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I. Introduction

I.1 Tissue Factor – the principal initiator of coagulation

The type I membrane protein tissue factor (TF) is the major cellular initiator of the clotting process and its primary role is to maintain hemostasis. In contrast to the TF initiated extrinsic pathway of coagulation the factor XII-mediated intrinsic pathway was not believed to play an important role for coagulation. However, factor XII-mediated fibrin formation was recently found to be essential for the formation and stabilization of platelet-rich occlusive thrombi in vivo (Renne T, 2005).

In TF initiated coagulation (extrinsic pathway of coagulation) the zymogen plasma factor VII (VII) binds to its cofactor TF, which is expressed on the cell surface. Factor VII subsequently undergoes proteolytic activation by VIIa, IXa, Xa and thrombin, which are present in trace amounts in the circulation. The TF/VIIa complex is formed. This initiator complex of coagulation cleaves and thereby activates the zymogens plasma factors X and IX (belonging to the intrinsic pathway of coagulation) by limited proteolysis. The serine proteases Xa and IXa participate in a series of membrane dependent proteolytic reactions leading to thrombin generation, fibrin deposition, and clot formation (Fig.1). IXa binds to its cofactor VIIIa on the negatively charged cell surface of the activated platelets and activates X. This membrane bound complex is called Xase complex. Xa assembles with its cofactor Va to form a complex together with negatively charged phospholipids, such as phosphatidylserine (PS) on the membrane of activated platelets that converts prothrombin to thrombin. Thrombin is the central serine protease of the coagulation network, which in turn cleaves soluble fibrinogen, forming an insoluble fibrin polymer or clot. It also impairs coagulation by activating factors V, VIII and XI, and moreover is a strong platelet agonist.

The transaminase factor XIII, which is crucial for the stabilization of fibrin polymers, also undergoes proteolytical activation by thrombin. Although association of VIIa and TF is greatly enhanced in the presence of calcium ions and negatively charged phospholipids forming a complex with the γ-carboxyglutamic acid residues of the protease domain of VIIa, neither factor is absolutely essential for the interaction (Sabharwal AK, 1995; Ruf W, 1991). Although the low amidolytic activity of VIIa is enhanced up to 100-fold in the presence of TF (Higashi S, 1992), membrane anchoring is not essential for this to occur (Ruf W and Kalnik MW, 1991). In contrast, the activation of X and IX is highly dependent on membrane anchoring (Neuenschwander PF, 1993), and is supported by negatively charged phospholipids (Edgington TS, 1991; Krishnaswamy S, 1992; Fiore MM, 1994).

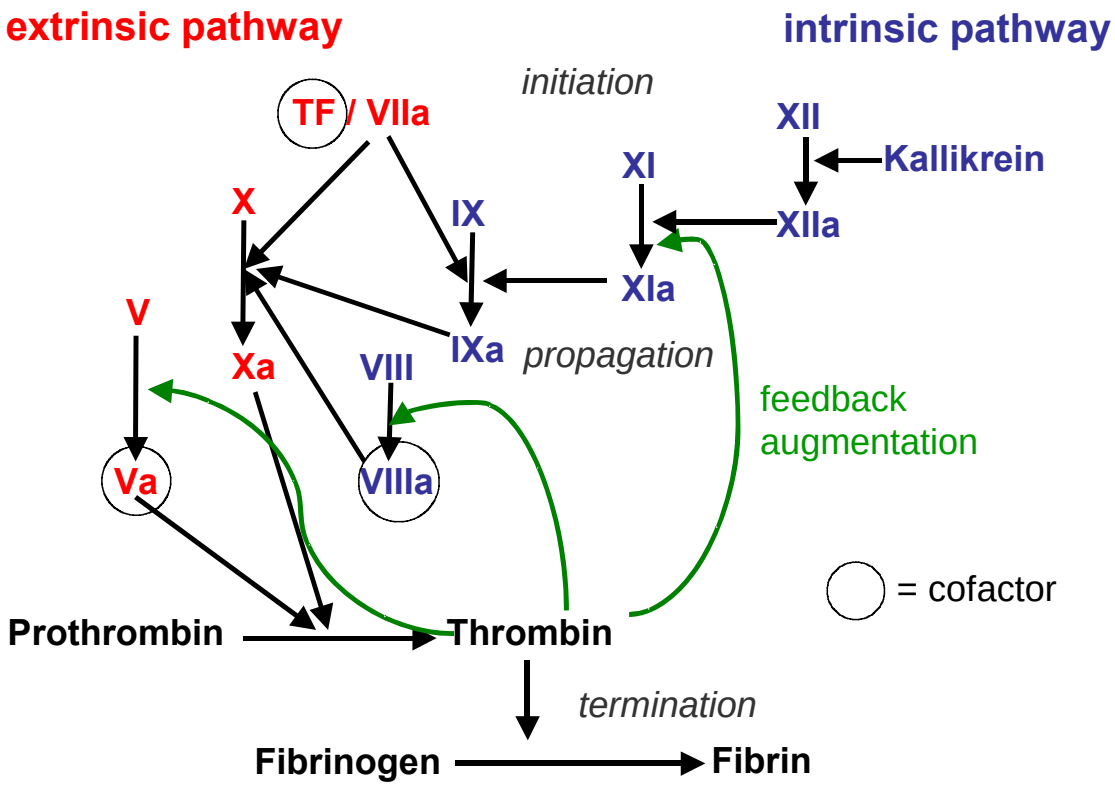

Fig.1: Schematic view of the coagulation network subdivided in an initiation phase, a propagation phase and a termination phase.

I.2 Regulation of blood coagulation

Blood coagulation is tightly regulated to generate a local fibrin clot at the site of vascular injury without compromising blood flow inside the vasculature. To achieve this, a complex network of positive and negative feedback reactions have evolved that result in controlled fibrin deposition and platelet activation only at the site of vascular injury (Gomez K, 2006). To fulfil this role TF is expressed constitutively in subendothelial tissues (vascular smooth muscle cells and fibroblasts), thereby protecting the vertebrate organism from infection and lethal blood loss in case of injury. During evolution several mechanisms have evolved regulating the initiation, propagation and termination phases of coagulation inside the vasculature and restricting coagulation to the site of injury.

The initiation phase of coagulation is regulated by the trivalent Kunitz-type inhibitor Tissue Factor Pathway Inhibitor-1 (TFPI) (Fig.3). TFPI is forming a quarternary high affinity complex with TF, VII / VIIa and X / Xa (Dickinson CD, 1997). This inhibitory complex prevents the diffusion of Xa into the prothrombinase complex and at the same time inhibits VIIa. Thus thrombin, the central protease of the coagulation network, cannot be generated.

The propagation phase of the coagulation cascade is controlled by serpins, such as antithrombin III, heparin cofactor II and by the anticoagulant protein C pathway. Serpins inhibit the activated plasmatic coagulation factors (such as Xa, IXa and thrombin) irreversibly by covalent binding to their active site serine. Activated Protein C cleaves and thereby inactivates the coagulation cofactors VIIIa and Va (cofactors in the activation of X and prothrombin) resulting in the down regulation of the activity of the coagulation system (Dahlbäck B, 2005).

The termination phase is regulated by the plasmin-dependent fibrinolysis pathway and its inhibitors, the serpin antiplasmin and the thrombin activatable fibrinolysis inhibitor (TAFI), which protects the fibrin clot against lysis (Mosnier LO, 2006).

I.3 The structural biology of TF

The transmembrane protease receptor TF is a 47 kDa glycoprotein receptor and it is a member of the class 2 cytokine receptor family. TF is most closely related to the interferon-α, interferon-γ and IL-10 receptors (Bazan, 1990). The extracellular part of TF is constituted by the tandem association of two fibronectin type III-like modules (residues 1-209) and a flexible peptidyl strand (residues 210-220) that tethers the domain to the transmembrane anchor. The transmembrane segment consists of 23 amino acids and is followed by a short cytoplasmatic tail of 21 amino acids (Edgington, 1991). Each fibronectin type III-domain of the extracellular part is formed by two antiparallel β-sheets with immunoglobulin superfamily (IgSF)-type C2 topology (Stuart, 1995) as found in other cell surface receptors, such as in both domains of the human growth hormone receptor, in domain 2 of the cell adhesion molecule CD2 and in domains 2 and 4 of

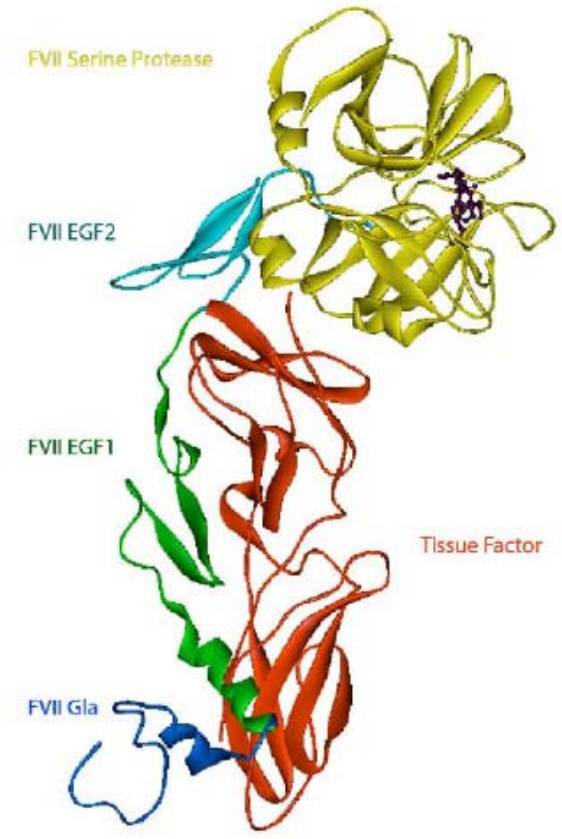

Fig.2: WebLab ViewerLite ribbon representation of the x-ray structure of the extracellular part of TF in complex with VIIa (Banner DW, 1996). TF is shown in red. The VIIa Gla domain is dark blue, VIIa EGF 1 domain in green, VIIa EGF 2 domain is light blue and the VIIa serine protease domain is yellow. The active site inhibitor is represented by ball and stick.

CD4. TF contains two disulfide bridges at positions 49-57 and 186-209 and one cytoplasmatic half-cysteine at position 245 that is acylated by palmitic acid or stearic acid (Bach RR, 1988). Human TF contains N-linked glycosylation sites at Asn 11, Asn 124 and Asn 137. In contrast to the four helix bundle ligands of the interferon and IL-10 receptor, TF binds the multidomain serine protease factor VII / VIIa with subnanomolar affinity and acts as a cofactor (Fig.2). Cell surface protease cascades are triggered by the regulation of protease receptors, such as the urokinase receptor of the fibrinolytic system (Ellis V, 1992) and TF (Ruf W, 1994).

1.4 Tissue Factor Pathway Inhibitor-1 – the physiologic inhibitor of the coagulation start

The 45 kDa glycoprotein TFPI is an important coagulation inhibitor, since it prevents the initiation phase by forming a quarternary high affinity complex with TF / VIIa and Xa. TFPI consists of a negatively charged N-terminus followed by three modules of Kunitz domains and a positively charged C-terminus (Fig.3). Mechanistically, TFPI first binds trace amounts of Xa (K_i = 4.4 nM) (Hackeng TM, 2006) and TFPI / Xa subsequently binds to the initiator complex TF / VIIa. Xa is bound by Kunitz domain 2 of TFPI and VIIa binds to Kunitz domain 1. Kunitz domain 3 is essential for the binding to cell surface proteoglycans and to lipoproteins.

TFPI was found to be expressed by endothelial cells of the microvasculature, megakaryocytes, platelets, monocytes and macrophages (Werling RW, 1993; Van der Logt, 1994). Inside the vasculature there are three different pools of TFPI that differ significantly in their structure and in their inhibitory activity (Broze, 1994). About 85% of the total TFPI amount is tightly bound to heparan sulfate-containing proteoglycans at the surface of

endothelial cells. 10% of the total TFPI circulates in plasma and is bound to lipoproteins (Sanders NL, 1985; Broze G, 1987). This fraction is mostly truncated and therefore consists of peptide fragments of different molecular weight (34-45 kDa). It is functionally inactive (Hansen JB, 1997). The heterogeneity results from C-terminal truncated forms at Kunitz domain 3 and from the formation of disulfide complexes with apolipoprotein A II (apo A II) (Novotny WF, 1989; Broze GJ, 1994). About 8% of the total TFPI amount is stored in platelet α-granules and is released after platelet activation with platelet agonists, such as thrombin or collagen (Novotny WF, 1989; Muller I, 2003). This fraction has an apparent molecular weight of 45-47 kDa and shows maximal inhibitory activity.

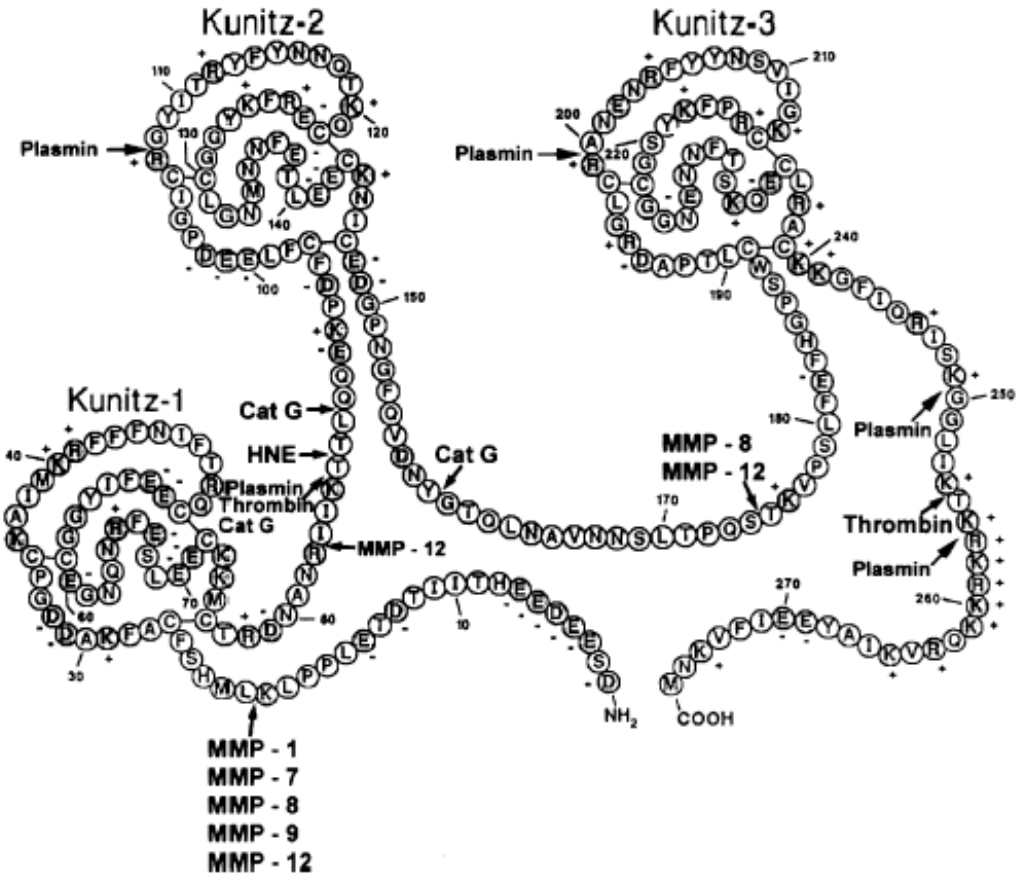

Fig.3: Sequence and modular architecture of TFPI-1. Proteolytic cleavage sites of thrombin, plasmin, cathepsin G (Cat G), neutrophil elastase (HNE) and matrix-metalloproteinases (MMP) (Cunningham AC, 2002).

I.5 Proteolytic cleavage of TFPI

In inflammation, wound healing and during infection stimulated human polymorphonuclear neutrophils (PMN) and, to a lower extent also human peripheral blood monocytes, release cationic serine proteases (neutrophil elastase (NE), proteinase 3 and cathepsin G (Cat G)) and matrix-metalloproteinases (MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-10, MMP-11) from their azurophilic granules that are capable of binding to the cell membrane of neutrophils (Campbell EJ, 1989; Owen CA, 1995; 1997; 1999). It was found that cell surface-bound NE is catalytically active and is resistant to inhibition by naturally occurring protease inhibitors, such as the serpin α_1-proteinase inhibitor (Owen CA, 1995).

In vitro studies have shown that the connecting regions between the Kunitz domains as well as the acidic N-terminal and basic C-terminal regions of recombinant human TFPI are very susceptible to limited proteolytic decomposition by NE, Cat G (Petersen LC, 1992; Higuchi DA, 1992) and MMPs (Belaaouaj AA, 2000; Cunningham AC, 2002). The degradation of TFPI by Cat G was found to be significantly slower than cleavage by NE (Higuchi DA, 1992). Serine proteases that are part of the coagulation cascade, such as thrombin (Ohkura N, 1997), factor Xa (Salemink I, 1998) and plasmin (Li A, 1998) also cause limited proteolysis of TFPI (cleavage sites Fig. 3). It was also found that the anticoagulant activity of TFPI was greatly reduced by limited proteolysis. Therefore, proteolytic inactivation of TFPI could be a mechanism capable of generating local procoagulant environments. These findings may represent a regulatory link between innate immunity and the coagulation start, since induction of coagulation accompanies the inflammatory response to a multitude of stimuli (Esmon CT, 2004; Opal SM, 2003). It is not yet established which proteases are responsible for TFPI decomposition in blood and if this degradation also occurs in the cellular context. It is also unclear whether this mechanism is relevant for the initiation of coagulation in vivo.

I.6 The procoagulant platelet-neutrophil microenvironment

Under in vivo conditions, platelets are rapidly assembled with different types of leukocytes in the developing thrombus (McEver RP, 2001). The initial contact corresponding to the tethering of platelets on the surface of neutrophils and monocytes is mainly mediated by the interaction of platelet P-selectin with PSGL-1, which is constitutively expressed by leukocytes. Therefore, only platelets have to be activated to be able to adhere to neutrophils and monocytes. Collagen-stimulated platelets were shown to express functionally active TF on their surface within minutes (Zillmann A, 2001) and they are known to express high amounts of anticoagulant TFPI (Novotny WF, 1988; Muller I, 2003). Neutrophils and monocytes were found to be essential as stimulators of the functional activity of TF associated with activated platelets and microvesicles (Muller I, 2003). Adhesion of platelets to neutrophils and monocytes has been proposed to result in the formation of a restricted microenvironment (Evangelista V, 1991). Inside this microenvironment (Fig.4) the access of plasma components, such as protease inhibitors, is restricted and therefore leukocyte derived proteases might inactivate the anticoagulant platelet TFPI and initiate TF procoagulant activity on the platelet surface (Engelmann B, 2003).

In addition, the formation of such platelet-neutrophil conjugates was found to be associated with various lethal disease states that are closely linked to inflammatory disorders. Among them are sepsis (Gawaz M, 1995), unstable angina (Ott I, 1997) and acute myocardial infarction (Michelson AD, 2001).

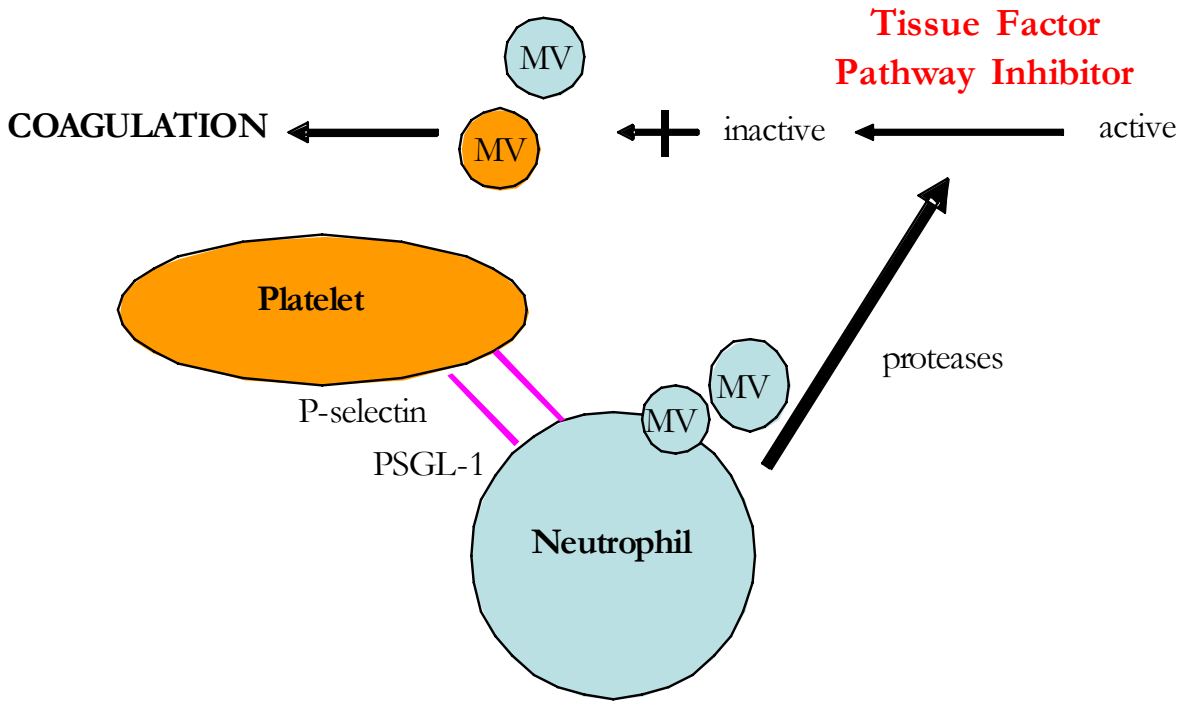

Fig.4: Cellular model for the intravascular tissue factor pathway modified according to Engelmann et al., 2003. Platelet activation leads to the exposure of TF on the cell surface, whereby the formation of the initiator complex of coagulation is enabled. Concomitantly, TFPI is released from the platelet α-granules and inhibits the initiator complex. Due to the concomitant presentation / activation of platelet adhesion molecules (P-selectin) platelets are enabled to interact with neutrophils via P-selectin / PSGL-1-interactions. Circulating and acutely shedded microvesicles (MV) are recruited to the platelet-neutrophil conjugates. Secreted neutrophil proteases could inactivate TFPI. Thereby, the functional activity of the TF associated with platelets and microvesicles might be enhanced.

