Activation mechanisms in vascular disease

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Chapter 1

General Introduction
Activation mechanisms in vascular disease
Vascular diseases often involve inflammation and coagulation, in which endothelial cell (EC) dysfunction plays a key role. In atherosclerosis chronic inflammation of the vessel wall can eventually result in local coagulation in the affected artery. In sepsis, acute inflammation may result in widespread coagulation in the microvasculature of various organs. Activation mechanisms underlying these processes are described in this general introduction and studied in detail in the following Chapters.

1.1 Atherosclerosis
Atherosclerosis is a chronic inflammatory disease of the arteries, resulting in clinical manifestations such as angina pectoris, heart attack and stroke. It is the major cause of morbidity and mortality in the western world. Atherosclerosis is a multifactorial disease which can involve hypertension, lipoprotein disorders and diabetes mellitus and external factors such as smoking and stress. The complexity of the disease is caused by the multiple (genetic and environmental) risk factors and implicated numerous cell types. The tissue involved is the arterial vessel wall which is composed of smooth muscle cells (SMCs), ECs and extracellular matrix proteins (ECM). SMCs are important to the elasticity of the vessel. ECs form the non-adherent lining of the vessel wall, which is the barrier between blood and tissue. The matrix proteins contribute to the sturdiness of the vessel wall.

Atherosclerosis is a lipid-driven process. When abundant low-density lipoproteins (LDL), containing cholesterol, remain in the circulation fatty streaks are formed in the vessel wall. The continuous uptake of LDL and its oxidative modification results in EC activation and induces an inflammatory response. The invasion of macrophages that initiate clearance of these lipids leads to progression of the inflammatory process, since the macrophages accumulate the lipids but are unable to digest it. These lipid-laden macrophages are now called "foam cells" and become fully activated and secrete cytokines and chemokines. The cytokines activate the medial SMCs and further stimulate the ECs. SMCs start to migrate and proliferate and move from the media to form an thickening intima. They lose their contractile phenotype and excessively produce ECM. This results in the formation of a fibrous cap to provide stability to the intimal plaque that has been formed so far. Progression of this process involves necrosis of foam cells, calcification of the core of the plaque and ultimately the unstable plaque may rupture, causing occlusion of the injured artery resulting in clinical manifestations as mentioned above (Figure 1).

Although the progression of the disease can span decades before becoming clinically relevant, the onset of atherosclerosis starts already at an early age. Lipid accumulation does not occur evenly throughout the arterial tree. Sites of non-laminar blood flow, so-called low shear stress areas, typically present at vessel branch points and bifurcations are prone to lipid accumulation in the vessel wall.
Therefore the formation of an atherosclerotic plaque is a local event, dependent on local factors and gene regulation.

**Figure 1. Development of atherosclerosis.** Monocytes adhere to activated endothelial cells that express adhesion molecules and penetrate the vessel wall (A). The monocytes differentiate into macrophages and take up lipids in the vessel wall. These activated macrophages secrete chemokines, cytokines and growth factors which attract smooth muscle cells (SMCs) from the media to the inflammatory region (B). The SMCs migrate and proliferate and form a fibrous cap to protect the lesion from rupture. A necrotic core develops where macrophages have died (C). When the lesion becomes vulnerable, the plaque can rupture and a thrombus is formed (D). Paintings by dr. A-J van Zonneveld.

### 1.2 Restenosis
Thickening of an atherosclerotic plaque can result in severe narrowing of the lumen of an artery, a condition which can become clinically relevant. A common treatment, called percutaneous transluminal coronary angioplasty (PTCA), consists of widening of the narrowed artery by using an inflated balloon catheter. Presently, many PTCA procedures are followed by placement of a stent. A large number of PTCA treated patients suffer from restenosis. Restenosis is the
process of re-formation of an intimal thickening after treatment of the initial atherosclerotic plaque. In this process, intimal thickening is not lipid driven, but is an uncontrolled wound healing process, involving new risk factors\(^3\). Luminal narrowing after PTCA can result from negative remodeling of the total vessel area and neointima formation, whereas after stent placement only neointima formation is observed\(^3\). Therefore, restenosis upon stent placement tends to be less symptomatic than after PTCA treatment alone. Although inflammatory cell types can strongly influence restenosis, the predominant cell type present in these neointimal lesions are SMCs, producing a large amount of ECM\(^3\). In summary, restenosis is a significant side effect of treatment of coronary atherosclerosis.

