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Coagulation and inflammation in experimental endotoxemia

in vitro and *in vivo*

-monitoring method and effects of nicotinamide

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Saltare necesse est

-dancing is necessary

ABSTRACT

In gram negative sepsis, endotoxin from the bacterial membrane elicits proinflammatory and procoagulant host responses. Sepsis and its frequent complication disseminated intravascular coagulation (DIC) are leading causes of morbidity and mortality in the intensive care. A pivotal mechanism in the pathogenesis of DIC is the expression of tissue factor (TF) on circulating monocytes after endotoxin encounter. Activation of the cascade systems of coagulation and inflammation can lead to a rapid deterioration in e.g. sepsis with procoagulant changes and microthromboses, resulting in DIC and multi organ failure. Monitoring of the degree of coagulation activation, to discover changes before clinical exacerbation, is critical.

We have established a global monitoring method for coagulation activity, clotting onset time (COT), based on free oscillating rheometry. The assay design mimicks the natural blood milieu, as the method is based on $CaCl_2$ repletion of blood or plasma, adding no artificial activators or inhibitors. The COT method proved to be a quick and reliable test, able to detect hypo- and hypercoagulation. It also showed promising results as a bedside monitoring method, able to detect the transient activation of coagulation caused by intravenous injection of endotoxin to healthy volunteers. We also demonstrate that the COT method is sensitive even to small changes in the amount of endotoxin induced monocyte surface TF. In neurotrauma patients, COT was a predictor of prognosis for the patients. This observation, however, is difficult to interpret and requires further investigation.

The vitamin B derivative nicotinamide was assessed for its potential modulating effects on endotoxin induced activation of coagulation and inflammation. Nicotinamide is a known PARP inhibitor. PARP is necessary for activation of the transcription factor NFκβ, responsible for transcription of many genes involved in the response to endotoxin, e.g. proinflammatory cytokines, giving a rationale for a potential beneficial effect of nicotinamide in endotoxemia. We demonstrate that nicotinamide is a potent inhibitor of three major endotoxin induced proinflammatory cytokines, IL-1β, IL-6 and IL-8, in addition to the previously known TNFα inhibiting effect. However, the dose dependent inhibition of endotoxin induced proinflammatory responses was shown unlikely to be due to PARP inhibition.

In endotoxin stimulated leukocyte suspensions as well as in whole blood, nicotinamide caused a dose dependent decrease of monocyte TF expression, describing a previously unknown inhibitory effect of nicotinamide on the procoagulant changes associated with endotoxemia. We have also demonstrated that the decrease of monocyte TF expression is at least partly caused by shedding from the monocyte surface. Our conclusion is that nicotinamide may have a therapeutic potential in modulating conditions associated with activation of coagulation and inflammation, such as in sepsis and DIC.

Key words: sepsis, endotoxin, tissue factor, cytokines, nicotinamide, coagulation, inflammation

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

I. Measurement of blood and plasma coagulation time using free oscillating rheometry.
Ungerstedt JS, Kallner A, Blombäck M
Scand J Clin Lab Invest 2002; 62(2): 135-140

II. Clotting onset time may be a predictor of outcome in human brain injury –a pilot study.
Ungerstedt JS, Grenander Å, Bredbacka S, Blombäck M
J Neurosurg Anesthesiol. 2003 Jan; 15(1): 13-18

III. Nicotinamide is a potent inhibitor of proinflammatory cytokines.
Ungerstedt JS, Blombäck M, Söderström T
Clin Exp Immunol 2003 Jan; 131 (1):48-52

IV. Whole blood coagulation activation after challenging healthy volunteers with intravenous endotoxin.
Ungerstedt JS, Soop A, Sollevi A, Blombäck M
Submitted

V. Nicotinamide inhibits endotoxin induced monocyte TF expression.
Ungerstedt JS, Heimersson K, Söderström T, Hansson M
In revision, Journal of Thrombosis and Haemostasis