I.7 Cellular microparticles

Microparticles (microvesicles) are small membrane vesicles (< 1μm in diameter) that are released from the plasma membrane of cells upon activation (Wiedmer T, 1991), during apoptosis (Aupeix K, 1997) and by shear stress (Reininger AJ, 2006). They constitute a heterogeneous population, differing in cellular origin, numbers, size, antigenic composition and functional properties. Microparticles are described to play a role in intercellular communication, immunity and coagulation (Hugel B, 2005). Microparticles support coagulation by the exposure of negatively charged phospholipids (PS) that are essential for thrombin generation and in the case of monocyte- and platelet-derived microparticles also by the exposure of TF (Muller I, 2003). Under physiologic conditions, about 80% of the plasma microparticles are derived from platelets (Berckmans RJ, 2001). The presence of microparticles has also been documented at sites of inflammation, such as the acellular lipid core of the atherosclerotic plaque (Mallat Z, 1999). Furthermore, increased numbers of circulating microparticles have been reported in patients with acute coronary syndromes (Mallat Z, 2000).

I.8 Tissue specific expression pattern of TF

TF is expressed in many tissues and it exhibits a distinct, nonuniform tissue specific pattern of expression. High levels of TF are detected in highly vascularized organs, such as the lung, brain and placenta (Fleck RA, 1990). Intermediate levels are found in the heart, kidney, intestine, testes and uterus. In contrast, low levels of TF are observed in the liver, spleen, skeletal muscle, and thymus. The cell types that express TF in these organs include

cardiomyocytes in the heart, bronchiolar and alveolar epithelial cells in the lung, astrocytes in the brain, and trophoblasts in the placenta (Eddleston M, 1993; Erlich J, 1999; Pawlinski R, 2002). The constitutive expression of TF in various tissues, such as the vasculature of the heart, may reflect a need for additional hemostatic protection in these tissues. In contrast, nonvital tissues that express low levels of TF, such as skeletal muscle, do not require additional hemostatic protection. These tissues appear to rely more on the intrinsic pathway of coagulation to maintain hemostasis. TF is constitutively expressed in the vascular wall, such as by fibroblasts of the adventitia and by smooth muscle cells of the media of arteries and veins. Endothelial cells probably do not express TF under physiologic conditions (Østerud B, 2006). This findings led Drake and coworkers to propose the popular concept of TF acting as a hemostatic "envelope" encapsulating the vascular bed. Rupture of the integrity of the envelope would trigger the clotting process instantly (Drake TA, 1989).

1.9 Intravascular TF

In recent years the envelope paradigm of TF expression and function has been challenged by the demonstration of intravascular TF (blood-borne or circulating TF) (Giesen PLA, 1999; Zillmann A, 2001; Muller I, 2003; Engelmann B, 2003). Induced expression of TF in cells within the vasculature is implicated in the pathogenesis of thrombosis in atherosclerosis, disseminated intravascular coagulation, malignancy and hyperacute rejection of xenografts (Wilcox JN, 1989; Levi M, 1999; Rickles FR, 2001; Robson SC, 1999) and it has been proposed that intravascular TF contributes to the propagation of the growing thrombus (Giesen PLA, 1999).

TF de novo-synthesis in monocytes was first reported in 1975 by Rivers and coworkers observing procoagulant activity in endotoxin-stimulated leukocyte suspensions (Rivers RP, 1975). TF expression on monocytes can be achieved by specific inflammatory stimuli, such as endotoxin (e.g. lipopolysaccharide (LPS)) (Gregory SA, 1989), phorbol esters (Lyberg T, 1981), C-reactive protein (Cermak J, 1993) and proinflammatory mediators, like tumor necrosis factor-α (TNF-α) (Conkling PR, 1988) and interleukin 1-β (IL-1β) (Herbert JM, 1992). Interestingly, platelets were found to regulate monocyte TF activity. In 1974, Niemetz and Marcus (Niemetz J, 1974) proposed that platelets enhance the procoagulant activity of white blood cells. This was also confirmed in monocyte cell cultures, in which isolated platelets added to monocytes enhanced LPS-induced TF activity (Lorenzet R,1986). Increased expression levels of monocyte TF might play a role in sepsis (Drake TA, 1993; Lupu C, 2005) and it was found that patients with unstable and stable coronary syndromes exhibit elevated levels of TF expression on circulating monocytes (Leatham EW, 1995).

In rapidly processed blood (to avoid the activation of TF gene transcription), TF was barely noticeable in neutrophils by TF-specific ELISA measurements and no TF procoagulant activity could be detected (Muller I, 2003). This observation is in accordance with the findings of Østerud and coworkers, who failed to detect TF antigen on neutrophils in stimulated whole blood (Østerud B, 2000). However, there is emerging evidence that neutrophils might be able to express TF under certain inflammatory conditions (Maugeri N, 2006; Ritis K, 2006).

Blood eosinophils were found to store TF, which is mainly embodied in their specific granules and exposed on their cell membrane after cell activation (Moosbauer C, 2006). Eosinophils are the cells with the highest TF content in blood under resting conditions. They contain approximately one forth of the TF molecules compared to fully activated

monocytes. The observations indicate TF as one of the critical mediators of the initial eosinophil migration across the activated endothelium (Moosbauer C, 2006).

There is strong evidence that platelets contain preformed TF, which is released within 5 minutes after collagen type I stimulation. Platelet TF contributes to the collagen-triggered activation of blood coagulation (Zillmann A, 2001). Immunoelectron microscopy showed TF antigen localized in the α-granules and the open canalicular system of resting platelets. The ability of activated platelets to trigger the initiation of coagulation was low. This suggests that platelet TF is cryptic (Maynard JR, 1975). One reason for the low TF procoagulant activity on activated platelets could be the concomitant release of TFPI, the physiologic inhibitor of the initiator complex of coagulation (Novotny WF, 1988). The presence of TF in platelets was confirmed by several authors (Camera M, 2003; Engelmann B, 2006). It is still a matter of debate whether TF is transported to platelets by leukocyte-derived microparticles (Del Conde I, 2005) and / or if the spliceosome of proplatelets that extend from megakaryocytes might potentially be capable of translating TF from pre-mRNAs (Denis MM, 2005; Schwertz H, 2006).

Microparticles support coagulation by exposure of negatively charged phospholipids that are essential for thrombin generation. In the case of monocyte- and platelet-derived microparticles their main, and probably central procoagulant function is the exposure of TF (Muller I, 2003). Under physiologic conditions, 80% of the plasma microparticles are derived from platelets (Berckmans RJ, 2001). TF was detected on platelet-derived microparticles and in vitro generated platelet microparticles (Muller I, 2003). Apparently, the filopodia of activated platelets are the preferential sites for the formation of TF-positive microparticles (Leon C, 2004). In vitro generated monocyte-derived microparticles (Satta N, 1994) and circulating monocyte-derived microparticles (Falati S, 2003) were shown to expose TF on their membrane. Circulating monocyte microparticles are able to adhere to activated endothelial cells and to activated platelets by P-selectin / PSGL-1 interactions and

interestingly they were found to play a significant role in fibrin stabilization of the nascent thrombus by the delivery of procoagulant TF.

A substantial part of total soluble TF in plasma has been suggested to be constituted by an alternatively spliced human TF (Bogdanov VY, 2003). However, the procoagulant activity of soluble TF is rather low compared to full-length TF.

I.10 The encrypted or latent state of TF

TF encryption has been suggested as the post-translational suppression of TF procoagulant activity on the cell surface (Bach RR, 2006). The discrepancy between TF antigen and the expression of TF procoagulant activity has previously been observed in a variety of cell types (Maynard JR, 1977; Walsh JD, 1991; Drake TA, 1989). A stimulus is required to uncover the latent proteolytic activity of the encrypted TF-VIIa complex (Bach RR, 1990). Until now several mechanisms were proposed to activate the encrypted TF: freezing and thawing, sonication, protease treatment, phospholipase treatment, non-ionic detergents, apoptosis, complement, and Ca^{2+}-ionophores (Bach RR, 1996; 2006). There is a significant variation among these methods with respect to the level of TF procoagulant activity evoked as well as to secondary effects on cell structure.

The nature of TF de-encryption is unclear. One mechanism leading to de-encryption of latent TF is the treatment of cells with Ca^{2+}-ionophores. This leads to an increase in cytosolic Ca^{2+} which in turn causes a disruption of PS asymmetry. PS is no longer sequestered on the inner leaflet of the plasma membrane. This does not necessarily mean that TF de-encryption is coupled to PS exposure, but it has been known for a long time that PS accelerates coagulation reactions on membrane surfaces (Lentz BR, 2003). However, Wolberg and coworkers discovered that Ca^{2+}-ionophore treatment of cells induces changes

in TF procoagulant activity that could not fully be reduced to the basal level by saturating concentrations of the PS-binding protein annexin V. This indicates that the increase in TF activity after ionophore treatment does not solely result from increased PS exposure (Wolberg AS, 1999).

It has been reported that after treatment of human pericytes with Ca^{2+}-ionophore the TF procoagulant activity increased but the prothrombinase complex assembly and function were not affected. Therefore it is reasonable to assume that Ca^{2+}-ionophore treatment does not only result in membrane alterations, but may actuate intracellular processes that lead to covalent modifications, dimerization, and/or conformational changes in the TF molecule to increase its cofactor activity (Bouchard BA, 1997). Bach and Moldow suggested a mechanism for the Ca^{2+}-ionophore-induced TF de-encryption resulting in a change in TF quarternary structure (Bach RR and Moldow CF, 1997). They propose that during de-encryption of TF by Ca^{2+}-ionophore inactive TF dimers are converted to procoagulant TF monomers. This model runs counter to a well established dogma. Self-association usually results in the activation of cell surface receptors. It also was demonstrated that TF dimerization does not inhibit TF procoagulant activity, which contradicts the model proposed by Bach and Moldow (Donate F, 2000).

Another model of TF de-encryption is based on the association of TF with distinct lateral membrane domains. It was recently demonstrated that palmitoylation of cytoplasmic cysteines can target integral membrane proteins to lipid rafts (Zacharias DA, 2002). TF is such a palmitoylated integral membrane protein. Disruption of lipid rafts by methyl-β-cyclodextrin extraction results in an increase in the basal expression of TF procoagulant activity (Dietzen DJ, 2004). However, the meaning of this finding is unclear because there is increasing evidence that cholesterol extraction impairs a cells' ability to expose PS (Kunzelmann-Marche C, 2002).

I.11 Potential role of disulfide switching in human TF

Increasing evidence indicates that disulfide bonds do not only play an important role in stabilizing the tertiary structure of proteins, but also control protein function (Haworth NL, 2006). The cofactor function of TF could be regulated by a labile disulfide regulating its cofactor function for VIIa and possibly its receptor function for X. In a site directed mutagenesis study it was shown that the disulfide bridge at position 186-209 of TF ($TF_{186S/209S}$) is required to maintain the cofactor function of TF for VIIa. Serine substitutions of the cysteines at position 49 and 57 ($TF_{49S/57S}$) resulted in a TF protein that was able to initiate coagulation as efficiently as the wild type molecule (Rehemtulla, 1991). Harlos and coworkers described a disulfide bond at position 186-209 that is characteristic of the class 2 cytokine receptor family. This disulfide lies at the very end of the membrane proximal fibronectin type III domain and links adjacent strands F and G. The sulphur atoms of this disulfide bond point away from the surface of the β-sheet towards the solvent. This is unusual, because in most β-sandwiches of IgSF domains the disulfide bonds point to the internal of the fold (Harlos K, 1994). Such unusual disulfides linking strands in the same β-sheet are named cross-strand disulfides (CSD) (Fig.5). This geometry of CSD results in a high torsional energy across the bond (Hogg PJ, 2003). The more strain there is on a disulfide bond the more readily it is cleaved. Disulfide bonds that straddle strands in the same β-sheet are relatively rare in proteins. Hogg and Wouters found such

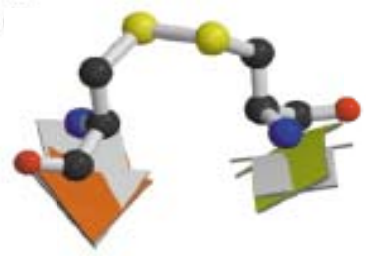

Fig.5: The disulfide linking C186/C209 of TF is accomplished by distorting the β-sheet so that the strands are tilted towards each other. The distortion imparts a high torsional energy on the bond. (MOLSCRIPT and Raster 3D software)

CSD in mammalian cell surface receptors (e.g. TF, thrombomodulin, growth hormone receptor, erythropoietin receptor, interferon-γ receptor and interleukin receptors). These authors propose that the function of some of these proteins might be controlled by cleavage of their cross-strand disulfide bond. They suggest that CSD can be reduced or oxidized reversibly by cellular oxidoreductases (e.g. protein disulfide isomerase, thioredoxin, glutaredoxin). Mechanistically the action of these enzymes is characterized by a thiol-disulfide exchange reaction. This was successfully established for the CD4 receptor on CEM-T4 cells (a thymocyte-derived cell line) (Matthias LJ, 2003). However, regulation of the TF procoagulant activity by a reversible, oxidoreductase-mediated cleavage of the CSD at position 186-209 has not yet been thoroughly investigated. Regulation of TF by such a disulfide switch might be of major interest in nearly all branches of clinical medicine, since many pathologies are related to coagulation disorders and venous and arterial thrombosis are the leading causes of mortality in industrialized countries.

I.12 Aims of the investigation

Increasing evidence indicates an important role of intravascular TF in the pathogenesis of lethal diseases, such as disseminated intravascular coagulation (DIC), arterial and venous thrombosis, acute myocardial infarction (AMI) and stroke. Blood-borne TF was detected on stimulated monocytes, activated platelets and their microparticles. Most of the TF molecules present on blood cells however, are not functionally active (encrypted or latent state of TF). Therefore it is of major interest to characterize on a molecular level why TF is cryptic and to reveal the underlying mechanisms that activate TF.

In the present study the following central questions were addressed:

1. Do isolated platelets exhibit TF procoagulant activity after activation?

2. Is the initiation of intravascular coagulation triggered by the proteolytic decomposition of platelet TFPI by neutrophil surface proteases?

3. Is there an intramolecular disulfide switch in the TF molecule triggering its procoagulant activity?

4. Which oxidoreductases are capable of regulating this thiol-disulfide exchange?

II. Materials and Methods

II.1 Materials

II.1.1 Instruments

Items	Companies, Type
Bacterial Incubator	Heraeus B 6200
Bacterial shaker	New Brunswick Scientific innova 4330
Cell culture incubator	Köttermann
Cell culture microscope	Carl Zeiss
Culture Hood	Heraeus, LaminAir, HLB 2472
Developing machine	AGFA
Electrophoresis power supply EPS600	Pharmacia
Electrophoresis unit, small	Bio-Rad Mini-Protean II
ELISA Reader Dynatech MR 7000	Dynatech Laboratories
Horizontal electrophoresis gel	MWG Biotech
Mega centrifuge	Heraeus, Omnifuge 2.0 RS
Midi-MACS LS column	Miltenyi Biotec
Mini-MACS column	Miltenyi Biotec
Mini MACS cell separator	Miltenyi Biotec
Miniprotean 3 gel cast	Bio-Rad
Multiphor II blotting device	Amersham Pharmacia Biotech
pH meter	HANNA instruments HI 221
Photometer	Tecan RainBow
Rotina 35 R	Hettich
Shakers	Heidolph, Unimax 2010, Edmund Bühler
Scale	BP2100S, BP310S, Sartorius
Thermocycler	Biozyme, MiniCycler^{TM}, MJ Research
Thrombelastograph RoTEG	Dynabyte
Table centrifuge mikro 22 R	Hettich
Ultra centrifuge	Beckman, L8-60M
Vortex	Scientificc Industries Genie-2
Water bath	GFL 1083, Amersham-Buchler

II.1.2 Reagents, pharmaceuticals and general material

Items	Companies
1kb DNA ladder, ready load	Invitrogen
100 bp DNA ladder	New England Biolabs
Acetic acid	Roth
Acrylamide (30%) / Bisacrylamide (0.8 %)	Roth

Agarose, ultra pure	Life Technologies, Inc.
Ammonium peroxydisulfate (APS)	Sigma
Beriplex P/N 500	Aventis Behring
Bovine serum albumin (BSA)	Sigma
Bradford Reagent	Bio-Rad
Bromphenolblue	Sigma
n-Buthanol	Roth
Calciumchloride, $CaCl_2 \cdot 2H_2O$	Sigma
Dextran solution, 25%	Sigma
Dimethylsulfoxide (DMSO)	Sigma
Disodiumhydrogenphosphate, Na_2HPO_4	Merck
Dithiothreitol (DTT)	Sigma
dNTP mix	Roche
ECL Western blotting detection reagents	Amersham Biosciences
Elastase Inhibitor III, MeOSuc-Ala-Ala-Pro-Val-CMK	Calbiochem
Ethanol	Roth
Ethanolamine	Sigma
Ethylenediaminetetraacetic acid	Sigma
Ethylenediaminetetraacetic acid Na-salt (Na-EDTA)	Sigma
Ethanol	Roth
Ethidiumbromide	Life Technologies, Inc.
EZ-Link Sulfo-NHS-Biotin Reagents	Pierce
Ficoll-Paque™ Plus	Amersham Biotech
fMLP	Sigma
D-Glucose	Merck
Glutathione (red.) (GSH)	Calbiochem
Glycerol	Sigma
Glycine	Roth
Heparin-Natrium	Ratiopharm
HEPES	Roth
Hirudine, Revasc	Aventis Pharma, Novartis
Iloprost	Schering
Ionomycin (A23187)	Sigma
Isopropanol	Roth
Isopropylthiogalactopyranoside (IPTG)	Roth
Lipopolysaccharide from *E. coli*	Sigma
Magnesiumchloride-hexahydrate, $MgCl_2 \cdot 6H_2O$	Sigma
Magnesiumsulfate, $MgSO_4$	Fluka
Manganchloride, $MnCl_2 \cdot 4H_2O$	Fluka
2-Mercaptoethanol	Sigma
Nα-(3-maleimidylpropionyl)biocytin (MPB)	Molecular Probes
MOPS	Sigma
Nitrocellulose membrane	Amersham Biosciences
Phosphatase-inhibitor cocktail	Roche
Potassiumacetate	Merck
Potassiumchloride, KCl	Sigma
Potassiumdihydrogenphosphate, KH_2PO_4	Merck

Protease-inhibitor cocktail	Roche
Protein marker, Page Ruler	Fermentas
Rubidiumchloride	Fluka
Sodiumacetate hexahydrate	Merck
Tri-Sodiumcitrate	Roth
SDS ultra pure	Roth
S2222, chromogenic substrate	Haemochrom Diagnostica
Sodiumhydrogencarbonate, $NaHCO_3$	Sigma
Sodiumdihydrogenphosphate, NaH_2PO_4	Merck
Sodiumhydroxide, NaOH	Sigma
Streptavidin-agarose beads	Sigma
TEG caps	ROTEM
TEMED	Roth
Thromborel S	Dade Behring
Tris-(hydroxymethyl)-aminomethane (Tris-base)	Roth
Triton-X100	Sigma
Tween 20	Sigma
Whatman 3MM Papier	Schleicher & Schuell
X-ray film	Fuji
Zeba Desalt Spin Columns	Pierce

II.1.3 Cell culture materials

Items	**Companies**
Ampicillin	Sigma
Bacto-Agar	Roth
Culture flasks	Falcon
FuGene 6 Transfection Reagent	Roche
Fetal bovine serum	Invitrogen-Gibco
Kanamycin	Sigma
MEM-alpha medium	Invitrogen-Gibco
Pemicillin/Streptomycin (100x)	Invitrogen-Gibco
Trypanblue	Sigma
Trypsin/EDTA	Invitrogen-Gibco
Yeast extract	Life Technologies Inc.

II.1.4 Enzymes and Proteins

Items	**Companies**
Annexin V, recombinant	BD Biosciences Pharmingen
Apyrase grade VII (from potato)	Sigma
Cathepsin G from human leukocytes	Sigma
Chondroitinase ABC, *Proteus vulgaris*	Calbiochem
Collagen, Type I	Horm, Nycomed
Corn Trypsin Inhibitor	Calbiochem
DNase I	Sigma

Factor X, human	Haemochrom Diagnostica
Glutaredoxin, recombinant, *E. coli*	Calbiochem
Human Neutrophil Elastase	Calbiochem
Pfu Taq Polymerase	Stratagene
Protein-Disulfide Isomerase (PDI)	Sigma
Ribonuclease I, *E. coli*	Fermentas
Sal I restriction endonuclease	New England Biolabs
Soluble Tissue Factor 1-219	Kindly provided by PD Dr. Victor Magdolen, Klinikum rechts der Isar, Technische Universität München
Streptavidin, horseradish peroxidase conjutated	Pierce
Streptavidin-Agarose, *Streptomyces avidinii*	Sigma
T4 DNA Ligase	New England Biolabs
Thioredoxin, recombinant, *E. coli*	Calbiochem
α-Thrombin	Sigma
Xba I restriction endonuclease	Fermentas

II.1.5 Antibodies

Items	Antigen	References
Anti-human CD14 magnetic MicroBeads	CD14	Miltenyi Biotec
Anti-human CD15 magnetic MicroBeads	CD15	Miltenyi Biotec
Goat anti-human TFPI (C-20) polyclonal antibody	TFPI	Santa Cruz (sc-18713)
Goat anti-mouse IgG, horseradish peroxidase conjugated	Mouse IgG, carboxyterm.	Calbiochem (#401253)
Mouse Anti-Glutathione monoclonal antibody	Glutathione	Virogen (101-A)
Mouse anti-goat IgG, horseradish peroxidase conjugated	Goat IgG, carboxyterm.	Santa Cruz (sc-2354)
Mouse anti-human Tissue Factor-VIC7 monoclonal antibody	TF	Dr. rer. nat. Sybille Albrecht, Pathologisches Institut, Technische Universität Dresden

Mouse anti-human Tissue Factor-VD8 monoclonal antibody	TF	Dr. rer. nat. Sybille Albrecht, Pathologisches Institut, Technische Universität Dresden
Mouse anti-human Tissue Factor-VIC12 monoclonal antibody	TF	Dr. rer. nat. Sybille Albrecht, Pathologisches Institut, Technische Universität Dresden
Mouse anti-human Tissue Factor 5G9 monoclonal antibody	TF	Kindly provided by Dr. Robert F. Kelley, Genentech, CA, US
Mouse anti-rat PDI monoclonal antibody (IgG2a), (clone RL90), (cross reacts with human, mouse and hamster PDI)	PDI	Affinity BioReagents (MA3-019)
Mouse anti-rat IgG2a monoclonal antibody, (clone R2A-2)	Isotype control	Sigma (R 0761)

II.1.6 Kits

Items	Companies
Gene Tailor™ Site-directed mutagenesis kit	Invitrogen
Qiagen gel extraction kit	Qiagen
Qiagen plasmid kit (midi, maxi)	Qiagen
QiaQuick PCR purification kit	Qiagen

II.1.7 Phagmid

Items	Companies
pBK-CMV	Stratagene

II.1.8 PCR-primers

All primers were synthesized by the MWG-Biotech AG.