1.3 Sepsis
Sepsis is generally known to be induced by gram-negative bacteria, notably by their product endotoxin also known as lipopolysaccharide (LPS). The most common and lethal infection is meningococcal septicemia. In addition, it is clear that also gram-positive bacteria can induce sepsis\(^4\). Sepsis belongs to the group of diseases that are collectively known as the systemic inflammatory response syndrome. An acute systemic inflammatory reaction can result in shock, disseminated intravascular coagulation (DIC), multiple organ failure and eventually death. Extensive acute inflammation can induce activation of the coagulation cascade, resulting in fibrin formation in the microvasculature. Thrombi can be detected in the capillary network in critical organs such as the kidney, lung and liver, eventually leading to organ failure. Upon coagulation, the symptoms observed in patients suffering from DIC are often bleeding, since widespread coagulation has consumed most of the coagulation factors. Not surprisingly, apart from anti-inflammatory treatment, new forms of sepsis treatment are related to anti-thrombotic agents, such as antithrombin III (ATIII), protein C and tissue factor pathway inhibitor-1 (TFPI-1)\(^5,6\).

2 Inflammation and coagulation in vascular disease
2.1 Inflammation in sepsis and atherogenesis
The innate immune system is first activated upon inflammation, which involves phagocytes, notably neutrophils and monocytes/macrophages. The activated complement system facilitates pathogen recognition by these phagocytes. However, if the phagocytes are not sufficient to prevent disease then the adaptive immune system is activated, involving the lymphocytes that induce a specific antibody response.

Well known chemotactic proteins to attract phagocytes to the site of injury are Interleukin 8 (IL-8), Gro-\(\alpha\) and monocyte chemoattractant protein 1 (MCP-1). These proteins are produced and secreted by locally activated tissues. Activated phagocytes can produce cytokines, such as interleukin-1\(\beta\) (IL-1\(\beta\)) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) that activate surrounding tissue cells and enhance the
inflammatory response. The cytokines interleukin-2, -4 and -6 and interferon-γ are involved in lymphocyte recruitment. In atherosclerosis the innate immune response is primarily activated while in sepsis both innate and adaptive immunity play a crucial role. Therefore, all cytokines mentioned above are abundantly present in sepsis while IL-1β and TNF-α are the principal cytokines involved in atherosclerosis. Most of these cytokines and chemokines are regulated by the nuclear factor-κB (NF-κB) pathway. Upon activation of the cell the inhibitor of this pathway (I-κB) dissociates from the NF-κB complex, so that NF-κB can translocate to the nucleus to initiate transcription. This group of nuclear factors regulates transcription of a wide variety of inflammatory and stress related proteins and can be induced by numerous cytokines as well. Among the downstream regulated genes of the NF-κB pathway are also various adhesion molecules to facilitate directed adhesion of the leukocytes, or coagulation-related factors such as tissue factor (TF) and plasminogen activator inhibitor 1 (PAI-1).

2.2 Coagulation- and fibrinolysis-related factors in sepsis and atherogenesis

Four coagulation-related proteins that are involved in both atherosclerosis and sepsis are TF, thrombin, TFPI-1 and PAI-1. The first two are initiators in these vascular diseases and the latter two are their cognate inhibitors. Elevated PAI-1 plasma levels have been reported as markers of an unfavorable prognosis in both diseases, predicting an enlarged chance to myocardial infarction or fatal outcome after onset of septic shock. These problems are related to thrombin-mediated fibrin formation initiated by TF. Increased levels of PAI-1 inhibit thrombolysis, thus sustain the existence of the occluding fibrin clot. In sepsis, coagulation is prevented by anti-thrombotic drugs to prevent DIC, whereas upon myocardial infarction fibrinolysis is stimulated by giving thrombolytic drugs.

In sepsis, TF-mediated coagulation is considered an important process in the pathophysiology of the disease. Even though TF is possibly involved in multiple actions including signaling, it is the key initiator in fibrin formation. Factor VII (FVII) binds to TF and may get activated (FVIIa). Factor X can also bind to TF and when this binding takes place it can be cleaved and thus activated by FVIIa. Activated FX can in turn activates Factor V, an together these latter coagulation factors activate prothrombin by cleavage. Thrombin, the last serine proteinase in the cascade, induces fibrin formation by cleavage of fibrinogen. This is known as the extrinsic coagulation cascade (Figure 2). Fibrin formation can be further enhanced by TF/FVIIa and thrombin to induce the second pathway of coagulation, the intrinsic coagulation cascade. TF/FVIIa/Fxa complexes can be rapidly neutralized by TFPI-1, inhibiting the extrinsic coagulation cascade.