ABBREVIATIONS

aPTT	activated partial thromboplastin time
ARDS	adult respiratory distress syndrome
COT	clotting onset time
CRP	C-reactive protein
CTI	corn trypsin inhibitor
CV	coefficient of variation
DIC	disseminated intravascular coagulation
ELISA	enzyme linked immunosorbent assay
F1+2	prothrombin fragment 1+2
FOR	free oscillating rheometry
GOS	glasgow outcome scale
ICAM-1	intercellular adhesion molecule-1
LBP	LPS binding protein
IL-	interleukin
LPS	lipopolysaccharide (endotoxin)
MAP kinase	mitogen activated protein kinase
MFI	mean fluorescence intensity
MOF	multi organ failure
NFκβ	nuclar factor κβ
PAI-1	plasminogen activator inhibitor-1
PARP	poly (ADP) ribose polymerase
PBS	phosphate buffered saline
PT (INR)	prothrombin time (international normalized ratio)
SEM	standard error of the mean
TAFI	thrombin activatable fibrinolysis inhibitor
TAT	thrombin-antithrombin complex
TBS	tris buffered saline
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TNF	tumor necrosis factor
t-PA	tissue type plasminogen activator
u-PA	urokinase type plasminogen activator

CONTENTS

INTRODUCTION

Hemostasis

Hemostasis includes all processes that stop or prevent bleeding and thrombosis. Hemostasis comprises intravascular cells, soluble coagulation factors and inhibitors, products released from or attached to the endothelium, and factors of the fibrinolysis system. The pathogenesis of thrombosis was outlined by Virchow in 1856. He described the triad leading to thrombus formation; injury to the vessel wall, disturbances in blood flow, and activation of blood constituents (1).

Primary hemostasis

The primary hemostasis leads to formation of a platelet plug, a process occurring within one minute after vessel injury. Briefly, after injury to a blood vessel with disruption of the endothelial lining, there is a short, reflectory vasoconstriction. Platelets encounter the wounded vessel wall exposing collagen and von Willebrand factor, to which they adhere. Adhesion of platelets induces activation, shape change, forming of pseudopodia and large amounts of glycoprotein IIb-IIIa (gpIIb-IIIa) transported to the platelet surface. Binding of fibrinogen to its receptor gpIIb-IIIa results in platelet aggregation and formation of a platelet plug.

Secondary hemostasis -coagulation

The secondary hemostasis then takes place, resulting in thrombin generation, whereafter thrombin converts fibrinogen to fibrin. Most of these processes require the presence of phosphatidylserine and Ca^{2+}. The classical view of coagulation as an enzyme system with a series of activation and inhibition steps was described in 1964 as a "waterfall" (2) or "cascade" (3) system. The system was divided into an extrinsic, TF initiated pathway and an intrinsic, surface or collagen induced pathway (Figure 1).

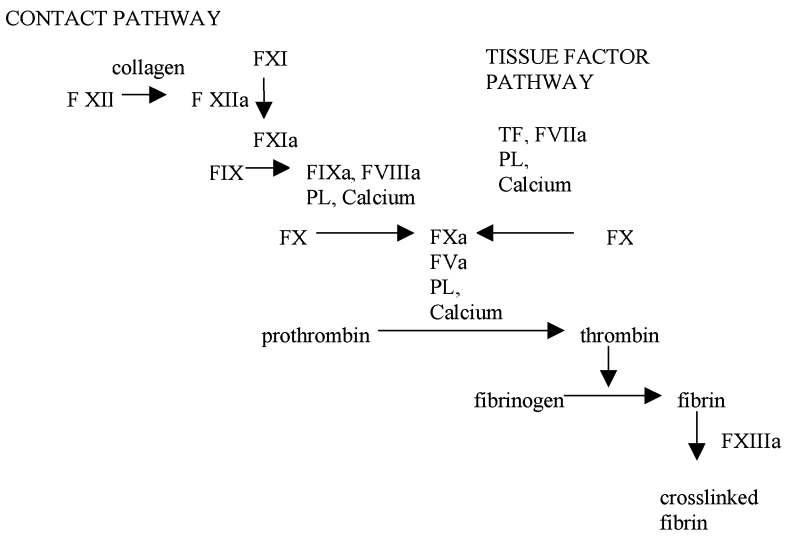


Figure 1. The coagulation cascade.

Recently however an increased understanding of the role of blood cells participating in coagulation has lead to a somewhat modified description of blood coagulation, not as an enzymatic protein cascade, but rather a cell based model consisting of three overlapping stages (4, 5) (Figure 2).

