Leukocytes and Coronary Artery Disease

Experimental and Clinical Studies

BY

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ABSTRACT

Tissue factor (TF) is the initiator of the coagulation cascade. Monocytes do not normally express TF, but can be induced to do so by certain stimuli. Aberrant TF expression is important in the thrombotic complications of bacterial sepsis, certain malignancies and coronary artery disease (CAD). In this thesis, regulation of monocyte TF by cytokines and by interactions with other vascular cells were studied, as well as the activation of blood cells, inflammation and coagulation in CAD patients and the association of the pro-inflammatory cytokine interleukin (IL)-6 with prognosis in unstable CAD.

In a whole blood experimental system, the anti-inflammatory cytokine IL-10 was shown to suppress lipopolysaccharide-induced TF expression in monocytes, whereas IL-4 and IL-13 did not, contrary to previous *in vitro* findings. Activated platelets also induced monocyte TF in whole blood in a P-selectin-dependent manner, causing a rapid surface exposure of TF independent of mRNA formation. The differentiated monocytic cell line U-937 displayed different kinetics of platelet-stimulated TF induction.

In co-culture with cytokine-activated human coronary artery endothelial cells, U-937 cells expressed TF, and also IL-6. The endothelial cells up-regulated their production of IL-10. Simvastatin, enalapril and dalteparin, all commonly used drugs in CAD treatment, suppressed TF induction but did not alter cytokine expression in co-cultures.

In unstable CAD, there was an activation of both coagulation and inflammation compared to stable CAD that coincided with an increased activation of platelets and leukocytes. Women had different patterns of cellular activation than men, indicating differences in pathogenetic mechanisms.

Plasma levels of IL-6 above 5 ng/L proved to be a strong, independent marker for increased risk of death in a 6-12 month perspective in patients with unstable CAD. This risk was significantly reduced by an early invasive strategy.

Key words: Tissue factor, monocytes, platelets, cytokines, endothelial cells, unstable coronary artery disease, coagulation, inflammation, biochemical markers, IL-6, risk stratification.

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*To my mother
and to Dan*

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I.   Lindmark, E., Tenno, T., Chen, J. and Siegbahn, A. IL-10 inhibits LPS-induced human monocyte tissue factor expression in whole blood.
      Br J Haematol 1998;102:597-604.

II.   Lindmark, E., Tenno, T. and Siegbahn, A. Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression.
      Arterioscler Thromb Vasc Biol 2000;20:2322-2328.

III. Lindmark, E. and Siegbahn, A. Tissue factor regulation and cytokine expression in monocyte - endothelial cell co-cultures. Effects of a statin, an ACE-inhibitor and a low-molecular-weight heparin.
      Submitted.

IV.   Lindmark, E., Wallentin, L. and Siegbahn, A. Blood cell activation, coagulation and inflammation in men and women with coronary artery disease.
      Thromb Res 2001;103:249-259.

V.   Lindmark, E., Diderholm, E., Wallentin, L. and Siegbahn, A. Relationship between interleukin-6 and mortality in patients with unstable coronary artery disease.
      JAMA 2001;286:2107-2113.

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List of abbreviations

ACE-inhibitors	Angiotensin converting enzyme inhibitors
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CABG	Coronary artery bypass grafting
CAD	Coronary artery disease
CHO	Chinese hamster ovary
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanate
FRISC	Fragmin and Fast Revascularisation during Instability in Coronary artery disease
gp	Glycoprotein
HCAEC	Human coronary artery endothelial cells
HLA-DR	Human leukocyte antigen-DR
HUVEC	Human umbilical cord vein endothelial cells

ICAM-1	Intercellular adhesion molecule-1
IFN-γ	Interferon-γ
IL-	Interleukin-
LDL	Low density lipoprotein
LFA-1	Leukocyte function-associated antigen-1
LMW-heparin	Low molecular weight heparin
LPS	Lipopolysaccharide
LRP	LDL-receptor related protein
MAC-1	Monocyte adhesion complex-1
MCP-1	Monocyte chemotactic protein-1
MFI	Mean channel fluorescence intensity
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
NF-κB	Nuclear factor-κB
PAF	Platelet activating factor
PBS	Phosphate buffered saline
PCA	Procoagulant activity
PDGF	Platelet-derived growth factor
PE	Phycoerythrin
PF4	Platelet factor 4
PSGL-1	P-selectin glycoprotein ligand-1
PTCA	Percutaneous transluminal coronary angioplasty
RANTES	Regulated upon activation normal T-cell expressed presumed secreted
RT-PCR	Reverse transcriptase polymerase chain reaction
sIL-2Rα	soluble IL-2 receptor α
TAT	Thrombin-antithrombin complex
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGF-β	Transforming growth factor β
TIMP	Tissue inhibitor of matrix metalloproteinases
TNF-α	Tumour necrosis factor α
TRA	Thrombin receptor activator
VCAM-1	Vascular adhesion molecule-1
VLA-4	Very late antigen-1

INTRODUCTION

Haemostasis

When a blood vessel is damaged, the site of disruption must be rapidly sealed to prevent loss of blood. This process is called haemostasis, and is generally divided into *primary* and *humoral* haemostasis [1]. The first involves vasoconstriction and the adhesion, activation and subsequent aggregation of platelets, forming a platelet plug on the lesion. The second is coagulation: an enzymatic cascade resulting in the formation of a fibrin meshwork in which blood cells get trapped, building a clot or *thrombus*. The clot is later dissolved by another protease reaction, the fibrinolysis, which also prevents the vessel from being occluded by the clot during its formation. In order for the blood to stay fluid within the circulation, a delicate balance between the carefully regulated systems of coagulation and fibrinolysis is needed. Disturbances in either system will cause a tendency towards either thrombosis or bleeding, respectively.

Platelets

Platelets are small, anucleate cells formed by cytoplasmic fragmentation of the megakaryocytes in the bone marrow. Their primary function is to control haemorrhage. Platelets contain two predominant types of granules, which store a large variety of proteins and vasoactive substances [2]. The α-granules hold several of the proenzymes of the coagulation and fibrinolytic cascades, as well as platelet specific proteins like platelet factor 4, P-selectin and platelet-derived growth factor (PDGF) [3,4]. The dense bodies, or δ-granules, contain ADP and ATP, calcium ions, pyrophosphate and serotonin, among other substances [2]. On their surface, platelets constitutively express several kinds of receptors, and more appear upon activation [5]. Platelets are discoid when resting, but can rapidly change shape in response to activation by rearrangement of microtubules and actin filaments.

When vessel injury occurs, the platelets rapidly adhere to the subendothelial collagen by linkage of the glycoprotein receptor gpIa/IIa and to the von Willebrand factor by the gpIb-IX-receptor [5,6]. The adhesion results in an outside-in signalling, which cause the platelets change shape; they spread across the lesion in order to cover it. The gpIIb/IIIa integrin, the fibrinogen receptor on the platelet surface, changes its conformation and thereby its affinity for the plasma protein fibrinogen. This results in aggregation of the platelets by bridging of fibrinogen between the gpIIb/IIIa-receptors [5,6]. The activation resulting from the adhesion and aggregation causes secretion and concomitant release of platelet granule proteins, some of which further potentiate the activation of the platelets. Moreover, adhesion molecules such as P-selectin (CD62P) and CD40 ligand (CD154) are exposed on the surface and mediate binding to leukocytes and endothelial cells [7,8].
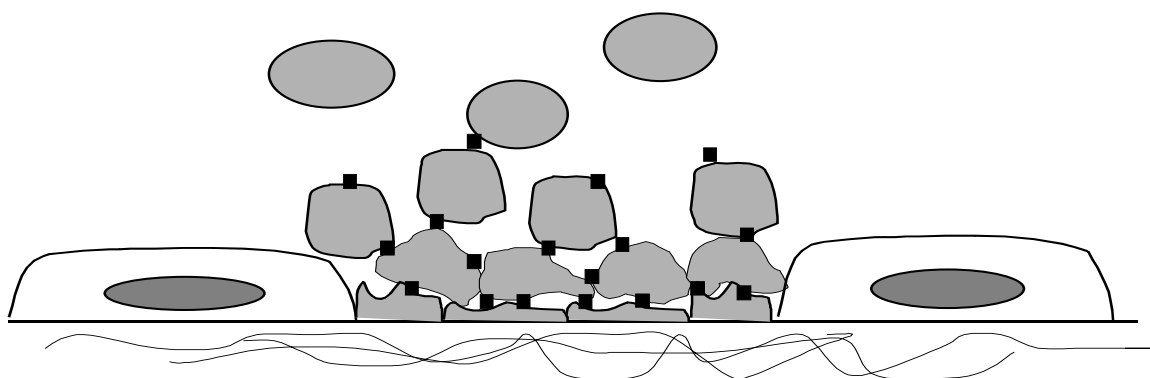
Figure 1. The formation of a platelet plug at the site of vessel injury. Black squares symbolise fibrinogen.

Important platelet activating agents other than adhesion are shear stress, thrombin, ADP, adrenaline, serotonin, platelet activating factor (PAF), thromboxane A_2 and angiotensin II. Of these, ADP, serotonin and thromboxane A_2 are released by the platelets themselves, thus forming an autocrine activation loop. Nitric oxide (NO) and PGI_2, prostacyclin, are the most important among the physiological platelet inhibitors. Both are produced and released by the endothelium.

The coagulation cascade

The process of blood coagulation is a true cascade type of reaction, where a series of active coagulation enzymes activate pro-enzymes (coagulation factors II, V, VII, VIII, IX, X, XI, XII and XIII) by proteolytic cleavage, resulting in manifold amplification in each step [1, 9-11]. There are two classical pathways of coagulation: the *intrinsic* and the *extrinsic* pathways. The names derive from the fact that the intrinsic, or contact activation, pathway only uses factors within the circulation, whereas the extrinsic pathway needs an extravascular protein, tissue factor (TF), in order to be initiated. It is thus synonymously named the tissue factor-dependent pathway, and is thought to be the predominant mechanism by which coagulation is initiated *in vivo*.

The TF-dependent coagulation cascade starts with the exposure of extravascular TF when a blood vessel is damaged. Trace amounts of active, circulating FVII, i.e. FVIIa [12], bind to TF, and this complex can now cleave FVII, FIX and FX into their active forms. FXa can cleave very small amounts of FII, prothrombin, into thrombin, which in turn activates co-factors V and VIII. These bind to activated platelets in the platelet plug, where a favourable phospholipid environment on the platelet membrane surface hosts the formation of the tenase complex (FIXa-FVIIIa) and the prothrombinase complex (FXa-FVa). These enzyme complexes greatly enhance the generation of FXa and thrombin, and render the cascade TF-independent after this step. Thrombin is the final effector enzyme of the coagulation process, cleaving the plasma protein fibrinogen into fibrin monomers, which polymerise into the fibrin meshwork that constitutes the clot under the influence of FXIIIa, also activated by thrombin.

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There are several endogenous modulators of the coagulation cascade. Tissue factor pathway inhibitor, TFPI, is produced and secreted by endothelial cells [13,14]. It binds to FXa, and this complex subsequently docks to and inhibits the TF-FVIIa complex. Antithrombin inhibits mainly thrombin and FXa, and their mutual binding is facilitated by proteoglycans tethered to the vessel wall [15]. Endothelial cells also express thrombomodulin on their surface, which binds thrombin, forming a complex that activates protein C. Together with its co-factor protein S, activated protein C degrades FVa and FVIIIa.
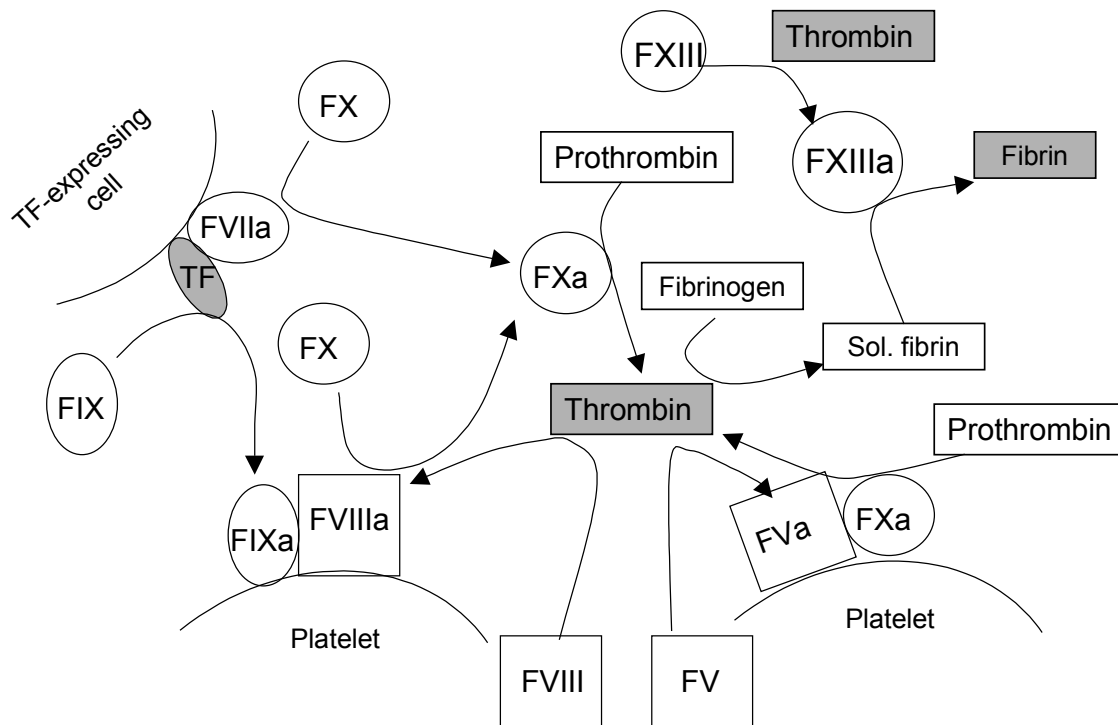
Figure 2. The coagulation cascade. The initiating molecule, TF, the key enzyme, thrombin, and the final product, fibrin, are highlighted.

Fibrinolysis

The process of degrading the fibrin clot and dissolving the thrombus is termed fibrinolysis [16]. This mechanism also helps control neoformation of thrombi. The key enzyme of the fibrinolytic system is plasmin, which degrades fibrin into D-dimers. Plasmin is the result of proteolytic cleavage of the proenzyme plasminogen by t-PA, tissue-type plasminogen activator. The fibrinolytic capacity is controlled at two levels; plasminogen activator inhibitor (PAI-1) binds to and inhibits t-PA, and antiplasmin forms an inactive complex with plasmin.

Tissue factor

Tissue factor (TF) is a 47-kDa, transmembrane glycoprotein and the main initiator of the coagulation cascade *in vivo* [17,18]. It is constitutively expressed by cells surrounding the blood vessels, and abundantly so in richly vascularised organs such as the placenta, the brain and the lungs. It is also present in organ capsules, thus forming a haemostatic barrier to prevent loss of blood in case of vessel damage. TF expression can be induced in some, usually TF-negative cells. In some pathological conditions, aberrant expression of TF causes thrombotic complications. In recent years, several additional functions of TF have begun to be elucidated.

Protein structure and gene regulation

TF consists of 263 amino acids, 219 of which make up the extracellular domain. The membrane-spanning segment is 23 amino acids, leaving a short cytoplasmic tail of 21 amino acid residues. The extracellular part has two disulfide bonds and is glycosylated in 3 places. It is the TF region responsible for the binding of FVIIa and for the protease activity of the TF-FVIIa complex. TF has been classified as a member of the class II cytokine/haematopoietic growth factor receptor family, also including interferon (IFN)-α, IFN-γ and interleukin 10-receptors.

The TF gene is located on chromosome 1 and spans 12,4 kbp, excluding the promoter and enhancer elements [19,20]. It contains 6 exons separated by 5 introns. The promoter region exhibits binding sites for the transcription factors Sp-1, AP-1, Egr-1 and NF-κB [21]. The organisation of the promoter allows for the TF gene to be regulated in either a constitutive or an inducible manner. The Sp-1 sites are thought to be responsible for basal TF expression in cells such as fibroblasts and epithelial cells. The Egr-1 and Sp-1 sites are involved in the serum response region, SRR [22], whereas the AP-1 and NF-κB sites constitute the LPS-response element, LRE [23]. The NF-κB family of transcription factors reside in the cytosol, bound to the inhibitory protein family I-κB. These modulating proteins are the targets for many pro-inflammatory stimuli, which by phosphorylation of a serine residue direct them towards degradation, thus allowing translocation of NF-κB into the nucleus [24]. Where it is not constitutively expressed, TF is considered an 'immediate early' or 'primary response' gene, and mRNA levels usually peak 1-2 hours after stimulation. TF mRNA is unstable and is rapidly degraded. TF protein synthesis is thus regulated mainly at the transcriptional level.

Tissue factor induction and expression

Cells in contact with flowing blood do not normally express TF; however, blood monocytes as well as vascular endothelial cells can be induced to TF expression by a variety of stimuli [18]. Many studies have focused on monocytes isolated from blood, for example by density gradient centrifugation, and subsequently incubated in cell culture medium with various substances. Lipopolysaccharide (LPS; endotoxin) from Gram-negative bacteria is the most potent and most widely studied inducer [25].

Others include immune complexes [26-28], complement [29,30], oxidised LDL [31-33], cholesterol [34] and cytokines like TNF-α [35], interleukin (IL)-1α, IL-1β and MCP-1 [36-38]. PDGF-BB and the acute phase protein C-reactive protein (CRP) [37,39,40] have also been shown to induce monocyte TF. In studies performed in a whole blood environment, however, LPS and immune complexes are so far the only soluble, naturally occurring agents that have been reported to directly cause TF induction [41]. Moreover, it was shown that the presence of platelets and granulocytes potentiated this induction [42]. This speaks for the importance of extending the studies of human blood cells to include their natural environment; an *ex vivo* model system. One study has claimed a TF-inducing potential of IL-6 and IL-8, both in isolated monocytes and in whole blood [43], but this finding has not been verified by other investigators. It is likely, though, that endogenous cytokines may potentiate the TF expression induced by some other agent [41].

Induction of TF in monocytes by LPS exhibits substantial variations between individuals, but is more reproducible when measured repeatedly over time in monocytes from the same subject. This is referred to as the high- and low responder phenomenon, and has been demonstrated both in whole blood [41] and in isolated mononuclear cells [37], and involves both TF antigen expression and activity. It seems that different responsiveness to LPS *in vitro* is due to differences in the monocytes themselves, whereas in whole blood it may involve co-stimulating cells like platelets and granulocytes.

Monocytes can also be stimulated to TF production by adhesion to a surface [44] or to other cells. In this context, P-selectin on platelets and ICAM-1 or other adhesion molecules on endothelial cells are now rather well documented TF inducers. This kind of cell-cell cross talk is probably of greater general importance *in vivo* than are the soluble TF-inducing agents, and is thought to play a key role in atherosclerosis and the complications thereof. It is further reviewed in the section "Vascular cell cross talk".