II.1.8.1 Cloning primers

Primer	Restriction Site	Sequence
outer TF5' (5'-primer)	-	CCAACTGGTAGACATGGAGAC
out TF3'ch (3'-primer)	-	CAGTAGCTCCAACAGTGCTCC
Sal5'TFclone (5'primer)	Sal 1	CGACGC**GTCGAC**ATGGAGACCCCTGCCTG
XbaITFrevcloning (3'primer)	Xba I	GC**TCTAGA**TTATGAAACATTCAGTGGGGAG

II.1.8.2 Site-directed mutagenesis primers

Primer	Mutation	Sequence
C49S forward	C49S	CAGGAGATTGGAAAAGCAAA**AGC**TTTTACACAAC
C49S reward		TTTGCTTTTCCAATCTCCTGACTTAGTGCT
C57S forward	C57S	TTACACAACAGACACAGAG**AGT**GACCTCACC
C57S reward		CTCTGTG-TCTGTTGTGTAAAAGCATTTGCT
C186S forward	C186S	GGATAAAGGAGAAAACTAC**AGT**TTCAGTGTTC
C186S reward		GTAGTTTTCTCCTTTATCCACATCAATCAA
C209S forward	C209S	GTACAGACAGCCC-GGTAGAG**AGT**ATGGGCCAGG
C209S reward		CTCTACCGGGCTGTCTGTACTCTTCCGGTTAAC

II.1.9 Bacterial strains and cell lines

Strain / Cell line	Species	Classification	Reference
DH5α	*E. coli*	Bacteria	Invitrogene
Chinese Hamster Ovary Cells (CHO)	*Hamster*	Mammals	LGC Promochem
Pulmonary Artery Vascular Smooth Muscel Cells (PAVSMC)	*Human*	Mammals	Cambrex

II.1.10 Bacterial and cell culture media

Freeze Medium for CHO cells
40% MEM-α
50% FBS
10% DMSO

Luria-Bertani (LB) Medium
10 g/l Trypton
5 g/l Yeast extract
10 g/l NaCl
pH 7.5
For LB-Agar plates add 1.5% (w/v) of Bacto-Agar.

Minimum Essential Medium (MEM) alpha (for CHO cells)
For formulation see Gibco-Invitrogen

Psi broth medium
20 g/l Trypton
5 g/l Yeast extract
5 g/l Magnesiumsulfate
pH 7.5

RPMI-1640 Medium (for monocytes and monocytic cell lines)
See Gibco-Invitrogen

II.1.11 Buffers and solutions

Antibody buffer
0.13% Na-EDTA
0.15% BSA
Dissolved in PBS

Blocking buffer
5% BSA in TBS/T

Blotting buffer
150 mM Glycine
20 mM Tris
0.1% SDS
20% Methanol

Buffer P1
50 mM Tris-HCl, pH 8.0
10 mM EDTA
10 mg/ml RNase A

Buffer P2
10% SDS
200 mM NaOH

Buffer P3
3 M Potassium acetate, pH 5.5

Buffer QBT
15% Ethanol
0.15% Triton X-100

Buffer QC
2.0 M NaCl
50 mM MOPS, pH 7.0
15% Ethanol

Buffer QF
1.25 mM NaCl
50 mM Tris-HCl, pH 8.5
15 % Ethanol

Ca^{2+}/Hepes
10 mM Hepes, pH = 7.4
100 mM $CaCl_2$

Cell lysis buffer
50 mM Tris, pH 8.0
150 mM NaCl
5 mM EDTA
1% Triton X-100

10 x DNA-Gel Loading Buffer
40% (w/v) saccharose
0.25% bromphenolblue
0.25% xylencyanol, use as 1x solution

EDTA buffer
50 mM Tris-HCl
20 mM EDTA
1 mg/ml BSA

Gel buffer (500 ml)
3 M Tris-HCl, pH 8.45
0.3 % SDS
(dissolve in 300 ml and adjust to pH 8.45 with HCl)

Hank's Balanced Salt Solution (HBSS)
0.4 mM KH_2PO_4
0.6 mM $MgSO_4$
5.4 mM KCl
1.3 mM $CaCl_2 \cdot 2H_2O$
0.5 mM $MgCl_2 \cdot 6H_2O$
5.6 mM α-D-Glucose
0.3 mM Na_2HPO_4
137 mM NaCl

4.2 mM $NaHCO_3$
pH 7.4

Phosphate-Buffered Saline (PBS)
136 mM NaCl
2,6 mM KCl
10 mM NaH_2PO_4
1.5 mM KH_2PO_4, pH 7.4

PBS / EDTA
100 ml PBS (10x), pH 7.4
1 ml Na-EDTA (0.5 M stock, pH 8.0)
900 ml ddH_2O

Resuspension buffer
138 mM NaCl
2.7 mM KCl
12 mM $NaHCO_3$
0.4 mM NaH_2PO_4
1 mM $MgCl_2 \cdot 6H_2O$
5 mM D-Glucose
5 mM Hepes, pH 7.35

Running buffer (for SDS-PAGE)
25 mM Tris
250 mM Glycine
0.1% SDS

4x SDS-loading buffer (for SDS-PAGE) (Laemmli buffer)

10 ml	1M	Tris-HCl, pH 6.8
23 ml	10%	Glycerol (87%)
10 ml	10% (w/v)	SDS
2 ml		2-Mercaptoethanol
4 ml	0.5 %	Bromphenolblue

Separating buffer (4x)

75.0 ml	2 M	Tris-HCl, pH 8.8
4.0 ml	10 % (w/v)	SDS
21.0 ml		ddH_2O

Transformation buffer I (Tfb I)

0.588 g	30 mM	Potassiumacetate, pH 5.8, adjusted with acetic acid
2.42 g	100 mM	Rubidiumchloride
0.294 g	10 mM	Calciumchloride
2.0 g	50 mM	$MnCl_2 \cdot 4H_2O$
30 ml	15% (v/v)	Glycerol

200 ml total volume

Transformation buffer II (Tfb II)

0.21 g	10 mM	MOPS, pH 6.5, adjusted with NaOH
1.1 g	75 mM	Calciumchloride
0.121 g	10 mM	Rubidiumchloride
15 ml	15% (v/v)	Glycerol

100 ml total volume

1x Tris-Acetate-EDTA (TAE)
40 mM Tris-HCl
40 mM Acetic acid
2 mM EDTA, pH 7.8

10x Tris-Buffered Saline (TBS)
400 mM Tris-HCl
1.37 M NaCl

TBS/T
1x TBS + 0.1% Tween 20

II.2 Methods

II.2.1 Cell isolation techniques

II.2.1.1 Blood recovery

Venous blood was obtained from healthy donors (age 18-35 years), who did not take drugs acting on the coagulation system for at least 14 days. The blood was anticoagulated with tri-sodiumcitrate, hirudine or heparine-sodium, respectively. All experiments performed with human blood were approved by the local ethic commission.

II.2.1.2 Isolation of platelets

Blood obtained from healthy donors (anticoagulated by tri-sodiumcitrate, 12.5 mM) was centrifuged in 10 ml centrifuge tubes at 1300 rpm for 15 minutes at 24°C. 2 ml platelet-rich plasma (PRP) were aspirated from each centrifuge tube and filled into a separate centrifuge tube. Apyrase grade VII (0.475 U/ml) and iloprost (10 ng/ml) were added to each 2 ml

sample of PRP and the tubes containing PRP were centrifuged at 1300 rpm for 10 minutes at 24°C. The supernatant was discarded and the platelet pellet was resuspended in resuspension buffer.

II.2.1.3 Preparation of platelet supernatant

1 x 10^{10} isolated platelets per ml resuspension buffer were stimulated with 0.1 U/ml thrombin and 8 µg/ml type I collagen for 30 minutes at 37°C. The stimulated platelets were centrifuged at 12600 rpm for 30 min at 24°C and the pellet was separated from the supernatant.

II.2.1.4 Isolation of peripheral blood monocytes (PBM)

Buffy coats obtained from the blood of healthy donors (anticoagulated by tri-sodiumcitrate (12.5 mM) and hirudine (1 µg/ml)) were diluted with 2 volumes of calcium-free PBS, and the suspension was underlayered with low-endotoxin Ficoll-Paque^{TM} Plus. After centrifugation for 25 minutes at 420g, the interphase was collected and washed with antibody buffer. The pellet of white blood cells was taken up in 2 ml of antibody buffer and 200 µl of CD14 microbeads were added to the suspension and incubated for 15 minutes at 4°C. A Midi-MACS LS column was placed into a magnetic field and equilibrated with antibody buffer. The CD 14 microbeads treated white blood cell suspension was applied to the column and the column was washed three times with 3 ml antibody buffer. The monocytes were eluted from the MACS column with 8 ml of antibody buffer and centrifuged at 1300 rpm (15 minutes, 24°C). The supernatant was discarded and the monocyte pellet was taken up in RPMI-1640 medium to a final cell density of 3 x 10^6 monocytes per ml.

II.2.1.5 Isolation of polymorphonuclear neutrophils (PMN)

Blood was anticoagulated with Na-heparin (10 µl/ml) or tri-sodiumcitrate (12.5 mM) and inverted twice. Whole blood was added to 3% dextrane (in HBSS) in a ratio of 2 to 1. The cups were inverted twice and the separation of red blood cells and serum, containing other blood cells occurred in about 30 minutes at room temperature (RT). After separation the supernatant was removed with a sterile plastic Pasteur pipette and it was layered on top of 7 ml low-endotoxin Ficoll-Paque^{TM} Plus in a 15 ml Falcon. The Falcons were spun for 30 minutes at 1200 rpm and RT. The supernatant was removed and the pellet was resuspended in 1 ml of RPMI-1640 medium. All suspensions were pooled into a new 15 ml Falcon, which was filled up to 10 ml RPMI-1640 medium. Then it was centrifuged for 10 minutes at 1000 rpm and RT. The supernatant was removed and the pellet was resuspended in 1 ml pyrogen-free water for hypertonic lysis of residual red blood cells. After 20 to 40 s of gentle resuspension, 10 ml HBSS were added and subsequently the cells were centrifuged for 10 minutes at 1000 rpm at RT. If residual red blood cells were present, the same step was repeated several times.

Alternatively another PMN isolation method was applied. Blood was anticoagulated with tri-sodiumcitrate (12.5 mM) and centrifuged for 15 minutes at 1300 rpm and RT. The PRP was removed and the buffy coat (interphase of white blood cells) was transferred to a new centrifuge tube. After centrifugation at 1300 rpm for 10 minutes at RT the layer of white blood cells was transferred to a Falcon tube and filled up with antibody buffer to 2 ml of total volume. Per 2 ml of white blood cells 200 µl of anti-human CD15 magnetic MicroBeads were added and incubated for 15 min at 4°C. The Mini-MACS columns were equilibrated with at least 2 ml of antibody buffer and the samples of white blood cells were diluted by adding 600 µl of antibody buffer. After applying the samples onto the columns the columns were washed fourfold with 500 µl of antibody buffer to remove all other blood cells. Subsequently the columns were removed from the magnetic field and the PMN were

eluted by applying 3 x 2 ml of antibody buffer. The PMN were pelleted by spinning the Falcon tube at 1000 rpm for 10 minutes at RT and resuspended in 1 ml of resuspension buffer.

II.2.1.6 Stimulation of isolated blood cells

For inducing TF expression of isolated monocytes 3 x 10^6 monocytes per ml RPMI-1640 medium were stimulated with 10 ng/ml LPS for 5 hours at 37°C. For monocyte microparticle formation 3 x 10^6 isolated monocytes per ml resuspension buffer were stimulated with 10 µg/ml LPS for 16 hours at 37°C.

For the formation of neutrophil microparticles 3 x 10^6 PMN per ml resuspension buffer were stimulated with 100 nM fMLP for 2 hours at 37°C.

For immunoblots 3 x 10^8 platelets were stimulated with 0.1 units/ml thrombin and 12 µg/ml collagen type 1. For functional assays platelets were stimulated with 12 µg/ml collagen or 3 µM A23187 Ca^{2+}-ionophore in 2 mM Ca^{2+}-containing resuspension buffer.

TF expression in PAVSMC was induced by treatment of the cells with 1 unit/ml thrombin for 4 hours at 37°C.

II.2.1.7 Isolation of microparticles derived from stimulated blood cells

After stimulation of isolated blood cells (platelets, neutrophils, monocytes), the samples were centrifuged for 15 minutes at 4500 rpm and RT. 250 µl of the obtained supernatant was filled in each Eppendorf-tube and it was centrifuged for 30 min at 12600 rpm and 24°C. Afterwards, 225 µl of the supernatant were removed and 25 µl were left on the bottom of the tubes. Subsequently, the microparticles of each tube were washed with 225 µl PBS, vortexed and again centrifuged for 30 min at 12600 rpm and 24°C. After the centrifugation, all the supernatant was removed and the microparticle pellet of each tube was resuspended in 25 µl resuspension buffer.

II.2.2 Cell culture techniques

II.2.2.1 Bacterial cell cultures

Transformed DH5α-bacteria were selected on LB plates with ampicillin (100 µg/ml) or kanamycin (50 µg/ml) for 24 hours. For the preparation of overnight mini-cultures one colony was picked and inoculated in LB medium with the appropriate antibiotic and shaken overnight at 37°C. The overnight mini-culture was then used to prepare glycerol stocks or to isolate and purify plasmid DNA for the transfection of eukaryotic cells. For the storage of transformed bacteria a glycerol stock was prepared by growing the bacteria to an OD of 0.8 at 600 nm. Then 500 µl of the bacterial culture was added to 500 µl of 80% glycerol and mixed thoroughly. The stocks were immediately frozen at -80°C.

II.2.2.2 Preparation of competent DH5α-cells

A 20 ml pre-culture was grown in LB medium overnight at 37°C and 180 rpm. The next day 1 ml from the pre-culture was inoculated in 100 ml of psi broth medium and cultivated at 37°C and 180 rpm to an OD of 0.5 at 600 nm. The culture was put on ice for 15 minutes. The cells were pellet at 5000 x g for 5 minutes and the supernatant was removed. The pellet was resuspended in 40 ml of Tfb I per 100 ml of bacterial cell culture and again kept on ice for 15 minutes, followed by centrifugation at 5000 g for 5 minutes. The supernatant was removed the bacterial cell pellet was resuspended in 4 ml Tfb II per 100 ml of culture and put on ice for 15 minutes. Subsequently 50 µl aliquots were prepared in 1.5 ml tubes and immediately frozen in liquid nitrogen and kept in –80°C.

II.2.2.3 Transformation of competent bacteria

The competent bacteria were thawed on ice. About 40 ng of ligated DNA or purified phagmid DNA were added to 50 µl of competent cells, mixed carefully and kept on ice for

additional 20 minutes. The bacteria were heat shocked at 42°C for 90 seconds, then 1 ml of LB medium was added and the transformation samples were shaken at 37°C for 30 minutes. A selection of transformed bacteria was achieved by plating the bacterial suspensions onto antibiotic containing agar plates. Only these bacteria which had taken up the plasmid, containing an antibiotic resistance cassette, were able to grow on the plate.

II.2.2.4 Cultivation of Chinese Hamster Ovary cells

The Chinese Hamster Ovary (CHO) cells were cultured in 75 cm^2 cell culture flasks containing MEM-α medium supplemented with 100 µl penicillin/streptomycin (100x) mingled with 10% fetal bovine serum (FBS) at 5% CO_2 (37°C). The cells were cultured until a confluence of approximately 90% was achieved. The cells were washed with 5 ml of PBS and released from their culture flask by adding 2 ml of PBS/EDTA and subsequent incubation for 20 minutes at 37°C. Freezing cultures were prepared by spinning the cells at 1000 rpm for 15 minutes and resuspending them in 1 ml of freeze medium for CHO cells per flask of confluent cells. Aliquots were set into an ethanol-filled container and frozen gradually at –80°C.

II.2.2.5 Transfection of Chinese Hamster Ovary cells

One day before transfection, the cells were treated with trypsin/EDTA and split into a new culture flask to become 80% confluent. For each culture flask to be transfected, 36 µl of FuGene 6 transfection reagent was diluted into serum free MEM-α medium. Subsequently 15 µg of DNA were added. The transfection mixture was prepared in such a way that the total volume was 800 µl and it was incubated for 45 minutes. Then the transfection mixture was added dropwise into each of the cell cultures and they were mixed thoroughly. Finally the transfected cell cultures were incubated for 16 hours at 37°C. During that time the protein expression from the transfected plasmid should proceed.

II.2.3 DNA techniques

II.2.3.1 Electrophoresis of DNA on agarose gels

Double stranded DNA fragments can be separated according to their length on agarose gels. Agarose was added to 1 x TAE to a final concentration of 0.7-2.0%. The mixture was boiled in the microwave until the agarose was completely molten. The agarose was cooled down to about 50°C before ethidium bromide was added to a concentration of 5 µg/ml and poured into the gel tray. DNA gel loading buffer was added to the samples and they were applied to the gel. Electrophoresis was performed in 1 x TAE at 3-8 V/cm. The DNA fragments were visualized in the gel by UV-light.

II.2.3.2 Isolation of DNA from agarose gels (Qiagen gel extraction kit)

This protocol was designed for the extraction of DNA fragments from 0.7-2.0% standard agarose gels in TAE or TBE buffer. DNA molecules were adsorbed to Qiagen silica columns. All non-nucleic acid impurities, such as agarose, proteins, salts and ethidium bromide were removed during the washing steps. The desired DNA band was excised from the gel under the UV-light. The gel slice was weighed and 5 volumes of buffer QG were added to one volume of gel for DNA fragments from 100 bp to 4 kb, for DNA fragments > 4 kb, 2 volumes of buffer QG plus 2 volumes of ddH_2O were added, and then incubated for 10 minutes at 50 °C to solubilize the agarose. The solubilized agarose was resuspended by vortexing and the sample was applied to the Qiagen silica columns to bind DNA. The sample was centrifuged for 30 seconds, then the column was washed with 500 µl of buffer QG and subsequently twice with buffer PE. Thereafter it was centrifuged for additional 30 seconds to remove residual alcohol from the column. The column was span for 1 min to elute the DNA in 30-50 µl of Tris-HCl or ddH_2O.

II.2.3.3 Purification of plasmid DNA (QIAquick PCR purification kit)

This protocol was designed to purify single- or double-stranded PCR products or DNA plasmids ranging from 100 bp to 10 kb. DNA adsorbs to the silica matrix in the presence of high salt concentrations while contaminants pass through the coloumn. The impurities were removed by washing steps and the DNA was eluted with Tris-HCl or ddH_2O. Five volumes of buffer PB was added to one volume of the contaminants and mixed. A QIAquick spin column was placed in a collection tube, the mixed sample was added to the column and centrifuged for 30-60 seconds. The flow-through was discarded and the column was placed back into the same collection tube. 0.75 ml buffer PE was added to the column and it was centrifuged for 30-60 seconds. The flow-through was discarded and the column was placed back into the same collection tube. The column was spun for one additional minute at maximum speed and placed in a clean 1.5 ml microfuge tube. 30-50 µl of elution buffer (EB) or ddH_2O were added to the centre of the column and it was centrifuged for one minute. The purified DNA was stored at –20°C.

II.2.3.4 Maxi-preparation of plasmid DNA (Qiagen plasmid maxi kit)

Bacterial cultures containing plasmids or recombinant plasmids were grown in 50 ml LB medium overnight in a 37°C-incubator shaking at 180 rpm. The bacteria were harvested and the DNA plasmids were isolated by using the Qiagen plasmid maxi kit. The extraction method applied is based on Birnboim's alkali lysis principle. The bacterial pellet was resuspended in 10 ml of buffer P1. 10 ml of buffer P2 were added and mixed gently. Then the lysate was incubated at RT for 5 minutes, 10 ml of chilled buffer P3 were added, mixed immediately and incubated on ice for further 20 minutes. The suspension was centrifuged for 30 min at 4000 rpm and 4°C and the supernatant was filtered over a folded filter. The supernatant was applied to an equilibrated QIAGEN-tip 500 and it was allowed to enter the resin by gravity flow. The QIAGEN-tip was washed twice with buffer QC. The DNA was

eluted with 15 ml of buffer QF. This procedure resulted in the isolation of a DNA-salt pellet, which was precipitated by 0.7 volumes of isopropanol (10.5 ml), and centrifuged at 4000 rpm for 30 minutes. The obtained pellet was washed twice with 70% ethanol and air-dried at RT. The pellet was then carefully solved in ddH_2O and quantified.

II.2.3.5 Measurement of DNA concentration

DNA concentrations were determined with a UV spectrophotometer measuring the absorbance (A) at a wavelength of 260 nm. The absorption of 1.0 at a wavelength of 260 nm corresponds to a double stranded DNA concentration of 50 µg/ml. The ratio of $A_{260\ nm}$ / $A_{280\ nm}$, which is a measure of the DNA purity, was over 1.8. This means, that the DNA preparations were pure from proteins.

II.2.3.6 DNA sequencing

All sequencing reactions were performed by SeqLab (Göttingen, Germany). The evaluation of all sequencing results were done with the program *Chromas*.