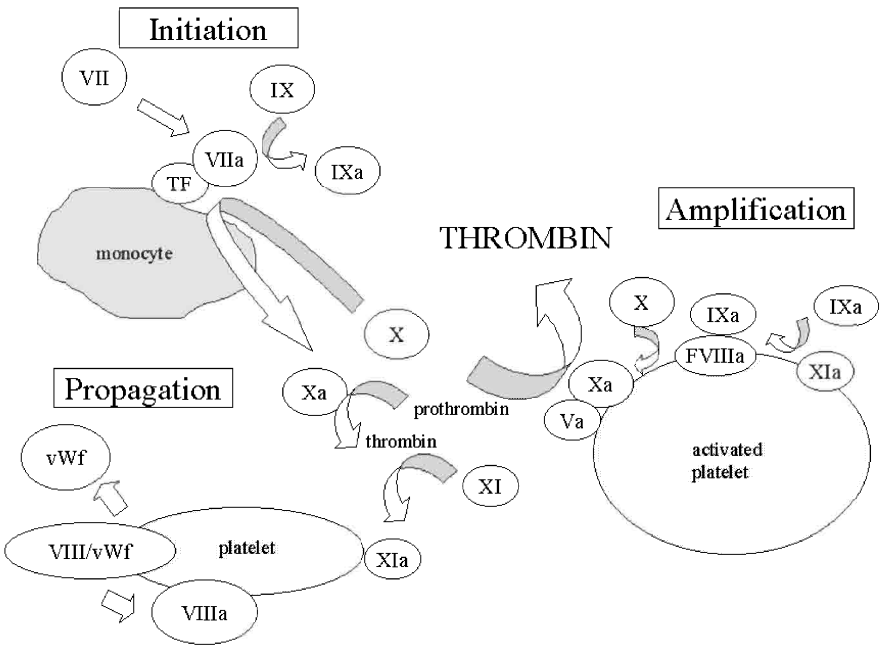


Figure 2. The cell based model of hemostasis consisting of three steps, initiation, amplification and propagation. vWF is von Willebrand factor.

The cell based model of coagulation

TF is the main physiological trigger of the coagulation cascade (6, 7) and is not normally present in the circulation but exposed after vessel injury or upon monocyte activation. In the initiation step of coagulation, circulating small amounts of activated factor VII (VIIa) (8) binds to TF (9, 10) whereafter the TF/factor VIIa complex activates factor X to factor Xa. The TF/factor VIIa complex can also activate factor IX to IXa (11), after which factor IXa together with VIIIa activates factor X to Xa. Factor Xa then complexes with factor Va on the TF expressing cell, and converts small amounts of prothrombin to thrombin.

The amplification stage occurs on the platelet surface. The small initial amount of thrombin that is formed before the TFPI-factor Xa complex inhibits TF/factor VIIa is sufficient to activate platelets,

that release α-granulae containing e.g. factor V. A key step is that thrombin also activates factors V and VIII.

The third step is propagation and occurs on the platelet surface. Factor IXa (previously generated by TF/factor VIIa complex or on the platelet surface by factor XIa) binds to activated platelets in complex with factor VIIa and activates factor X. The formation of factor Xa is the rate-limiting step of coagulation. Factor Xa binds factor Va on the platelet surface, yielding a massive production of factor Xa and a thrombin burst.

Thrombin is an enzyme with many functions. It converts fibrinogen into fibrin monomers, and activates factor XIII needed for the subsequent fibrin monomer polymerization into a stable network. Thrombin further promotes coagulation by activating platelets and TAFI, the latter to protect the clot from being lysed. In addition, thrombin activates the protein C anticoagulant system, limiting the subsequent thrombin formation.

The cell-based model offers explanations to hitherto unsolved questions like why direct activation of factor X by TF/factor VIIa does not compensate for the deficiency of factors VIII or IX in hemophilia. It is hypothesized that hemophiliacs indeed produce enough factor Xa, but on the TF expressing cell surface. Factor Xa cannot move to the platelet, as it will be rapidly inhibited by antithrombin or TFPI. By contrast, since the process of antithrombin inhibiting factor IXa is slow and TFPI does not inhibit IXa, factor IXa can move from the TF expressing cell to the activated platelet surface where coagulation is amplified (4, 5).