The importance of TF-mediated coagulation in sepsis is illustrated by the observations that antibodies directed against TF could prevent DIC or lethal septic shock in animal studies. Monocytes are considered the crucial cell type...
regarding stimulation of DIC in sepsis, since they are known to synthesize TF upon LPS stimulation\(^{10,11,21,22}\). In addition, granulocytes are abundantly present in affected organs during inflammation in sepsis and have been reported to carry TF on their surface as well\(^{23-27}\). Still, the role of granulocytes in sepsis and whether they actually synthesize TF is controversial\(^{28}\) and will be further discussed in Chapter 6.

**Fibrinolysis**  
- tPA / uPA  
- PAI-1  
- VN  
- plasminogen → plasmin  
- FDPs ← fibrin ← fibrinogen  

**Atherosclerosis**  
- SMC proliferation  
- Tissue factor  
- FXa ← FX  
- TFPI-1  
- FVa ← FV  

**Coagulation**  
- FVIIa  
- ATIII  
- prothrombin  

*Figure 2.* A schematic representation of interaction between coagulation, fibrinolysis and atherosclerosis. Tissue factor is the main initiator of the extrinsic coagulation pathway resulting via multiple steps in thrombin activation and fibrin formation. Fibrin can be degraded by factors from the fibrinolytic pathway. An inhibitor of this latter pathway is plasminogen activator inhibitor 1 (PAI-1). In the presence of cofactor vitronectin (VN), PAI-1 can also inhibit thrombin. The function of thrombin as mitogen for smooth muscle cells (SMCs) could be involved in atherosclerosis. Tissue factor pathway inhibitor type 1 (TFPI-1) and antithrombin III (ATIII) are inhibitors of the coagulation cascade. tPA and uPA are the tissue type and urokinase type plasminogen activators, respectively. FVII, FX and FV are coagulation factors VII, X and V, respectively. The "a" indicates activated protein. FDPs are fibrin degradation products.

Although PAI-1 is best known for its function in fibrinolysis (Figure 2), it has also been implicated in many different processes relevant to atherogenesis. One of these processes involves inhibition of thrombin by PAI-1 in the presence of the cofactors heparin or vitronectin\(^{29-31}\). These interactions are presumably not relevant in plasma since the genuine thrombin inhibitor ATIII is a more rapid inhibitor and abundantly present\(^{32}\). Yet, in the atherosclerotic vessel wall thrombin is present and active while ATIII is not encountered at this location. Thrombin acts as a mitogen for SMCs in vitro\(^{33-35}\) and is thought to induce proliferation of SMCs during the development of atherosclerosis and restenosis\(^{36}\). This is corroborated by numerous animal models of intima formation where hirudin, a specific thrombin inhibitor, reduced intimal thickening\(^{37-39}\). In addition, the thrombin receptor PAR1
is abundantly present in atherosclerotic lesions. PAR1 stimulation with thrombin receptor agonist peptide (TRAP) resulted in activation of NF-κB. Moreover, thrombin-mediated SMC proliferation could be inhibited by antisense p65 (NF-κB) treatment. Since PAI-1 is locally synthesized by activated ECs, SMCs and macrophages it could be a potential inhibitor for thrombin in the vessel wall, provided vitronectin is present (Figure 2). Indeed, vitronectin is available in the vessel wall and, in addition, shown to co-localize in human atherosclerotic lesions with PAI-1 and thrombin. Hence, the circumstances are met for PAI-1/VN control of thrombin-mediated SMC proliferation. Prothrombin and vitronectin are derived from plasma as well as the other components of the intrinsic- and extrinsic coagulation pathway that are encountered in diseased vessels. TF can be locally expressed by activated macrophages and ECs. This suggests that prothrombin can be activated in the diseased vessel wall and active thrombin can cleave its receptor PAR1, that is present on intimal SMCs, to induce proliferation. Support for this mechanism is reported by adenoviral overexpression of TFPI1, the inhibitor of the TF pathway, in models of atherogenesis. A decrease in lesion formation is observed due to inhibition of TF, which presumably resulted in reduced thrombin-mediated intima formation. When thrombin is inactivated by PAI-1/VN by forming a covalent ternary complex, then this complex can be internalized by a scavenger receptor known as low density lipoprotein-related receptor (LRP) 45. LRP is widely expressed by cells in atherosclerotic lesions (Figure 3). In contrast to the pro-atherogenic role of PAI-1 in plasma, the latter mechanism would suggest an anti-atherogenic role for PAI-1 in the vessel wall.