II.2.3.7 Polymerase Chain Reaction (PCR)

All oligonucleotid primers used in the PCRs were synthesized by MWG Biotech (Germany) and delivered in lyophilized form. The oligonucleotides were dissolved in sterile water to obtain a 100 pM solution. From the primer solutions the experimental mixtures for conventional PCR, sequencing and site directed mutagenesis were prepared. The coding regions of full length human TF were amplified from a HL-60 cDNA. For the amplification of the TF cDNA with outer primers the reaction was performed in the presence of 5 pM oligonucleotide primers (5'-primer: CCAACTGGTAGACATGGAGAC; 3'-primer: CAGTAGCTCCAACAGTGCTCC), 20 mM of each of the four deoxy-nucleoside triphosphates (dNTPs), and 2.8 units of Pfu Turbo DNA polymerase in 20 µl

buffer supplied by the manufacturer at an annealing temperature of 59.5°C. For the amplification of TF cDNA containing a Sal I / Xba I-restriction site (bold sequence) inner primers (5'-primer: CGACGC**GTCGAC**ATGGAGACCCCTGCCTG; 3'-primer: GC-**TCTAGA**TTATGAAACATTCAGTGGGGAG) were used with the outer primers' amplification product as a template and an annealing temperature of 65.0°C. All other reaction conditions were the same as in the previous reaction. The PCRs were performed according to the following protocol:

Step 1:	*initial denaturation*	*94.0°C for 2 min*
Step 2:	*denaturation*	*94.0°C for 20 seconds*
Step 3:	*annealing*	*appropriate annealing temperature for 1 min*
Step 4:	*elongation*	*72.0°C for 1 min*
Step 5:	*closing the cycle and back to step 2*	
Step 6:	*final elongation*	*72.0°C for 5 minutes*
Step 7:	*End*	*keep at 4°C*

II.2.3.8 Restriction digests of DNA fragments

Digestion of DNA with restriction endonuclases was performed according to the manufacturer's instructions using recommended buffer systems and the appropriate reaction temperatures. Generally, 1 unit of enzyme was used to digest 1 µg of DNA. Plasmid DNA was usually digested for 1-2 hours. Completion of the reactions was monitored by agarose gel electrophoresis.

II.2.3.9 Ligation of DNA fragments

The plasmid DNA and the DNA fragments were prepared by cutting with suitable restriction enzymes and subsequent purification. A 1:3 molar ratio of vector to insert DNA fragments were incubated with 1 unit of T4 DNA-ligase in 1x ligation buffer in a total volume of 20 µl overnight at 16°C. The next day the mixture was heated for 10 minutes to inactivate the T4 DNA-ligase.

II.2.3.10 Construction of the protein expression phagmid pBK-CMV-TF

cDNA obtained from HL-60 cells was amplified by polymerase chain reaction (PCR) as described before. A DNA fragment of 0.9 kb was obtained as PCR product by 31 cycles of 40 seconds at 94°C, 60 seconds at 65°C, and 60 seconds at 72°C with a thermal cycler. After the fragment was purified using the QIAquick PCR purification kit and the Qiagen gel extraction kit, it was ligated to the Sal I / Xba I-digested pBK-CMV phagmid with T4 DNA-ligase and transformed into calcium-competent *E. coli DH5-α* cells. A white transformed colony formed on LB medium containing 50 µg/mL ampicillin (LBamp) and IPTG was inoculated and grown overnight in LBamp (3 mL). The picked clone was sequenced to verify, that no random mutation had occurred during the PCR reactions. The cloned construct, named pBK-CMV-TF, was subsequently transformed into calcium-competent *E. coli DH5-α*-cells and selected by its ampicillin resistance on LBamp agar plates and a 1 l culture was grown in LBamp to amplify and isolate the cloned product with the Plasmid Maxi Kit.

II.2.3.11 Site-directed mutagenesis of TF C49S, TF C57S, TF C186S, TF C209S, TF C49S/C57S and TF C186S/C209S in pBK-CMV-TF

Site-directed mutagenesis was performed using the Gene Tailor^{TM} Site-Directed Mutagenesis Kit. The method is used to exchange one single amino acid within a

polypeptide chain or to remove or insert up to five amino acids in one single reaction. The pBK-CMV-TF phagmid was methylated to insure that the parental plasmid that is not containing the desired mutation will be degraded by the transformed bacterial strain. This will greatly increase the efficiency of the mutagenesis reaction. The primers were designed as described in the manufacturer's booklet containing the desired mutations (see material section). Thereby it is important the primers to be overlapping and containing protruding ends to achieve a recircularization of the vector. For the mutagenesis PCR of the methylated template, Platinum® Pfx polymerase was used, which has a very reliable proof reading activity for long elongation reactions. After mutating and amplifying the construct, the transformation was carried out according to the protocol supplied by the manufacturer. Subsequently the mutated constructs were sequenced and correctness of the inserted mutations was verified.

II.2.4 Protein analyses

II.2.4.1 Determination of protein concentrations

All protein concentrations were determined employing the Bio-Rad Assay, which is based on the Lowry method. 5µl of the protein sample was pipetted into a 96 well microtiter plate then 25 µl of reagent A and 200 µl of reagent B were added. It was incubated for 12 min to allow the colour reaction to reach its end point. Afterwards, the absorbance of the samples at 700 nm was measured with a photometer. The obtained absorbance values were transformed into protein concentrations by a BSA standard curve, recorded with BSA solutions of known protein concentration.

II.2.4.2 UV-Spectroscopy

Protein concentrations can be determined by UV-Spectroscopy, since proteins show a maximum of absorbance at about 280 nm due to their aromatic constituents (F, Y, W). If the sequence of a certain protein is known, its extinction coefficient at 280 nm (ε_{280}) can be calculated by applying the software *protparam* that is available at www.expasy.ch. Therefore, the protein concentration c can be calculated using the Lambert-Beer equation $A = \varepsilon\ c\ d$, d representing the path length of the cuvette and A the measured absorption of the protein solution. For all measurements a cuvette with a path length of 1 cm and a *Perkin Elmer* UV-Spectrophotometer was used. The recorded UV spectra gives information on the purity of a protein solution and the possible occurrence of protein aggregates.

II.2.4.3 Circular Dichroism-Spectroscopy

Circular Dichroism-Spectroscopy (CD) is a biophysical method that measures the angle of which the plane of polarized light is changed after such light is passed through a solution containing a chiral substance. Amino acids are containing chiral centers and therefore CD-Spectroscopy can be applied to proteins. The CD-Spectra of proteins are solely composed of the CD-Spectra of their single amino acid constituents and of their secondary structure elements. Therefore it is possible to calculate the composition of a proteins' secondary structure by spectra deconvolution. The deconvolution of protein spectra is achieved by the comparison of characterized protein spectra with a well known secondary structure compsition to the CD-spectrum of a sample protein solution. This can be calculated with the commercially available software *CDN*. CD-Spectra were recorded with a *JasCo* photometer at 25 °C and the cuvette that was used for all measurements had a path length of 0.1 cm.

II.2.4.4 Sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

30 µl of cell lysates of a defined total protein concentration were mixed with 10 µl of 4 x Laemmli buffer (optionally containing 2-mercaptoethanol as a reductant) and boiled for at least 3 min to denature all proteins. The SDS complexes were electrophoretically separated on a polyacrylamid gel of appropriate acrylamid percentage. Vertical gels were set in between two glass plates with a thickness of 1.5 mm. Gels (7.5%, 12%) were commercially available as ready gels from Bio-Rad or Pierce or they were poured in the appropriate percentage according to the following pipet scheme in between the fixed glass plates and polymerized for at least 30 min at RT:

compounds	7.5%	12%	16.5%	stacking gel
ddH_2O	2.43 ml	3.45 ml	-	3.05 ml
Separating buffer (4x)	1.25 ml	2.5 ml	-	1.25 ml
Gel buffer	-	-	3.5 ml	-
Acryl-Bis	1.25 ml	4.0 ml	3.5 ml	0.66 ml
10% APS	25 µl	50 µl	32,5 µl	25 µl
TEMED	3 µl	5 µl	3.25µl	5 µl
32% (v/v) Glycerol	-	-	3.5 ml	-

The gels are composed of a low percentage stacking gel, which is focussing all the proteins of the sample to enter the separating gel simultaneously and a separating gel that separates the proteins of the applied sample according to their apparent molecular weight. First the separating gel was poured in between the glass plates and n-buthanol was stacked on it during the polymerization. After half an hour the n-butanol was poured from the polymerized gel and the stacking gel was added to polymerize. A comb was inserted in between the glass plates to form the slots in which the samples were applied. After the gel was ready it was fixed in the gel chamber and the running buffer was filled into the

chamber. The protein samples were applied into the slots and the gel was run at about 100 V. The negatively charged SDS-protein complexes run into the direction of the anode at the bottom of the vertical gel. The proteins were separated according to their electrophoretic mobility ($\mu = v/E$), which largely depends on their molecular weight.

II.2.4.5 Immunoblot

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane using a wet transfer system. All components were soaked beforehand in transfer buffer and the nitrocellulose membrane was activated by incubation in the methanol containing transfer buffer for several minutes. The gel was placed on top of a sponge and a 3 mm Whatman filter paper, subsequently the nitrocellulose membrane was pressed onto the gel. The blotting sandwich was completed after the addition of a second filter paper and a sponge. Transfer in the cassette assembly was carried out at 270 mA for 90 minutes. The remaining protein binding capacity of the membrane was neutralized by incubation in blocking buffer for > 1 hour on a shaker. After the blocking procedure, the membrane was incubated for 90 minutes with a primary antibody which was diluted to a final concentration of 2.5 - 10 µg/ml in blocking buffer. The membrane was washed three times with washing buffer removing excess primary antibody unspecifically bound to the membrane. A peroxidase-conjugated, secondary antibody was used, binding to the F_c region of the primary antibody, which was diluted 1:1000 – 1:10000. Excess antibody was removed by washing three times with washing buffer. Protein-antibody complexes were detected on a x-ray film using the chemoluminescence reaction catalysed by the antibody-conjugated peroxidase. ECL solutions were used as a substrate for this reaction according to the manufacturer's protocol.

II.2.4.6 Nα-(3-maleimidylpropionyl)biocytin-labelling of the reduced cysteine residues in recombinant sTF_{1-219} and the extracellular protein domains of monocytes

40 ng recombinant sTF_{1-219} (TF extracellular domain) or 3 x 10^6 monocytes were resuspended in 170 µl PBS and incubated in 125 µM MPB for 30 minutes at room temperature. MPB is not able to pass the plasma membrane of intact cells. It binds to the free thiol groups of cysteines in the cells' extracellular protein domains, resulting in a covalently linked MBP-label. On one occasion, sTF_{1-219} was preincubated with thioredoxin, dithiothreitol, glutaredoxin or protein disulfide isomerase for 30 minutes at 37°C before labelling with MPB. Excess MPB was quenched with GSH (200 µM) for 30 minutes at RT, to exclude further MPB labelling of proteins after the cells were lysed. The labeled cells were washed three times with PBS and sonicated in 200 µl of ice-cold cell lysis buffer. Before the cells were sonicated, 25 µl protease-inhibitor cocktail and 25 µl phosphatase-inhibitor cocktail were added to each sample. Streptavidin-agarose beads were incubated with the cell lysates overnight on a rotating wheel to isolate the biotin-labelled proteins. The streptavidin-agarose beads were washed three times with cell lysis buffer, and the biotin-labelled proteins were released from the beads by boiling them in 30 µl of SDS-Laemmli buffer for 3 minutes under non-reducing conditions. Samples were resolved on SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. The TF antigen was detected by immunoblot with primary antibody concentrations of 3 µg/ml mouse-anti-human VIC12-TF-antibody and a 1:2000 dilution of horseradish peroxidase conjugated goat-anti-mouse IgG. Alternatively, the MPB-labelled, reduced form of recombinant sTF_{1-219} could be detected with streptavidin peroxidase, which binds specifically to the biotin label.

II.2.4.7 Biochemical detection of protein S-glutathionylation in membrane proteins

Reduced glutathione (GSH) was labelled with biotin by the reaction of the primary amine of GSH with N-hydroxysulfosuccinimide-biotin (NHS-biotin). The reaction was performed by adding stoichiometric amounts (10 mM) of NHS-biotin and GSH to PBS for 1 hour at RT. Unreacted NHS-biotin was quenched by the addition of 50 mM ethanolamine for 1 hour. The glutathionylation of extracellular domains of membrane proteins was performed by incubating the cells with 100 µM diamide and 125 µM biotin-GSH for 10 min at RT. Subsequently, the cells were centrifuged for 5 min at 1300 rpm, the supernatant was removed and 25 µl protease inhibitor cocktail and 212.8 µl cell lysis buffer were added to each sample. Then protein S-glutathionylation in sTF, in the TF of TF-overexpressing CHO cells and in stimulated VSMCs was detected (II.2.4.4).

II.2.4.8 Ellman's assay

Ellman's assay is used to determine the concentration of reduced thiol groups in a pure protein solution. First, a 3 mg/ml DTNB solution was prepared (pH > 7). The appropriate protein concentration should be at least 10 µM. 970 µl of the thiol-containing protein in Phosphate buffered solution (pH = 7-7.5) was mixed with 30 µl DTNB and incubated for 10 min at RT. The absorbance of the protein solution at 412 nm was measured and the absorbance of the blank (buffer plus DTNB) was subtracted. The extinction coefficient ε for the measurement is 13700 M^{-1} cm^{-1} (in Guanidinium, pH 7.4) or 14150 M^{-1} cm^{-1} (in H_2O, pH 7.4). Concentrations could be determined according to the Lambert-Beer equation.

II.2.5 Functional assays

II.2.5.1 Factor Xa formation assay

The formation of factor Xa is a measure for the functional activity of TF, since factor Xa is formed in the ternary initiator complex of blood coagulation, consisting of TF, factor VIIa and factor X. Factor VIIa either cleaves the zymogen factor X directly, thereby transforming it to the active protease factor Xa, or it cleaves the zymogen factor IX resulting in the formation of the active factor IXa, which in turn activates factor X by proteolytic cleavage. The formed factor Xa cleaves the chromogenic substrate S2222 (N-Benzyl-L-Ile-L-Glu-L-Gly-L-Arg-pNA·HCl) and thereby the cleavage product para-nitroaniline (pNA) is formed, which can be quantified by its absorption at 405 nm. The amount of pNA formed is directly proportional to the concentration of factor Xa. Therefore, the concentration of factor Xa can be determined photometrical. The coagulation factors that are necessary for the formation of Xa were added in form of the commercially available Beriplex, which besides the factors VII, X, IX and II also contains the anticoagulant zymogen protein C and the serpin inhibitor antithrombin. Thus, similar to the conditions in whole blood, not only procoagulant, but also anticoagulant components are included in this photometric assay. For the measurement of TF-derived procoagulant activity, isolated blood cells (platelets, neutrophils, monocytes) were incubated for 15 minutes together with their activators (collagen type I, A23187) at 37°C. During the incubation time a 96 well plate was prepared by adding 50 µl of an 8 mM $CaCl_2$ solution to each well. In each measurement a standard curve was prepared from recombinant TF. The chromogenic substrate together with the coagulation factors was prepared as a stock solution, mixing 3370 ml resuspension buffer, 400 µl of S2222 and 225 µl of Beriplex. 100 µl of the stock solution was added to each cell sample in every well and the absorption

values were measured photometrically during 30 minutes in 6 intervals in an ELISA reader and expressed as a concentration of factor Xa in units/ml.

II.2.5.2 Two-stage factor Xa formation assay

To further characterize TF procoagulant activity, this assay was used to show that factor Xa formation proceeded via the extrinsic pathway. Therefore, 20 µl $CaCl_2$ (50 mM) were added into each well of a microtiterplate and incubated for 3 minutes at 37°C. The standard of recombinant TF (Recombiplastin®) was prepared as follows: 20 µl of recombinant TF were diluted in 180 µl of resuspension buffer. 100 µl from this stock were diluted in 100 µl of resuspension buffer and vortexed. Then 100 µl were taken from the previous dilution and again it was diluted in 100 µl of resuspension buffer. Thus, seven 1:2 dilutions were prepared, the last dilution being the first point in a standard curve of six points. After incubation, 20 µl of recombinant TF standard solution and of the samples were pipetted into the appropriate wells. Subsequently, 2.8 µl of recombinant human factor VIIa (1µM) was added in each well. The microtiterplate was shaken for 15 minutes on a shaker. Afterwards, 15.84 µl human factor X was added to each well and it was shaken for 30 minutes at 37°C. 10 µl of the chromogenic substrate S2222 was added to each well and the colour reaction was incubated for 30 minutes at 37°C. After 30 minutes the reaction was stopped by the addition of 20 µl of EDTA buffer to each well. The absorption values of the samples were determined as described above.

II.2.5.3 Thrombelastography (TEG)

Thrombelastography (TEG) is a state of the art method to measure the fibrin formation rate in whole blood and therefore allows functional examination of the blood coagulation system under conditions that are close to the *in vivo* situation. The principle of TEG depends on a stamp, which is turning slowly 4.75° forwards and backwards in a small tube

containing blood. If a stimulus induces the formation of fibrin polymers the blood resistance increases, which restricts movement of the stamp. This increasing resistance is time dependent and is recorded by a detection system and visualized in graphic form. The method measures the rate of fibrin formation (coagulation time; CT) and the rate of thrombus growth (clot formation time; CFT). For TEG experiments blood was collected in a syringe containing tri-sodiumcitrate (12.5 mM) as anticoagulant and corn trypsin inhibitor (32 µg/ml) to exclude the initiation of blood coagulation via the intrinsic pathway. 269.1 µl whole blood pre-stimulated for 5 minutes at 37°C with collagen (10 µg/ml) were pipetted into a TEG cap, 5.2 µl anti-PDI antibody (400 µg/ml), 30 µl microparticles and 60 µl Ca^{2+}/Hepes were added.

II.2.5.4 Statistics

Statistical significance (marked with *) was tested by applying the Mann-Whitney-rank-sum-test comparing the mean values of n independent experiments. p values that were < 0.05 were considered to be significant. Values were expressed as mean +/- standard deviation.

III. Results

III.A The procoagulant activity of platelet TF

III.A.1 Collagen-stimulated platelets expose TF procoagulant activity

TF is known to be stored in platelet α-granules and is rapidly exposed by platelet activation (Muller I, 2003). It is largely unknown whether the platelet TF is functionally active. Therefore, we investigated the procoagulant activity of rapidly isolated platelets after stimulation. Stimulation with type I collagen caused a significant increase of the TF procoagulant activity of isolated platelets. This was found in both the colorimetric Xa formation assay carried out with a coagulation factor concentrate (containing factors X, IX, VII, protein C and protein S) (Fig.6) and in the colorimetric two-stage Xa formation assay (only depending on recombinant factor VIIa and isolated factor X) that excludes activation of factor X via the intrinsic pathway (not shown). To further corroborate that the procoagulant activity detected on activated platelets was TF-dependent, we added a functionally inhibitory anti-TF antibody (VIC7) and the chloromethylketone-inactivated TF ligand factor VIIai (Fig.6). Both the anti-TF antibody and VIIai caused a five fold decrease of procoagulant activity. These findings indicate that platelet activation results in a moderate increase in surface TF activity.

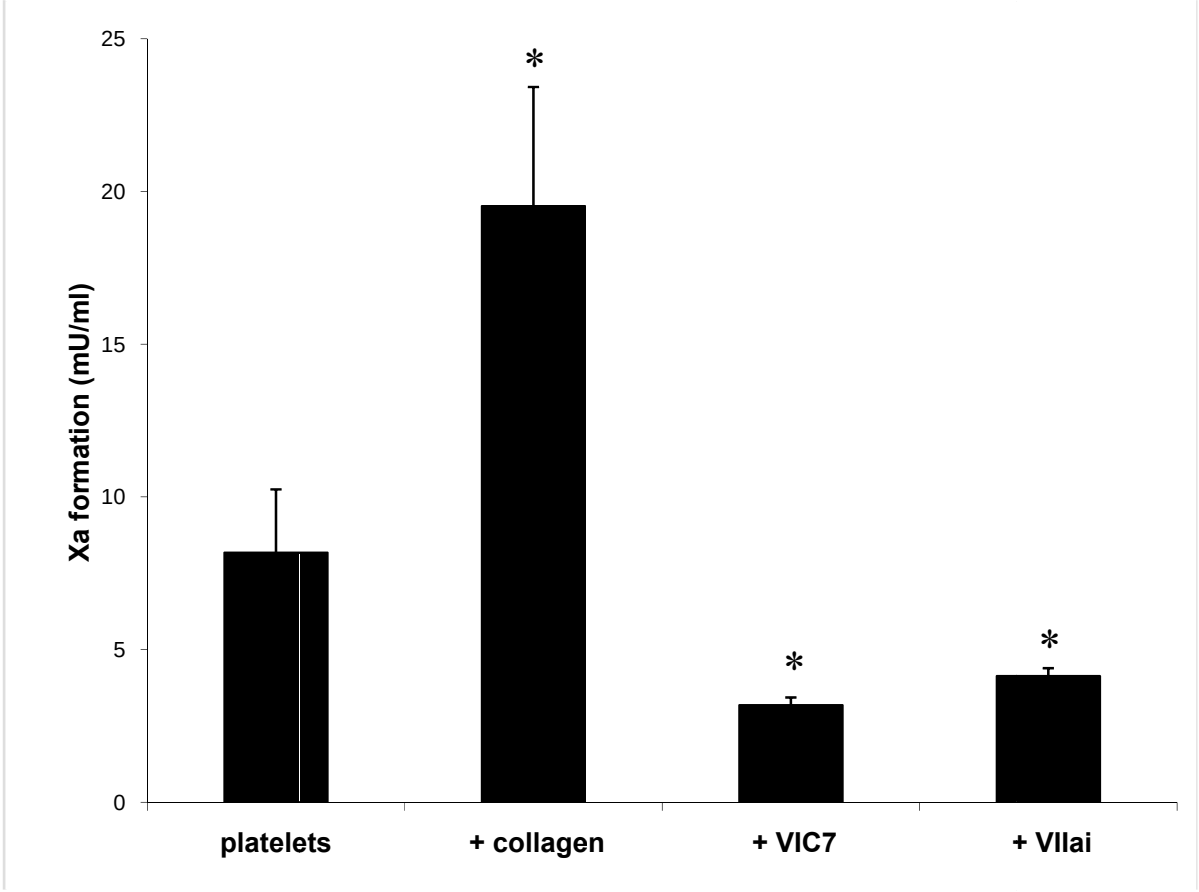

Fig.6: Procoagulant activity of collagen-stimulated (12 µg/ml) isolated platelets ($6x10^8$/ml). Factor Xa formation was detected using the coagulation factor concentrate (n = 5, mean +- standard deviation, $p < 0.001$). Stimulated platelets were pre-incubated with the functionally inhibitory anti-TF-VIC7 antibody (10 µg/ml) or with active site inhibited VIIa (VIIai) (10 nM) to show TF dependence.