Contact activation

The contact system or intrinsic route of coagulation activation is initiated by contact with collagen or in vitro by negatively charged surfaces. This pathway is thought to be of little importance for activation of coagulation in vivo, but may be of importance in vivo, e.g. in activation of fibrinolysis (12, 13) and in blood pressure regulation in severe sepsis (13-15).

Coagulation inhibitors

Aberrant coagulation is prevented by several endogenous soluble and endothelial cell factors that inhibit circulating active proteases distant from the localized thrombus, and prevent coagulation to occur on an intact endothelium.

Antithrombin is a serine protease inhibitor that mainly inactivates factor Xa and thrombin, but also factors IXa, XIa, XIIa and kallikrein. The inhibition of thrombin is 1000 fold accelerated in the presence of heparin (16). Antithrombin circulates in great molar excess to protect from aberrant thrombin formation. The endothelial cell membrane heparan sulfate binds and enhances the inhibitory activity of antithrombin.

Thrombin bound to thrombomodulin on the endothelial cell surface activates protein C to activated protein C (APC) which, in combination with its cofactor protein S cleaves and inactivates factors Va

and VIIIa (17). The protein C system is influenced by many factors, and is downregulated by cytokines in sepsis (18).

TFPI is produced and secreted by endothelial cells (19). TFPI binds to factor Xa, a complex that binds and inhibits the TF/factor VIIa complex.

Fibrinolysis

The function of the fibrinolytic system is to maintain a normal hemostatic balance, without aberrant forming or thrombi, reviewed by Collen (20). The most important enzyme in the fibrinolytic system is plasmin, that degrades fibrin molecules, producing fibrin D-dimers, a marker of fibrinolysis or fibrin turnover. The plasminogen precursor is converted to plasmin by t-PA or u-PA, while PAI-1 is the major inhibitor of t-PA. TAFI is activated by thrombin and thereafter downregulates the fibrinolytic system (21).

Tissue factor

Function

TF is the main physiologic initiator of coagulation (6, 7). It is a 47 kDa transmembrane glycoprotein, structurally unrelated to the rest of the coagulation proteins, classified as a member of the cytokine receptor superfamily (22, 23). TF is a receptor for coagulation factor VII and VIIa. The potential of the cell surface TF/VIIa complex to serve as a catalytic unit activating factor IX and factor X (11) is restricted to phosfatidylserine-associated surfaces (24).

Apart from its central role in coagulation, many other functions of TF have been described. The function as a signaling receptor was first described in 1995 (25). TF/VIIa binding induces changes in cytosolic Ca^{2+} and MAP kinase activation, altering the expression of a number of genes, including upregulation of the TF gene (26-29). The TF signaling may be of great physiological importance, as TF is important for regulation of embryonic angiogenesis, as TF -/- mice die in utero, due to a defective vascular development (30, 31).

In several disease states, TF expression causes thrombotic complications and the level of expression correlates to negative outcome. TF is abundantly expressed within atherosclerotic plaque (32) and there is increasing evidence for a key role of TF in coronary artery disease (33). High TF expression is found on monocytes of sepsis patients (34) and high TF activity is associated with negative outcome in patients with meningococcemia (35). TF has a role in cell migration and tumor metastasis (36). TF has also been shown to exert proinflammatory properties (36-38).

Constitutive TF expression

TF is constitutively expressed in all organs of the body, particularly in well vascularized organs as the brain, lung and placenta (39-41).

Constitutive TF expression is however restricted to extravascular cells (39, 41), thus normally TF is separated from the circulating blood by the endothelium. Upon vessel injury and endothelial damage, subendothelial TF is exposed and initiates activation of coagulation.

Induction and regulation of vascular cell TF expression

Lymphocytes and granulocytes have not been shown to express TF (42, 43). Endothelial cells have been shown to express TF after stimulation, but this is thought to be an exclusive in vitro phenomenon (44). Since there are great discrepancies between effects seen in monocyte suspensions compared to whole blood, which more reflects the in vivo situation, findings in cell suspension experiments should always be confirmed in whole blood models or in vivo (45). Human peripheral monocytes isolated from whole blood have been shown to express TF after stimuli with a number of agents, e.g. endotoxin, immune complexes, CRP, C5a (anaphylatoxin) and the proinflammatory cytokines IL-1β, IL-6, IL-8 and TNFα (44). These are however in vitro findings, and possibly the agents listed above are capable of upregulating TF only in already preactivated cells (46).