Many reports show TF induction in cultured endothelial cells of various origins, by stimuli similar to those for monocytes [18]. However, these findings are limited to *in vitro* systems. *In vivo*, studies in animal models have failed to demonstrate endothelial TF production in response to LPS [45,46], and segments of human saphenous veins were also found to remain TF-negative when perfused with LPS or thrombin [47]. In a recent report, a repressive cis-acting element in the TF promoter was found that appeared to be active selectively in endothelial cells [48], thus supporting a non-coagulant state.

There is currently a controversy regarding the ability of neutrophils to express TF. A small sepsis study on baboons has been able to demonstrate TF-expressing neutrophils in the liver [49], but studies in human cells fail to induce neutrophilic TF with LPS [50]. Although TF-positive neutrophils have been observed in thrombus formation studies using human blood [51], it seems likely that it stems from engulfment of TF-positive cells or vesicles.

TF that has been exposed on the cell surface is subsequently removed, by shedding or by internalisation. In the internalisation process, TFPI plays a role by binding to both to the TF-FVIIa complex and, with its C-terminal domain, to LDL-receptor related protein, LRP [52]. This protein in turn drags the entire TF-FVIIa-TFPI complex into clathrin-coated pits, from where the complex is internalised and degraded. TF may also be shed. Investigators have detected 'blood-borne' TF in the form of TF-positive microvesicles, and suggest that these vesicles have been shed from leukocytes [51].

Clinical relevance of aberrant tissue factor expression

The most prominent clinical condition where TF is involved is disseminated intravascular coagulation (DIC), a severe complication of sepsis. It is caused by LPS-induced TF expression by peripheral blood monocytes, and the resulting hypercoagulable state. In coronary artery disease (CAD), TF is abundantly expressed within the atherosclerotic plaque [53]. It is exposed to the blood when the plaque ruptures, thus causing thrombosis. TF-positive endothelial cells overlying the plaque have also been demonstrated [54], as well as TF-expressing, circulating monocytes [55,56]. Another pathological condition where TF-mediated thrombosis is a common complication is cancer. Elevated TF expression has been detected in malignant cells, both in solid tumours [57] and in leukaemia blast cells [58], and has been associated with thrombotic events in patients. Also in Crohn's disease, an inflammatory bowel disorder, monocytic TF has been implicated to cause hypercoagulability [59].

Tissue factor activity and cellular localisation

Procoagulant activity is a term that denotes the ability of the TF-FVIIa complex to cleave its natural substrate FX. Although primarily transcriptionally regulated, TF surface exposure and activity may also be controlled at other levels. Early studies in the 1970's reported the existence of a latent form of TF in fibroblasts [60], and more recent investigations have demonstrated a marked and rapid increase in procoagulant activity in cells treated with a calcium ionophore, too soon after challenge to allow for *de novo* protein synthesis [61,62].

There are two prevailing theories regarding the regulation of pre-existing TF. Intracellular TF has been demonstrated both by cell extract gradient centrifugation [63] and by staining intact cells with TF antibodies and labelled FVIIa [64]. However, one group showed the existence of TF homodimers on the cell surface, and that TF activity increases upon dissociation of these dimers, thus suggesting an encryption/de-encryption model of regulation [62]. The phospholipid microenvironment and membrane exposure of phosphatidylserine also seems to be important for TF activity [65-67]. Not unlikely, the form of post-translational TF regulation may vary between cell systems, and the nature of TF latency *in vivo* is so far largely unresolved.

Tissue factor suppression

Stimulated TF expression in monocytes can be down-regulated by several agents. IL-10, IL-4 and IL-13 are generally considered as anti-inflammatory cytokines, although their functions are very complex and include both stimulatory and inhibitory effects on different cell types within the immune system. *In vitro*, on isolated monocytes in culture, they have all been shown to attenuate LPS-, CRP, MCP-1 or IL-1α/β-induced TF [38,40,68-73]. However, the previously observed differences in experimental systems prompted for an investigation of their effects in a whole blood model.

IL-10 has been implicated as a potential therapeutic agent, since it inhibited inflammation and coagulation and in human sepsis studies [74,75] and prevented lethal endotoxaemia in mouse models [76,77]. Other substances reported to suppress TF synthesis are retinoic acid [78,79], which also has been demonstrated to protect from lethal septic shock in rats [80], nitric oxide (NO) [81] and pentoxifylline, an agent that increases intracellular levels of cyclic AMP [82].

Drugs of various kinds may also influence monocyte TF expression. Aspirin is rather well characterised as a suppressor of LPS-induced TF *in vitro* [83,84]; however, in whole blood its effect is potentiating [85-87]. This is yet another example of the differences between these experimental systems. The two drug categories of angiotensin converting enzyme (ACE)-inhibitors and statins comprise the most recent additions to the group of TF-suppressing drugs. Their direct effects have been demonstrated in LPS- or oxidised LDL-stimulated, isolated monocytes and macrophages [88-90]. Moreover, *in vivo* lowering of soluble TF [91] as well as attenuation of monocyte TF in cardiac transplant recipients [92] have been reported. These findings are of considerable importance, since these classes of drugs are commonly used in the treatment of coronary artery disease, but on other indications. Knowledge of their additional beneficial effects in vascular biology may increase their area of use in this field.

The other functions of tissue factor

In recent years, extensive new knowledge has been gained regarding the possible physiological roles of TF. It now seems beyond doubt that TF is a true receptor, transmitting a secondary signal within the cell. Binding of FVIIa to TF has been shown to induce cytosolic Ca^{2+}-oscillations and MAP-kinase activation as well as the subsequent transcription of genes coding for growth factors and proteins regulating cellular reorganisation [18,93 and the references therein].

TF seems to play an important role in migration and tumour metastasis. In a mouse melanoma model, TF transfection of the tumour cells promoted metastasis by a mechanism requiring both the intracellular part as well as a functional extracellular part of TF [94,95], and this requirement has also been reported for a model using Chinese hamster ovary (CHO) cells [96]. Recently, it was shown that FVIIa binding to fibroblast TF reduced the PDGF-BB concentration needed for directed migration by 100-fold, and that this event was dependent on phospholipase C activity but also

required the protease activity of the TF/FVIIa complex [97]. Moreover, other investigators report that upon ligation of TF, the intracellular part binds actin-binding protein 280 (ABP-280, filamin) and thereby becomes linked to the cytoskeleton [98]. In one study, soluble TF was shown to exert strong effects on the migration of smooth muscle cells [99].

A role in angiogenesis has also been suggested for TF. Indeed, in several studies in TF knock out mice, investigators observed that $TF^{-/-}$ embryos died in utero [100-102]. The yolk sac showed a defective vasculature, the embryos were growth retarded and their blood vessels were deficient in pericytes or smooth muscle cells. It has also been reported that even minute amounts (~1% of normal) of TF expression by a minigene rescued $TF^{-/-}$ mouse embryos, and that this rescue was dependent of TF activity but independent of the intracellular part [103]. Together, these studies suggest that TF is essential for normal embryonal blood vessel development. It seems, however, that this is depends somewhat on the genetic background of the null mice: one study has reported a small but significant proportion of $TF^{-/-}$ mice embryos that survived until delivery, showing a normal macroscopic and microscopic phenotype [102]

In light of the increasing evidence of TF being more than the initiator of the coagulation cascade, it is reasonable to consider TF as a potential target in more aspects that coagulopathies. However, it also calls for caution, as interference with TF may affect yet unknown functions of the cells.

Vascular cell cross talk

Leukocytes are mobile, both in the blood stream and by transversing the endothelium into the extravascular tissue. They respond to chemotactic stimuli, guiding them in the direction of an ongoing inflammatory process. They interact with each other as well as with other vacular cells, such as endothelial cells and smooth muscle cells. The interplay, or cross talk, consists of both direct cell-cell contact and secretion of soluble mediators, mainly cytokines and chemokines, which then act in a paracrine manner. Platelets also interact with leukocytes and endothelium, not only at the site of an acute vascular injury. To increase the understanding of the pathogenetic mechanisms of atherosclerosis, studies of vascular cell cross talk are important.

Platelet-leukocyte complexes

Platelets that are disturbed from their resting state readily adhere to monocytes and neutrophils. This was first discovered in the early 1960's [104], and the phenomenon was for many years termed 'platelet satellitism'. *In vivo*, this association between platelets and leukocytes occurs in sites of haemorrhage [105] or in inflamed tissues [106,107], and the interaction may facilitate the functions of both cell types in these pathological conditions [108]. Several studies in recent years have focused on platelet-leukocyte interactions. *In vitro*, the complexes formed are dynamic and rapidly reversible. Monocytes are favoured over neutrophils by the platelets, showing faster and more extensive complex formation [109]. The adhesion is mediated by different

receptor/counter-receptor couplings, depending on the degree of platelet activation. In moderately activated platelets, fibrinogen bridging between gpIIb/IIIa on the platelet and CD11b/CD18 (Mac-1) on the leukocyte is thought to be predominant [110], whereas P-selectin – PSGL-1 (P-selectin glycoprotein ligand-1) mediates the interaction with fully activated platelets [111]. CD40 ligand is another protein, which was recently found to be expressed on strongly activated platelets [8] and that may play a role in platelet-monocyte cross talk. Other molecules, such as ICAM-2 on the platelet surface, might mediate leukocyte adhesion, but so far little is known about their role.
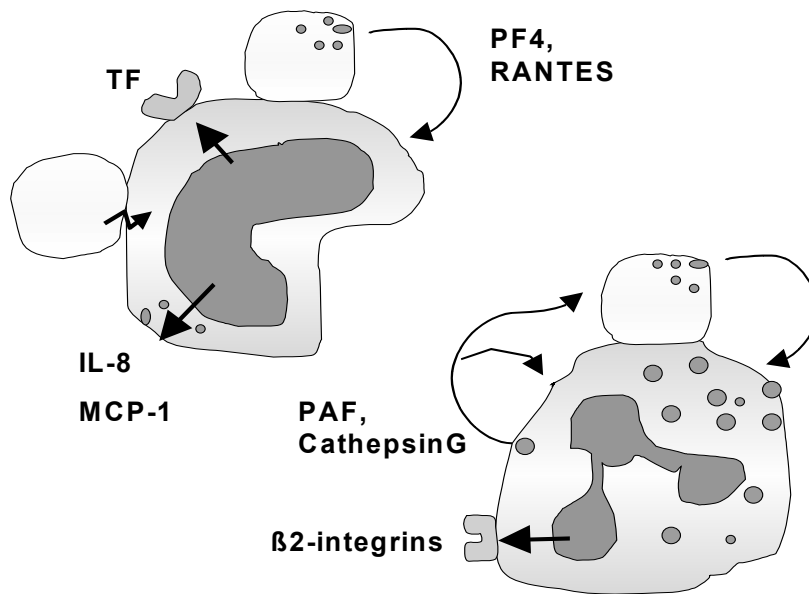
Figure 3. Platelet – leukocyte interactions.

Activated platelets have been shown to induce both TF [112,113] and cytokine (IL-1β, MCP-1 and IL-8) secretion [114,115] in monocytes by P-selectin dependent mechanisms. Monocyte TF induction by P-selectin tethering has been verified by use of P-selectin-transfected CHO cells [112]. In mouse neutrophils, PSGL-1 cross-linking was demonstrated to up-regulate the β2-integrin CD11b/CD18, thereby enhancing their adhesion to ICAM-1. In human neutrophils, CD11b up-regulation as well as L-selectin shedding as a result of incubation with platelet membranes has been reported [116]. Moreover, release of cathepsin G, a strong platelet agonist, by activated neutrophils can cause a potentiation of platelet activation [117]. Activated platelets have also been shown to induce superoxide anion release by both monocytes and neutrophils through P-selectin ligation [118,119]. These studies support earlier observations of a platelet-dependent granulocyte enhancement of LPS-induced monocyte TF expression [42].

Increased levels of circulating platelet-neutrophil complexes has been reported in patients with unstable coronary artery disease [116] and acute myocardial infarction [115], and platelet-monocyte complex formation was increased in stable CAD patients [120]. In conditions exhibiting enhanced platelet activation, this is more likely to be reflected by platelet-leukocyte complexes than by circulating, activated platelets, considering the rapid and dynamic nature of complex formation [121].

Monocyte - endothelial cell interactions

Like neutrophils and T-lymphocytes with them, monocytes transmigrate across the endothelium at the site of infection or inflammation to participate in the immune response in the inflamed tissue. The endothelial cells in the region of ongoing inflammation are activated, with concomitant up-regulation of adhesion molecules on their surface [122,123]. Monocytes in the blood stream first associate with the selectin group of adhesion molecules, namely P-selectin and E-selectin (*tethering*). This induces *rolling* of the monocytes, where they are slowed down to roll along the endothelial lining with continuing association and dissociation of selectins and their monocytic ligands. The monocytes can now 'sense' locally sequestered chemoattractants and pro-inflammatory cytokines, and respond to them by conformational activation of adhesion receptors. They flatten and spread on the endothelium in a step called *firm adhesion*, where another class of molecules, the immunoglobulin-like adhesion molecules ICAM-1 and VCAM-1, on the endothelial cells are involved. ICAM-1 binds to mainly two cognate ligands on the monocyte, namely the β2-integrins CD11b/CD18 (MAC-1) and LFA-1 (CD11a/CD18), whereas VCAM-1 has VLA-4, a β1-integrin, as a counter-receptor. The following *diapedesis*, the transendothelial migration, is also mediated by these molecules. Directed by the chemotactic gradient, the monocytes can now migrate through the extracellular matrix in the tissue towards the inflammatory foci. It has been suggested that the process of extravasation activates the monocytes and participates in inducing their differentiation into macrophages [122]. This process occurs continuously in normal vessels under non-inflammatory conditions, where low-rate monocyte efflux from the blood stream ensures maintenance of the macrophage population in a given tissue compartment.

Since monocyte recruitment to the arterial wall intima is a specific event in atherosclerosis, monocyte-endothelial cell interactions have been widely studied *in vitro* in recent years. Co-culture of monocytes with endothelial cells has been shown to result in TF induction, either in the monocytes [124,125], in the endothelial cells [126] or in both cell types [127,128]. There has also been discordance regarding the adhesion molecules primarily involved in this induction. One group observed increased monocytic TF following cross-linking of Lewis[x], a ligand for E-selectin, and partial inhibition of TF in co-cultures in the presence of an anti-E-selectin antibody [125,129]. Others, however, favour ICAM-1, and this molecule has been demonstrated to up-regulate TF in monocytes using either activated endothelial cells [124], activated
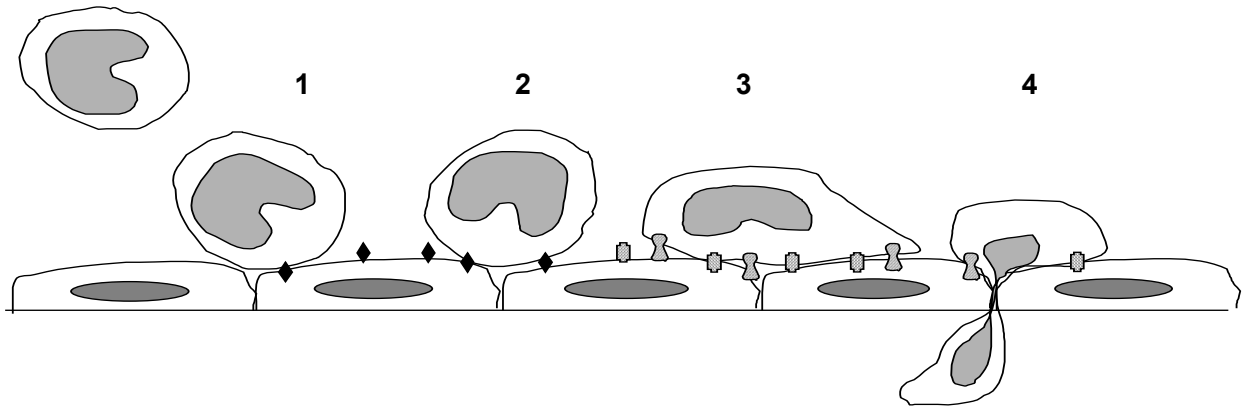
Figure 4. The four steps of monocyte extravasation. 1. Tethering 2. Rolling 3. Spreading 4. Diapedesis. Black rhombi represent P-selectin or E-selectin ligation to PSGL-1 or LewisX. Striped figures symbolise ICAM-1 - CD11b/CD18 and VCAM-1 - VLA-4 binding.

smooth muscle cells [130] or transfected CHO cells [130]. Engagement of CD11b/CD18 on monocytes by cross-linking is also known to induce procoagulant activity [131,132], but so is engagement of VLA-4, the cognate ligand for VCAM-1 [132,133]. On the whole, no conclusive evidence has so far been presented that definitely favours one pathway over the other. The differences in results may stem from different experimental systems; cell types/cell lines, culture media, serum content and other conditions. Then again, it is not unlikely that more than one adhesion molecule couple may cause TF induction *in vivo*, depending on the prevailing local conditions and the presence of other cells and co-stimulatory factors.

Not surprisingly, cytokine secretion is also induced in monocyte-endothelial cell co-cultures. Recently, Zohlnhofer *et al* elegantly showed IL-1β-dependent induction of IL-6 production by the endothelial cells, resulting from monocyte adhesion to VCAM-1 [134]. Earlier, Lukacs *et al* had demonstrated ICAM-1-dependent induction of MIP-1α in monocytes upon co-culture with endothelial cells [135], as well as secretion of IL8 and MCP-1 in the co-cultures [136]. Others have reported increased amounts of IL-1β and TNF-α in co-culture supernatant [126]. Monocyte TNF-α up-regulation has also been demonstrated in receptor cross-linking studies [132].

In vitro, the most commonly used type of endothelial cell is the human umbilical cord vein endothelial cell, HUVEC, probably due to the convenient availability of umbilical cords and the fairly straight forward isolation procedure. However, there are substantial differences in characteristics between venous and arterial endothelial cells, and even between arterial endothelial cells of different origin [137,138]. Therefore, it

is difficult to estimate how much of the results from HUVEC experiments that actually apply in coronary arteries.

CD40 ligand – a central role in vascular cell cross talk

CD40 ligand (CD40L, CD154), a member of the TNF gene superfamily, is expressed predominantly on activated CD4+ T-helper cells, but in recent years, it has been shown to exist in its functional form also on smooth muscle cells, macrophages, endothelial cells, activated basophils [139,140] and activated platelets [8]. Its receptor, CD40, is expressed primarily by B-lymphocytes, but by a variety of other cell types as well, including epithelial cells, monocytes, macrophages and activated endothelial cells [140]. For several years, CD40-CD40L-mediated signals were thought to be of importance mainly in contact-dependent T-cell/B-cell cross talk in the humoral immune response. More recently, however, the involvement of CD40-CD40L interactions in inflammatory processes such as atherosclerosis has received great attention, as it is becoming increasingly clear that this receptor-ligand couple indeed has a variety of functions in the body.