We investigated whether the effect of factor Xa formation induced by collagen activated platelets could be blocked by inhibitors of the major platelet collagen receptors, GPVI and $\alpha_2\beta_1$-integrin. We found that the platelet procoagulant activity could be blocked completely by pre-incubation of the platelet suspension with a functionally inhibitory GPVI antibody (Fig.7). Pre-incubation with the soluble domain of GPVI as well as the antibody directed against the $\alpha_2\beta_1$-integrin partially inhibited the procoagulant activity.

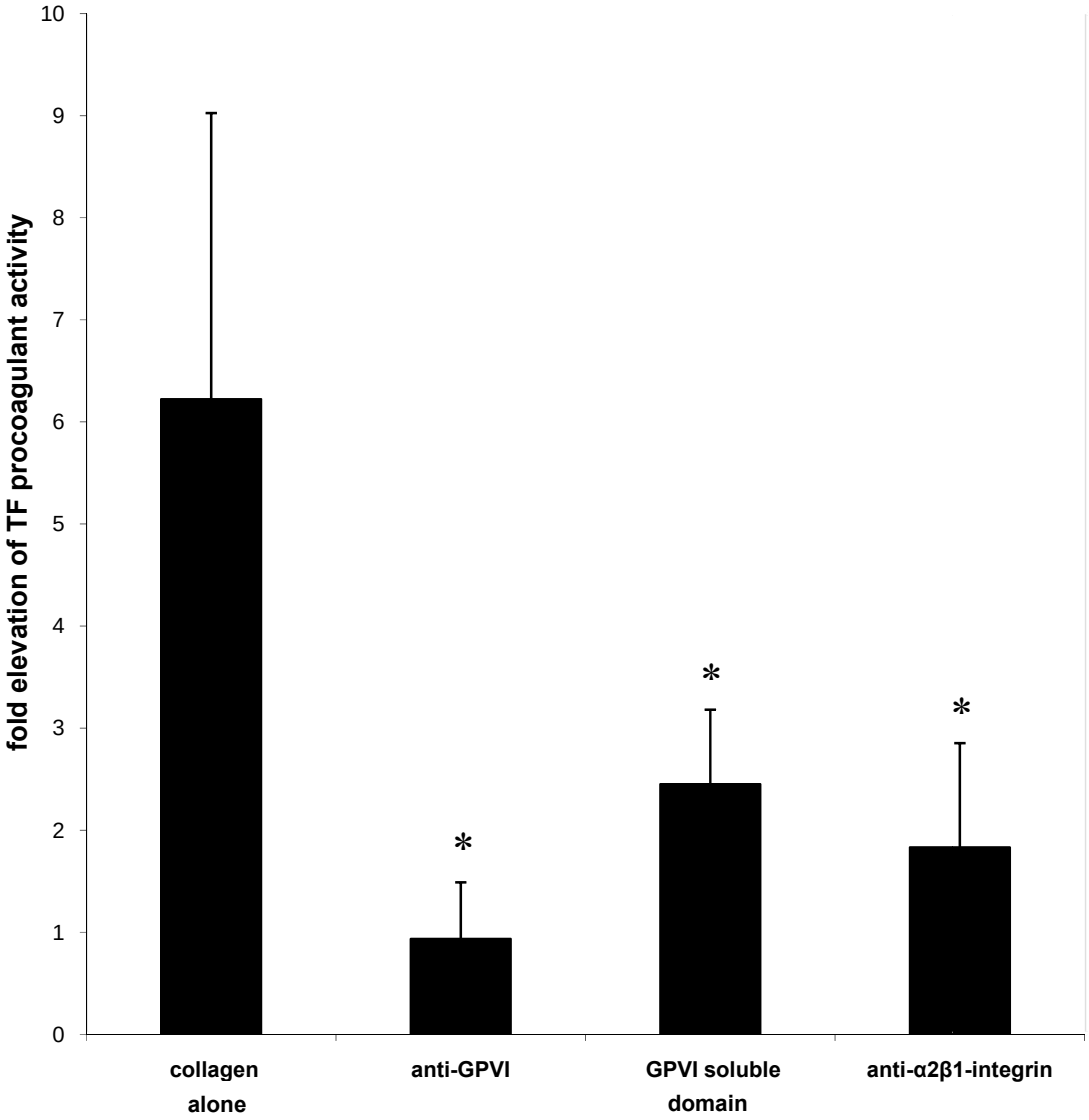

Fig.7: Procoagulant activity of collagen-stimulated (12 µg/ml) isolated platelets ($6x10^8$/ml). The concentrations of anti-GPVI inhibitory antibody, GPVI soluble domain and anti-α2β1-integrin antibody were in all cases 10 µg/ml (n = 3–9, means +- standard deviation, $p < 0.05$ vs collagen alone, coagulation factor assay).

Activated platelets are known to expose negatively charged phospholipids on their outer membrane leaflet. The TF activity measured on activated platelets was strongly decreased by preincubation of the activated platelets with annexin V, which specifically binds to negatively charged phospholipids in a Ca^{2+}-dependent manner (Fig.8).

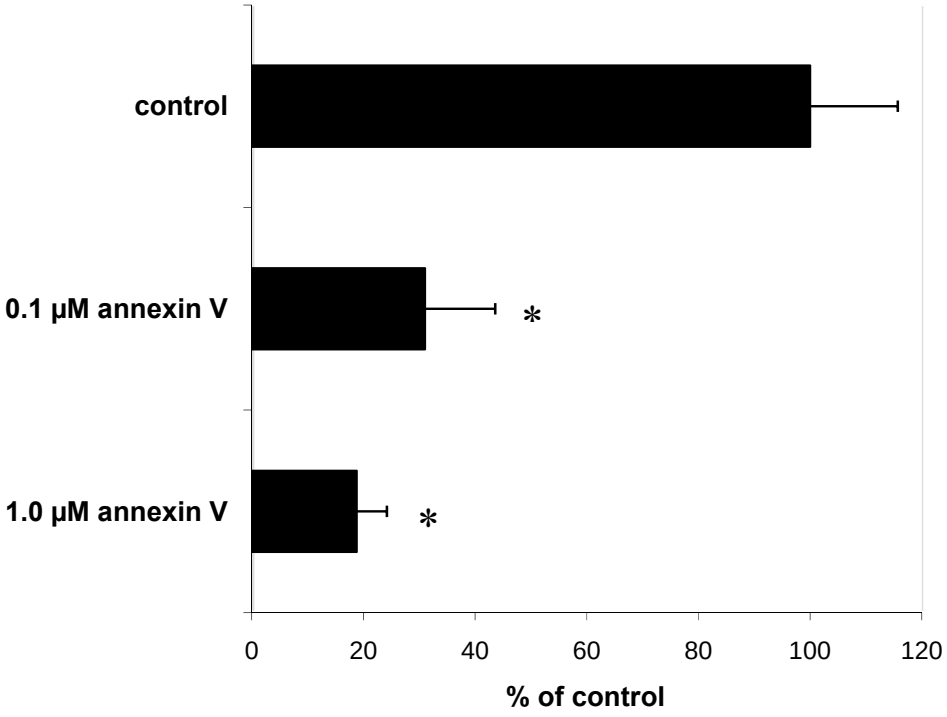

Fig.8: The procoagulant activity of collagen-stimulated (12 µg/ml) isolated platelets ($6x10^8$/ml) is decreased by annexin V in a concentration-dependent fashion (n = 3, $p < 0.01$, means +- standard deviation, coagulation factor assay).

In addition, we found that exposure of procoagulant activity on the surface of collagen-stimulated platelets is a rapid process, which is maximal after about two minutes (Fig.9).

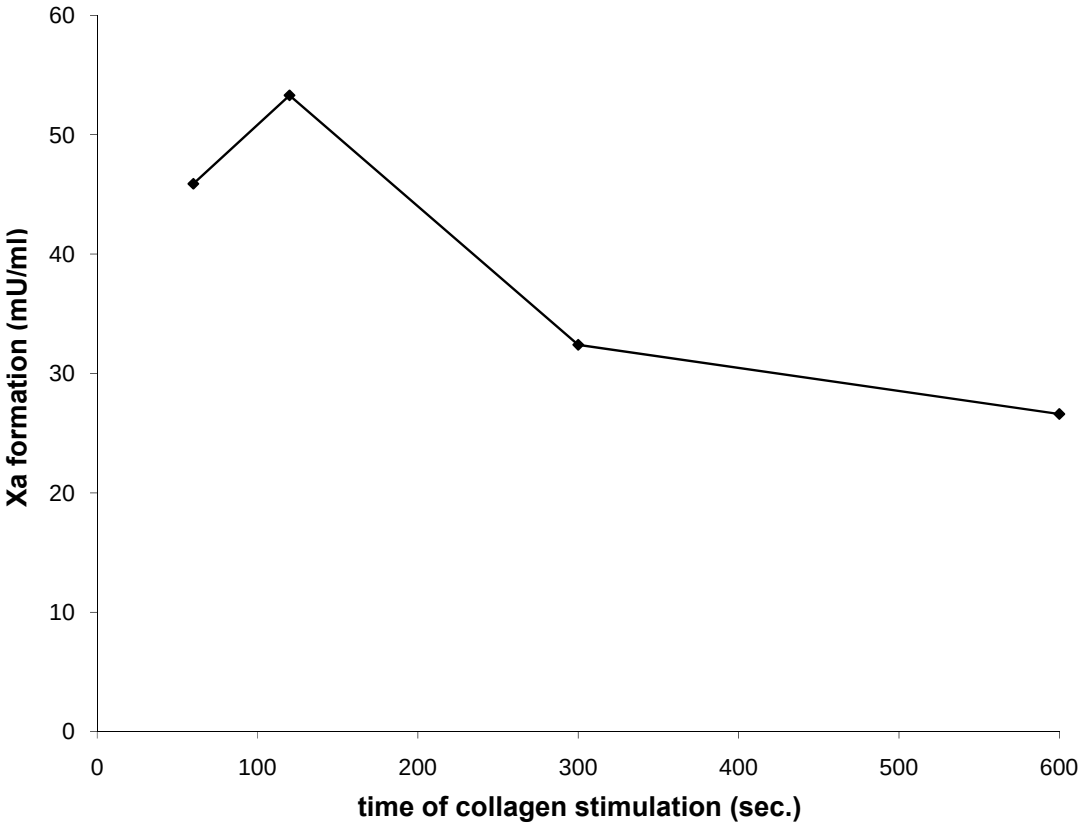

Fig.9: Time dependence of the procoagulant activity on the surface of collagen-stimulated (12 µg/ml) isolated platelets ($6x10^8$/ml) (coagulation factor assay). Representative experiment for a total of 8 experiments.

III.A.2 The TF procoagulant activity in collagen-activated platelets is largely encrypted

From our experiments on the functional activity of TF we hypothesize that TF procoagulant activity in platelets is largely encrypted. Indeed the total procoagulant activity of resting and collagen-activated platelets is lower than the procoagulant activity of Ca^{2+}-ionophore-treated (A23187) platelets (Fig.10). Ca^{2+}-ionophore (Kauffman RF, 1980) is known to de-encrypt TF procoagulant activity in various cell types (monocytes, HL-60 cells, fibroblasts)

(Bach RR, 1990; Drake TA, 1989). De-encryption of TF procoagulant activity in isolated platelets, however, is novel. After platelet activation with A23187 most of the TF procoagulant activity was found in the supernatant (Fig.10), whereas after collagen stimulation most of the TF procoagulant activity could be detected in the platelet pellet (Fig.10). Most likely A23187 strongly induces microparticle formation and therefore TF procoagulant activity is mostly recovered in the platelet supernatant.

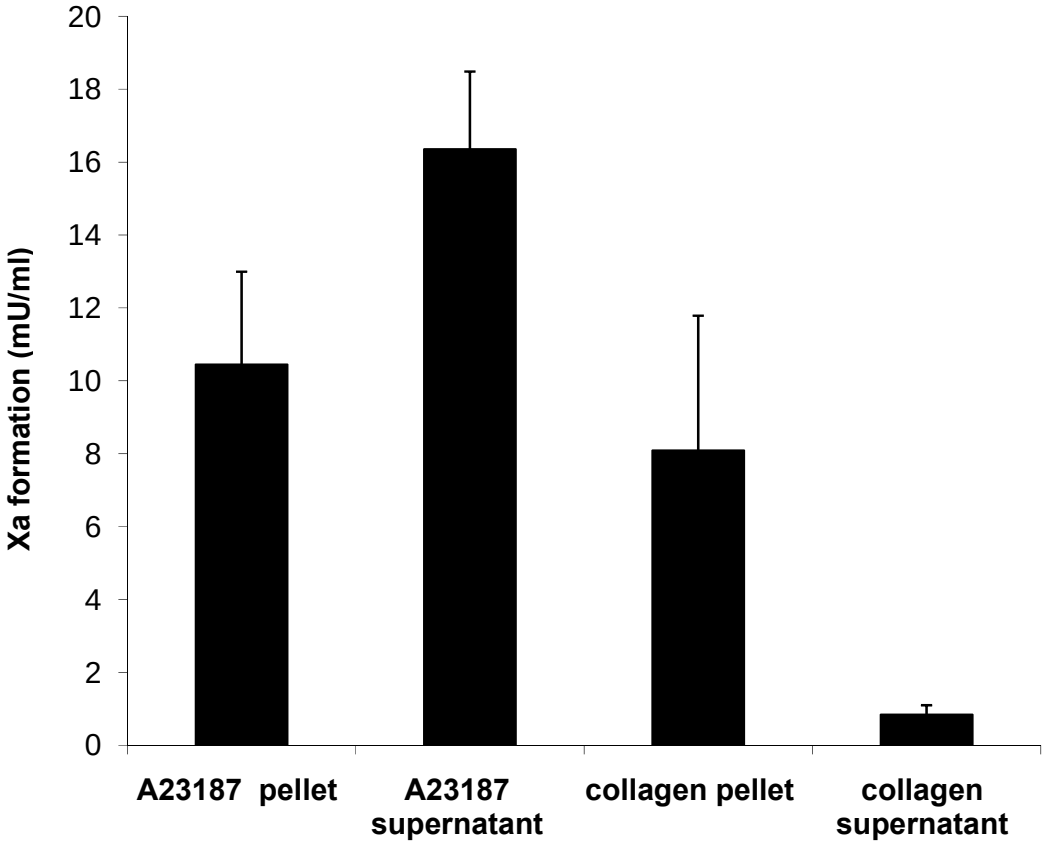

Fig.10: Activation of TF procoagulant activity in isolated platelets ($6x10^8$/ml). Xa formation was measured 120 seconds after platelet activation with Ca^{2+}-ionophore A23187 (10 μM) or collagen (12 μg/ml) with the coagulation factor assay (n = 4).

After activation with A23187 platelet procoagulant activity was rapidly released (Fig.11). In the supernatant, a high activity was already observed after 15 seconds, which strongly increased thereafter. In the pellet, the highest activity was already detected after 15 seconds.

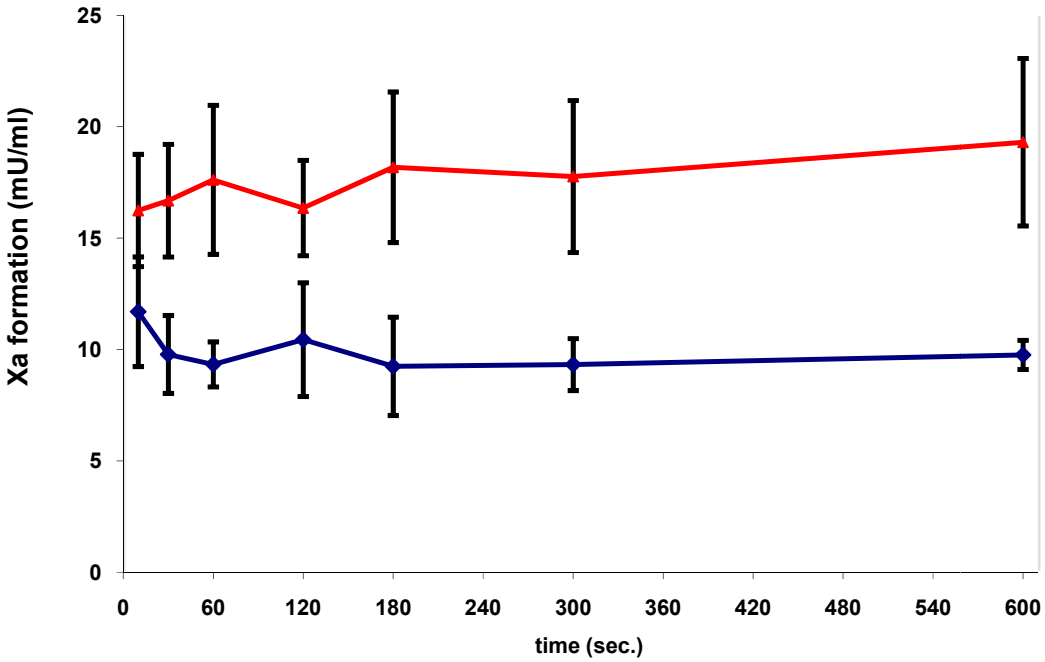

Fig.11: Time dependence of the release of procoagulant activity in the supernatant (red line) and the pellet (blue line) of A23187-stimulated (10 μM) isolated platelets ($6x10^8$/ml) (n = 8, coagulation factor assay).

III.B Decomposition of platelet TFPI by neutrophil serine proteases

III.B.1 Human polymorphonuclear neutrophils and isolated human neutrophil elastase (NE) evoke TF procoagulant activity

In 2003, Müller and coworkers described an increase of platelet TF procoagulant activity in collagen-stimulated platelet-leukocyte conjugates (Müller I, 2003). It has been known for several decades that phagocytic myeloid cells, such as neutrophils and monocytes bear cell-surface bound NE and Cathepsin G (CG), which are resistant to inactivation by plasmatic protease inhibitors (Campbell EJ, 1989; Owen CA, 1995; 1997; 1999). To test whether NE can initiate intravascular coagulation, collagen-stimulated isolated platelets were treated with increasing concentrations of NE. Thereby, a dose-dependent elevation in TF procoagulant activity was detected (Fig.12).

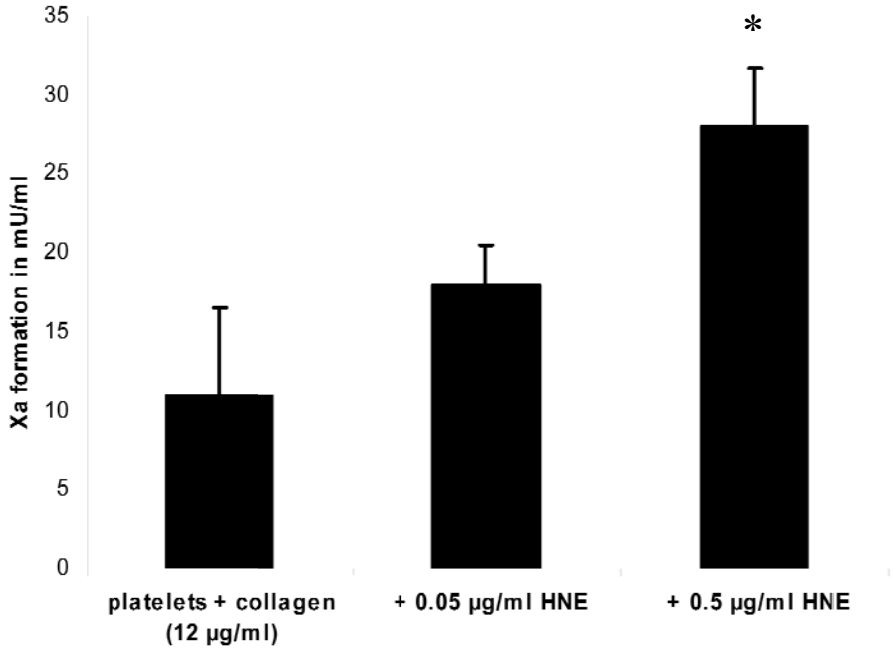

Fig.12: Dose-dependent release of procoagulant activity in collagen-stimulated isolated platelets ($6x10^8$/ml) by low concentrations of NE ($n = 4$, $p < 0.01$, coagulation factor assay).

III.B.2 NE is surface associated on myeloid blood cells and their microparticles

To investigate whether NE is present on microparticles released from activated polymorphonuclear neutrophils, flow cytometry experiments were performed in collaboration with Olivier Gasser (Immunonephrology Laboratory, University Hospital Basel / Switzerland). NE was found to be bound to the surface of PMA-activated neutrophils (Fig.13), confirming previous findings (Campbell EJ, 1989; Owen CA, 1995; 1997; 1999).

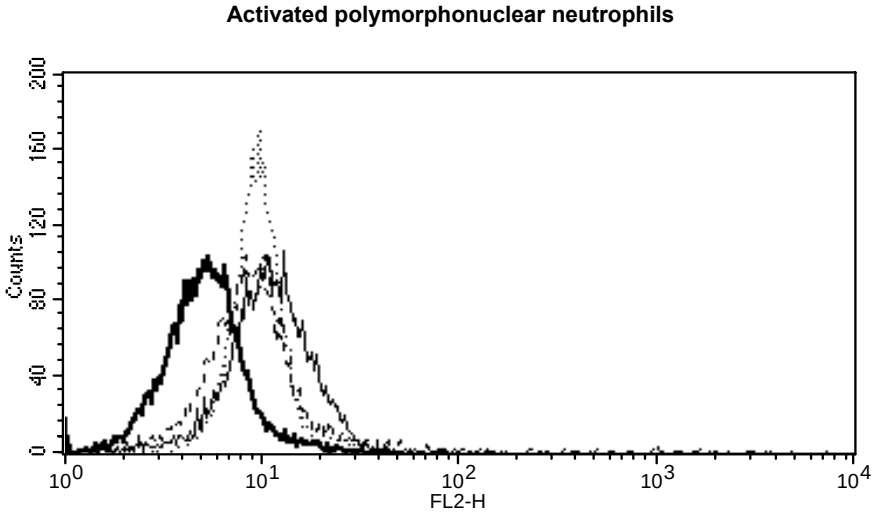

Fig.13: FACS analysis of PMA-activated isolated polymorphonuclear neutrophils. The bold line indicates an isotype control. The thin line indicates the activation marker CD11b and the thin, dashed line indicates CD11a. The bold, dashed line indicates the NE specific fluorescence signal.