The current view is that in vivo, endotoxin is the physiological inducer of monocyte TF expression (44), an induction that may be potentiated by granulocytes and platelets (47, 48). The levels of TF may vary between individuals, a phenomenon called high and low responser phenomenon (49). Interaction of monocytes with platelets via P-selectin (50), endothelial cells via ICAM-1 or CD40 (51, 52) and leukocytes via CD40 (53, 54) can induce affect TF activity, thought to be of great importance in the development of atherosclerosis (55). Inhibition of monocyte TF is exerted by several antiinflammatory cytokines, e.g. IL-4, IL-10 and IL-13, in vitro as well as in vivo (56-58).

Monocyte TF is an immediate early gene, with mRNA levels peaking 1-2 hours after stimulation. Until recently, TF was thought to be primarily regulated on the transcriptional level (59, 60). Evidence is now accumulating of other regulatory pathways, as monocyte surface TF expression is seen already 15 minutes post stimulus, before mRNA can be detected (55, 61). Whether the preexisting TF is stored intracellularly or encrypted in the cell membrane is not well understood. Staining of cells with TF antibodies and labeled factor VIIa has detected intracellular pools of these proteins (62), while there is other evidence of encrypted forms of TF (63). Recently, circulating TF microparticles, originating from platelets, erythrocytes and leukocytes, have been demonstrated in peripheral blood of healthy volunteers (64, 65), showing procoagulant activity through a non TF dependent pathway (65). Increased levels of monocyte derived microparticles with strong procoagulant activity have been found in patients with sepsis and septic shock compared to controls (66). The role of TF microparticles however is presently unclear.

After exposure to the cell surface, TF is removed by shedding or by internalization. TFPI can bind the factor VIIa/TF complex and mediate its internalization and degradation (67). There is also evidence of an additional, TFPI independent process of internalization of TF (68, 69). Alternatively, TF can be

shedded from the cell surface. It is hypothesized that the TF containing microparticles are microvesicles shedded from the monocyte surface (64).

Endotoxin

Lipopolysaccharides (LPS, endotoxins) are surface structures on the outer membrane of gram negative bacteria. They consist of the membrane anchoring lipophilic region lipid A, a hydrophilic polysaccharide part with a core and the O-specific chain.

The lipid A moiety of endotoxin is a potent activator of the innate immune system (70). Endotoxin is the major mediator of the pathogenesis of gram negative sepsis and septic shock (71), and endotoxins are widely used in model systems of gram negative sepsis, e.g. in animals challenged with lethal doses of live E Coli, in cell culture systems, and in experimental models where small amounts of purified endotoxin is injected to healthy volunteers. Upon administration of a low dose endotoxin to healthy volunteers, a variety of inflammatory mediators are released from various cell types. Activated macrophages, lymphocytes, mast cells, keratinocytes and endothelial cells produce e.g. proinflammatory cytokines that contribute to the homeostatic changes in hemodynamics, ventricular function and pulmonary gas exchange.

Monocytes

Monocytes are circulating blood cells, which upon chemoattractant stimuli are recruited to transmigrate through the endothelium to the underlying tissue, and turn into tissue macrophages at the site of the inflammatory process. Activated monocytes contribute to the pathology of many diseases. In sepsis and DIC monocytes participate both in the activation of coagulation and in the inflammatory responses to endotoxin.