Activated T-lymphocytes have for many years been known to induce TF expression and procoagulant activity in monocytes *in vitro* by a contact-dependent mechanism [141-143]. More recently, the induction was shown to be mediated by coupling of CD40L on the T-cells with CD40 on the monocytes or macrophages [139,144]. The same effect was seen in endothelial cells [145]. Activated platelets have also been reported to trigger both TF expression and chemokine secretion in endothelial cells by CD40-CD40L interaction [8,146]. Furthermore, ligation of CD40 on monocytes and endothelial cells *in vitro* has been observed to result in increased expression of adhesion molecules, secretion of cytokines and matrix metalloproteinases as well as enhanced survival and tumouricidal activity (monocytes) [140,147 and the references therein]. It has also numerous functional consequences in other CD40-expressing cell types [140].

In situ immunohistochemical analyses on sections of human carotid atheroma have revealed expression of both CD40 and CD40L in the shoulder region of the plaques [139] (see below for review of atherogenesis). In a model of atherosclerosis using hyperlipidemic mice, CD40L interference by a specific antibody reduced the size and lipid content of the initial atherosclerotic lesions as well as the number of infiltrating leukocytes [148]. Moreover, disruption of the CD40L gene in a similar mouse model revealed an even more profound reduction in advanced lesion size and a different plaque composition compared to mice with an intact gene [149]. Recently, the same two groups confirmed that even delayed antibody treatment, in established atherosclerosis, inhibited further progression and induced a more stable plaque phenotype [150,151]. These studies provide evidence for an important role for CD40L in the progression of atherosclerosis.

Coronary artery disease

Cardiovascular disease is the principal cause of death in the westernised world, and coronary atherosclerosis is its predominant cause. Atherosclerosis is an inflammatory process in the vessel wall [152], and when present in the coronary arteries, it often leads to restriction of the blood flow to the myocardium or to the development of thrombosis. Depending on the degree of vessel occlusion by the thrombi, the myocardium may be irreversibly damaged from the resulting ischemia, giving rise to a myocardial infarction. The understanding of the pathogenesis of atherosclerosis is continuously growing, with fundamental research in vascular biology supporting careful epidemiological studies. New approaches on risk stratification and treatments are emerging, and increasing knowledge is being gained regarding potential drug targets in the process of atherogenesis and its complications.

The atherosclerotic plaque

The atherosclerotic plaque, or *atheroma*, is a focal, eccentric thickening of the arterial wall, with an accumulation of lipids and inflammatory cells [153,154]. It is characterised by a central *lipid core* covered by a layer of extracellular matrix proteins, the *fibrous cap*. Around the lipid core and in the cap, a varying degree of infiltration by smooth muscle cells, T-lymphocytes and lipid-laden macrophages, or *foam cells*, can be seen. Mast cells may also be present in the plaque. The formation of atherosclerotic lesions is a complicated process, not all of which is completely understood.

Initiation of atherosclerosis

The atherogenetic process starts already in early life. It develops slowly, and usually doesn't cause clinical symptoms until several decades later [152,155]. When undisturbed, the vascular endothelium possesses properties that modulate haemostasis and vascular tone. It is also a selective permeability barrier towards plasma lipoproteins, and regulates the proliferation of the underlying smooth muscle cells. In case of inflammation, it can be induced to up-regulate adhesion molecules and secrete chemoattractants in a number of possible combinations, resulting in the recruitment of specific subsets of leukocytes to the underlying tissue. Under laminar blood flow, this homeostasis usually functions. However, in regions of lower shear forces or turbulent flow, such as branching points, bifurcations and the convex site of bending arteries, the balance is disturbed. Together with other factors, such as elevated and modified low-density lipoprotein (LDL), free radicals caused by smoking, genetic alterations, hypertension, high glucose levels or infectious microorganisms, the endothelium in these regions may become dysfunctional in the above mentioned properties [152]. These are the sites where atherosclerosis arises.

The earliest phase of atherosclerosis, the *fatty streak*, is characterised by the recruitment of mononuclear leukocytes, monocytes and T-lymphocytes, into the intima of the vessel wall [152,154]. The monocytes then engulf lipid and transform into so-

called macrophage foam cells. Receptors specific for the modified forms of LDL, *scavenger* receptors, have been identified in macrophages. The increased adhesion and extravasation of monocytes is a result of adhesion molecule up-regulation. Early increased expression of VCAM-1 has been observed on the endothelial cells on sites of lesion formation in hypercholesterolemic rabbits [156,157]. In human endothelial cells overlying atheromata, P-selectin is up-regulated [158]. Neither of these adhesion molecules is constitutively expressed on normal endothelium. In more advanced plaques, the ICAM-1 expression is also increased compared to normal endothelial cells [159].

Increased permeability of the dysfunctional endothelium allows an augmented flux of LDL into the arterial intima [155]. LDL particles may be modified by oxidation in the intima, a microenvironment secluded from circulating antioxidants. Oxidised LDL and the reactive oxygen species that arise during its oxidation have a number of toxic and pro-inflammatory effects on the cells in the lesion.

Nitric oxide (NO) is a potent vasodilator and inhibitor of platelet activation and is produced by the endothelium. However, it is now understood that it also has anti-inflammatory actions. These are exerted by inhibiting activation of the transcription factor NF-κB through induction and stabilisation of IκB [155]. NO has been shown to reduce monocyte adhesion, inhibit VCAM-1 gene expression and cytokine-induced secretable endothelial products [160]. This action of NO is likely to contribute to maintaining an anti-atherogenic profile, and is compromised along with vasodilation in abnormally functioning endothelium.

Progression of atheroma

As the atherosclerotic lesion progresses, the macrophages and T-cells are joined by smooth muscle cells. These migrate from the media, attracted by PDGF and TGF-β, both produced by activated endothelial cells [152,154,155]. The smooth muscle cells produce extracellular matrix proteins, mainly fibrillar collagen type I and III, that soon form a capsule, a *fibrous cap*, separating the interior of the atheroma from the vessel lumen. The inner part of the plaque is characterised by the *lipid core*, which is soft and weak and contains a mixture of foam cells, accumulated lipids, cholesterol esters and cellular debris. It is rich in extracellular and surface-bound TF and highly thrombogenic. The central parts of the lipid core are practically acellular, resulting from foam cell apoptosis and necrosis. It is sometimes also called the necrotic core.

The atheroma expands at its shoulder regions, where the lesion meets the normal vascular wall, by means of continued leukocyte adhesion and entry as well as continued lipid accumulation [152,154,155]. Neovascularisation at the base of advanced plaques may provide a second site of leukocyte entrance. The shoulder region of the plaque is a site of intense inflammatory activity [154,155]. Cellular activation has been verified by positive immunostaining for major histocomaptibility complex (MHC) class II (HLA-DRα antigen). Activated T-cells express CD40L on their surface and secrete IFN-γ - two key mediators of inflammatory stimuli [154,155].

IL-2 and RANTES have also been identified in human atheromata [161,162]. Macrophages/foam cells, activated by CD40 ligation as well as by IFN-γ and oxidised LDL, express TF and many inflammatory cytokines, such as IL-1β, TNF-α and IL-6. Endothelial cells and smooth muscle cells are activated by the inflammation and participate in it by producing chemoattractant substances like MCP-1, IL-8 and specific lymphocyte-attracting chemokines, and by a continued up-regulation of their adhesion molecules [154,155].

Plaque stability

Although generally displaying the hallmarks mentioned above, human atheromata are very heterogeneous in appearance. The size of the lipid core varies, as does the thickness and cellular content of the fibrous cap. The latter depends on the balance between synthesis and breakdown of matrix proteins [163-165]. PDGF and TGF-β can increase collagen synthesis by smooth muscle cells, and both these cytokines are found in the plaque. On the contrary, IFN-γ decreases synthesis of matrix proteins, as well as interfere with smooth muscle cell proliferation and promote apoptosis. T-lymphocytes produce IFN-γ, and indeed, an inverse relationship between collagen gene expression and the presence of T-cells has been observed. There is also evidence of accelerated degradation of matrix components in vulnerable plaques [163-165]. The family of matrix metalloproteinases (MMPs), i.e. collagenases, elastases and stromelysins, are synthesised by activated macrophages and foam cells as well as by smooth muscle cells. Their activities are regulated by tissue inhibitors of matrix metalloproteinases, TIMPs. Exposure of smooth muscle cells to inflammatory cytokines induces the expression of MMPs without altering the basal expression of TIMPs, suggesting a net imbalance in favour of matrix protein degaradation [155].

The vulnerability of the plaque, i.e. its predisposition to rupture, is determined by the mechanical strength of the fibrous cap to resist circumferential tensile stress and intrinsic mechanical forces [165,166]. The thickness of the fibrous cap is inversely related to the tensile stress in the cap, as is the degree of stenosis – the circumferential stress of the plaque decreases with increasing stenosis severity. This partly explains the fact that many less stenotic plaques are rupture-prone. Most plaques rupture at their shoulder region, the site of the highest circumferential stress, and also of the greatest inflammatory activity and highest rate of collagen breakdown. Vulnerable plaques are typically characterised by a thin fibrous cap, a substantial lipid core, few smooth muscle cells and a high density of macrophages and T-lymphocytes [164]. In contrast, more stable plaques often display a thick, dense fibrous cap with numerous smooth muscle cells, a small or absent lipid core and little infiltration of leukocytes. These lesions are often more stenotic, but less rupture-prone. Instead, they may cause a superficial thrombosis due to endothelial erosion, resluting from the increased shear stress when the flowing blood is forced past the obstructing lesion. However, any combination of the above mentioned characteristics can be found, even within the same individual.
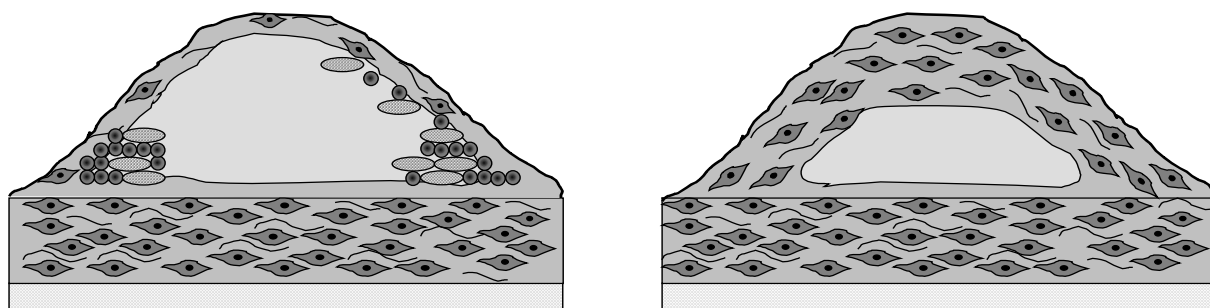
Figure 5. Morphology of an unstable (left) and a stable (right) atheroma.

Clinical manifestations of coronary artery disease

Atherosclerotic lesions develop slowly and often remain clinically silent for many years. As the intima focally thickens, the vessel compensates this at first by dilating, a process called *remodelling*, in order to minimise the effect on the blood flow [152]. When the atheroma has reached a size where the artery can no longer compensate by dilating, it may start to intrude into the vessel lumen and disturb the flow of blood. At this point, it may also cause clinical symptoms in the form angina pectoris, arising from the inability of the artery to provide the myocardium with sufficient amounts of oxygenised blood. Stable angina pectoris, or 'effort angina', causes symptoms when the oxygen demand of the heart muscle is increased, e.g. during exercise. The extent of the symptoms may vary greatly depending on the degree of stenosis, and may be more or less incapacitating.

The unstable form of angina pectoris is considered an acute coronary syndrome, and arises either from a worsening of previously stable disease (crescendo angina) or as suddenly oncoming symptoms in patients without records of CAD. The patients present with episodic chest pain at rest. The ischemia is most often caused by a thrombus, which has formed as a result of plaque rupture and exposure of the blood to the thrombogenic lipid core [163,164]. The thrombus may occlude a considerable part of the vessel lumen, and thus cause severe obstruction of the blood flow. The unstable episode may regress by itself if the endogenous fibrinolytic system succeeds in dissolving the thrombus. If the thrombus completely occludes the vessel, preventing all blood flow, a myocardial infarction may develop [167]. This can be of varying severity, depending on the time until clot lysis. A transient occlusion usually limits the myocardial damage and results in a 'non-Q-wave' myocardial infarction, whereas a permanent occlusion of the vessel may give rise to a large, transmural infarction.

Unstable angina pectoris and non-Q-wave myocardial infarction are classified together as unstable coronary artery disease.

Not all plaque ruptures give rise to acute clinical symptoms. Autopsy studies clearly indicate that plaque fissuring in coronary arteries often occurs in the absence of clinical events [155]. Several explanations are possible. The local flow conditions may determine whether the thrombus will remain mural or become occlusive, i.e., larger stenoses are more likely to be complicated with occlusive thrombi. Variable thrombogenicity of the plaque content as well as the activity of the endogenous haemostatic and fibrinolytic systems may affect the rate of thrombus formation and lysis. In the absence of an associated clinical event, the mural thrombus is incorporated in the atherosclerotic lesion, causing rapid progression in plaque size and lumen reduction.

Asymptomatic stenosis

Stabile angina

Unstable CAD

Myocardial infarction
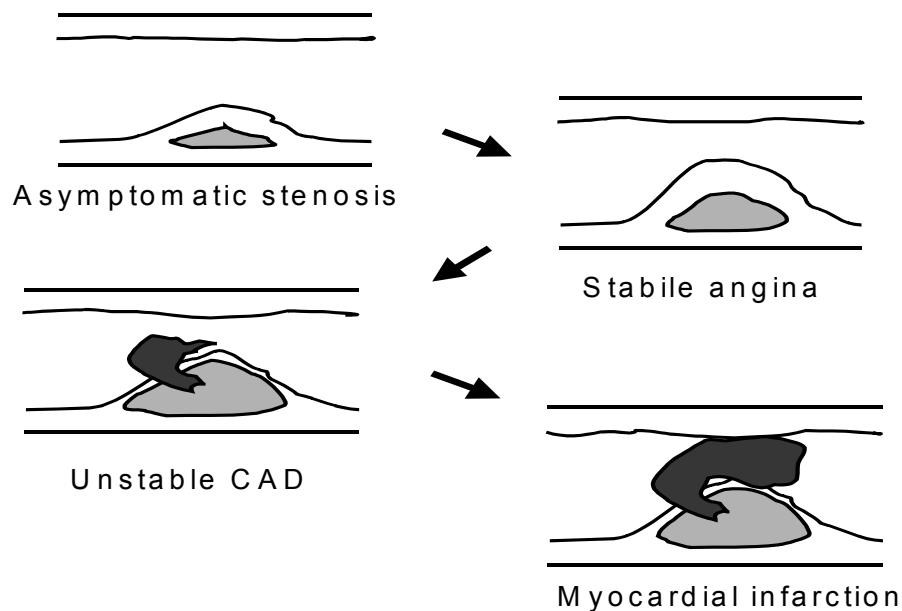
Figure 6. The various manifestations of coronary artery disease.

Systemic markers of unstable coronary artery disease

The endothelial dysfunction, the inflammatory process in the vascular intima and the propensity of thrombus formation, which all are associated with unstable coronary artery disease, also give rise to a peripherally measurable biological response. A number of studies on patients with unstable CAD have shown raised plasma levels of coagulation activation markers, such as prothrombin fragments 1+2, thrombin-antithrombin (TAT) complex [168] and fibrinopeptide A [169,170]; of inflammatory markers such as CRP [171-173], fibrinogen [171] and various cytokines [173-175]; of soluble adhesion molecules [176-179] and of soluble TF [175,180]. Increased

activation of circulating T-lymphocytes and monocytes has also been demonstrated [55,143,181], as well as activated platelets in free form [182] or in complexes with granulocytes [116]. These biochemical abnormalities are more rarely seen in stable angina pectoris.

These findings emphasise the many factors influencing the development of unstable CAD. It is interesting to speculate about the cause-consequence relationship between local and systemic inflammation – does a systemic inflammatory condition drive the process of atherosclerosis towards an unstable plaque phenotype, or is it the inflammatory activity in the plaque itself that gives rise to the systemic response? As yet, these questions remain unanswered. Enough is known about the potential of vascular cell cross talk to raise the possibility of active participation, whether primary or secondary, of circulating blood cells and soluble mediators in the pathogenesis of unstable CAD. However, it is difficult to draw any general conclusions from the studies performed to-date, since most of them investigate only a few parameters in their respective material, and some of the results have been contradictory. Moreover, most studies have been performed on a predominantly male patient group. Therefore, more studies are needed that analyse a large number of biochemical and cellular markers, and also that compare male and female patients.

Risk stratification in unstable coronary artery disease

Biochemical signs of myocardial damage, such as increased plasma levels of troponin-T or -I, have an additive value to clinical and etiological variables when it comes to estimating the prognosis of a patient with unstable CAD. The risk of developing a myocardial infarction, fatal or non-fatal, in a shorter or longer time perspective, increases several-fold with raised levels of troponin-T [183]. Interestingly, the inflammatory acute phase proteins CRP and fibrinogen have also proved to predict future cardiac events. Large cohort studies have been able to determine an association between elevated levels of circulating CRP or fibrinogen with increased risk for cardiac events or death, both in patients that have experienced an episode of unstable CAD as well as in apparently healthy individuals [184-187]. Even with a follow-up of 4 years in the FRISC (FRagmin during InStability in Coronary artery disease) trial, increasing levels of both these markers were seen to correlate with an increased risk for mortality in unstable CAD patients [188].

IL-6 is a cytokine with both pro- and anti-inflammatory effects on many cell types throughout the body. It is important in the regulation of the immune response, affecting both B-cell immunoglobulin production and T-cell cytotoxic activity [189]. IL-6 affects platelet production and reactivity and also endothelial function. It is the only substance known to induce synthesis of all of the acute phase proteins by the liver. Large, prospective studies on healthy populations have shown that IL-6 plasma levels in the upper quartile of the considered 'normal' range are predictive of an increased risk of premature death or future myocardial infarction, independent of other cardiovascular risk factors including CRP [190]. As yet, its prognostic value in

unstable CAD patients has only been investigated in one small study, where it was observed to correlate to short-term coronary events [191].

The fact that inflammatory markers carry prognostic information speaks for the concept of atherosclerosis as an inflammatory disease with both local and systemic components. The use of biochemical markers to identify high-risk patients can be useful for customising treatment strategies for patients with unstable CAD.