Figure 3.
A schematic representation of thrombin activation and inhibition in the vessel wall. Apart from the function as coagulation factor, thrombin is also a mitogen for smooth muscle cells (SMC). In the vessel wall prothrombin can be activated indirectly by tissue factor (TF) and induce SMC proliferation via its receptor PAR. Locally synthesized plasminogen activator inhibitor 1 (PAI-1) can inhibit thrombin in the presence of vitronectin (VN) and form a ternary complex.

This complex can be cleared by the low-density lipoprotein receptor related protein (LRP), which is a scavenger receptor present on the surface of smooth muscle cells (SMC) and macrophages (Mo). The endothelial cells (EC) form the barrier of the vessel wall. In the circulation PAI-1 is predominantly the inhibitor of tissue type plasminogen activator (tPA) to prevent fibrinolysis.
The complexity of intima formation is illustrated by the fact that multiple other pro- and anti-atherosclerotic factors are encountered in the diseased vessel wall. A number of processes, other than discussed above, where PAI-1, vitronectin and thrombin are involved are now described. PAI-1 inhibit urokinase-type plasminogen activator (u-PA), thereby inhibiting u-PA-induced migration by plasmin or plasmin-mediated metalloproteinase activation. Cellular adhesion and detachment is also influenced by PAI-1 binding to VN, since the \( \alpha_v \) integrins and the urokinase receptor (u-PAR) binding sites on VN overlap with the PAI-1 binding site. Many different studies associate fibrin with induced intima formation, in which thrombin, PAI-1 and VN can obviously play a role.

3. Model systems

3.1 Human cell culture models

To simplify a complex disease it might be useful to focus on a single cell type and study its response to various stimuli relevant to the disease. Baseline growth is determined by the culture media to maintain a quiescent (healthy) phenotype. To mimic an atherogenic response many cytokines or a combination thereof can be applied to induce cell activation. IL-1\( \beta \) or TNF\( \alpha \) serve as typical atherogenic stimuli for activation of cells. A more complex stimulus, which mimics the in vivo situation, is the supernatant derived from macrophages that were activated with oxidized LDL. Apart from IL-1\( \beta \) and TNF-\( \alpha \), the macrophage supernatant contains additional factors, including also inhibitors of activation to regulate the activation process.

Human ECs can be isolated from the vein of an umbilical cord, the so-called HUVECs. They are dissociated from the vessel wall by the protease trypsin, collected and cultured for several generations. SMCs can be cultured from remnants of saphenous vein redundant from by-pass surgery. These cells grow out from these explants and are cultured extensively to obtain large quantities of these cells. Monocytes are purified by elutriation from the cell fraction of human blood. When primary monocytes are cultured they become adherent and spontaneously differentiate into macrophages. Upon further stimulation of these macrophages with oxidized LDL, these macrophages produce numerous cytokines and chemokines.

3.2 Murine atherosclerosis model

When studying a complex disease such as atherosclerosis, the results obtained in tissue culture experiments should be tested in vivo. Genetically altered mice can be informative to use for this purpose. Normally mice do not develop atherosclerosis even when fed a high fat/cholesterol diet. However, during the past decade a number of genetically altered mice have been generated, that are disturbed in their lipid metabolism. For example, mice that lack the Apolipoprotein
E (ApoE) gene develop severe atherosclerosis, even without a high fat/cholesterol diet. ApoE is present on LDL particles and is recognized by specialized receptors in the liver to clear LDL from the blood. When ApoE is absent, LDL remains in the circulation and will eventually be cleared by other tissues such as the blood vessels from the arterial tree. A mutant form of ApoE was discovered in a large Dutch family of familial dysbetalipoproteinemia of which the members are prone to atherosclerosis. This mutant human gene was called ApoE*3-Leiden and was introduced into mice. The transgenic mice indeed develop atherosclerosis during 6-24 weeks of a high fat/cholesterol diet. Atherosclerosis in the ApoE*3-Leiden mice is a lipid-driven process, inducing macrophage-rich lesions throughout the arterial tree, containing few SMCs. The lipid-laden macrophages show typical foam cell morphology. In atherosclerotic lesions from these mice the role of macrophages and lipids during the development of atherosclerosis are highlighted.