The endotoxin effects are exerted via endotoxin binding first to circulating LPS binding protein, LBP, whereafter the complex binds to the monocyte surface receptor CD14 (72, 73). Nuclear transcription factor NFκβ/Rel complex activation is induced and transcription of several genes is initiated, including genes coding for proinflammatory cytokines (74) and TF (59, 60). Upon monocyte activation by inflammatory stimuli, the β2 integrin CD11b/CD18 expression on monocytes and granulocytes quickly increases due to preformed, intracellularly stored CD11b/CD18 (75, 76). CD11b/CD18 binds to endothelial cell adhesion molecules during the inflammatory response in preparation for transendothelial migration. CD11b/CD18 is involved in the proinflammatory and procoagulant responses to endotoxin, as its expression is elevated on circulating monocytes in septic patients (34, 77) and in patients with ischemic disease (78). Activated monocytes expressing CD11b/CD18 induces procoagulant activity. CD11b/CD18 binds and proteolytically activates factor X (79), regulated by

conformational rearrangement of the CD11b/CD18 complex (80), providing an alternative TF independent initiation of the coagulation cascade.

Cytokines

Cytokines are soluble proteins produced by a variety of cell types, e.g. leukocytes and macrophages. They mediate biological effects systemically or locally. By binding to specific target cell surface receptors with high affinity, low cytokine concentrations are needed to elicit biological responses. Cytokines play a major role in initiation, propagation and regulation of immune and inflammatory responses, by acting as mediators that control growth and differentiation of immunocompetent cells (81). A specific cytokine can have multiple effects on different cell types, and different cytokines can act on the same cell population to induce similar or opposite effects. Cytokines often influence the synthesis and function of other cytokines. There are proinflammatory cytokines (TNFα, IL-1β, IL-6, IL-12 and IFNγ), antiinflammatory (IL-4, IL-10, IL-13), and naturally occurring soluble inhibitors of proinflammatory cytokines (TNF-receptors, soluble IL-1-receptors and IL-1-receptorantagonists). In general, cytokines do not regulate normal homeostasis, but have their major biological role during host defense against external challenge.

The proinflammatory cytokines play a central role in the patophysiology of gram negative sepsis (82), and have been demonstrated to appear early after endotoxin injection in healthy volunteers (83).

In animal models of gram negative sepsis, the plasma levels of TNFα correlates with the severity of symptoms, but this does not seem to be the case in septic patients. Many studies have measured circulating cytokines in sepsis patients (84) and the individual cytokine response following exposure to endotoxin, e.g. in gram negative sepsis, shows considerable variation (84, 85). Despite this individual or subpopulation variation, there are a number of reports on proinflammatory cytokines contributing to disease severity, organ failure and poor outcome in sepsis and septic shock (85-88).

Sepsis and disseminated intravascular coagulation

The two systems of inflammation and coagulation both play important protective roles in response to infection. However, they can also contribute to pathogenesis. Sepsis is the consequence of bacteria colonizing the vasculature. Bacteria derive either from an endogenous infectious focus, or from an outer source, e.g. trauma or surgery. If the ethiological agens is gram negative bacteria, the cellular response is elicited by endotoxin (lipopolysaccharide, LPS) from the bacterial membrane. After endotoxin stimulation, monocytes are the main effector cells mediating procoagulant and proinflammatory responses. Monocytes express TF and produce proinflammatory cytokines, giving rise to the clinical symptoms, e.g. fever, caused by the endogenous pyrogens IL-1β and IL-6. Adult respiratory distress syndrome, ARDS, is a complication of respiratory failure caused by increased vascular permeability and leakage of intravascular fluid and inflammatory cells into the pulmonary

interstitium. ARDS is a part of the multiple organ failure syndrome (MOF), a complication characterized by microthrombosis, bleeding and inflammation in different organs, especially highly vascularized organs i.e. the brain, lungs, kidney, liver, skin and adrenal glands. Another feared complication is disseminated intravascular coagulation, DIC, which is a part of MOF but also can contribute to or even cause MOF. DIC is a consumption coagulopathy terminating in simultaneously occurring thromboses and hemorrhage in the microvasculature. Clinically overt DIC may occur in 30-50% of patients with gram negative sepsis (89, 90). Sepsis and its complications are leading causes of morbidity and mortality in the intensive care (91). The mortality in advanced septic shock with oliguria and peripheral cyanosis is 75-100% regardless of treatment. However, if the patient receives adequate treatment in an early phase of sepsis and intense monitoring, the mortality is around 20%. DIC was first descried by Landois in 1875, who gave human blood transfusions to dogs, and thromboses in the mesenteric vessels (92). The initial phase of DIC is characterized by a massive systemic activation of coagulation leading to intravasal fibrin formation and microthrombi, with secondary hemorrhages. There is risk of developing a syndrome with simultaneously occurring microvascular thromboses and bleeding.
DIC is not in itself a disease but a syndrome, always secondary to an underlying disease, most frequently sepsis. Clinical conditions associated with DIC are listed in Table 1.