Interestingly both microparticles derived from activated polymorphonuclear neutrophils and from activated peripheral blood monocytes were found to expose significant amounts of NE on the surface, while microparticles derived from activated platelets were devoid of NE (Fig.14).

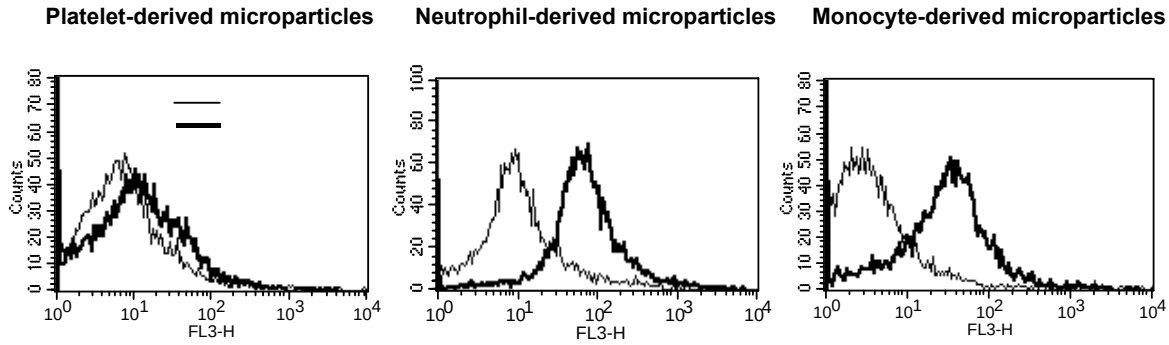

Fig.14: FACS analysis of microparticles derived from activated platelets, neutrophils and monocytes. The thin line indicates the fluorescence signal of the biotinylated isotype control antibody (detection with streptavidin-Cy5) and the bold line indicates the fluorescence signal of the NE-specific antibody.

III.B.3 Cell surface association of serine proteases results from polar interactions with glycosaminoglycans and with nucleic acids

The surface association of NE may result from polar interactions of positively charged patches on the enzyme surface with the negatively charged glycosaminoglycans (GAGs) on the neutrophil cell membrane (Kostoulas G, 1997). To test this, isolated neutrophils were pre-treated with chondroitin-4,5-sulfate which is a major component of neutrophil GAGs and with chondroitinase ABC, an enzyme degrading GAGs. Additionally, they could also result from the negatively charged backbone of nucleic acids (DNA, RNA), that are released by activated neutrophils within neutrophil extracellular traps (NETs) (Brinkmann V, 2004). Therefore, activated neutrophils were also pre-treated with nucleases (DNase I, RNase A) to degrade the NETs. Subsequently, platelet-neutrophil conjugates were investigated for their procoagulant activity to test whether surface association of NE is required for proteolytic inactivation of platelet TFPI. Procoagulant activity was found to be

diminished in platelet-neutrophil conjugates after pre-treatment of neutrophils with chondroitinase ABC or chondroitin-4,5-sulfate (Fig.15). This finding suggests that GAGs contribute to the surface association of NE, thereby providing a procoagulant microenvironment at the interface of platelet-neutrophil conjugates.

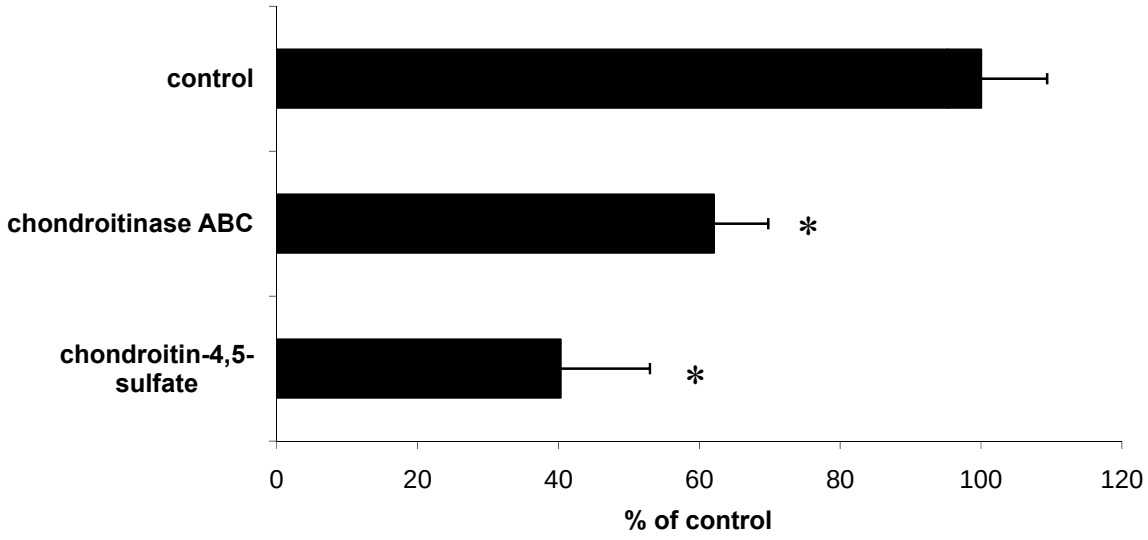

Fig.15: Neutrophils ($1x10^6$) were pre-treated with chondroitinase ABC (1U/ml) and chondroitin-4,5-sulfate (10 mg/ml). Subsequently they were washed and incubated with $1x10^7$ platelets (activated by collagen) to form platelet-neutrophil conjugates ($n = 4$, $p < 0.01$, coagulation factor assay).

Additionally, it was found that the increase in TF activity in platelet-neutrophil conjugates could be prevented by pre-treating the neutrophils with RNase A but not with DNase I (Fig.16). This finding suggests that NE might be mainly associated with the negatively charged backbone of RNA, rather than with that of DNA in NETs.

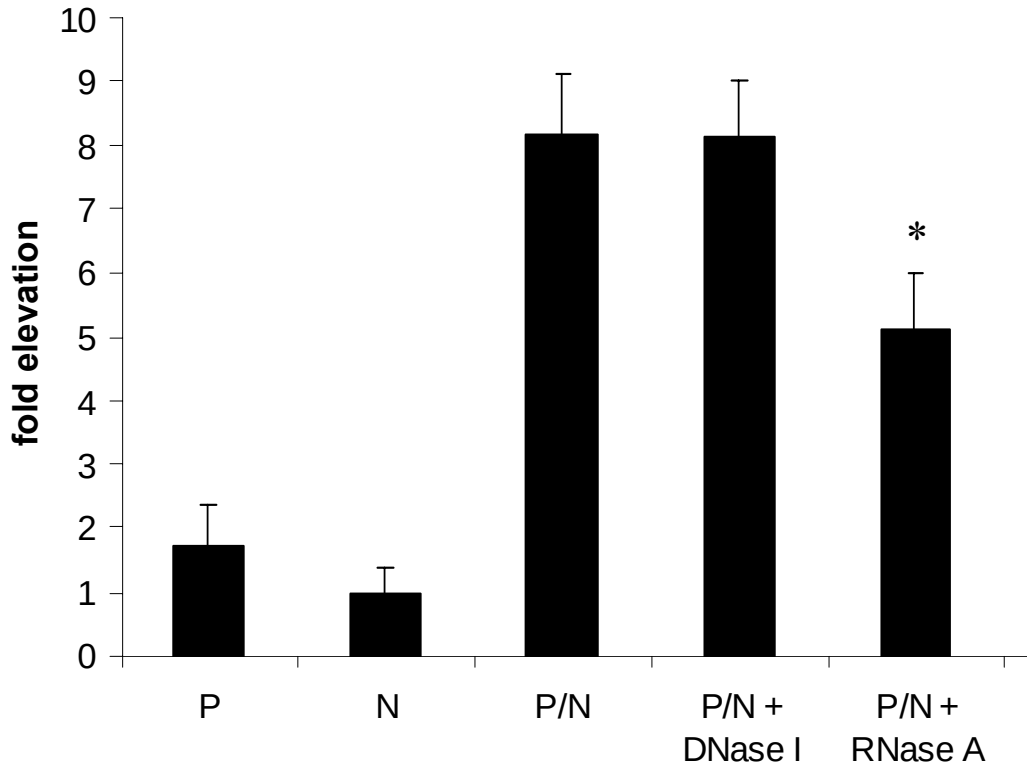

Fig.16: Control or DNase I (1U/ml) and RNase A (1U/ml) pre-treated isolated neutrophils ($1x10^6$) were incubated with activated platelets ($1x10^7$) and their procoagulant activity was measured ($n = 5$, $p < 0.05$, coagulation factor assay).

III.B.4 Platelet TFPI is degraded by NE in platelet-neutrophil conjugates

To further establish the role of neutrophil surface proteases in the initiation of intravascular coagulation we analysed the presence of TFPI in supernatants of platelet-neutrophil conjugates. In immunoblots of the supernatants efficient degradation of platelet TFPI was detected (Fig.17). To identify the neutrophil proteases responsible for this decomposition, isolated polymorphonuclear neutrophils were pretreated with specific protease inhibitors. TIMP-2 is a specific inhibitor of all MMP, whereas the applied chloromethylketone (CMK) is specific for human NE. α_1-antichymotrypsin (α_1-ACT) specifically blocks cathepsin G

(CG). TIMP-2 did not show any effect on the degradation of platelet TFPI in the platelet-neutrophil conjugates. The NE-specific CMK however blocked the degradation of platelet TFPI almost completely and pre-treatment of isolated neutrophils with α_1-ACT partially inhibited TFPI degradation (Fig.17). This indicates that NE and, to a lower extent CG, degrade TFPI and thereby might initiate intravascular coagulation on the surface of activated platelets.

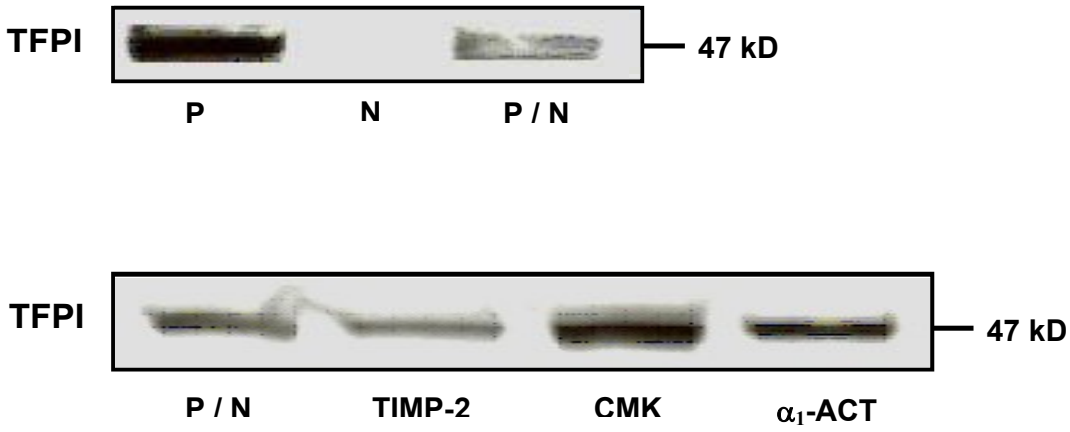

Fig.17: Degradation of platelet secreted TFPI in platelet-neutrophil conjugates (P/N) detected by Western blot analysis. Isolated neutrophils (N) were pretreated with 1 mM TIMP-2, a NE-specific CMK or the CG-specific protease inhibitor α_1-ACT.

The TFPI degradation products detected in immunoblots from suspensions of activated platelets and neutrophils and the degradation products achieved by limited proteolysis of the supernatant of stimulated platelets with isolated NE were of the same size and intensity, indicating that NE is the major protease that degrades TFPI in the cellular system (Fig.18).

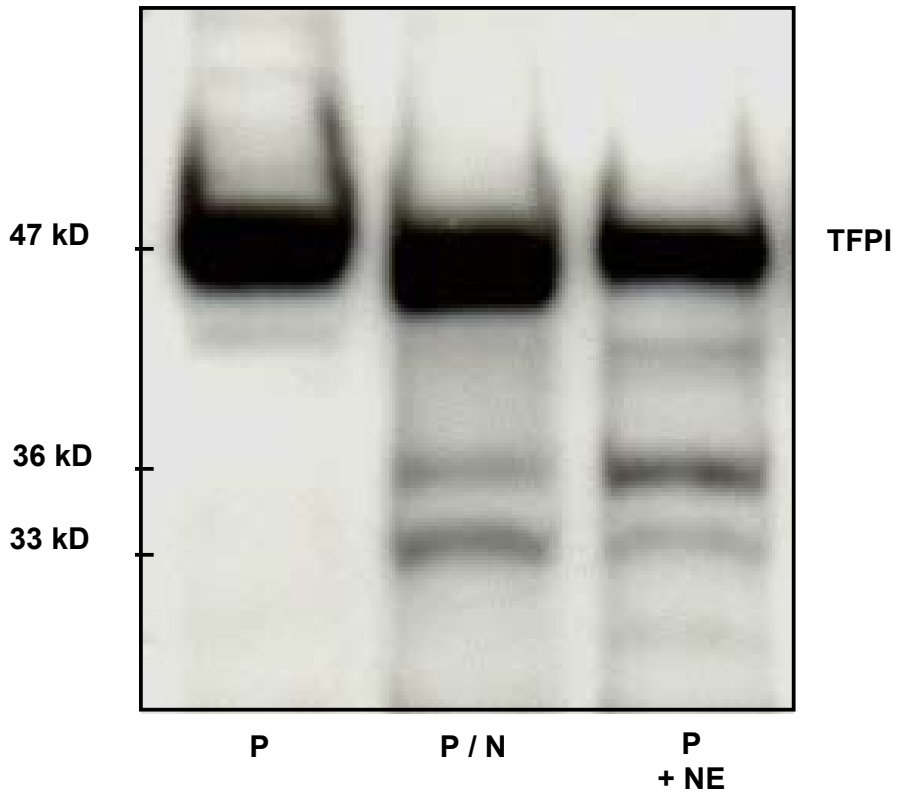

Fig.18: Degradation products obtained in platelet-neutrophil conjugates and those obtained by limited proteolysis of platelet supernatants with isolated human NE (15 μg/ml) detected by Western blot analysis.

In addition, isolated microparticles derived from fMLP-activated polymorphonuclear neutrophils (N-MP) were also capable of degrading platelet TFPI in a NE-specific manner when added to stimulated platelets. Degradation of platelet TFPI by N-MP could be prevented by pre-treatment of the N-MP with an NE-specific inhibitor (not shown).

III.C A disulfide switch in the TF molecule regulates its procoagulant activity

III.C.1 TF contains a labile disulfide that is essential for its procoagulant function

To assess the contribution of extracellular cysteines for TF activity, we substituted the cysteines C49/C57 and C186/C209 by serine. In C186S/C209S TF procoagulant activity was completely lost (Fig.19). Substitutions of C49 and C57 by serine had no effect on the procoagulant activity (Fig.19). Treatment of the C49S/C57S mutant with thioredoxin (TRX) or dithiotreitol (DTT) decreased the procoagulant activity. Phenylarsine oxide (PAO), a compound that binds to vicinal thiols thereby mimicking a disulfide, increased the procoagulant activity of the C49S/C57S mutant (Fig.19). This suggests that the oxidized state of the C186/C209 disulfide pair represents the active state of TF (in accordance with Ahamed, 2006).

To register the thiol states of TF we performed MPB-immunoblot experiments. MPB selectively recognizes free thiol groups. The oxidoreductase TRX reduced the labile disulfide bond in the extracellular TF domain (sTF), whereas addition of PAO oxidized the disulfide (Fig.20). This finding implies a physiological role for TRX in the inactivation (encryption) of TF procoagulant activity.

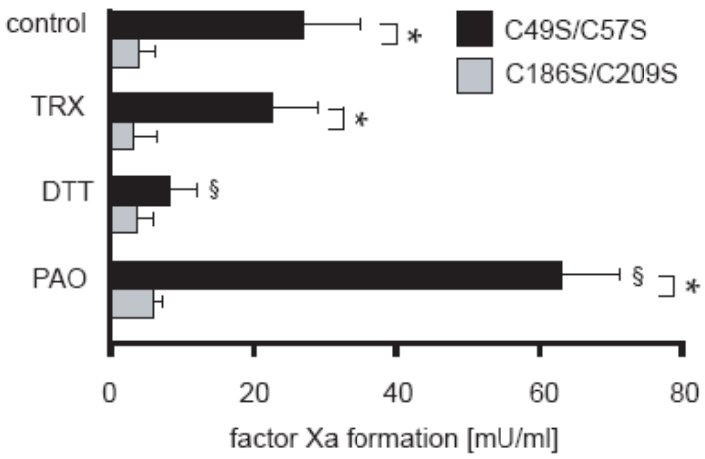

Fig.19: TF cysteine mutants (C49S/C57S, C186S/C209S) were treated with 10 µM TRX, 1 mM DTT or 100 µM PAO respectively (coagulation factor assay).

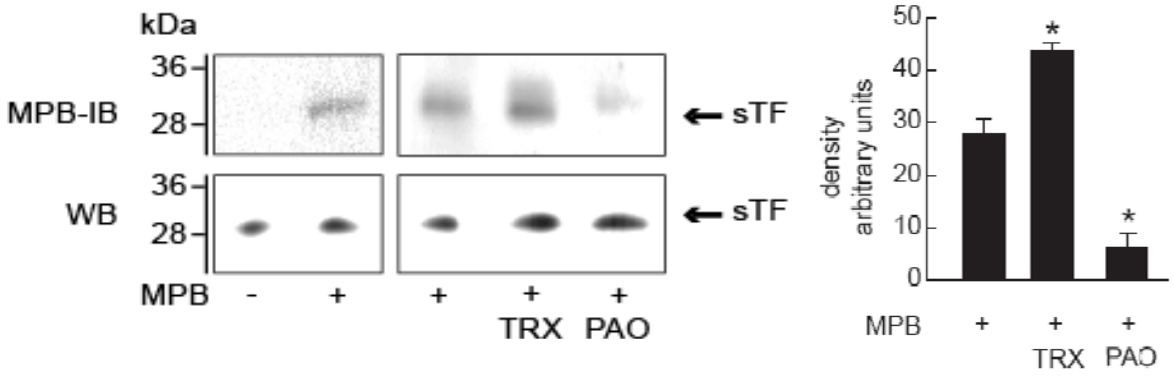

Fig.20: MPB-Immunoblot characterizing the dithiol oxidation state in the extracellular domain of sTF after treatment with TRX and PAO (left panel). Reduced dithiols in sTF were reacting with the thiol reactive compound MPB. A pull-down with streptavidin-agarose beads was performed, selecting for reduced dithiols in sTF. According to the control immunoblot MPB incorporation did not affect the binding of VIC12. The mean density of the sTF bands of n = 3 experiments was evaluated by using Scion Image software (right panel) and statistically analyzed by the Mann-Whitney-rank-sum-test ($p < 0.05$).

III.C.2 Protein Disulfide Isomerase oxidizes the C186/C209 pair

Activated platelets, which are known to decrypt TF, secrete protein disulfide isomerase (PDI). PDI is located to the external surface of the platelet plasma membrane (Chen K, 1995; Essex DW, 1995). This oxidoreductase could be involved in the regulation of the thiol state of TF. PDI was indeed found to oxidize the dithiol species of the C186/C209 pair of the sTF to its functionally active disulfide state (Fig.21). About 70 % of the active site cysteines of PDI were determined to be in the oxidized state (quantification of free PDI thiols by the colorimetric Ellman's assay). The supernatant of collagen-activated platelets (P-SN) is also capable of oxidizing sTF free dithiols (Fig.21). The oxidation of TF free dithiols to the disulfide could also be demonstrated on LPS-stimulated isolated monocytes, which are known to express encrypted TF on their cell surface (data not shown), indicating that also cell TF is present in the reduced state.

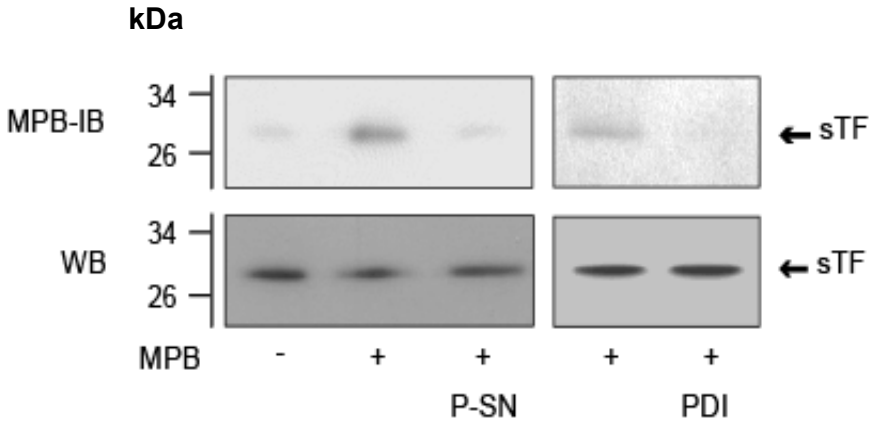

Fig.21: MPB-immunoblot characterizing the dithiol oxidation state in the sTF after treatment with the supernatant of activated platelets (P-SN) and recombinant PDI. Treatment with P-SN or PDI did not influence binding of VIC12 (control Western blot).