Treatments of unstable coronary artery disease

Medical treatment of unstable CAD can be said to follow three main directions: anti-ischemic, anti-thrombotic, and medication to prevent recurrence or worsening of the disease. Anti-ischemic therapy aims at relieving the symptoms, by reducing the work load of the heart and by vasodilatation of the coronary vessels. This is achieved by drugs like betablockers, ACE-inhibitors, nitrates and calcium antagonists [192]. Anti-thrombotic drugs have two main targets: reducing platelet reactivity and aggregability and suppressing coagulation. Acetyl salicylic acid (ASA, aspirin) and clopidogrel are both platelet antagonising agents and are used as a baseline long-term treatment in most patients. GpIIb/IIIa (fibrinogen receptor) inhibitors can be used for short-term treatment during an angioplasty intervention. For anticoagulation, heparin and, lately, low-molecular-weight (LMW-) heparin are the most commonly used drugs. Both inhibit FXa and thrombin [193]. When it comes to prevention of disease progression, medical therapy of unstable CAD resembles that of the stable form of the disease in the sense that the same risk factors are targeted [194]. High levels of blood cholesterol and triglycerides can be reduced by lipid-lowering drugs like statins; something that greatly improves prognosis following acute myocardial infarction. So does treatment with ACE-inhibitors, a class of drugs used to treat hypertension and cardiac failure.

Lately, an evolving insight is being gained that some of these drugs may have other beneficial effects in atherosclerosis than just their primary indication. The anti-inflammatory properties of aspirin have been known for many years. More recently, the statins, inhibitors of the enzyme HMG-CoA reductase, have proven to exert a multitude of effects on cells, unrelated to their suppression of cholesterol biosynthesis. They have been shown to have immunomodulatory properties [195], to be anti-inflammatory [196-198], anticoagulant [89,199], anti-oxidant [200,201] and angiogenic [200] as well as to improve vascular function [202-204]. They also seem to have a stabilising effect on unstable atheromata, inducing a thickening of the cap and a reduction in leukocyte infiltration and activity [205]. There are emerging implications of a role of angiotensin II in the pathogenesis of CAD, since this effector peptide of the renin-angiotensin system has been shown to induce many pro-atherogenic responses in vascular cells [206]. Its presence has also been demonstrated in human atherosclerotic plaques [207], and the myocardium of patients with unstable CAD [208]. ACE-inhibitors suppress the formation of angiotensin II, and have been shown to reduce plasma levels of TF and MCP-1 in patients with myocardial infarction [209], as well as inhibit lipopolysaccharide-induced TF in monocytes *in vitro* [88]. LMW-

heparin has, in addition to its anticoagulant mode of action, also been ascribed anti-inflammatory [210-213] and anti-adhesive [214,215] properties.

Recently, the FRISC II (Fragmin and Fast Revascularisation during Instability in Coronary artery disease II) randomised, prospective trial concluded that, if not contraindicated, a strategy of early revascularisation resulted in a better prognosis for the patient than did a primarily medical treatment strategy, even with the addition of long-term anticoagulant therapy with LMW-heparin [216]. Revascularisation of a stenosed coronary artery can be done in two ways: by percutaneous transluminal coronary angioplasty (PTCA, or percutaneous coronary intervention) or by coronary artery bypass grafting (CABG). PTCA is a less traumatising procedure, where a catheter containing an inflatable tip is inserted in the coronary artery through the groin, via the aorta. The tip is then inflated in the stenosed region of the vessel, and the atherosclerotic lesion is forced to give way, being pressed into the vessel wall. Often, an endoluminal *stent* is placed over the lesion to keep the vessel dilated. CABG surgery is an open-heart procedure, using extracorporeal circulation during the operation. In CABG, the stenosed vessel is replaced by a segment of saphenous vein from the leg and ligated to the inner thoracic mammary artery.

However, even if the patient undergoes revacularisation, the need for medication that prevents progression of the atherosclerotic process remains. The use of, in particular, statins seems very promising, and new potential targets for therapy are continuously being discovered. "Never more than now has basic research been more essential to increase our ability to cure" [155].

AIM

The general aim of this project was to further study the regulation of monocyte tissue factor expression, by cytokines and by other contact with cells, and to explore its possible association to the activation of coagulation and inflammation in patients with coronary artery disease. Also, the relationship between inflammation and outcome in unstable CAD patients was studied. More specific aims were:

♦ To establish a whole blood experimental system to study the regulation of monocytic tissue factor expression, and -

♦ - to investigate the effects of anti-inflammatory cytokines on LPS-induced TF expression in this system.

♦ To study the mechanism of monocyte TF expression induced by activated platelets, *in vitro* as well as in whole blood.

♦ To establish a co-culture system to investigate monocyte-endothelial cell interactions, and -

♦ - to investigate the induction of tissue factor and cytokines in this system and the effects of some commonly used drugs in CAD treatment.

♦ To study the cellular and biochemical patterns of coagulation and inflammation in patients with stable and unstable coronary artery disease, and to compare male and female patients.

♦ To evaluate the potential association between plasma levels of the pro-inflammatory cytokine IL-6 and outcome over 6-12 months in patients with unstable coronary artery disease, and investigate the effects of treatment strategy in this context.

PATIENTS, MATERIALS AND METHODS

The whole blood system

Blood was drawn from a forearm vein by a 21G needle using EndoTube ET (Chromogenix) endotoxin free heparin (paper I) or Vacutainer (Becton Dickinson) citrate (0,13M, paper II) blood collection tubes. Stasis was released after puncture, and for platelet studies, the first tube was discarded and the second one used for experiments. Aliquots of blood were placed in sterile polystyrene tubes and any agents requiring pre-incubation, such as cytokines or inhibitory antibodies, were added. In platelet activation experiments, a gpIIb/IIIa-inhibitor, fradafiban (Boehringer Ingelheim) was present to prevent platelet aggregation. After 5-15 minutes of incubation, the stimulus was added. In paper I, 5 ng/mL of LPS was used. In paper II, platelets were activated by addition of 20 µM of thrombin receptor activator, TRA (Sigma Chemical). The tubes were then further incubated in 37^oC, 5% CO_2 for 15 minutes to 3 hours. In paper I, IL-10 was in some experiments added 30 minutes-1 hour subsequent to initial LPS exposure. The tubes were gently rotated every 30 minutes of incubation to prevent complete sedimentation of the cells.

Cells and cell culture

Maintenance

In papers II and III, the monocytic cell line U-937 was used after differentiation by 72-96 hours' exposure to 0,1 µM of vitamin D_3. Differentiation resulted in growth arrested, CD14-positive cells with low basal TF expression [217]. The cells were also positive for CD11b and CD40. U-937 were maintained in RPMI 1640 medium supplemented with 7,5% fetal bovine serum (FBS) and 1% glutamine + antibiotics (100 IU/mL of penicillin and 50 µg/mL of streptomycin), and split twice a week. Differentiation as well as experiments were performed in the same medium with 10% FBS. For co-culture experiments with endothelial cells, antibiotics were omitted during co-culture.

In paper III, primary human coronary artery endothelial cells (HCAEC 5346) were purchased from Clonetics and cultured in EGM-2 MV medium from the same manufacturer. The cells were split every 3-4 days and medium was changed every other day.

Co-cultures

In paper II, vitD$_3$-differentiated U-937 were co-cultured with human platelets, purified from platelet-rich plasma by gel-filtration on a Sephacryl S-1000 column, with or without previous stimulation of the platelets with 1 U/mL of thrombin. In some experiments, platelets or U-937 cells were pre-incubated with neutralising antibodies before co-culture. Incubation of U-937 cells with buffer eluate (Tyrode's/HEPES buffer) from the Sephacryl column was used as a control.

In paper III, HCAEC were seeded into gelatine-coated 12-well cell culture plates and grown to confluence. 24 hours prior to co-culture, half of the EGM-2 MV medium was exchanged for RPMI 10% FBS, 1% glutamine, and 1000 U/mL of IFN-γ was added to some wells. In experiments including drugs, 5 μg/mL of simvastatin (Merck & Co), 20 μM of enalapril (Renitec®, Merck & Co) or 10 U/mL of dalteparin (Fragmin®, Pharmacia) were added 1 hour prior to co-culture, to HCAEC- or differentiated U-937 cultures separately, and were subsequently present throughout the experiment. Before addition of monocytic cells, the HCAEC cultures were washed twice in warm medium, which was carefully and completely removed after each wash, to ensure removal of all IFN-γ. U-937 cells were added to HCAEC at a concentration of 2×10^5 cells/mL, in RPMI 10% FBS, 1% glutamine. The co-cultures were then further incubated for 18 hours in 37°C, 5% CO_2.

Tissue factor mRNA analyses

For isolation of total mRNA from whole blood (papers I and II), RNEasy / QIAamp RNA Blood Mini Kit (Qiagen) was used. Isolation of total RNA from U-937 cells (paper II) was done with Trizol reagent [218]. RNA concentration was determined using a UV-spectrophotometer at 260 nm, and the integrity of the 28S and 18S ribosomal RNA was assessed in an ethidium bromide-stained agarose gel. The TF mRNA was the subjected to reverse transcription as previously described in detail [219]. In paper I, we used a quantitative RT-PCR that had previously been established in the group, using an *in vitro*-synthesised internal standard RNA [219]. In paper II, the Taq-Man® real time PCR assay and an ABI Prism 7700 Sequence Detection System (Perkin Elmer Applied Biosystems) were used [218].

Flow cytometry

Flow cytometry is a technique in which fluorochrome-conjugated antibodies are used to stain the antigen to be studied, and the light emitted from the stained cells is detected by a sensor in the instrument. The flow cytometer is equipped with a laser beam, which irradiates the cells as they pass through a flow cell, one at a time. The laser light is then scattered in two ways: deflected in the forward axis (forward scatter) depending on the size of the cell, and bounced off the cells in an approximately right angle (side scatter) when it encounters membrane structures within the cells. Side scatter can therefore be said to reflect the granularity of the cells. The different fluorochromes that can be conjugated to the antibodies emit light of different wavelength, thus enabling the detection of up to four antigens in a single sample. In these papers, only two antigens at a time are studied, using the fluorochomes fluorescein isothiocyanate (FITC; green) and phycoerythrin (PE; orange). Different types of cells can be distinguished by their different forward and side scatter properties, or by staining with a cell-specific antibody, e.g. anti-CD14 for monocytes and anti-CD4 for T-helper cells. In the generated histograms, digital gates can be constructed around the cell population of interest, and the computer can then create a histogram of the fluorescence emitted from this population. Two parameters are
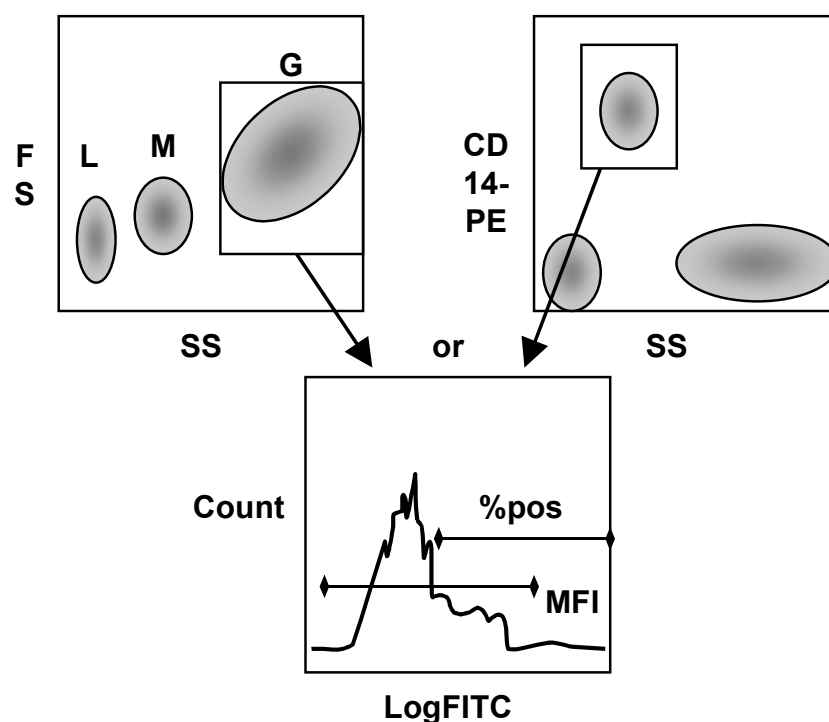
Figure 6. The principle of flow cytometry histogram analysis.

measured: a percentage of 'positively stained cells', related to a sample stained with an irrelevant antibody of the same isotype, and mean channel fluorenscence intensity (MFI), from which the background fluorescence of cells + isotype control antibody is subtracted. The percentage parameter reflects how many cells in the population that express the antigen in question, and MFI tells something about the amount of antigen expressed by each cell. A Coulter Epics XL flow cytometer (Coulter Electronics) was used for all flow cytometer analyses in this thesis.

Whole blood (papers I, II and IV)

100 µl of blood, fresh or incubated, was added to 60 µl of phosphate buffered saline (PBS) with 1% bovine serum albumin (BSA) containing saturating concentrations of the appropriate fluorochrome-conjugated monoclonal antibodies. For staining of leukocyte surface antigens, the mixtures were then incubated for 30 minutes on ice, in the dark. For detection of platelet-leukocyte complexes and their content of platelet activation antigens, the samples were incubated for only 15 minutes and at room temperature. At the end of the incubation, samples were washed twice in PBS and the supernatant was aspirated. The erythrocytes were lysed and the other cells fixed using Coulter Whole Blood Lysing Reagents, then washed again twice and resuspended in PBS/BSA and kept on ice until analysis. For platelet-leukocyte complexes, the red cells were lysed and the leukocytes fixed without prior washing. The monocyte

population was identified by gating on the CD14-positive cells. Platelet-leukocyte complexes were defined as positive staining for gpIIb/IIIa within the CD14-positive monocyte population, or within the granulocyte population defined by scatter characteristics.

Cell cultures (papers II and III)

Cells in suspension (differentiated U-937 alone and incubated with platelets) were collected and washed into PBS 0,1% BSA. HCAEC cultures and co-cultures with U-937 were washed carefully with PBS 1% BSA to remove non-adherent cells, then treated with HEPES/EDTA/BSA buffer for 20 minutes at 4^oC followed by 10 minutes at 37^oC (method from [8]). The cells were then loosened from the plate by vigorous pipetting and washed into PBS 1% FBS. To 100 µl of cell suspension, primary, unconjugated mouse anti-human TF antibodies were then added, and the samples were incubated for 30 minutes on ice, in the dark. Thereafter, they were washed again twice and incubated for another 30 minutes on ice with FITC-conjugated secondary rabbit anti-mouse IgG F(ab)$_2$-fragments before final washing and resuspension. In mixed cell samples, a third incubation step with PE-conjugated anti-CD14 antibody was performed. For U-937-platelet complexes and their degree of activation, the cells were stained with direct-conjugated antibodies against gpIIb/IIIa, P-selectin and CD40L.

For staining of intracellular antigens, the cells must be permeabilised to allow entrance of the antibodies into the cell. For this purpose, the Cytofix/Cytoperm™ kit from BD Pharmingen was used, following to the manufacturer's protocol. Blocking control experiments with non-conjugated antibodies of the same clone were carried out with one of the antibodies (anti-IL-6) used, and conjugated isotype control antibodies were used throughout.

Procoagulant activity

To whole blood samples, 0,5 mM EDTA was added, the blood then mixed with an equal amount normal saline and layered on top of a Lymfoprep density gradient (Nycomed) (method from [220]). After centrifugation, the mononuclear cell band was harvested, washed twice in PBS 0,1% BSA and resuspended in PBS alone. Aliquots containing approximately 50 000 monocytes were placed in the wells of a 96-well microtitre plate, and procoagulant activity (PCA) was measured in a two-stage amidolytic assay where a chromogenic substrate, S-2222 (Chromogenix), is cleaved by FXa, which in turn is activated from FX by the TF-FVIIa complex. A reaction mixture containing coagulation factors in the form of the factor concentrate Prothromplex-T™ TIM 4 (Immuno), S-2222 and 2 mM of CaCl$_2$ was added to the wells and the change in absorbance at 405 nm following a 30 minute-incubation at 37^oC was determined.

For measurement of PCA in U-937 cells, the same method was used, but in the presence of 10 µM of ionomycin. The reaction was started by the addition of CaCl$_2$.

Endotoxin contamination

All reagents, culture media and buffers were regularly screened for endotoxin contamination using CoaTest® Endotoxin chromogenic limulus amoebocyte lysate (LAL)-assay (Chromogenix). For whole blood, final concentrations were always below 10 pg/mL. For cell culture in paper II, slightly higher values were accepted (<100 pg/mL), since U-937 are not as sensitive to LPS as monocytes are, and since a buffer control was always included. Only sterile plastic-ware was used, and pipet tips were autoclaved before use. The bench and pipets were consistently wiped with surface disinfectant.

Patients

Paper IV

In this study, 26 patients with unstable CAD were recruited according to the inclusion and exclusion criteria of the FRISC II trial (see below), except that most patients had not yet received dalteparin at the time of sampling. 40 patients with stable angina pectoris were recruited from the list for elective PTCA or, in a few cases, coronary angiography. Exclusion criteria for these patients were the same as for unstable patients, with the addition of myocardial infarction or episodes of instability within the last 6 months. The characteristics of the patient study groups are presented in Table 1. For flow cytometry analyses, blood from 16 normal, healthy volunteers among the laboratory personnel was obtained, none of which were smokers, had diabetes or hypertension or were on ASA or lipid-lowering medication.

Paper V

Study V was a substudy of the FRISC II trial, which was a prospective, randomised, double-blind and placebo-controlled multi-centre study which included 3489 patients in 58 Scandinavian hospitals between June 1996 and August 1998. It evaluated the benefit of an early invasive treatment strategy compared to a non-invasive strategy, and also investigated the potentially beneficial effects of a prolonged (3 months) treatment with LMW-heparin (dalteparin). The details of the study protocol and the main results have been published elsewhere [216,221].

Patients were eligible for inclusion if they suffered increasing or rest symptoms of cardiac ischemia verified by ECG or at least one biochemical marker above normal range (creatinine kinase (CK)-MB, troponin-T, catalytic activity of CK, CK-B or CK-MB), with the last episode within 48 hours before the start of dalteparin or heparin treatment. Exclusion criteria were: raised risk for bleeding episodes, anaemia, indication for treatment in the past 24 hours with thrombolysis, angioplasty in the previous 6 months, being on a waiting list for a coronary revascularisation procedure, other acute or severe cardiac disease, renal or hepatic insufficiency, known clinically relevant osteoporosis, other severe illness, hypersensitivity to randomised drugs, anticipated difficulties with co-operation or participation in this or another clinical trial. Patients with previous open-heart surgery, advanced age (>75 years) or other

disorders that made randomisation to early revascularisation inappropriate, were excluded from randomised intervention strategies but were still assigned dalteparin or placebo.

