affects atherosclerosis minimally or not in early atherosclerosis development. To study the role of TNFα in advanced and complex atherosclerotic lesions we crossbred TNFα-deficient mice onto an APOE*3-Leiden background (**chapter 6**). To induce atherosclerosis development the mice were fed a cholesterol-rich diet. Mice deficient for TNFα and their control littermates, showed comparable levels of plasma cholesterol and triglycerides and the systemic inflammatory parameters, serum amyloid A (SAA) and soluble intercellular adhesion molecule-1 (sICAM). Although absence of TNFα did not affect the quantitative area of atherosclerosis, mice deficient for TNFα had a higher relative number of early lesions and a lower relative number of advanced lesions. In addition, the advanced lesions in TNFα deficient mice showed a decrease in necrosis and an increase in apoptosis. Hence, TNFα stimulates the formation of lesions towards an advanced

phenotype, with more lesion necrosis and a lower incidence of apoptosis.

Peroxisome proliferator-activated receptors (PPAR) are nuclear receptors present in several organs and cell types. PPAR alpha and gamma are the two main categories of these receptors, which are both characterized by their ability to influence cell proliferation, differentiation, apoptosis, and inflammation as well as lipid metabolism and glucose homeostasis via transcriptional activation or repression of target genes or via DNA-binding-independent pathways. In atherosclerosis PPAR-α and PPAR-γ activation results in reduction of atherogenic triglycerides and systemic plasma inflammatory proteins, raise HDL levels and improve insulin resistance. At a cellular level, PPARα/γ agonists act on most cell types involved in atherosclerosis reducing their involvement in the tissue response associated with lesion development. In **chapter 7** the combined PPARα/γ agonist tesaglitazar was investigated on its anti-atherogenic effects in APOE*3Leiden mice with normal and reduced insulin sensitivity. APOE*3-Leiden transgenic mice were fed either a low-fat (LF) or high-fat (HF) insulin-resistance-inducing diet. In both LF and HF-fed mice, one group received a high-cholesterol supplement. A second group received the same HC diet, additionally supplemented with tesaglitazar. A third control group received a low cholesterol supplement, resulting in plasma cholesterol levels similar to those of the tesaglitazar-group. In this study we showed that tesaglitazar has anti-atherosclerotic effects, analyzed both by cross-sectioning at the level of the aortic root and by *en face* analysis of the aortic arch. These anti-atherosclerotic effects go beyond plasma total cholesterol lowering, and were more pronounced in animals on high-fat diet. In addition, tesaglitazar treatment reduced inflammatory parameters as plasma SAA levels, the number of adhering monocytes, and NFκB activity in the vessel wall. The mechanism by which tesaglitazar exerts its anti-atherosclerotic actions beyond plasma cholesterol lowering could therefore be associated with its anti-inflammatory effects.