After incubation of LPS-stimulated monocytes with increasing concentrations of recombinant PDI an increase in TF procoagulant activity was observed (Fig.22). This increase was even stronger, when isolated LPS-stimulated monocytes were first incubated with recombinant human glutaredoxin (GRX) and subsequently with PDI. This suggests that protein S-glutathionylation potentially encrypts monocyte TF. The PDI-induced procoagulant activity was blocked by addition of the thiol alkylating agent dithionitrobenzoate (DTNB) or by pre-incubation of the stimulated monocytes with a neutralizing anti-TF antibody. Control experiments proved that the PDI-induced increase in procoagulant activity was both dependent on the TF vicinal thiols (alkylating free thiol groups on monocytes with DTNB blocked TF procoagulant function) and on the TF function (binding of a functional inhibitory antibody with an epitope close to C186/C209 prevents binding of VIIa). These findings imply a major role for PDI in the activation of encrypted TF and suggest the presence of a protein S-glutathionylated mixed disulfide TF species on LPS-stimulated monocytes.

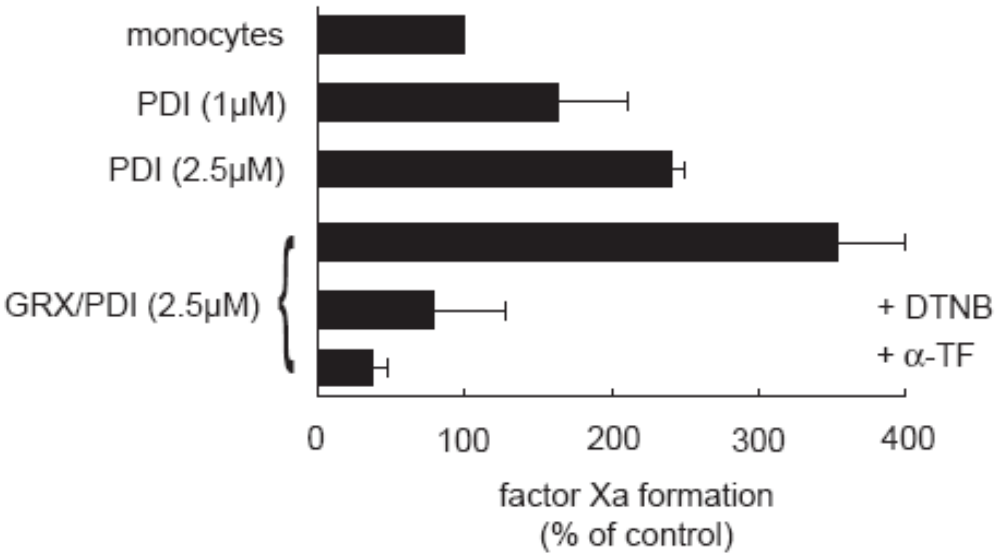

Fig.22: Increase in TF procoagulant activity of LPS-stimulated monocytes after incubation with increasing concentrations of recombinant PDI and of recombinant human GRX followed by PDI incubation (n = 4, coagulation factor assay).

In whole blood TEG experiments the clotting time of collagen-activated blood was delayed by an anti-PDI antibody when compared to the appropriate buffer control and the IgG2a isotype control (Fig.23). This indicates a significant role for the PDI-triggered activation mechanism of TF in the whole blood system.

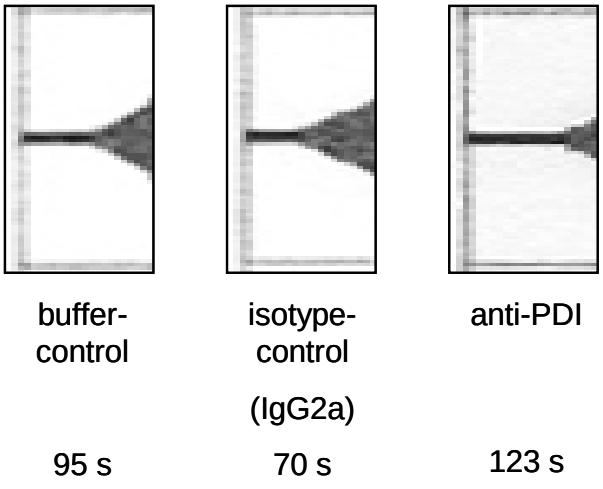

Fig.23: TEG experiment of collagen-activated whole blood (collagen activates intravascular TF) incubated with equal volumes of resuspension buffer, IgG2a isotype antibody or functional inhibitory anti-PDI antibody (n = 3). The clotting time of one representative experiment is indicated in seconds.

Treatment of sTF with GRX increased the reduced fraction of the labile disulfide pair, whereas pre-incubation with GRX and subsequent treatment with PDI increased the formation of disulfides (Fig.24). This suggests the presence of constitutive protein S-glutathionylation of sTF.

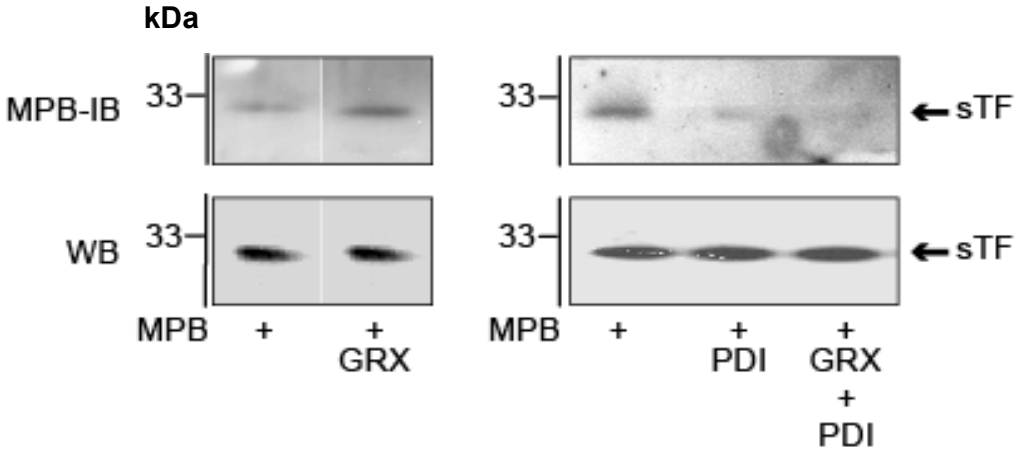

Fig.24: MPB-immunoblot characterizing the dithiol oxidation state of sTF. sTF was pre-treated with GRX, PDI or GRX plus PDI. The addition of GRX or PDI did not affect MPB incorporation of TF (control Western blot).

III.C.3 Glutathionylation of C186/C209 vicinal thiols of TF

Cysteine residues in proteins can be glutathionylated if they are surface exposed and if the binding of gluthathione is facilitated by an appropriate electrostatic protein surface. We tested, whether TF might undergo this common cysteine modification. In a CHO cell system transfected with full-length TF we found that wild type TF (wt) could be glutathionylated by reaction with biotin-labeled glutathione (biotin-GSH), whereas the C186S/C209S mutant could not (Fig.25). The C186/C209 disulfide pair is solvent accessible and has all the structural requirements to undergo protein S-glutathionylation. However, the C49/C57 disulfide pair does not undergo a reaction with biotin-GSH, since no signal was detected in the C186S/C209S TF mutant (Fig.25, right panel).

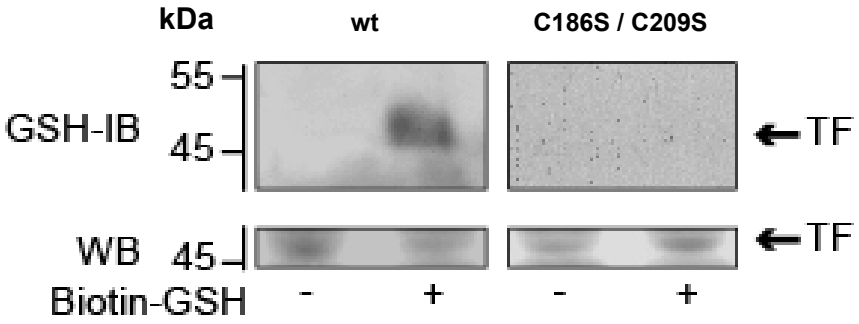

Fig.25: GSH-immunoblot detecting in vitro protein S-glutathionylated TF on transfected CHO cells. Cells were incubated with 125 µM biotin-GSH and 100 µM diamide that is acting as an oxidant. After cell lysis a biotin-selective pull-down with streptavidin-agarose was performed. TF antigen was detected with the VIC12 anti-TF antibody. According to the control Western blot, binding of VIC12 was not affected by treatment of sTF with biotin-GSH or the serine mutation.

In Pulmonary Artery Vascular Smooth Muscle Cells (PAVSMC), which are known to express high levels of TF antigen, the labile cysteine pair of the TF molecule was also found to be susceptible to in vitro protein S-glutathionylation (Fig.26). This protein S-glutathionylation could be reversed by subsequent treatment of the cells with low concentrations of GRX or DTT, indicating that biotin-GSH is covalently bound to TF via a disulfide linkage.

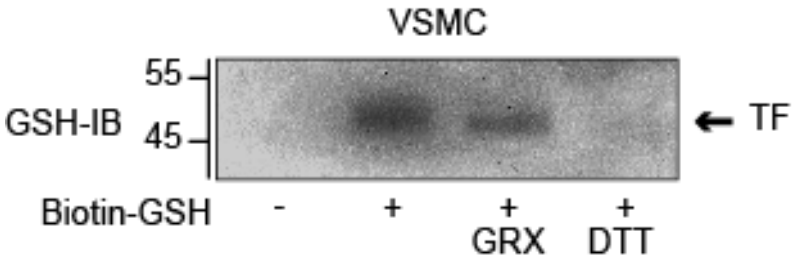

Fig.26: GSH-immunoblot detecting in vitro protein S-glutathionylated TF on PAVSMC. A biotin-selective pull-down with streptavidin-agarose was performed and TF was detected with the anti-TF antibody (VIC12).

III.C.4 In vitro protein S-glutathionylation of TF is reversible

To test whether cell lysate containing the oxidoreductases GRX and PDI might regulate the protein S-glutathionylation of TF we examined its incorporation of biotin-GSH. Thereby, we could distinguish whether GRX and PDI act as a reductant or as an oxidant. We observed that pre-treatment of sTF with CHO cell lysate prevented the incorporation of biotin-GSH into TF (Fig.27). In addition, the reductants DTT and GRX also prevented biotin-GSH incorporation. Moreover, protein S-glutathionylation of sTF was slightly reversed by PDI.

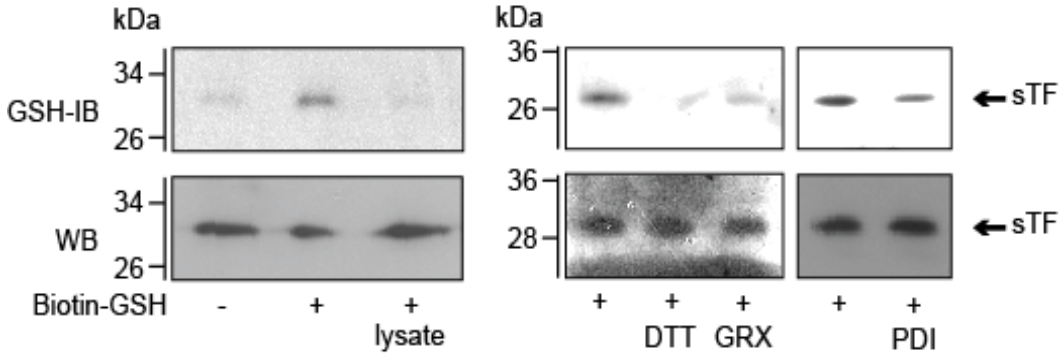

Fig.27: GSH-immunoblot detecting in vitro protein S-glutathionylation of TF. A biotin-selective pull-down with streptavidin-agarose was performed and TF was detected with the VIC12 anti-TF antibody. According to the control Western blot, binding of VIC12 was not affected by treatment of sTF with biotin-GSH, CHO cell lysate, DTT, GRX and PDI.

IV. Discussion

IV.1 TF procoagulant activity of activated platelets

Recent studies indicate that there is a pool of TF in the blood circulation, so-called intravascular or blood borne or circulating TF (Giesen PL, 1999; Engelmann et al., 2003). Giesen and co-workers reported blood borne TF in thrombi formed on collagen-coated glass slides that were exposed to flowing human blood. The collagen-coated glass slides were devoid of TF, implying that TF procoagulant activity is derived from human blood. TF in the formed thrombi was found to be active, since abundant fibrin was detected. Independently, our research group identified TF in peripheral human blood from healthy donors. TF antigen appeared on the surface of collagen-activated platelets adhering to leukocytes (Zillmann A, 2001). This is a rapid process, since TF antigen was already detectable after 5 minutes of stimulation. Therefore, de novo synthesis of TF is not possible. It is still a matter of debate how platelets acquire TF. According to one hypothesis TF is transported to platelets by leukocyte-derived microparticles (Del Conde I, 2005). Alternatively, the spliceosome of proplatelets that extend from megakaryocytes is capable of translating TF from pre-mRNAs (Denis MM, 2005; Schwertz H, 2006). It was shown that intravascular TF was competent to start coagulation and TF antigen was detected in isolated platelets, whereas no TF antigen was detectable in isolated resting neutrophils and rapidly isolated monocytes (Zillmann A, 2001). However, this study did not contain direct evidence for the presence of TF procoagulant activity on the surface of activated platelets.

We detected a rapid increase in TF procoagulant activity of isolated human platelets after a 10 minute stimulation with collagen type I (in agreement with Camera, 2003). When the isolated platelets were pre-incubated with a functional inhibitory anti-TF antibody, the procoagulant activity was completely blocked. This shows that the formation of factor Xa

by activated platelets is TF dependent. TF procoagulant activity was also substantially blocked by pre-incubation of isolated platelets with a functional inhibitory anti-GPVI antibody, but only slightly blocked by pre-incubation with an antibody directed against $\alpha_2\beta_1$-integrin. This implicates a pivotal role for GPVI-dependent kinase signalling in the surface exposure of TF on the platelet membrane.

IV.2 Encryption of platelet Tissue Factor activity

TF encryption indicates the post-translational suppression of TF procoagulant activity on the cell surface (Bach RR, 2006). A discrepancy between TF antigen and the expression of TF procoagulant activity has previously been observed in a variety of cell types (Maynard JR, 1977; Walsh JD, 1991; Drake TA, 1989). Treatment of cells with the Ca^{2+}-ionophore A23187 is known to de-encrypt TF procoagulant activity (Bach RR, 1996; 2006).

Treatment of platelets with Ca^{2+}-ionophore resulted in a three times higher procoagulant activity compared to collagen-treated platelets, indicating that about two thirds of the total platelet TF procoagulant activity might be encrypted in collagen activated platelets. While most of the procoagulant activity was found in the pellet after collagen treatment, nearly no activity was observed in the supernatant. However in Ca^{2+}-ionophore treated samples most of the TF activity was found in the supernatant, which might be due to the formation of procoagulant platelet microparticles (Henriksson CE, 2006).

The underlying mechanism leading to the increase of TF activity after treatment with Ca^{2+}-ionophore is not understood (Bach RR, 2006). It was suggested that the exposure of the cofactor phosphatidylserine (PS) contributes only partially to the increase of TF procoagulant activity in LPS-stimulated monocytes and non-transformed human dermal fibroblasts (Wolberg AS, 1999). In our experiments, the increase in platelet procoagulant

activity evoked by the treatment with Ca^{2+}-ionophore could not be fully suppressed in the presence of saturating concentrations of the PS binding protein annexin V (Fig.8), indicating that the increase in TF procoagulant activity did not result solely from increased exposure of PS. Therefore, other yet unknown posttranslational mechanisms have to be involved in encrypting TF procoagulant activity on the cell surface of platelets.

IV.3 Neutrophil surface proteases trigger the TF procoagulant activity in platelet-neutrophil conjugates

TFPI is the physiologic inhibitor of the initiator complex of coagulation. Recently, a glycosyl phosphatidylinositol (GPI) anchored variant of TFPI was characterized on the surface of cultured endothelial cells (Zhang J, 2003). A GPI anchor was predicted and identified for the TFPI splice variant β lacking Kunitz domain 3 but not for the TFPI splice variant α. TFPI-α was found to be the most abundant form expressed by endothelial cells. It was described recently that TFPI-β is responsible for the bulk of the cellular VIIa/TF inhibitory activity (Piro O, 2005). However, in our study we focussed on TFPI-α. This variant was found to be by far the predominant splice form in platelets (Bidzhekov K, TFPI mRNA analysis, personal communication).

Recombinant human TFPI-α is known to be highly susceptible to limited proteolysis by neutrophil serine proteases, such as NE and CG (Petersen LC, 1992; Higuchi DA, 1992) and further to coagulation proteases like thrombin (Ohkura N, 1997) and plasmin (Salemink I et al, 1998) as well as to MMP (Belaaouaj AA, 2000; Cunningham AC, 2002). In previous studies performed in our laboratory an increased TF procoagulant activity was detected in platelet-neutrophil conjugates compared to isolated neutrophils or collagen-activated platelets (Müller I, 2003). Based on these data a model for the initiation of

intravascular coagulation was suggested (Engelmann B, 2003). In this model, neutrophil proteases were proposed to inactivate platelet TFPI in a microenvironment formed by platelet-neutrophil conjugates (Fig.4). The platelet-neutrophil microenvironment is mainly dependent on P-selectin / PSGL-1 interactions formed between activated platelets and polymorphonuclear neutrophils (Müller I, PhD thesis; McEver RP, 1997; Palabrica T, 1992). Platelet-neutrophil adhesion (firm adhesion) is further stabilized by fibrinogen bridges formed by the activated platelet receptor GPIIb/IIIa and the leukocyte receptor Mac-1 (CD11b/CD18) (Spangenberg P, 1993).

To experimentally address this hypothesis, collagen activated platelets were treated with increasing concentrations of isolated human NE. A concentration dependent increase in platelet TF procoagulant activity was noted. This finding implies that NE is able to trigger the release of TF procoagulant activity on activated platelets. Since NE is the major serine protease stored in the azurophilic granules of neutrophils it appears likely that this enzyme is also responsible for the increased TF procoagulant activity in platelet-neutrophil conjugates. In immunoblot experiments of the supernatants obtained from platelet-neutrophil conjugates, rapid degradation of platelet TFPI was noted (Fig.17). This was markedly reduced by pre-treatment of neutrophils with a NE-specific CMK inhibitor. This suggests that in platelet-neutrophil conjugates TFPI is most efficiently degraded by NE. In confirmation of this conclusion, the degradation products obtained in platelet-neutrophil conjugates migrated at the same size as those obtained by treatment of activated platelets with isolated human NE (Fig.18).

In addition, we observed degradation of platelet TFPI by neutrophil-derived microparticles in the supernatant of collagen-activated platelets (data not shown). This finding could suggest a novel role for these plasma components in the initiation of intravascular coagulation.

Since TF and its physiologic inhibitor TFPI are both stored in the platelet α-granules and are exposed on the membrane after platelet activation, it appears likely that TF in activated platelets is rapidly inactivated by the formation of a quarternary complex consisting of TF/VIIa and Xa/TFPI. If the concentrations of functionally active TFPI are substantially lowered by NE, the whole coagulation cascade may in principle proceed on the membrane surface of activated platelets. This would enable coagulation to take place exactly at the site of the growing thrombus. Hence the thrombus could be efficiently stabilized by the fibrin polymers.

IV.4 Characterization of the procoagulant microenvironment formed between activated platelets and polymorphonuclear neutrophils

Since a procoagulant microenvironment was found to be formed mainly by P-selectin / PSGL-1-interactions between activated platelets and polymorphonuclear neutrophils (Müller I, PhD thesis) we decided to further investigate the conditions leading to a local intravascular coagulation start.

In this context, it was of major interest to test whether NE is bound to the surface of activated neutrophils and their microparticles or if it is simply secreted into the surrounding media. We detected NE on the surface of activated neutrophils and on the surface of microparticles derived from activated neutrophils and monocytes by FACS analysis. NE is able to bind negatively charged cell surface structures once released from the azurophilic granules of myeloid cells. Proteoglycans, glycosaminoglycans and neutrophil extracellular traps (NETs) are negatively charged structures present on the surface of activated neutrophils. Proteoglycans and glycosaminoglycans contain negatively charged sulfate groups (Hornebeck W, 1994) and the backbones of nucleic acids, which are contained in

the NETs of activated neutrophils (Brinkmann V, 2004) consist of negatively charged phosphate esters. NE possesses a highly asymmetric distribution of positively charged residues that are solvent exposed (Navia MA, 1989), which enables them to bind to polyanionic surfaces. Cell surface bound NE could be removed by pre-treatment of neutrophils with chondroitinase ABC and by chondroitin-4,5-sulfate. NE could also be removed from the surface of PMA-activated neutrophils by treatment with RNase A, but not with DNase I. As a consequence of the NE removal, the proteolytic activity of the neutrophils that maintains TF activity was decreased. These findings suggest a major role for different polyanionic cell surface molecules for the inactivation of TFPI.

TFPI decomposition may play a significant role in pathologies associated with acute and chronic inflammation. In atherosclerosis chronic inflammation of the vessel wall is induced by transmigration of monocytes into the vascular wall and their differentiation to macrophages and foam cells. An atherosclerotic plaque is formed. Rupture of a specific type of plaque leads to arterial thrombus formation, acute myocardial infarction and stroke (Fuster V, 2005). Sepsis is another inflammatory disorder resulting in activation of coagulation. It is associated with widespread blood clotting in various organs (disseminated intravascular coagulation) (Creasey AA, 1993). Both, arterial thrombosis and septic shock are leading causes of mortality in many countries.

Disseminated intravascular coagulation is a frequent complication that can finally lead to multiple organ failure. It is becoming increasingly clear that coagulation and innate immunity have coevolved early in eukaryotic development, and that these systems continue to function as a highly integrated unit following tissue injury (Opal SM, 2003). In disseminated intravascular coagulation during sepsis leukocytes are activated (Grisham MB, 1988) and the surface expression of platelet adhesion molecules is increased (Gawaz M, 1995). The occurrence of platelet-leukocyte aggregates was also found to be increased in this study. We therefore assume that the neutrophil-dependent proteolytic mechanism of

coagulation activation might play a role in the development of disseminated intravascular coagulation and multiple organ failure.

