Calcification and C-reactive protein in atherosclerosis : effects of calcium blocking and cholesterol lowering therapy

Trion, A.

Chapter 8

Summary and general discussion

Cardiovascular disease (CVD) is still the leading cause of mortality and morbidity in Western society. Atherosclerosis is the primary etiologic factor underlying CVD. Atherosclerosis is characterized by an accumulation of lipids, extracellular matrix components, inflammatory cells and calcium precipitates in the vessel wall.

Vascular calcification is a prominent feature of atherosclerosis. However, the mechanisms underlying vascular calcification are still obscure. The major objective of the work described in the first part of this thesis was to elucidate the mechanisms involved in atherosclerotic calcification. **Chapter 2** summarizes the literature on calcification in atherosclerosis and the involvement of vascular smooth muscle cells (VSMCs) in this process. Vascular calcification was previously thought of as a degenerative, end-stage process of vascular disease. However, since the detection of bone-associated proteins, such as osteonectin, osteocalcin and matrix Gla protein in calcified vascular tissues, calcification is considered to be an organized, regulated process similar to mineralization in bone tissue. VSMCs are currently considered to be responsible for the formation of vascular calcifications. Apoptosis of VSMCs appears to be a key factor in this process, while other factors including cell-cell interactions (macrophages and VSMCs), lipids, and plasma inorganic phosphate levels modulate the calcification process. To further study the process of VSMC calcification we developed and characterized an *in vitro* model of rat aortic VSMC calcification (**Chapter 3**). We used neonatal rat aortic VSMCs since these cells resemble a proliferative phenotype which is similar to the VSMC phenotype observed in atherosclerotic plaques. β-Glycerophosphate and high calcium levels emerged as important determinants of *in vitro* calcification of neonatal rat aortic VSMCs.

High concentrations of plasma cholesterol, in particular low-density lipoprotein (LDL) cholesterol, are among the principal risk factors of atherosclerosis, and the process of atherosclerosis has previously been described as an accumulation of lipids in the vessel wall. Since atherosclerosis is now regarded as a complex and multifactorial disease, lipid lowering is no longer the single focus of anti-atherosclerotic research. Other targets are now under investigation, such as direct effects on the vessel wall.

A number of drugs currently used in the treatment of CVD have been shown to have significant pleiotropic actions. These drugs include angiotensin-converting enzyme (ACE) inhibitors, statins, and calcium antagonists (CAs). CAs, originally developed and applied as anti-hypertensive drugs, have been implicated to have anti-atherosclerotic effects which are not related to blood pressure-lowering effects. So far however, animal studies and clinical trials have not been able to establish these anti-atherosclerotic effects convincingly.

To investigate whether pharmacotherapy influences vascular calcification, we have studied whether a calcium antagonist (amlodipine) and a statin (atorvastatin) and their combination may influence VSMC calcification (**Chapter 4**). Incubation of neonatal rat aortic VSMCs with various concentrations of amlodipine had no effect on calcification at all. However, incubation of VSMCs with atorvastatin resulted in a dose-dependent increase of calcium deposition. At a concentration of 10 μmol/L atorvastatin calcification was increased 2-fold. Combining the treatments resulted in a 2-fold increase in calcification which is similar to the increase observed for atorvastatin alone. As the highest concentration of atorvastatin used (50 μmol/L) had pro-apoptotic effects, the pro-calcification effect of atorvastatin may be related to atorvastatin-induced generation of apoptotic bodies that act as a starting point around which calcification occurs.

Based on the results described in **chapters 3**, **and 4**, we conclude that neonatal rat aortic VSMCs are capable of producing calcific deposits under specific culture conditions, and this *in vitro* model of VSMC calcification allows the study of arterial wall calcification and the factors, including pharmacotherapeutics, influencing it. Furthermore, amlodipine was shown to have no effect on the process of VSMC calcification, whereas atorvastatin had a stimulatory effect on this process.