The study design is schematically shown in Figure 7. All patients received dalteparin subcutaneously for at least 5 days, until an exercise test had been performed or until revascularisation. Thereafter, they entered the double-blind treatment with twice-daily subcutaneous injections with dalteparin or placebo until 90 days after entry. The direct invasive strategy required coronary angiography within a few days of enrollment, aiming for revascularisation within 7 days of the start of open-label treatment. Revascularisation was recommended in all patients with an obstruction of at least 70% of the diameter of any artery supplying a substantial proportion of the myocardium. Percutaneous coronary intervention was recommended if there were one or two target lesions, and coronary artery bypass surgery was preferred in patients with three-vessel or left main artery disease. In the non-invasive strategy, coronary angiography and, if appropriate, revascularisation, was recommended in patients with refractory or recurrent symptoms despite maximum medical treatment or severe ischemia on an exercise test before discharge. During follow-up, invasive procedures were considered, irrespective of randomised strategy, for all patients with incapacitating symptoms, recurrence of instability or myocardial infarction. Aspirin was given to all patients on admission, and beta-blockade was given unless contraindicated. Cholesterol-lowering with statins, angiotensin-converting-enzyme inhibitors for left-ventricular dysfunction and aggressive antidiabetic treatment were given according to current treatment guidelines.

All patients and controls gave informed consent to participate in the studies. The investigations conformed to the Declaration of Helsinki and were approved of by the local ethics committees.

Blood sampling and analysis

In paper IV, blood was drawn as described in the section 'The whole blood system', and in samples for flow cytometry, antibody labelling of the blood was carried out within 10 minutes. In paper V, venous blood samples in EDTA Vacutainer® tubes were taken from all patients at randomisation, at a median of 39 hours after onset of the last episode of chest pain. In both studies, plasma was prepared within 30 minutes of collection by centrifugation, then aliquoted and stored at $-70^{o}C$ until analysis.

Cell surface activation markers were analysed by flow cytometry as described in 'The whole blood system'. Measurements of IL-6, IL-10, soluble IL-2 receptor, soluble P-selectin and soluble TF were performed using commercial enzyme-linkes immunosorbent assay (ELISA) kits (Quantikine and Parameter from R&D Systems and Imu-Bind from American Diagnostica; paper IV). IL-6 and IL-10 were quantified using the manufacturer's 'high sensitivity' kits. FVIIa and soluble fibrin were analysed using quantitative spectrophotometric assays (Staclot VIIa-rTF from Diagnostica

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┌─────────────────────────────────────────┐
│ Admitted patients with chest pain and signs │
│ of ischemia fulfilling inclusion criteria   │
└─────────────────────────────────────────┘
                    │
                    ▼
        ┌──────────────────────────────────────┐
        │ Contraindications to early revascularisation? │
        └──────────────────────────────────────┘
         ╱                                    ╲
        ╱                    ┌──────────────────────────────────┐
       ╱                     │ 2457 patients with no contraindications; │
      ╱                      │ randomised in interventional study       │
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┌──────────────────┐  ┌──────────────────────┐  ┌──────────────────────┐
│ 666 contraindications │  │ 1235 patients assigned │  │ 1222 patients assigned │
│ 366 late inclusions   │  │ non-invasive strategy  │  │ invasive strategy      │
└──────────────────┘  └──────────────────────┘  └──────────────────────┘
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      │ 2267 patients randomised  │              │ Randomisation to      │
      │ in medical study          │              │ dalteparin/placebo    │
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  │ 1140 assigned │  │ 1127 assigned │
  │ dalteparin    │  │ placebo       │
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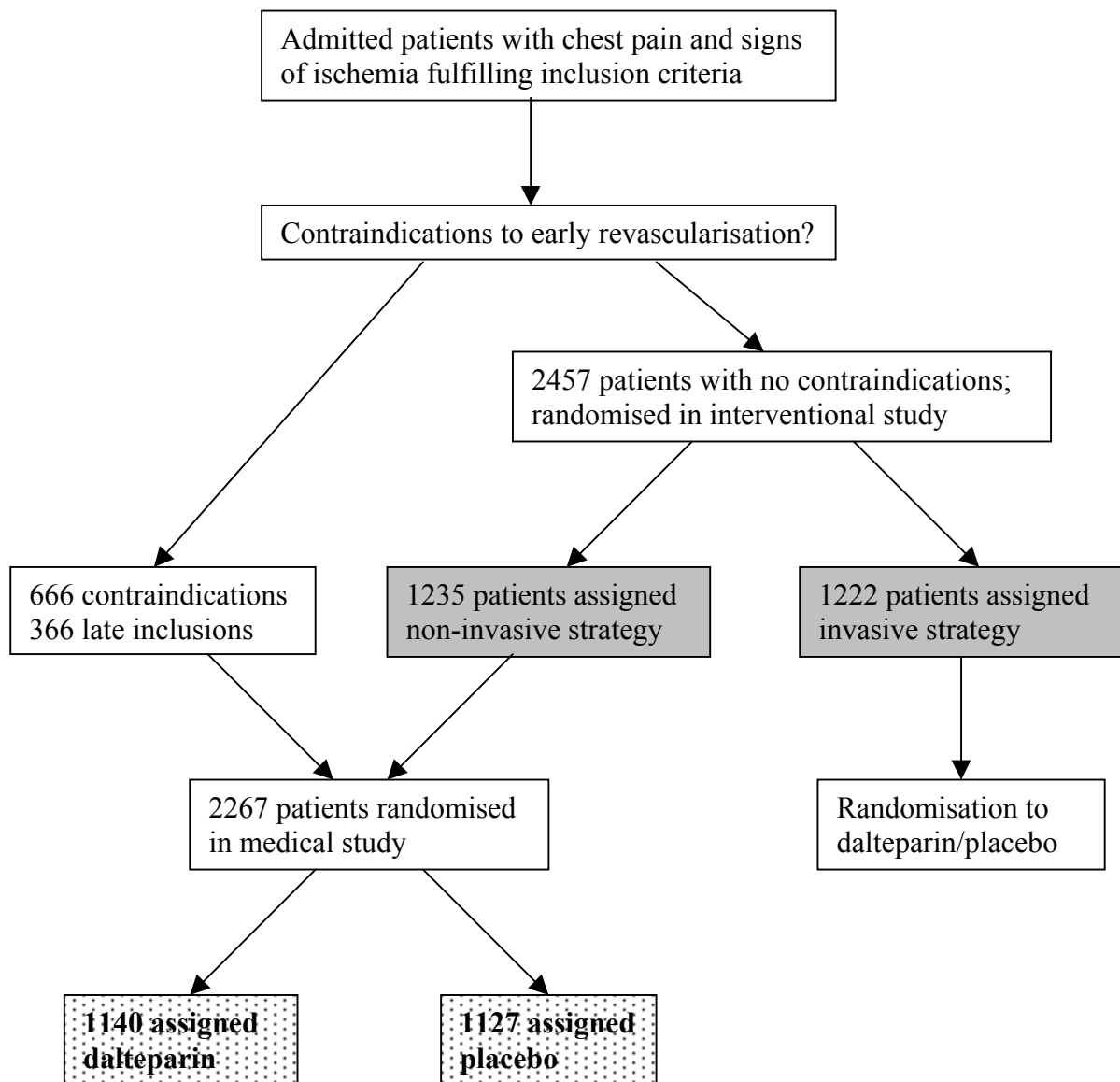
Figure 7.
Overview of the main FRISC II trial. Patient groups that were compared
to each other in paper V are shaded alike.

Stago and Berichrom FM from Behring Diagnostics). CRP measurements (paper IV) were carried out using the IMMULITE Automated Analyzer® and kit (DPC® Diagnostic Products Corporation), as were the IL-6 analyses in paper V. In paper IV, the normal ranges stated by the assay manufacturers were used, except for FVIIa and soluble TF, where plasma from 21 age-matched controls was analysed.

Statistical analyses

In all experimental studies, Student's t-test for dependent (papers I and II) or independent (paper III) was used to assess statistical significance between different data sets. In paper IV, Mann-Whitney U-test was applied. In paper V, Pearson χ^2-analysis was used to test significance of the overall degree of association. Forward stepwise logistic regression analysis was used to adjust for established risk indicators regarding mortality and for evaluating factors that could contribute to elevated IL-6 levels. P-values of less than 0,05 were considered statistically significant. Analyses were performed using Statistica ™ (StatSoft) or SPSS version 10.0 software.

The whole blood system and TF regulation by cytokines (paper I)

The whole blood experimental system was first evaluated using LPS as a stimulating agent. As little as 5 ng/mL of LPS proved sufficient for maximal response, and a basal incubation time of 2 hours was chosen based on the good induction achieved in this time together with the relatively low background TF expression. We also found longer incubation times unsuitable for this system, since the cells sediment, thus lacking proper gas exchange [68]. In a material of blood from 20 healthy individuals, monocyte TF was measured in fresh cells and in incubated cells with or without LPS-stimulation. A direct-conjugated anti-human TF antibody was used, 4508CJ (American Diagnostica). Fresh monocytes expressed very low amounts of TF: mean 3,1 ± standard deviation 1,5% positively stained cells. Incubation increased the spontaneous TF expression (12,2 ±8%). The TF induced by LPS tended to vary a great deal between individuals (52,5 ±17%), which is in accordance with the previously observed high- and low responder phenomenon. The individuals with the highest background TF also presented the highest TF-values after LPS-stimulation.
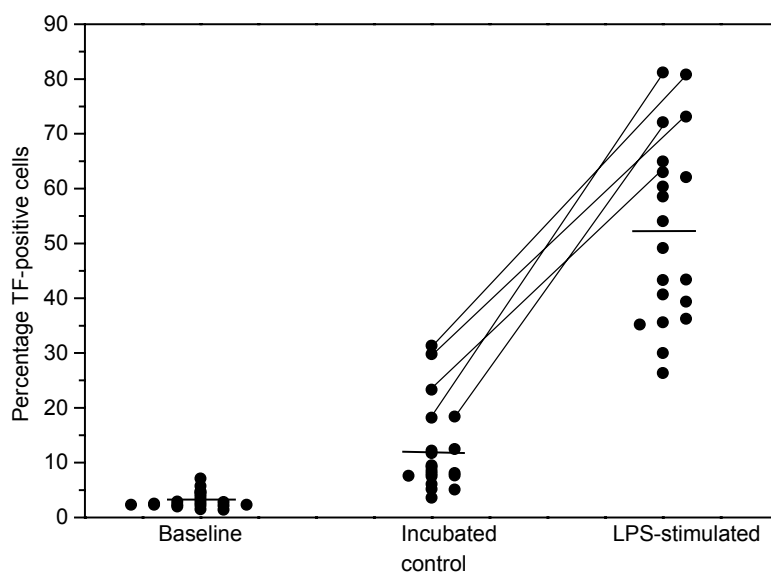

Figure 8. Tissue factor expression by human monocytes as measured by whole blood flow cytometry analysis. Mean values are indicated with horizontal bars. The samples that exhibited the highest spontaneous TF-induction after incubation were also highly responsive to LPS, as shown by lines connecting data points from the same series.

With this as a background, we tested the effects of IL-10, IL-4 and IL-13, all previously shown to attenuate monocyte TF expression *in vitro*, on LPS-induced TF in whole blood. A dose-response curve was constructed for IL-10, whereas the concentrations of IL-4 and IL-13 were chosen based on the previous *in vitro* experiments by Ernofsson *et al* (20 ng/mL and 50 ng/mL, respectively) [40]. IL-10 attenuated the TF induced by LPS in monocytes in a dose-dependent manner, with a maximum inhibition of $60 \pm 18\%$ achieved at a final IL-10 concentration of 5 ng/mL. IL-4 and IL-13 did not reduce monocyte TF; on the contrary, they even increased antigen expression in some experiments. If added together with IL-10, they both decreased the inhibitory effect seen with IL-10 alone. Yet, neither of them caused TF induction on their own or otherwise affected background values.

The flow cytometry results were confirmed by TF mRNA analysis, in which the potentiating effect of IL-4 and IL-13 was even more striking. Assays of mononuclear cell procoagulant activity were also performed. Here, we observed that IL-10 caused a stronger attenuation of TF activity than it did of the antigen expression. IL-4 and IL-13 did not alter LPS-induced TF activity.
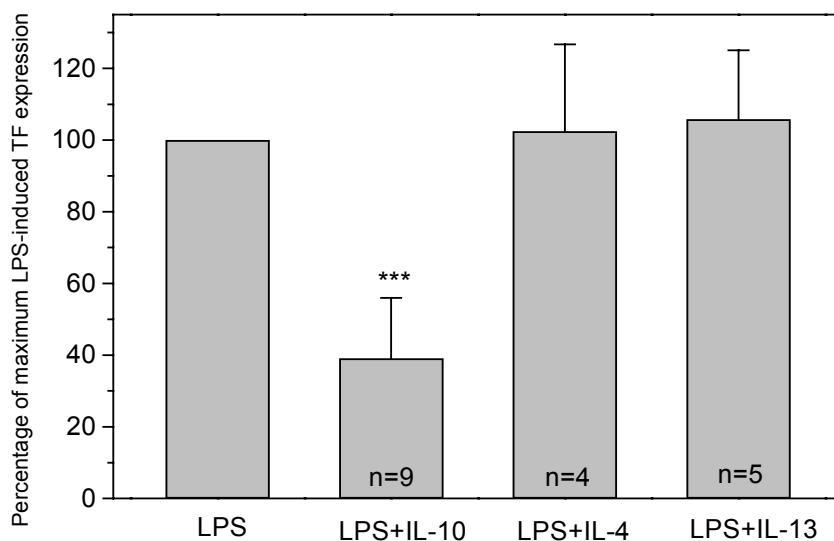

Figure 9. The effect of IL-10 (5 ng/mL), IL-4 (20 ng/mL) and IL-13 (50 ng/mL) on LPS-induced monocyte TF expression. Values compared to LPS without cytokine in the same series and expressed as mean ± SD.

The effects of IL-10 on LPS-induced monocyte TF in whole blood was not surprising. It confirmed previous *in vitro* studies on TF in isolated monocytes [38,40,70,72,73], as well as human and animal studies of inflammation and coagulation in experimental endotoxaemia [74,76,77]. IL-10 exerted its strongest effect when added prior to or shortly after LPS challenge. When added later into the LPS-stimulation, its inhibitory capacity was considerably reduced. The mechanism of IL-10 inhibition is not fully understood, but seems to be exerted mainly at the transcriptional level by inhibition of

nuclear translocation of NF-κB [222]. The spontaneous background TF observed in some of our experiments was not influenced by IL-10, indicating that this induction acted through pathways different from those of LPS. In view of what is known of TF induction by cell-cell cross talk, where platelets, granulocytes and T-lymphocytes have all been shown to influence TF expression in monocytes [41,42,141], interaction with other, activated cells is probably the cause for background TF. It also explains why the responsiveness to IL-10 varied in our experiments, and why complete inhibition at the antigen level was not achieved. This assumption is supported by a study by Warnes *et al* [223], published at the same time as paper I, where the addition of neutralising antibodies against P-selectin and monocyte B7 (ligand of T-lymphocyte CD28) in combination with IL-10 resulted in enhanced suppression of LPS-induced TF in whole blood compared to IL-10 alone. Also, Pradier *et al* observed that monocyte TF induction by CD40-CD40L in mixed lymphocyte reactions was IL-10 resistant [144].

It is interesting to note that the effect of IL-10 on TF activity was more pronounced and less subject to individual variations. This supports the theory that TF may be expressed on the surface while being catalytically inactive. It also indicates that IL-10 may have an overall more predictable effect on LPS-induced hypercoagulability.

The potentiating effects of IL-4 and IL-13 on TF mRNA induction by LPS are intriguing. They are in complete opposition of most current knowledge of the effects of these cytokines on monocytes [38,40,69,71,224]; most of which, however, is based on experiments with isolated cells. Possibly, general cell activation could account for some of the effects seen in our system. These questions remain to be addressed in forthcoming experiments. However, our results stress the importance of extending studies of blood cells to include the whole blood environment.

The whole blood experimental system obviously differs from the situation with isolated cells by the presence of other blood cells as well as their possible secretion products and all the endogenous plasma proteins. These conditions make the system less defined and less controllable than cell culture. Differences between the systems are seen both at the kinetics and the dose-response curve of LPS-induced TF, whole blood requiring lower concentrations and shorter incubation time to reach peak TF levels. Of course, one must recognise that the whole blood system is not an *in vivo* environment. Possible effects of the anticoagulant (heparin in this study) must be taken into account, as well as the plastic surface of the tube and the lack of circulation and active gas exchange during incubation. This could cause artefactual cell activation, particularly in donors with primed cells, e.g. an ongoing immune response. However, this is certainly true for isolated blood cells in culture as well, and the benefit of studying the cells in their own natural environment makes the whole blood model an important experimental system.