IV.5 Disulfide switch of TF regulates initiation of intravascular coagulation on monocytes – potential role for TF encryption

Thiol-disulfide exchange is an emerging mechanism of cell surface protein regulation (Hogg PJ, 2003). From its x-ray structure (Harlos K, 1994) TF was found to contain a labile disulfide bond that could potentially be subject to rapid disulfide switching (Hogg PJ, 2003; Schmidt B, 2006). TF is a member of the class 2 cytokine receptor family (Bazan JF, 1990) and its x-ray structure revealed an unusual disulfide bridging cysteine 186 with cysteine 209 inside an antiparallel β-sheet. This disulfide bridge is characteristic of the cytokine type 2 receptor family. It lies at the very end of domain 2 and links adjacent strands F and G. The sulphur atoms of this S-S bond point away from the surface of the β-sheet towards the solvent (Harlos K, 1994). It has been established by site directed mutagenesis experiments that the integrity of this energetically unfavourable disulfide in TF is required for the binding of factor VII (Rehemtulla A, 1991), which defines its role as the cofactor responsible for the coagulation start (Ruf W, 1998). Residues close to the allosteric disulfide have been identified to interact with factors IX and X (Kirchhofer D, 2000).

To assess the role of extracellular cysteines for TF function, we generated different mutants with cysteine to serine substitutions. Experiments performed with the C49S/C57S and C186S/C209S mutants showed that the C186S/C209S double mutant was devoid of TF procoagulant activity. The C49S/C57S double mutant of TF, in contrast, was functionally active. The procoagulant activity of the C49S/C57S mutant could be partially decreased by treatment with the oxidoreductase TRX, which is known to reduce disulfide bonds to

vicinal thiols. Indeed TRX has been shown to reduce factor VIII and other coagulation factors (Savidge G, 1979). The TF procoagulant activity of C49S/C57S was profoundly decreased by the chemical disulfide reductant DTT. Treatment of the C49S/C57S double mutant with PAO that is known to coordinate with vicinal thiols increased TF activity. Coordination of vicinal thiols with PAO mimics a disulfide linkage, which is likely functional, suggesting that oxidation of the C186/C209 pair de-encrypts TF.

These functional data could be confirmed in thiol-selective immunoblot experiments in which the amount of reduced dithiols in sTF was analysed in a semiquantitative manner. sTF exhibited proper folding (Andersson D, 2001). Indeed nearly the same content of secondary structures was observed as to be deduced from the protein's x-ray structure. The fold of sTF was tested by recording a circular dichroism (CD) spectrum and the content of secondary structure was calculated by deconvolution of the spectrum. With this method it could be excluded that sTF is denaturated by reduction of the C186/C209 disulfide with 1 mM DTT (Appendix A2).

In contrast to TRX, the addition of PDI turned out to increase the TF procoagulant activity of isolated LPS-stimulated monocytes. Inhibition by the anti-TF antibody and by treatment of the cells with DTNB unambiguously demonstrated activation of TF by thiol-disulfide exchange. Moreover, thiol-selective immunoblots analysing the amount of free thiol groups of PDI-treated sTF and cell TF are in agreement with the conclusion that PDI oxidizes TF. Recently, TF was found to co-immunoprecipitate PDI, which confirms that TF is a substrate for PDI (Ahamed J, 2006). Our findings thus imply a major role for PDI in the de-encryption of latent TF procoagulant activity on the cell surface of monocytes and other cell types that express TF constitutively (Fig.22, 23, 24). Collagen-activated platelets were also found to have an oxidative effect on the allosteric C186/C209 dithiol of sTF. Activated platelets are known to release thiol isomerases that are capable of regulating disulfide modifications (Essex DW, 2001). In addition, activated platelets are known to de-encrypt

the TF procoagulant activity of monocytes (Østerud B, 2001). According to our results this finding can now be explained by the release of platelet PDI that catalyses thiol-disulfide interchange of monocyte TF.

IV.6 Protein S-glutathionylation of TF – a potential safety device

Protein S-glutathionylation is currently emerging as a common posttranslational cysteine modification of various proteins and it is a main form of protein S-thiolation (Ghezzi P, 2005). The term protein S-glutathionylation indicates the formation of mixed disulfides between proteins and glutathione (GSH). The thiol oxidoreductase glutaredoxin (GRX) reverses protein S-glutathionylation by a thiol / disulfide exchange mechanism (Ghezzi P and Bonetto V, 2005). Proteins of the plasma membrane of cells exposed to blood, such as TF are at the interface between an oxidizing (plasma) and a reducing environment (cytoplasm) (Fahey RC, 1977). In particular, protein S-glutathionylation is associated with the stabilization of extracellular proteins and the protection of proteins against irreversible oxidation of critical cysteine residues (Biswas S, 2005).

After incubation with GRX and subsequent PDI addition, the TF activity of monocytes was significantly higher than after treatment with PDI alone. Based on these results, we hypothesize that monocyte TF might be protein S-glutathionylated and that glutathionylation could be involved in TF encryption. We were able to confirm S-glutathionylation of TF by different methods (thiol-selective immunoblots of sTF, *in vitro* glutathionylation of vascular smooth muscle cells and of TF cysteine mutants in a CHO cell system).

Cell lysis is known to de-encrypt the TF activity in various cell types (Drake TA, 1989). Hence we were interested if CHO cell lysates that are completely devoid of TF are able to

reverse the protein S-glutathionylation of glutathionylated sTF. In fact, the lysate of CHO cells strongly diminished protein S-glutathionylation of sTF. This suggests that the release of thiol oxidoreductases during cell rupture, such as GRX and PDI, could activate latent TF. In the case of vascular injury cell lysis of several vascular wall cells occurs, such as fibroblasts, vascular smooth muscle cells and endothelial cells. Thereby, thiol oxidoreductases could act as a response to injury preventing lethal blood loss of the organism by activation of encrypted TF. Damage of vascular wall cells also occurs during atherogenesis (Schecter AD, 1997). This could indicate a significant role for thiol oxidoreductases in the initiation of intravascular coagulation during the rupture of atherosclerotic plaques.

Recently, platelet deposition, TF accumulation and fibrin generation were observed in a mouse cremaster muscle arteriole after laser-induced endothelial injury (Falati S, 2002). The observed fibrin formation could result from activation of intravascular TF and / or of vascular wall TF. Falati and co-workers favour a major proportion of TF carried by microparticles. The observed increase of TF antigen at the site of injury could result from TF-bearing platelet microparticles (Muller I, 2003) and from monocyte microparticles (Falati S, 2003) that are recruited to the site of injury via GPVI-mediated adhesion to collagen (Penz S, 2005; Suzuki H, 2003) or by P-selectin / PSGL-1 interactions. The TF-bearing microparticles subsequently adhere to the activated platelets in the growing thrombus that expose P-selectin on their surface. Thereby the integrity of the growing thrombus could be additionally stabilized by fibrin polymerisation (Falati S, 2003). The procoagulant activity of TF-bearing microparticles, when present alone, was found to be limited (Müller I, 2003; Engelmann B, 2006). In the murine thrombosis model the latent TF of platelet and monocyte microparticles might be rapidly activated by the thiol oxidoreductases locally released by damaged cells from the injured vessel wall and by activated platelets, which are the major component of the growing thrombus.

PDI and GRX are not the only proteins that are known to reside in the ER and may regulate the procoagulant activity of TF. Recently the ER chaperone 78-kDa glucose regulated protein (Grp78) was found to inhibit TF procoagulant activity by physically interacting with the extracellular domain of TF (Watson LM, 2003; Bhattacharjee G, 2005). Both, VIIa generation and Xa formation were blocked in Grp78 overexpressing cells. It was shown that Grp78 is present on the surface of endothelial cells and monocyte / macrophage-like cells in atherosclerotic lesions. Moreover, the secretion of factor VII and a mutant form of tissue plasminogen activator is increased in response to Grp78 overexpression, whereas von Willebrand factor and factor VIII secretion is decreased (Dorner AJ, 1988; 1990;1992).
These findings might suggest that both PDI and Grp78 are secreted from the ER after cell activation (Chen K, 1995; Bhattacharjee, 2005) and could play a significant role in the regulation of intravascular TF procoagulant activity.

IV.6 Model for the redox regulation of intravascular TF activity

Based on our findings on the regulation of TF activity by a disulfide switch of cysteines 186 and 209 we suggest a model (Fig.28) how TF procoagulant activity might be de-encrypted on the surface of microparticles and activated platelets inside the growing thrombus. Our mechanistic model would give an explanation for the observed rapid fibrin formation in the murine laser-injury thrombosis model (Falati S, 2002, 2003).
We hypothesize that both damaged cells and activated platelets release intracellular oxidoreductases (GRX, PDI) leading to a change of the redox state in the nascent thrombus. Subsequently mixed TF disulfides, such as glutathionylated TF are reduced by GRX leading to a reactive TF intermediate which contains a reduced C186/C209 disulfide pair. The conformation of this reduced TF state is well ordered (CD spectra, Appendix A2) and

therefore the dithiol in position C186/C209 is readily oxidized by PDI to the appropriate disulfide. From our experiments it appears that the oxidized disulfide species of TF represents the only TF species capable of initiating coagulation.

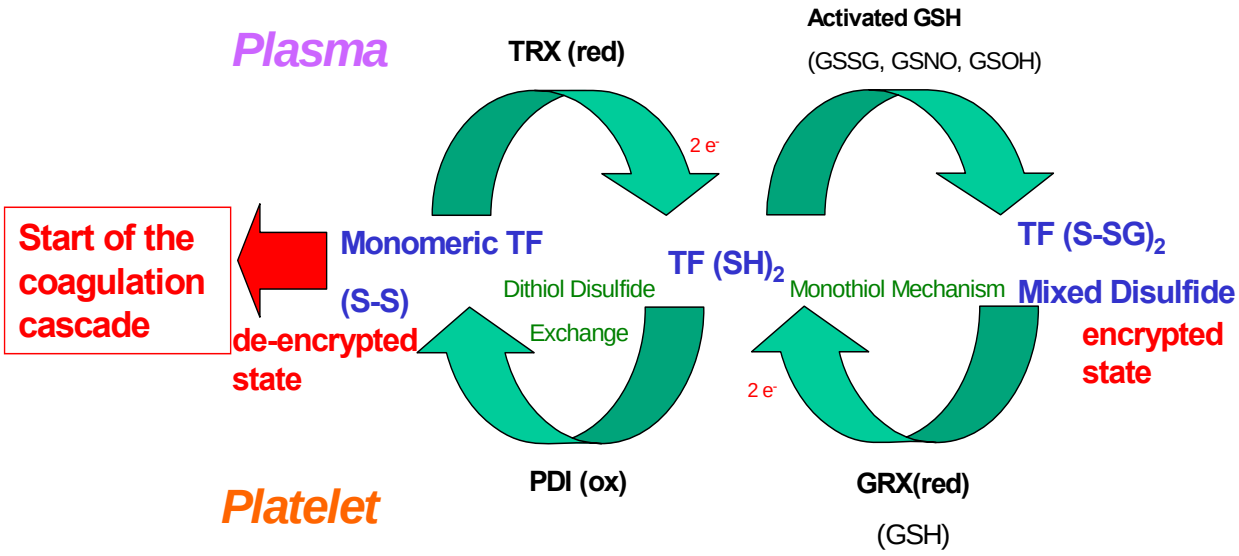

Fig.28: Model of redox regulation of TF. The oxidized C186/C209 disulfide pair (S-S) is suggested to represent the functionally active state of TF, whereas all other TF species are unable of initiating coagulation.

V.1 Summary

Both activated platelets and circulating microparticles were described to express tissue factor (TF), the principal initiator of coagulation, on their cell surface (intravascular TF). It is still not clear whether TF is functionally active on activated platelets. TF expressed on activated monocytes and various other cell types has been described to be functionally inactive (encrypted or latent TF). In the present study, cellular mechanisms are analyzed that could release the TF procoagulant activity of blood components.

Tissue factor pathway inhibitor-1 (TFPI) represents the main physiologic inhibitor of the coagulation start. It inhibits the ternary initiator complex of the extrinsic coagulation pathway by first binding the circulating factors X / Xa and subsequently interacting with VII / VIIa. We found that after stimulation with thrombin and collagen type I, TFPI was recovered in the platelet releasate and it was degraded by neutrophil elastase (NE) released from activated neutrophils. TFPI degradation was also induced by NE on neutrophil microparticles. We found that NE is bound to negatively charged macromolecules (proteoglycans, RNA) on the surface of activated neutrophils by polar interactions. Overall, we could provide substantial experimental evidence that upon interaction of activated platelets with PMN a microenvironment is formed, which allows the efficient degradation of TFPI by the PMN-associated serine protease NE. This cross talk between the innate immune system and the coagulation system might be of general importance in pathologies, such as sepsis, arterial and venous thrombosis and myocardial infarction.

TF-encryption has represented an unsolved problem for several decades. We reveal that thiol-disulfide exchange in the extracellular C186/C209 disulfide pair of TF triggers the TF procoagulant activity. Formation of the intramolecular C186/C209 disulfide activates TF procoagulant function, whereas reduction of the disulfide to the appropriate sulfhydryls and the formation of mixed disulfides (protein S-glutathionylation of TF) were found to

suppress its procoagulant function. TF activation is supported by the thiol isomerase protein disulfide isomerase (PDI) and it is facilitated by the reactive oxygen species (ROS) detoxifying enzyme glutaredoxin (GRX). Protein S-glutathionylation of TF was uncovered as one reversible mechanism preventing the intravascular coagulation start.

We suggest that thiol isomerases are injury-responsive signals driving coagulation through posttranslational cysteine modifications of TF. This mechanism could help to explain the augmented occurrence of vasoocclusive pathologies during ageing, where increasing concentrations of ROS might favour TF oxidation.

V.2 Zusammenfassung

Das Membranprotein Tissue Factor (TF) ist das zentrale Startermolekül der Blutgerinnung und wird schnell auf aktivierten Thrombozyten und auf Monozyten exponiert (intravasaler TF). Es ist noch unklar, ob TF auf aktivierten Plättchen funktionell aktiv ist. Der von stimulierten Monozyten und von verschiedenen anderen Zellen exprimierte TF wurde als funktionell inaktiv (encrypted oder latent) beschrieben. In der vorliegenden Studie wurden molekulare Mechanismen der TF-Aktivierung untersucht.

Tissue Factor Pathway Inhibitor-1 (TFPI) ist der wichtigste physiologische Inhibitor des Gerinnungsstarts. Er inhibiert den ternären Starterkomplex des extrinsischen Blutgerinnungssystems, indem er zunächst an den Faktor X / Xa und anschliessend an den TF gebundenen Faktor VII / VIIa bindet. Der im Überstand aktivierter Plättchen vorhandene TFPI wurde nachweisbar durch die neutrophile Serinprotease Neutrophile Elastase (NE) abgebaut. Auch mit Mikropartikeln aus Neutrophilen wurde ein Abbau beobachtet.

Unsere Ergebnisse bestätigen die Hypothese, dass durch die Interaktion von aktivierten Plättchen mit Neutrophilen ein Mikroenvironment gebildet wird, das den effektiven Abbau

von TFPI durch Neutrophilen-assoziierte NE ermöglicht und auf diese Weise den intravasalen Gerinnungsstart steuert.

Auf der Zelloberfläche exprimierter TF ist kryptisch (inaktiv). Die Ursachen für die TF Enkryption sind seit mehreren Jahrzehnten Gegenstand der Forschung und sind bislang ungeklärt. Wir fanden, dass ein Oxidoreductase-abhängiger Thiol-Disulfid-Austausch des extrazellulären C186/C209-Disulfidpaars von TF den Gerinnungsstart durch Monozyten induziert. Die Bildung der C186/C209 Disulfidbrücke aktiviert die prokoagulatorische Funktion im TF-Molekül, während Reduktion zu den entsprechenden Sulfhydrylen und die Bildung gemischter Disulfide (Protein S-Glutathionylierung von TF) dessen prokoagulatorische Funktion unterdrücken. Die Aktivierung von TF wird durch die Thiolisomerase Protein Disulfid Isomerase (PDI) gefördert, ein Prozess, der durch die Wirkung des reaktive Sauerstoffspezies (ROS) entgiftenden Enzyms Glutaredoxin (GRX) erleichtert wird. Die Protein S-Glutathionylierung von TF wurde als einer der reversiblen Mechanismen identifiziert, der den intravasalen Gerinnungsstart verhindert und dadurch zur Enkryption beiträgt. Unsere Ergebnisse lassen vermuten, dass Thiolisomerasen bei Gefässverletzungen als Signale wirken, welche die Gerinnung mittels posttranslationaler Cystein-Modifikationen des TF-Moleküls propagieren. Dieser Mechanismus ist möglicherweise am verstärkten Auftreten vasookklusiver Pathologien im Alter beteiligt, bei denen ROS die Oxidation von TF fördern könnte.

VI. References

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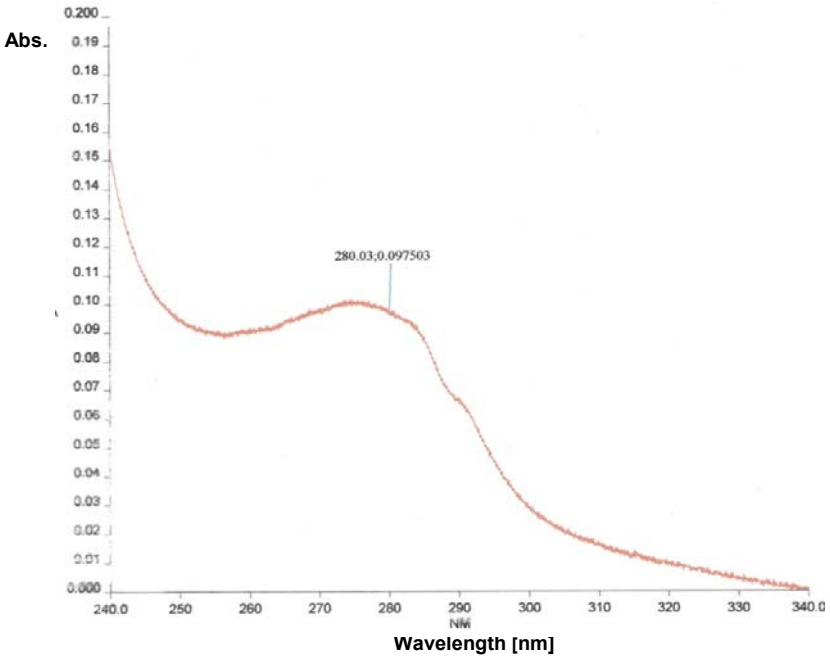
VII Appendix

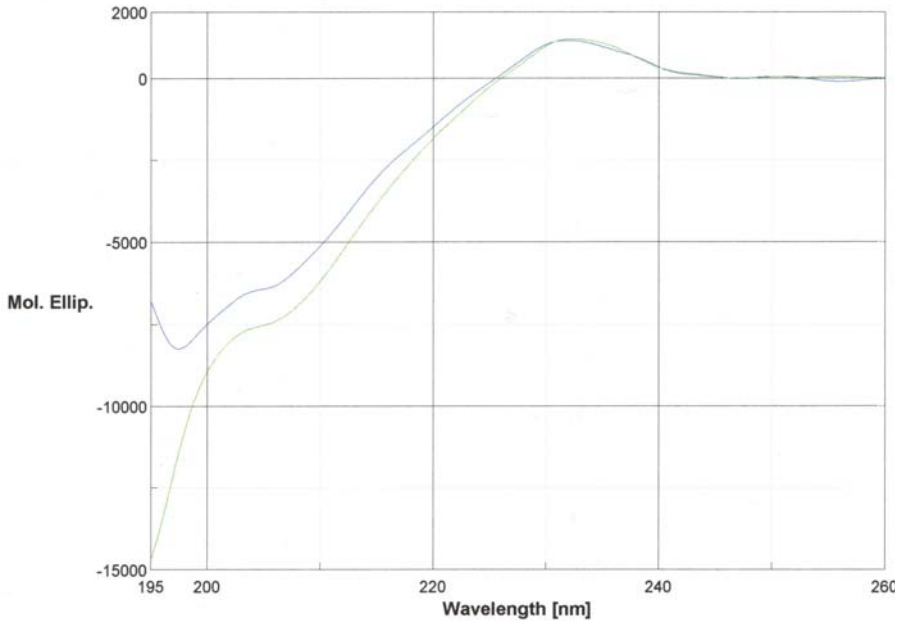
VII.A UV-spectrum and Circular Dichroism-spectra of the recombinant TF extracellular domain

A1



A1: UV-spectrum of sTF in PBS recorded with a Perkin-Elmer spectrophotometer.

A2



A2: CD-spectrum of sTF in PBS recorded with a JasCo Circular Dichroism spectrophotometer. Both the native (blue) and the 1.5 mM DTT- treated (green) sTF spectrum contain ordered structural elements that are similar to that determined in the x-ray structure.

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Publication List

Mittl P.R.E., Luthy L., **Reinhardt C.** and Joller H. 2003. Detection of High Titers of Antibody against *Helicobacter pylori* Cysteine-Rich Proteins A, B, C, and E in *Helicobacter pylori*-Infected Individuals. *Clinical and Diagnostic Laboratory Immunology* 2003;10(4):542-545.

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Reinhardt C, Grahl L, Bidzhekov K, Morgenstern E and Engelmann B. March 26-29th, 2006. Neutrophil surface proteases – activators of the intravascular Tissue Factor, 85th annual meeting of the German Physiological Society and Joint meeting of the Federation of European Physiological Societies, Munich, Germany (poster presentation). *Abstract was short listed for the FEPS Young Investigators Award poster session.*

Grahl L, von Bruehl M, **Reinhardt C**, Manukyan D, Altmann B, Dlugai S, Konrad I, Lorenz M, Mackman N, Massberg S, Engelmann B. July 6th-12th, 2007. Protein Disulfide Isomerase (PDI) uncovers Tissue Factor (TF)-dependent fibrin formation after vessel injury. Congress of the International Society on Thrombosis and Haemostasis, Geneva, Switzerland. *Abstract was awarded with an ISTH Young Investigators Award to Davit Manukyan.*

Hiermit erkläre ich, die vorliegende Arbeit eigenständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

München im Februar 2007,

Christoph Reinhardt