3.3 Murine restenosis model
SMCs are abundantly present in human atherosclerotic lesions, in contrast to the ApoE*3-Leiden mouse model. To study the role of SMCs in atherosclerosis, numerous murine models have been developed over the last decade. These models are based on some form of vascular injury, such as mechanical or electrical injury, oxidative stress or reduced flow. In the carotid artery ligation model a combination of these factors induces SMC proliferation and migration when the flow is completely disrupted by complete ligation of the left carotid artery near the distal bifurcation (Figure 4). This model mimics the vascular pathological process called restenosis. Restenosis is not a lipid-driven process and depends on vessel injury. In the carotid artery ligation model, the EC layer remains intact. In one to four weeks, intimal thickening takes place, leading to a lesion that consists predominantly of SMCs. The precise mechanism for the development of these lesions is presumably a combination of reduced shear stress (non-laminar flow), mechanical stress (ligature) and hypoxia (reduced oxygen supply), which will induce activation of cells in the vessel wall. This model is suited to study the response of SMC migration and proliferation in mice with a different genetic background.

PAI-1 deficient (PAI-1<sup>-/-</sup>) and vitronectin deficient (VN<sup>-/-</sup>) mice are used in our experiments to study their role in SMC proliferation. Both types of mice are viable and fertile and no abnormalities can be observed in healthy animals. However, when used in thrombosis models both types of deficient mice show reduced clot stability. This observation correlated with the findings that venous thrombosis was observed in PAI-1-overexpressing transgenic mice. The PAI-1<sup>-/-</sup> mice have been studied in various atherogenic models and conflicting data are reported on the pro- or anti-atherosclerotic functions of PAI-1. To resolve the role of PAI-1 in relation to SMC proliferation we have conducted carotid artery ligation experiments in PAI-1<sup>-/-</sup> and VN<sup>-/-</sup> mice in Chapter 5 of this thesis.
3.4 Murine sepsis model

Animal models of sepsis usually rely on infusion of bacteria, such as *Escherichia coli*, or their toxic product LPS. The dosage of bacteria or LPS will determine the symptomatic outcome of the disease. In this model mRNA expression levels have been determined for various coagulation-related genes, such as PAI-1, u-PA, TF and von Willebrand Factor. Due to organ- or region specific gene regulation, differential sensitivity to inflammation and coagulation is observed. Apart from expression levels for inflammatory and coagulation-related genes, protein distribution can be studied in this model. We focus on TF mRNA and protein expression in this murine sepsis model, which is described in Chapter 6 of this thesis.

4. Techniques for analysis of gene expression

To unravel complex biological processes and diseases, the initial goal is to identify the genes/proteins involved. This goal is greatly accelerated by "mining" the data generated by the Human Genome Project. Independently, Celera Genomics, a commercial company, and a public genome consortium surfaced most of the human genes, being collectively approximately 53,000 putative genes. Currently, approximately 60% of the putative genes found by Celera...
could be categorized into known gene families by sequence homology. We assume that this percentage will be similar for the genes obtained by the public genome consortium. Still, the precise function of most of these genes have to be further analyzed. Of the remaining 40% of putative genes no homology with any known sequences could be found. Therefore, high throughput techniques, such as DNA-microarray, are employed to characterize these genes to obtain more information about their gene expression patterns in cells or tissues under normal and diseased conditions. DNA-microarray is a screening method to monitor the involvement of a pre-selected set of genes or, in the near future, the global involvement of all known genes. Unique DNA sequences for these genes are spotted, and subsequently hybridized with various different populations of mRNA. Other techniques such as serial analysis of gene expression (SAGE) or differential display by reverse transcriptase polymerase chain reaction (DD/RT-PCR) are used to pre-select genes involved in a specific process by comparing mRNA derived from different conditions. SAGE is based on the sequencing of 3'-ends of a population of reverse transcribed mRNAs as will be further discussed in Chapter 2. DD/RT-PCR makes use of a combination of multiple designed primer sets to theoretically detect at least 80% of mRNA species present. Using SAGE or DD/RT-PCR, genes coding for known and unknown proteins can be obtained. These represent up- or down-regulated genes that are expressed when different mRNA pools are compared. SAGE has the advantage of looking at a general expression pattern within one condition and actually quantifies the number of identical transcripts. For SAGE extensive databases exist for comparison of results between researchers world-wide. This collection of data, that is publicly available, increases the interaction between researchers and expands the perspectives of the results produced by individual scientists.
General Introduction

References


66. Yamamoto K, Loskutoff DJ. Fibrin deposition in tissues from endotoxin-treated mice correlates with decreases in the expression of urokinase-