Platelet regulation of monocytic tissue factor expression (paper II)

The fact that activated platelets could induce monocyte TF in whole blood was previously shown by Amirkhosravi *et al* in 1996 [113], who also demonstrated the dependence of P-selectin for this expression. In 1998, however, it was discovered that platelets can express CD40L [8], another known inducer of TF in monocytes. The importance of this molecule in platelet-monocyte interactions had not been previously investigated. Therefore, in our next study, we investigated the mechanism by which platelets induce TF in monocytes, both in a whole blood environment and *in vitro*, using the monocytic cell line U-937 and platelets purified from plasma by gel filtration. The platelet-monocyte complexes formed upon activation of the platelets were detected by flow cytometry and analysed regarding their content of P-selectin and CD40L. Both in whole blood and in the cell line, platelet activation resulted in complexes that were positive for both these activation markers. In whole blood, activation of the platelets by TRA resulted in a very rapid exposure of TF on the surface of monocytes, which peaked already after 15 minutes' incubation with 41 $\pm18\%$ TF-positive cells, although ranging from 17% to 77%. After this, the TF-levels
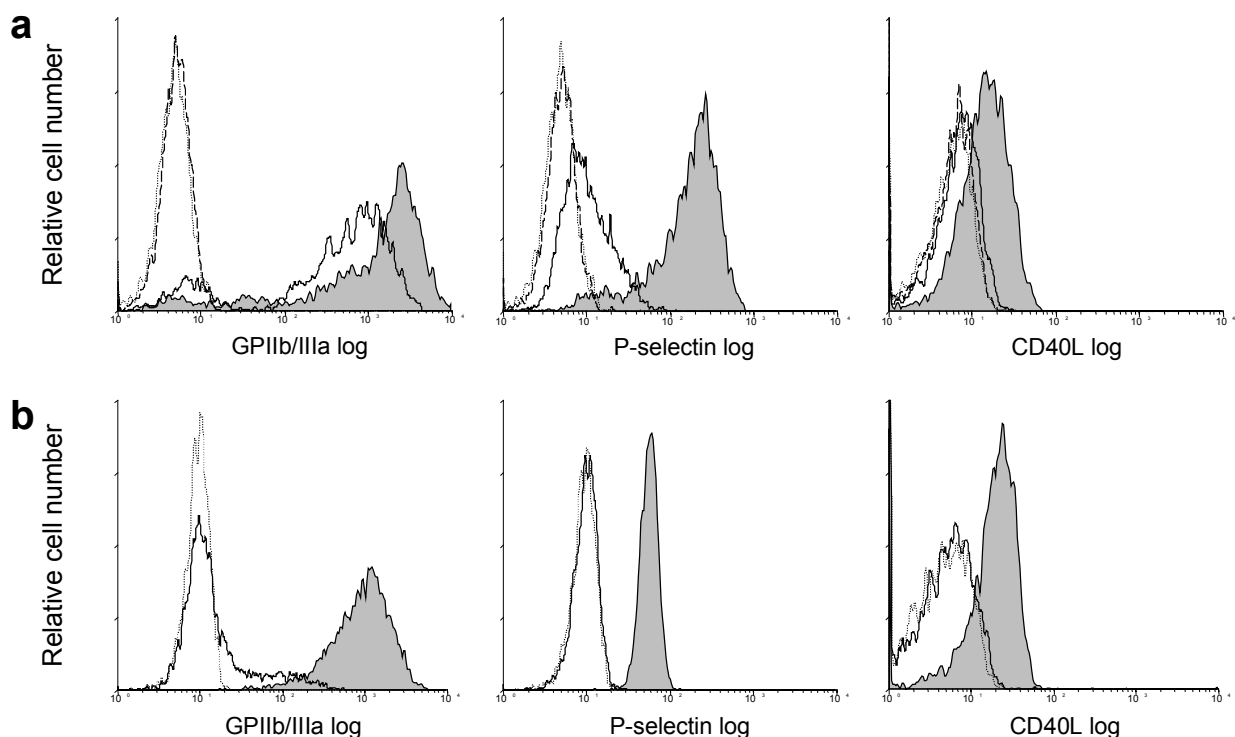

Figure 10. Flow cytometric characterisation of platelet-U-937 and platelet-monocyte complex formation and their content of platelet activation markers. (a) The expression of GPIIb/IIIa (presence of platelets), P-selectin and CD40L (activation markers) was examined on the U-937 cell population after 5 minutes of incubation with buffer (dashed line), platelets (solid line) or 1 U/ml thrombin stimulated platelets (grey area). (b) Monocyte (CD14-positive) staining for the same markers after 15 minutes' incubation of whole blood in the presence (grey area) or absence (solid line) of 20 µM of TRA. The staining with isotype control antibody is shown as a dotted line for both experiments. On the X-axis, the logarithmic fluorescence intensity for each marker is shown.

declined to 23 ± 12% over the following 2 hours of incubation. With LPS-stimulation (5 g/mL), the TF antigen levels increased gradually over time, with maximum levels at the end of the 2-hour incubation. Experiments with simultaneous mRNA analyses revealed that no TF mRNA was present at the time of peak antigen levels in TRA-stimulated blood, whereas LPS-stimulated blood followed the expected mRNA kinetics, peaking at 1 hour. TF antigen expression following TRA stimulation could be blocked to 51 ±16% ($p<0,001$) with neutralising antibodies against P-selectin and also to a certain extent (up to 30% inhibition, $p=0,02$) with anti-CD40L antibodies. Combining the two antibodies did not result in further inhibition compared to anti-P-selectin alone.
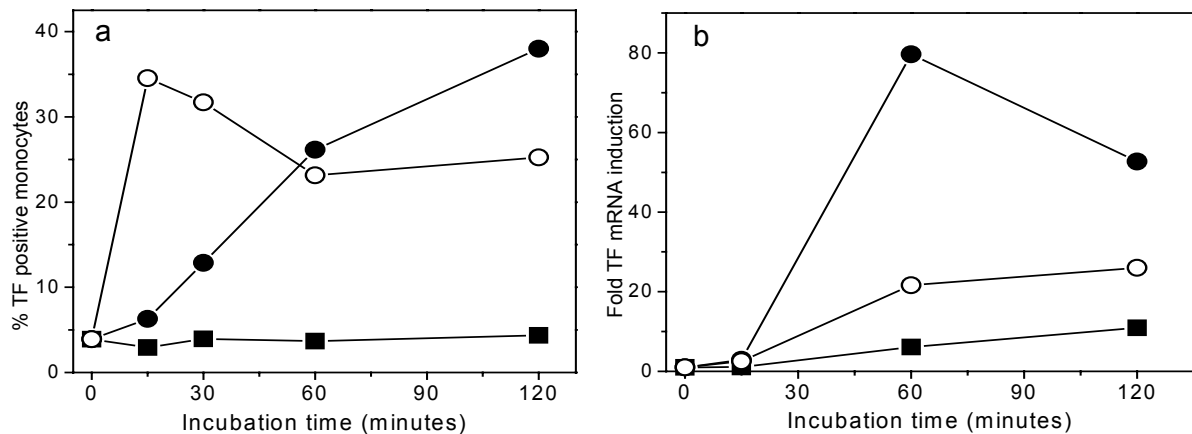

Figure 11. Kinetics of TF induction in whole blood by LPS (5 ng/ml, solid circles), TRA (20 μM, open circles) or in an unstimulated control (solid squares). (a) Flow cytometric analysis of monocyte TF antigen expression at the indicated timepoints of incubation. One representative experiment out of five. (b) Whole blood TF mRNA content at the indicated timepoints of incubation. One representative experiment out of two.

In this study, we showed for the first time an independence of *de novo* TF protein synthesis for the rapid TF exposure induced by activated platelets, suggesting that the TF protein was present in the monocytes prior to stimulation. The inability of IL-10 to inhibit platelet-induced TF (our unpublished data) also speaks for an up-regulation independent of gene transcription. Our results correlate well with those of Giesen *et al*, who demonstrated TF-containing microvesicles as well as TF-positive monocytes and neutrophils in blood from healthy individuals, and hypothesised that leukocytes are the source of blood-borne TF [51]. The same group has previously shown 3 cellular pools of encrypted TF in stimulated smooth muscle cells [64], raising the possibility that other cells might have a similar organisation. We favour the idea of intracellularly stored TF in resting blood monocytes, based on the fact that had TF been present on the surface in an encrypted form, we should have been able to detect it with at least one of the 7 different anti-TF monoclonal antibodies we have tested. This we have failed to do (data not shown).

In vitD$_3$-differentiated U-937 cells, incubation with activated, purified platelets induced an up-regulation of TF expression, with measurable TF antigen at 30 minutes

but with peak expression after 2 hours of incubation (antibody TF9-9C3, American Diagnostica). mRNA levels showed an early peak at 30 minutes, then declined to reach background levels again after 4 hours. Blocking studies were performed at the level of procoagulant activity after a 4 hour-incubation, where the presence of an anti-P-selectin antibody but not anti-CD40L suppressed the platelet-induced PCA (47 ±7% suppression). To clarify whether differentiated U-937 were able to express TF in response to CD40 ligation, co-culture experiments with isolated human lymphocytes (isolated from heparinised blood by Ficoll-Paque [Amersham Pharmacia Biotech] density gradient centrifugation and subsequent adherence of monocytes to a cell culture plate) were performed. The lymphocytes were then allogenically activated in the same way as in a mixed lymphocyte reaction. The induction of PCA in these co-cultures could be almost completely blocked by anti-CD40L antibodies.

P-selectin is obviously important for platelet-induced TF in monocytes, however, perhaps not altogether essential since we did not achieve complete inhibition with the P-selectin-neutralising antibodies, neither in whole blood nor in the cell line. There may be other adhesion molecules involved, whose role has not yet been investigated. The importance of CD40-ligation is difficult to estimate; possibly, CD40L enhances P-selectin-induced TF in whole blood, although it does not cause TF expression on its own. In differentiated U-937 cells, platelet CD40L was of no apparent importance in TF-induction, although the cells were responsive to T-cell CD40L. A possible explanation may be that platelets only express the membrane-bound form of CD40L, whereas T-cells also produce a shorter, soluble form of the molecule [225]; conceivably, the soluble CD40L is responsible for most of the effects seen in the U-937 - lymphocyte co-cultures. It also appears that platelets induce TF by different kinetics in the cell line system than in blood monocytes. U-937 cells might not possess intracellular stores of TF, but they also show a more rapid induction at gene level by platelets than blood monocytes do.

Monocyte-endothelial cell co-cultures (paper III)

Several studies in the past few years have investigated cellular effects of monocyte co-culture with HUVECs. Because of the previously observed differences between endothelial cells of different origin, we chose coronary artery endothelial cells for our system. These were stimulated with IFN-γ, which is thought to be a key cytokine in atherosclerosis and which selectively up-regulates ICAM-1 on the endothelial cells. We used vitD$_3$-differentiated U-937 cells as a model for monocytes, since these cells have been used successfully in previous co-culture and adhesion studies [125,226], and since the use of a cell line eliminates the problem with contaminating lymphocytes that might react allogenically to the HCAEC. Our aim was to investigate the expression of tissue factor and cytokines, namely TNF-α, IL-6 and IL-10, in this system, as well as the effects of the drug classes statins, ACE-inhibitors and LMW-heparin. All antigen measurements were done by flow cytometry as described in 'Patients, materials and methods'.

Tissue factor and cytokine expression

Resting U-937 as well as HCAEC expressed low or very low levels of TF (6,5% and 3,2% positively stained cell, respectively). Co-culture with resting HCAEC did not alter TF expression of either cell type. When cultured together with pre-stimulated endothelial cells, however, U-937 TF expression increased to 33,8% TF-positive cells ($p<0,001$), whereas TF on HCAEC did not change significantly (Figure 12).

We confirm previous studies that ligation to ICAM-1 induces TF expression in monocytes. However, resting HCAEC also express ICAM-1 and bind U-937, although at a lower level. U-937 adhesion to HCAEC, as estimated by the proportion of CD14-positive cells out of the total number of adherent cells in washed co-culture wells, was 52% in pre-stimulated cultures compared to 32% in resting ones. It thus seems that TF induction requires additional stimuli, provided by IFN-γ-activated endothelial cells but not by resting ones. Considering that expression of E-selectin, VCAM-1 and ICAM-2 was unaltered from IFN-γ stimuli or from co-culture, it is reasonable to believe that a soluble factor is the missing link.
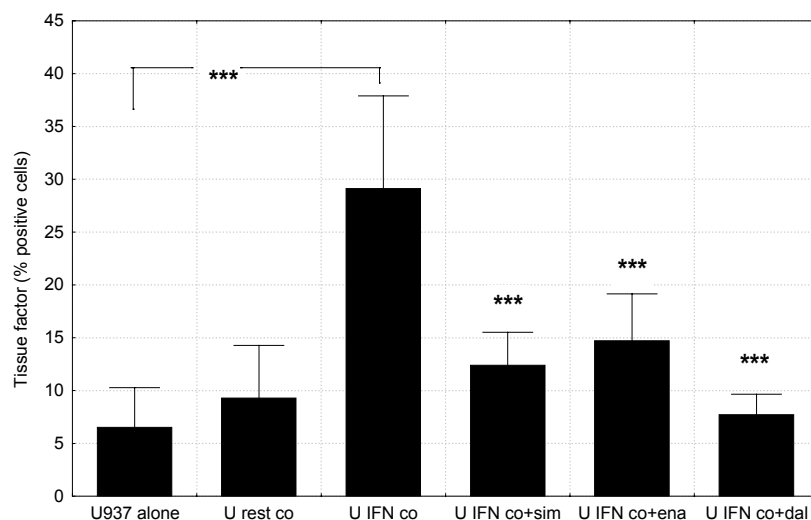

Figure 12. Tissue factor expression by U-937 cells cultures separately or together (co) with resting or IFN-γ-stimulated HCAEC, in the presence or absence of simvastatin (sim), enalapril (ena), and dalteparin (dal). n=13 (separate), n=8 (co-cultures without drugs), n=4 (co-cultures with simvastatin or dalteparin) and n=7 (co-cultures with enalapril). Columns show mean + SD.

When cultured separately, both differentiated U-937 and resting HCAEC expressed substantial levels of intracellular TNF-α, with 62,1% and 40% TNF-positive cells, respectively. HCAEC also expressed IL-6 and IL-10 at around 14% positive cells of each. IFN-γ stimulation significantly increased IL-10 expression to 23,7% ($p=0,02$), but did not alter the expression of the other two cytokines.

Resting co-culture resulted in a down-regulation of TNF-α by both cell types (8,7% TNF-positive U-937 and 26,2% positive HCAEC), but when the HCAEC had been pre-activated, TNF was only reduced in the monocytic cells. Co-culture with resting

HCAEC induced IL-6 expression in U-937 cells (27,6%, *p*<0,01) (Figure 13). When HCAEC were pre-stimulated, the expression was slightly lower, although not significantly so. For IL-10, the expression remained unaltered upon co-culture in U-937 and resting HCAEC, whereas IFN-γ-activated HCAEC increased their expression further to 42,2% (*p*<0,02 compared to stimulated HCAEC alone).
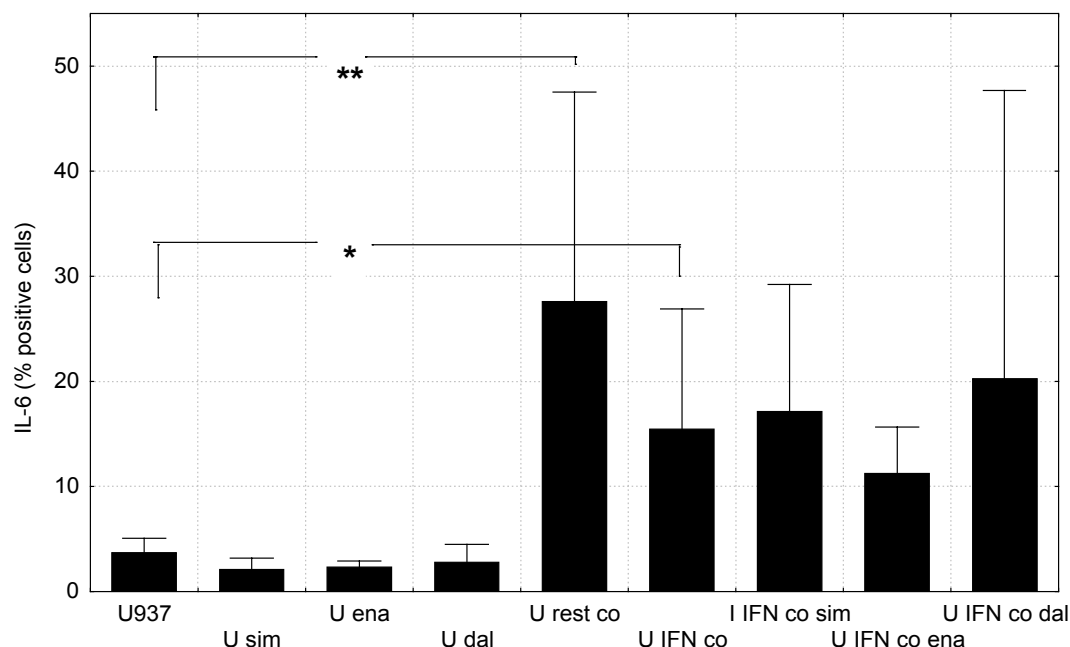

Figure 13. Intracellular expression of IL-6 in differentiated U-937 cells, cultured separately or together with resting or IFN-γ-stimulated HCAEC (co) in the presence or absence of simvastatin (sim), enalapril (ena), and dalteparin (dal). n=7 (separate cultures without drugs and pre-stimulated co-cultures without drugs), n=5 (U sim and U ena), n=4 (resting co-cultures), n=3 (U dal and co-cultures with sim, ena and dal). Columns show mean + SD.

The induction of IL-6 in the monocytic cells upon co-culture seems to be independent of pre-stimulation with IFN-γ, indicating that TF and IL-6 are induced by at least partly separate pathways in this system. In HCAEC, pre-stimulation reversed the down-regulation of TNF-α induced by co-culture, but instead allowed for a further increase in IL-10 expression. Further studies involving blocking of cytokines and/or their receptors are needed to elucidate the mechanisms of this phenomenon. Contrary to previous knowledge, we found IL-10 protein inside HCAEC. This was surprising and is difficult to explain. However, there were no other cells in the culture that could have secreted the cytokines, which might then have bound to receptors on HCAEC and been internalised. U-937 cells were not positive for IL-10, which speaks against antibody cross-reactivity with any common intracellular antigen. Also, IL-10 production by HCAEC fluctuated in response to drugs (see below) and co-culture, and even in the latter case, U-937 were more or less negative. This phenomenon requires further investigation, and we are currently planning new experiments to validate these results.

Effects of drugs

All three drugs tested suppressed the monocytic TF induction caused by co-culture with pre-stimulated endothelial cells, with 12,4% TF-positive cells after treatment with simvastatin, 16% with enalapril and 7,7% with dalteparin (Figure 12). They also significantly reduced basal TNF-α expression in U-937 cells to only 6,8-9,6% ($p<0,001$ for each). In IFN-γ stimulated HCAEC, however, only enalapril had this effect ($p=0,02$). This drug also significantly suppressed IL-6 and IL-10 levels in activated HCAEC, to 4,5% and 4,7% positive cells, respectively. In co-cultures, none of the drugs altered the cytokine expression, neither by U-937 nor by HCAEC.
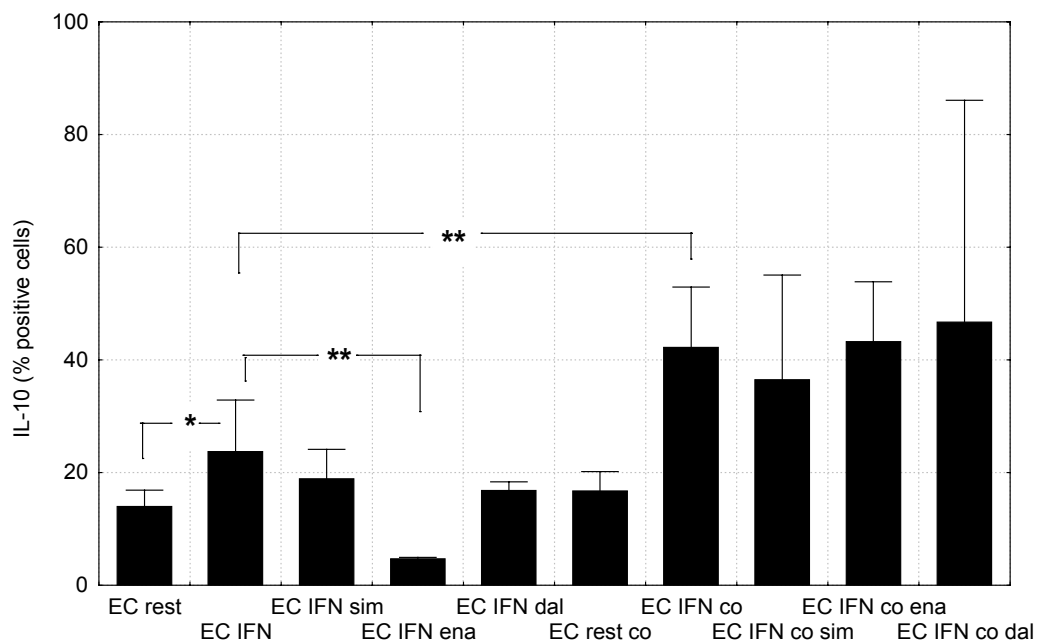

Figure 14. Intracellular expression of IL-10 in resting or IFN-γ-stimulated HCAEC, cultured separately or together differentiated U-937 cells (co) in the presence or absence of simvastatin (sim), enalapril (ena), and dalteparin (dal). n=7 (separate cultures without drugs and pre-stimulated co-cultures without drugs), n=4 (resting co-cultures), n=3 (all cultures with drugs present). Columns show mean + SD.