Recognition of the significant role of inflammation in the development of atherosclerosis has dramatically changed our understanding of the pathophysiology of CVD in the last decade. Elevated levels of the acute phase reactant C-reactive protein (CRP) have been consistently associated with CVD in prospective and cross-sectional clinical and epidemiological studies. The plasma level of CRP is considered to reflect the inflammatory condition of the patient and/or the vessel wall. In addition, several studies indicate a causal role of CRP in CVD.

The mechanism underlying the relationship between inflammatory variables, such as CRP, and CVD is complex and has not yet been fully elucidated. In **chapter 5** the literature on the involvement of CRP in CVD, and especially in atherosclerosis development, is summarized. Many *in vitro* studies have shown numerous effects of CRP on endothelial cells, smooth muscle cells, and monocytes, the majority of those effects contributing to pro-inflammatory and pro-atherosclerotic effects. However, the CRP concentrations used in these *in vitro* studies are generally much higher than the levels found in man, and the relevance of these data has yet to be elucidated. Therefore, we designed an *in vivo* study using ApoE*3-Leiden (E3L)/hCRP transgenic mice to test whether moderately elevated levels of CRP have an effect on atherosclerosis development in these transgenic animals (**chapter 6**). We found that mildly elevated levels of CRP in plasma do not contribute to the development of (early)

atherosclerosis in hypercholesterolemic E3L mice. Plaque size and severity were not increased in mice expressing CRP as compared to the non-CRP expressing controls, indicating that CRP has no direct effect on atherosclerosis development in this mouse model.

Whether the CA amlodipine was able to inhibit atherosclerosis development in ApoE*3-Leiden/hCRP transgenic mice was studied in **chapter 7**. Furthermore, it was investigated whether co-treatment with atorvastatin would synergistically enhance the anti-atherosclerotic effect of amlodipine. Finally, we also studied the potential lipid-lowering independent effects of atorvastatin. Although amlodipine treatment did not significantly lower cholesterol levels or blood pressure, it reduced lesion area by 43%. Combined treatment with atorvastatin and amlodipine reduced lesion area more extensively than either treatment alone, leading to a reduction of lesion area by 93%. The atorvastatin-treated mice did not have significantly less atherosclerosis when compared to the low-cholesterol control group, in which the plasma lipid levels were titrated to resemble the atorvastatin group, even though a reduction of 31% was observed.

In view of the results from **chapter 6 and 7**, we conclude that CRP does not have a direct effect on atherosclerosis development in ApoE*3-Leiden/hCRP transgenic mice. However, the association between CRP and cardiovascular disease is well established, and possibly CRP influences CVD risk through other mechanisms such as thrombosis, complement activation and revascularization after ischemia. Amlodipine was found to have an atheroprotective effect, even in the absence of blood pressure lowering. In addition, our results demonstrated a clear additive effect of co-treatment with amlodipine and atorvastatin on early atherosclerosis. We were unable to demonstrate a cholesterol lowering-independent anti-atherosclerotic effect of atorvastatin in E3L/CRP mice.

The studies reported in this thesis confirm the crucial role of VSMCs in atherosclerosis. VSMCs contribute to atherosclerosis development not only through proliferation, but also by producing the calcium deposits that are often found in atherosclerotic lesions. Atorvastatin, but not amlodipine was found to have a stimulatory effect on VSMC calcification. Treatment with statins has been demonstrated to stabilize atherosclerotic lesions. Statin treatment reduces the lipid content of the atherosclerotic lesion, and may stimulate calcification. These processes combined could be the mechanism by which atherosclerotic lesions are stabilized by statin therapy.

The *in vivo* studies have demonstrated that both amlodipine and atorvastatin have atheroprotective effects and that combining treatment with these drugs enhanced this effect. However, treatment with the drugs started before onset of disease. Therefore for co-

treatment to be effective in humans, patients at high risk for CVD could be started on the drugs before symptoms occur. Whether this is feasible and useful should be studied further.