Statins have over the past few years proved to possess a number of cell-modulating properties, beside their cholesterol-lowering effect. They inhibit both TF [90,89] and cytokine expression [196,197] in LPS-stimulated monocytes and macrophages, they reduce adhesion of monocytic cells to endothelium [227,226], increase endothelial nitric oxide synthase-levels [202] and prostacyclin production [203] and inhibit IFN-γ-induced expression of MHC II-complex in EC [195], among other reported actions. In our model, the presence of simvastatin strongly reduced TF expression by differentiated U-937 in co-culture. This may be the result of simvastatin actions either directly on the U-937 or on the endothelial cells, perhaps by increasing their NO-production [81]. Simvastatin had a striking effect on basal TNF-α production in U-937 cells, but not in HCAEC, suggesting that TNF-α is regulated by different pathways in these cells.

Napoleone *et al* recently showed that angiotensin II, yielded by intrinsic ACE-activity in monocytes, was important for LPS-induced TF-expression in these cells, since ACE-inhibitors as well as an angiotensin II-receptor blocker suppressed TF induction at the gene level [88]. Angiotensin II has also been found to activate the transcription factor NF-κB [206], which is thought to promote leukocyte adhesion and extravasation into the atherosclerotic lesion by influencing the expression of cytokines and adhesion molecules [206]. By this study, we demonstrated for the first time that the ACE-inhibitor enalapril inhibits monocytic TF expression induced by adhesion to activated endothelial cells, indicating a role for angiotensin II as a mediator of the TF up-regulation. Enalapril also reduced all intracellular cytokines measured in separate cultures of HCAEC and U-937. This finding adds new information regarding the ability of ACE-inhibitors to block TF induction, as well as suggesting other mechanisms by which these drugs may interfere with the progression of atherosclerosis.

The immunomodulatory properties of LMW-heparin have previously been sparsely investigated. However, LMW-heparin as well as unfractionated heparin has been shown to bind to CD11b/CD18 on leukocytes, thus inhibiting binding of other ligands [215]. It has also been reported to reduce leukocyte adhesion to endothelial cells [214], which we could confirm in this study, and we also observed that this was not the result of a down-regulation of CD11b/CD18 on the monocytic cells (data not shown). We also showed that dalteparin suppresses monocytic TF induction in the co-culture model, a previously unknown effect of LMW-heparin. This could possibly also be related to the blocking of the CD11b/CD18 integrin, conceivably by reducing its cross-linking even in adherent cells. Dalteparin reduced basal levels of TNF-α in differentiated U-937 cells, but not in HCAEC. Regarding this, previous investigations diverge: LMW-heparin has been shown to reduce or increase TNF-α secretion [210,228,229]. This may be a question of dosage - two studies show that only low doses of LMW-heparin has anti-inflammatory effects [211,212]. In any case, it speaks for a direct cellular effect of LMW-heparin, not dependent on reduction of adhesion.

None of the three drugs affected the cytokine content of the co-cultured cells. This is particularly surprising for enalapril, which reduced all cytokines in separately cultured HCAEC. The reason for this may be the short pre-incubation time. In previous studies on statins, the cells have been exposed to drugs for up to 48 hours prior to the experiment. This is relevant if the components of the activation-induced pathways are presumed to be present also in the resting cell, thus requiring some time to be down-regulated by the drug. However, Napoleone *et al* added ACE-inhibitors simultaneously with LPS, without pre-incubation [88], and in our separate HCAEC cultures, there were significant differences in intracellular cytokines at 18 hours when enalapril was present, suggesting a difference in induction pathways between separately cultured and co-cultured cells.

Lessons from papers I - III

The studies in papers I – III have furthered our understanding of monocyte TF regulation in several ways. We now know that IL-10 is as potent an inhibitor of LPS-stimulated TF induction in whole blood as it is in isolated cells, whereas IL-4 and IL-13 have a completely different effect in the whole blood environment. Also, we noted a more complete suppression of TF activity than of the TF protein expression by IL-10. This is interesting in view of the suppression of residual TF antigen expression by antibodies against P-selectin and B7 that was shown by other workers, and suggests that the TF resulting from cell-cell contact with platelets and T-cells might not be active. We didn't measure TF activity in whole blood in paper II, so as yet, this speculation remains unverified. However, IL-10 could not suppress platelet-induced TF in our system, a finding that verifies the results of Warnes *et al*.

The rapid exposure of TF on monocytes in response to platelet activation in healthy donor blood suggests a mechanism for propagation of thrombus formation, perhaps also in the context of atherosclerotic plaque erosion, where platelets bind to and aggregate on the denuded endothelium. Much is yet unknown of the regulation of this seemingly stored but latent TF, but the amounts of TF exposed on the monocyte surface varied substantially between individuals. It would be conceivable that patients with large storage pools of TF more rapidly develop occluding coronary thrombi in case of plaque erosion or rupture. IL-10 is a promising therapeutic agent in bacterial sepsis and inflammatory bowel disease, but does not seem to be of use where activated platelets cause monocyte TF exposure.

TF can also be induced in monocytes by adhesion to activated coronary endothelium, as shown in paper III. This induction appears, or at least still remains, after 18 hours of incubation, correlating with an up-regulation at the gene level. This is also the mechanism previously seen in $\beta2$- as well as $\beta1$-integrin-associated TF induction [133,130]. ICAM-1 is expressed on and within the atheroma, raising the possibility that monocytes adhering to the endothelium on the plaque may be induced to TF expression. TF induction could in our system be more or less blocked by three drugs commonly used in the treatment of CAD patients, namely statins, ACE-inhibitors and LMW-heparin. This suggests another beneficial effect of these drugs in CAD.

It is difficult to estimate the importance of the induction of IL-6 in monocytic cells seen upon adhesion to HCAEC, and the up-regulation of IL-10 in the endothelial cells. We do not know whether these events may occur *in vivo* or whether they are an *in vitro* phenomenon. Until further studies with cytokine blocking in this system have been performed, we can only conclude that it was an interesting observation that deserves further notice.

Blood cell activation, coagulation and inflammation in CAD (paper IV)

In paper IV, our aim was to study the activation pattern in blood cells, coagulation and inflammation in patients with stable versus unstable coronary artery disease, using a number of markers in the same patient material.

As cellular activation markers, monocyte TF, monocyte and granulocyte CD11b and T_H-cell HLA-DR (MHC II) were measured by flow cytometry. Circulating platelet-monocyte and platelet-granulocyte complexes were also estimated. Plasma markers analysed were soluble TF (which can be said to be a marker both for monocyte activation and coagulation), MCP-1, soluble IL-2 receptor α and soluble P-selectin reflecting cellular activation; FVIIa and soluble fibrin representing coagulation activation and CRP, IL-6 and IL-10 as pro- and anti-inflammatory markers.

Cell surface markers

Patients with unstable coronary artery disease (UA) displayed significantly higher levels of circulating platelet-monocyte as well as platelet-granulocyte complexes than did stable angina (SA) patients ($p<0,01$ for both), which in turn showed slightly but not significantly higher values than healthy controls. The raised levels in unstable patients were almost entirely due to the high degree of platelet activation in the female part of the patient group (median 23, interquartile range [14,6-66,8] in women versus 4,1 [1,8-22,6] in men for monocyte MFI). Stable patients had no difference between men and women (Figure 15).
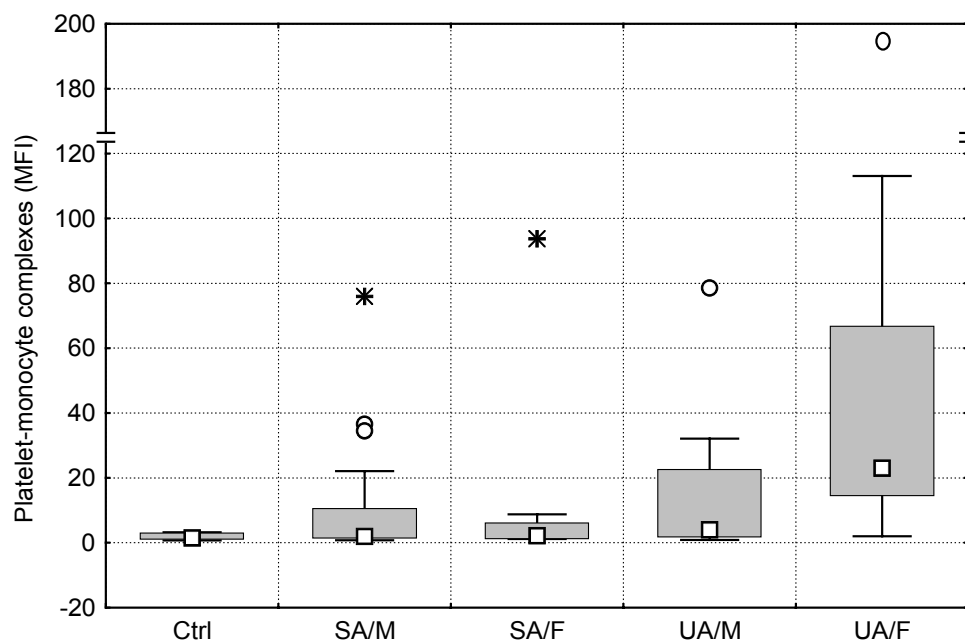

Figure 15. Platelet-monocyte complexes in whole blood as measured by monocyte mean fluorescence intensity for the platelet marker GPIIb/IIIa. Median values (open squares), 25th and 75th percentiles (box), non-outlier min and max (whiskers), ouliers (open circles) and extremes (snowflakes). Control group, stable and unstable males and females.

CD11b, part of the CD11b/CD18 complex, is up-regulated on monocytes and granulocytes upon activation, as is HLA-DR antigen on T-lymphocytes. In unstable CAD, both monocytes and granulocytes expressed significantly more CD11b than did cells of the control group ($p<0,01$ and $p<0,05$, respectively), but only the granulocytes differed from stable patient values (Figure 16). T-cells showed increased HLA-DR expression in both CAD groups (medians 11,9 and 10,4) compared to controls (median 8,0, $p<0,05$)), but did not differ from each other. Monocyte surface TF levels were not significantly raised in the patient groups.
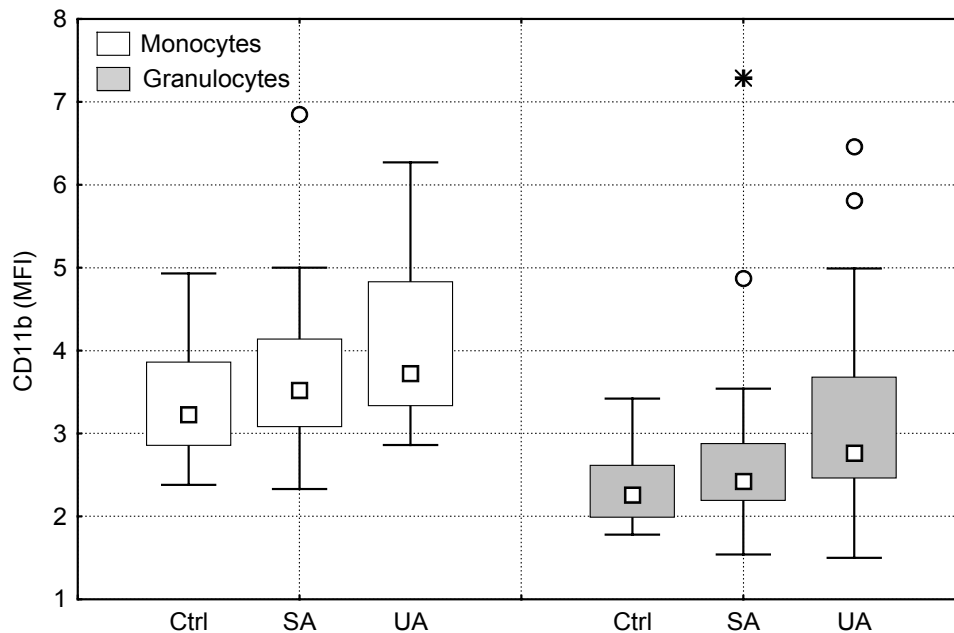

Figure 16. Leukocyte expression of CD11b in controls (ctrl), SA patients and UA patients. Median values (open squares), 25th and 75th percentiles (box), non-outlier min and max (whiskers), ouliers (open circles) and extremes (snowflakes). Open boxes represent monocyte data, shaded boxes are granulocyte values.

Biochemical markers

Neither of the patient groups presented levels of FVIIa that were above normal range, nor did they differ from each other. However, unstable patients had significantly higher amounts of soluble fibrin than did stable patients, and both groups were well above the normal range given by the assay manufacturer. As for soluble TF, both patient groups differed significantly from the controls (146 [104-188] and 116 [92-150] pg/mL in the patient groups, respectively, versus 76 [32-108] in the control group). Unstable patients tended to have higher levels than stable ones ($p=0,06$). Women demonstrated higher plasma TF levels than men, and this time the difference was most pronounced in the stable patient group ($p<0,001$). Unfortunately, our control material contained too few women to determine whether this gender-related difference also applied in a healthy population.

Almost all patients had plasma levels within the normal ranges of the cytokine and CRP assays. However, the amounts of these markers were significantly higher in unstable patients than in stable ones ($p<0,001$ for IL-6, $p<0,01$ for IL-10 and $p<0,01$ for CRP).
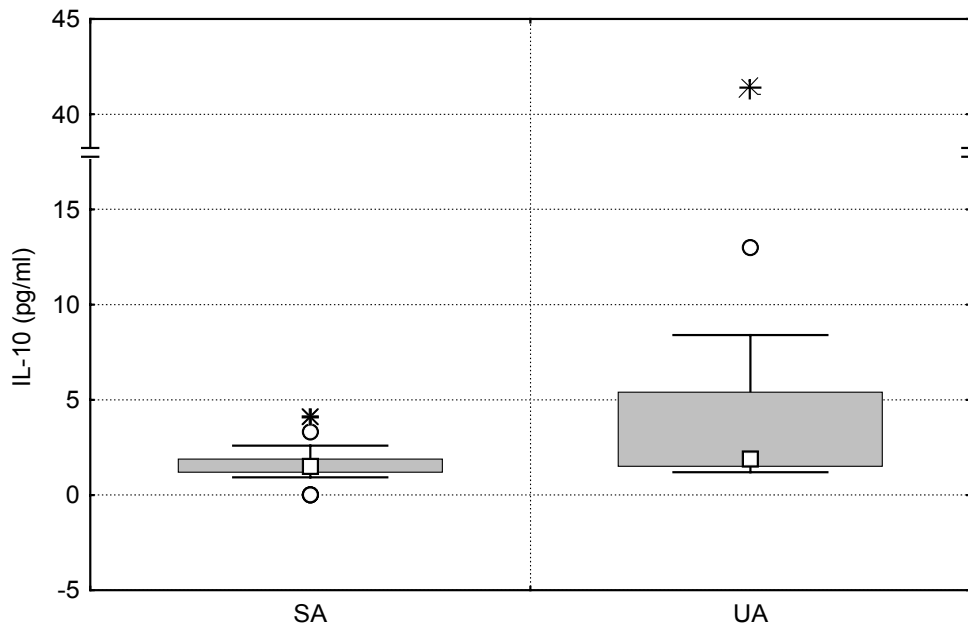

Figure 17. Plasma levels of IL-10 in stable versus unstable angina patients. Median values (open squares), 25th and 75th percentiles (box), non-outlier min and max (whiskers), ouliers (open circles) and extremes (snowflakes).

Soluble IL-2Rα was measured in order to reflect T-cell activation. Soluble P-selectin can be derived from both endothelium and activated platelets, and can be seen as a marker for present or previous activation of these cell types. MCP-1 is secreted by activated monocytes/macrophages, and has stimulatory effects on these cells. For sIL-2Rα and MCP-1, no significant differences could be seen between the patient groups, although unstable patients showed a tendency towards higher MCP-1 levels. Both markers were within their respective normal ranges. As for soluble P-selectin levels, these were slightly higher in the unstable patients, and this was again due to the female part of the unstable group (median and interquartile range for unstable women 44 [31-59], for unstable men 38 [29-42] and for stable women 34 [30-42] ng/ml). Overall, approximately 25% of the stable patients and 50% of the unstable patients were above normal range.

Discussion

In coronary artery disease, there are various differences in disease pattern between men and women with a variance in both symptoms and outcome [230,231], as well as in the effect of treatment [232]. It could even be conceivable that women have a different underlying pathophysiology to their vessel disorder. Several studies have shown that platelets of women with unstable CAD are more reactive *ex vivo* and require stronger forms of treatment in order to achieve ample inhibition [232]. It was

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proposed that platelets play a more important role in female CAD. We showed by this study that women presenting with unstable angina, in particular, have remarkably raised amounts of circulating platelet-monocyte as well as platelet-granulocyte complexes than men with the same syndrome, and also than women with stable angina. The same patient group also showed a trend towards higher levels of soluble P-selectin.

Platelets and leukocytes in complex are capable of activating one another. Even though an increased complex formation was seen in unstable women, levels of MCP-1 or IL-8 (data not shown) were not elevated, suggesting that the complexes did not result in any substantial monocyte chemokine secretion. P-selectin tethering to monocytes can also induce TF, as shown in paper II. This TF is probably shed and may appear in plasma as soluble TF. However, stable women displayed low amounts of complexes but high levels of soluble TF. Neither could any correlation be seen with monocyte surface TF, which was normal in all patients.

T-lymphocyte activation and proliferation has been demonstrated in recent studies of unstable coronary artery disease [181,233]. We confirm these results by the finding of increased numbers of CD4-positive T-cells expressing HLA-DR; in this study, however, in both patient groups. There was no difference between the groups regarding plasma levels of soluble IL-2Rα, either. We found higher levels of plasma IL-10 in unstable patients than in stable ones. This anti-inflammatory cytokine is secreted mainly by T_{H2}-cells, but also by monocytes/macrophages. In a way, it can thus be seen as an indicator of T-cell activation, which has arisen from antigen challenge or pro-inflammatory stimuli. IL-6 and CRP, both pro-inflammatory proteins, were also elevated in the unstable patients. However, there was no difference between men and women in inflammatory activity. Thus, inflammation does not seem to be the cause of increased soluble TF and platelet-leukocyte complexes. Both IL-6 and CRP have been reported to induce TF in monocytes [39,40,43]. We did not see any increase in surface TF, but both monocytes and granulocytes were activated in the unstable patients as indicated by increased surface CD11b expression. Monocytes may, at the time of sampling, already have shed or internalised their TF.

Increased levels of soluble fibrin, reflecting thrombin activity, indicated an ongoing activation of the coagulation cascade in patients with unstable CAD. Activation of coagulation may stem from several sources; plaque rupture is a likely candidate, however, one can not exclude the role of soluble TF or TF-containing microparticles shed from monocytes or endothelial cells. We failed to detect any raised levels of FVIIa in plasma - this could be explained either by local activation in the coronary vessels [170], or simply by the assumption that active FVII is consumed by binding to TF and subsequently inactivated.

In conclusion, there is an increased activation of blood cells, coagulation and inflammation in patients with unstable coronary artery disease, as compared to normal individuals as well as to stable angina patients. It is difficult to draw any conclusions regarding systemic effects of local events, or indeed the opposite. A more extensive

study would be required to elucidate more of the connections between coagulation, inflammation and cellular activation, something that again stresses the complexity of the pathology of coronary disorders. Albeit small, our study strongly indicates that the analytical patterns are different not only between stable and unstable disease, but also between men and women.

Interleukin-6 related to outcome in unstable CAD patients (paper V)

In 3269 patients of the FRISC II trial, IL-6 levels were measured in plasma samples taken at randomisation. The lower detection limit for the IMMULITE IL-6 assay was 5 ng/L, so this level was used as cut-off. 897 patients had plasma IL-6 concentrations above this cut-off, and 2372 patients had non-detectable levels, i.e. lower than 5 ng/L. IL-6 levels at randomisation were then analysed for association with outcome in the two main FRISC II study arms; the medical and the interventional trials (outlined in Figure x), where the impact of randomised treatment strategy was also evaluated. In the interventional cohort, patients were randomised that did not have contraindications to invasive therapy. The medically randomised cohort consisted of all patients not randomised to an early invasive strategy, i.e. those randomised in the interventional trial to receive a non-invasive treatment strategy and those with contraindications to invasive procedures.

The endpoints analysed were all-cause death and the composite of death or non-fatal myocardial infarction. The medical trial follow-up was 6 months, whereas the interventional trial had a follow-up of 12 months. The efficacy analyses of the follow-ups were point estimates, and included only patients with an adjudicated event or with recorded absence of the specific event until at least 170 days and 335 days of the respective follow-up period. All analyses were done according to intention-to-treat.

Interventional trial

As there was no influence of long-term dalteparin in the comparisons between the invasive and non-invasive groups, analyses of these cohorts were performed disregarding medical assignment.

For patients randomised to a non-invasive strategy, IL-6 levels higher than 5 ng/L at inclusion were associated with a 3,5-fold increase in probability of death at 12 months; 7,9% compared with 2,3% in patients with IL-6 levels less than 5 ng/L ($p<0,001$, Figure 18). In high-level patients, an early invasive strategy led to a 5,1% absolute or 65% relative reduction in 12-month mortality ($p<0,01$). At lower IL-6 levels, there was no significant difference in mortality between treatment strategies.

For the composite of death or myocardial infarction, elevated plasma IL-6 levels were not associated with any larger event proportion in the non-invasive group (risk ratio 1,25, 95% CI 0,92-1,68). Accordingly, an invasive strategy improved the endpoint outcome irrespective of IL-6 levels ($p<0,05$ for both).
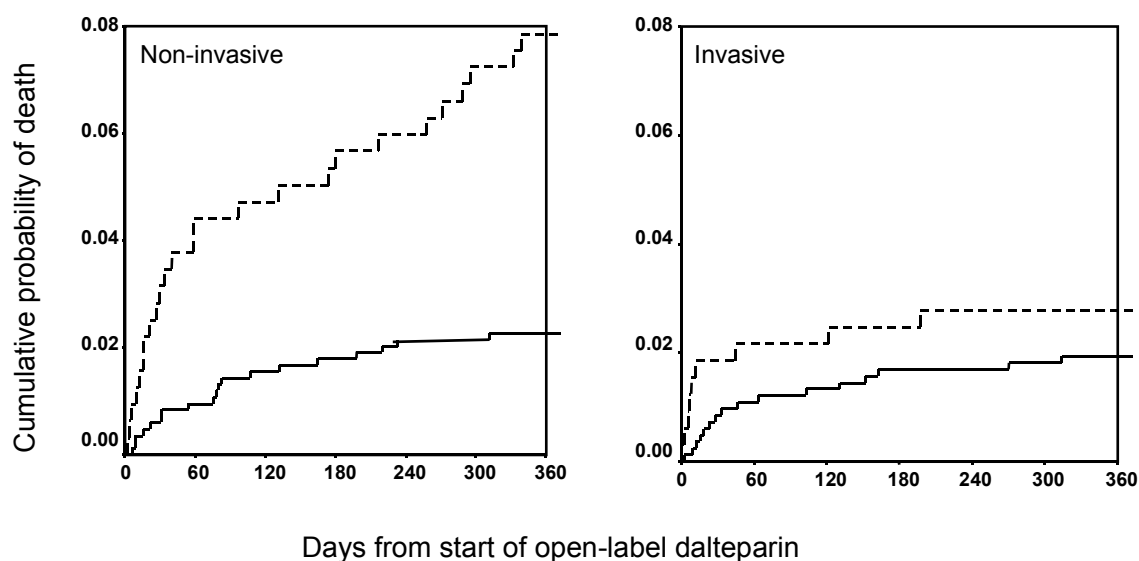
Figure 18. Mortality in the interventional study related to IL-6 levels. 12-month probability of death in the invasive and non-invasive cohorts subgrouped by randomised treatment strategy. Dashed line denotes plasma IL-6 levels >5ng/L, solid line denotes levels <5 ng/L.
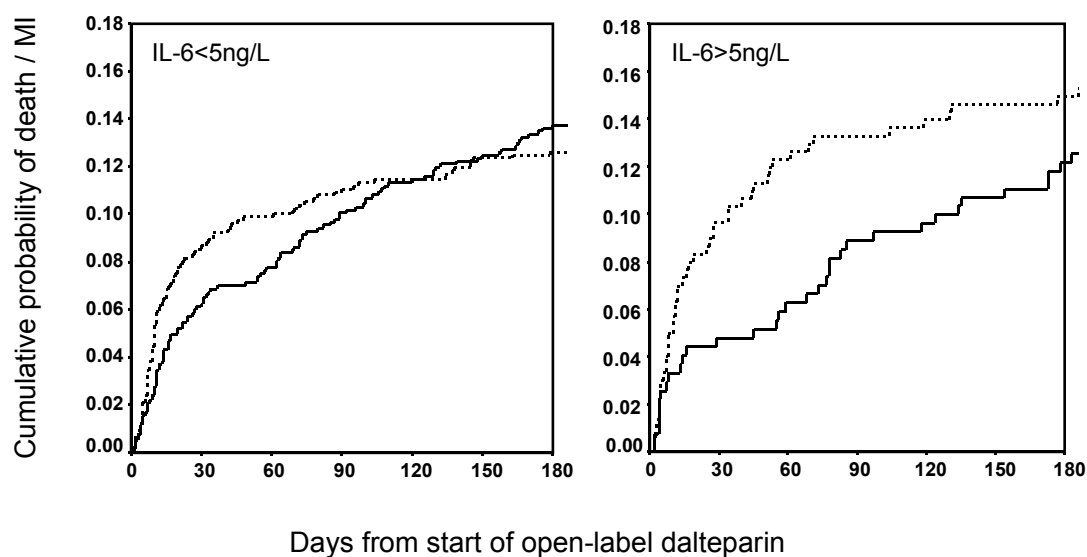

Figure 19. Death / MI related to medical assignment. 6-month probability of death or myocardial infarction in the medical study, subgrouped by IL-6 plasma levels, in relation to randomised treatment. Dotted line denotes placebo, solid line denotes dalteparin assignment.

Medical trial

In the non-invasive, placebo-treated group, patients with IL-6 levels of 5 ng/L or higher had a 6-month mortality of 7,9% versus 2,5% in patients with levels below the cut-off limit (*p*=0,001). At elevated IL-6 levels, assignment to dalteparin tended to reduce the risk to 4,4% (*p*=0,08). In patients with IL-6 lower than 5 ng/L, dalteparin did not influence 6-month mortality.

As for the combined end-point, higher plasma levels of IL-6 were not significantly associated with risk (RR 1,16; 95% CI 0,83-1,62 in the placebo group). Assignment to dalteparin did not reduce the composite of death or myocardial infarction at 6 months regardless of IL-6 levels. However, it significantly lowered the incidence of these events during the first 60 days of treatment in patients with elevated plasma IL-6 (*p*=0,01-0,04 at 30, 45 and 60 days), but not in those with lower levels (Figure 19).

Multivariable analysis

The independence of IL-6 as a predictor of mortality was assessed by forward, stepwise logistic regression analysis, for which established risk indicators as well as randomised treatments and interaction terms were evaluated. Only 7 covariates remained as independent predictors in the interventional trial and 5 in the medical trial. Plasma IL-6 levels remained significantly associated with increased mortality in both the interventional and the medical trial (Table 1).

Table 1

	Interventional study Death 12 months, n=2257			Medical study Death 6 months, n=2119		
Covariates	**Odds Ratio**	**95% CI**	**p-value**	**Odds Ratio**	**95% CI**	**p-value**
Age	1.07	1.0 - 1.1	<0.001	1.07	1.0 - 1.1	<0.001
Diabetes	4.07	2.4 - 6.9	<0.001	2.90	1.8 - 4.8	<0.001
Previous MI	1.83	1.1 - 3.1	0.03	1.79	1.1 - 2.9	0.02
ST-depression	1.89	1.1 - 3.3	0.02	--	--	--
ACE-inhibitors	3.15	1.9 - 5.3	<0.001	1.79	1.1 - 2.9	0.02
Invasive strategy	0.53	0.3 - 0.9	0.02	--	--	--
IL-6 > 5 ng/L	2.08	1.2 - 3.5	0.006	2.09	1.3 - 3.3	0.002

Covariates evaluated: age, gender, diabetes, hypertension, smoking, previous myocardial infarction, ST-depression at admission, use of statins, ACE-inhibitors and beta-blockers, cholesterol levels above 5.5 mmol/L, troponin-T levels above 0.1 µg/L, C-reactive protein levels above 10 mg/L at randomisation, IL-6 levels above 5 ng/L at randomisation, randomised treatment (invasive / non-invasive and dalteparin / placebo, respectively) and the interaction term IL-6 levels*randomised treatment.

Discussion

In this study, we showed that plasma levels of IL-6 is a powerful marker for identifying unstable CAD patients with increased risk of death in a 6 to 12-month perspective. IL-6 was independent of other risk indicators, including the biochemical markers troponin-T and C-reactive protein. We also demonstrated for the first time that elevated IL-6 levels independently identify patients whose risk of death would be considerably reduced by an early invasive approach. It thus seems that an invasive strategy is of choice in spite of the increased inflammatory activity in these patients. Assignment to prolonged treatment with subcutaneous dalteparin also tended to reduce the risk of death for patients with IL-6 levels above 5 ng/L. Furthermore, this subgroup of patients experienced a reduction in the composite endpoint during the first 60 days of dalteparin treatment, indicating that they could be protected also from myocardial infarction by LMW-heparin treatment while waiting for invasive treatment. This pattern was also seen in the main FRISC II medical study for patients with troponin-T levels above 0.1 μg/L [221].

Interestingly, IL-6 levels did not predict occurrence of the composite endpoint, which included non-fatal myocardial infarctions. The same phenomenon has been repeatedly noted for plasma levels of CRP. Considering that the majority of deaths within a year from a severe unstable episode are due to cardiac causes, it seems that elevated plasma levels of IL-6 reflect an inflammatory condition leading to a higher risk of fatal consequences of the index as well as of a subsequent myocardial infarction. Using stepwise logistic regression analysis, patients presenting with elevated IL-6 levels were characterised by a short angina history, no statin treatment on admission, low admission cholesterol levels, myocardial damage as indicated by raised troponin-T levels and inflammatory activity reflected by elevated levels of CRP. IL-6 levels above the cut-off were also more frequent with increased age. This rough approximation suggests that raised levels of IL-6 are more common in acute unstable conditions compared to a worsening of chronic CAD, and that both myocardial damage and general inflammation are involved. This leads to mind that this cytokine, present in the atheroma [207] and secreted by both endothelial cells, smooth muscle cells, macrophages and T-cells [189,207], may indicate a greater atherosclerotic burden and also an increased inflammatory activity in the plaques. As a result, these may be more vulnerable and prone to deeper fissuring, thus causing more severe thrombotic episodes leading to myocardial damage. The ischemic or necrotic myocardium could also be a source of cytokines [234-236]. The correlation between levels of troponin-T and IL-6 in this material was moderate but significant (Spearman's rho correlation coefficient=0.38, $p<0.001$). However, the predictive value of IL-6 was additive to but independent of troponin-T, which indicates that they stem from at least partly separate events.

One could also hypothesise that IL-6 reflects an ongoing low-grade inflammation, other than the atherosclerotic disease, and from there contributes to the progression of CAD. In two recent review articles, collected evidence and indices for a central role of IL-6 in the development of coronary heart disease are presented, taking into account the pleiotropicity of this cytokine and its wide range of actions, including effects on

platelets, endothelium, factors of metabolism and coagulation [237,238]. Likely, neither of the above explanations is exclusively correct - they may act alone or in combination with each other or with other factors depending on the individual.

The method of analysis that we used to measure plasma levels of IL-6 can be considered as rather insensitive. ELISA-based methods can detect levels of as little as 0.1 ng/L of IL-6, whereas we had a limit of detection already at 5 ng/L. Possibly, a more sensitive IL-6 assay method, allowing stratification into tertiles or quartiles, would have revealed more detailed prognostic information. We also relied on a single blood sample per patient, taken at varying time-points after the last episode of chest pain, which could be confounding given the relatively short half-life of IL-6 in plasma (4 hours). Still, while using 5 ng/L as a cut-off level, a pronounced difference in mortality was observed. As to the effect of sample time, only the latest sample time quartile had a slightly lower frequency of elevated levels. Thus, IL-6 adds important prognostic information, and this automated assay of IL-6 may provide a means for including IL-6 in a routine screening package for risk stratification of unstable CAD patients.

Reflections on the pathobiology of unstable CAD

Our results from papers IV and V further the understanding that atherosclerosis is indeed an inflammatory disease, a paradigm which is now increasingly accepted. IL-6 can be found in plaques; it is also detected at elevated levels in the plasma of patients with unstable CAD, and from there predicts a worse prognosis. As discussed above, IL-6 may be a cause or a consequence of the apparently more severe CAD of these patients. Study IV was unfortunately too small to examine the degree of correlation between cellular activation and plasma IL-6 levels, something which might contribute to a better understanding of the biochemical picture. In a report published in the same journal and issue as paper V, Zhang *et al* found a very strong correlation between blood levels and leukocyte content of myeloperoxidase (MPO) and prevalence of CAD [239]. The authors speculate that MPO may play a direct causal role in the development of atherosclerosis, since MPO is capable of LDL oxidation as well as reducing the bioavailability and function of nitric oxide, among other effects. Together with ours, these results indicate that in the circulation, more information about the atherosclerotic disease can be found than just markers of the local inflammatory process.

In conclusion, the connections between local coronary atherosclerosis and systemic effects, or systemic causes, remain incompletely understood, despite the great scientific progress in the area in recent years. Continued efforts to grasp the complex mechanisms underlying one of the major causes of death in the westernised world, with the concomitant discovery of new, potential therapeutic targets, may eventually bring us closer to controlling human biology for a life in good health.

SUMMARY

The studies presented in this thesis have shown that

♦ The whole blood experimental system has different properties than cell culture models. This can be understood also from the differences in kinetics and dose-response in LPS-stimulated monocyte TF induction. The whole blood model provides a valuable complement to studies on isolated leukocytes.

♦ IL-10 is a potent inhibitor of LPS-induced TF in whole blood monocytes, whereas IL-4 and IL-13 are not, contrary to *in vitro* findings. IL-10 inhibits TF activity to a larger extent than it does TF antigen expression.

♦ Activated platelets induce a rapid, mRNA-independent TF expression by monocytes in whole blood, indicating a latent storage pool of TF in these cells. The induction is largely dependent on P-selectin, and to some extent on CD40L on platelets.

♦ Differentiated monocytic cells, U-937, do not display the same kinetics of activated platelet-induced TF as blood monocytes, nor do they respond to the platelet form of CD40L.

♦ In co-culture with activated human coronary artery endothelial cells expressing increased amounts of ICAM-1, U-937 are induced to TF- and IL-6 expression, and the endothelial cells up-regulate their production of IL-10. In this system, TF expression is strongly suppressed by a statin, an ACE-inhibitor and a low-molecular-weight heparin. Cytokine expression in co-cultures is not affected by the drugs.

♦ In unstable CAD, there is an activation of both coagulation and inflammation compared to stable CAD that coincides with an increased activation of platelets and leukocytes. Cellular interactions may contribute to the systemic responses observed. Women have different patterns of cellular activation than men, indicating possible differences in pathogenetic mechanisms.

♦ A plasma level of IL-6 above 5 ng/L is a strong, independent marker for increased risk of death in a 6-12 month perspective in patients with unstable CAD. This risk is significantly reduced by an early invasive strategy. Elevated IL-6 levels also identifies patients that may benefit from prolonged treatment with LMW-heparin, e.g., while awaiting revascularisation.

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Addendum / Errata

Leukocytes and Coronary Artery Disease

by Eva Lindmark

Typing mistakes and other minor errors are not included in the list!

Page	Line	Comment
4	paper V	The full title should read 'Relationship between interleukin 6 and mortality in patients with unstable coronary artery disease. Effects of an early invasive or noninvasive strategy.'
5	2	Page number for 'Introduction' should be 8.
30	4 and 5	5 μM of simvastatin and 20 μg/mL of enalapril
33	19	The sentence beginning with 'The characteristics…' should be omitted.
52	14	Figure x = Figure 7
53	Fig 18	The figure legend should end with '<5 ng/L'.
72	Add	238. Yudkin J S, Kumari M, Humphries S E, and Mohamed-Ali V. Inflammation, obesity, stress and coronary heart disease: is interleukin-6 the link? *Atherosclerosis*. 2000(148);2:209-214. 239. Zhang R, Brennan M, Fu X, Aviles R J, Pearce G L, Topol E J, Sprecher D L and Hazen S L. Association between myeloperoxidase levels and risk of coronary artery disease. *JAMA* 2001(286);17:2136-2142.