Sepsis, severe infection (gram positive or gram negative bacteria, viruses e.g. HIV)
Trauma (multitrauma or neurotrauma)
Organ destruction (severe pancreatitis)
Malignancy
Obstetric conditions (abruptio placenta or amniotic fluid embolism)
Vascular abnormalities (large vessel aneurysms or vascular malformations)
Toxic or immunologic reactions (snake bite, transfusion reaction, transplant rejection)

Table 1. Clinical conditions that can be complicated by disseminated intravascular coagulation.

The consensus definition of DIC as proposed by the SSC/ISTH subcommittee is "DIC is an acquired syndrome characterized by the intravascular activation of coagulation with loss of localization arising from different causes. It can originate from and cause damage to the microvasculature, which if sufficiently severe, can produce organ dysfunction" (93). The mechanisms that lead to activation of coagulation, potentiated by the simultaneous depression of physiological inhibitory systems and to increased or impaired fibrinolysis in DIC, are only partly understood. Much knowledge of the pathogenesis of DIC is derived from experiments injecting lethal doses of E Coli to animals, or from injecting low doses of purified endotoxin to humans, baboons and chimpanzees.

Pathogenesis of sepsis-induced DIC

An overview of the pathogenesis of sepsis-induced DIC is shown in Figure 3.

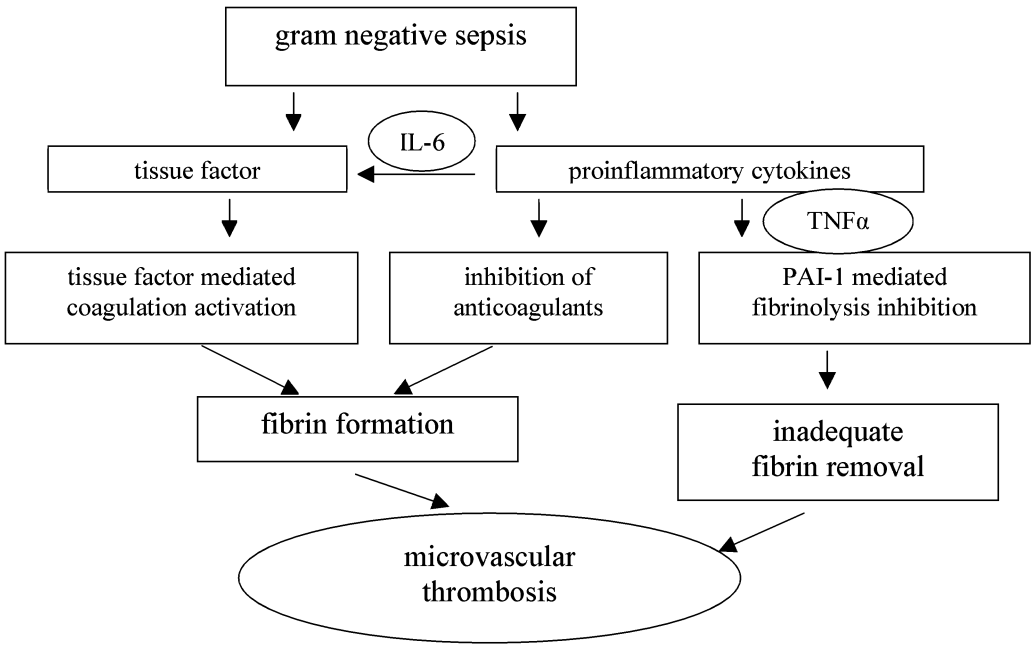


Figure 3. Overview of the pathogenesis of sepsis-induced DIC. See text for details.

The systemic activation of coagulation in DIC is mediated by TF. This was first described by Taylor, who showed that inhibition of the TF pathway attenuated coagulation activation and prevented death in baboons injected with a lethal dose E Coli (94). The importance of TF in sepsis and DIC has recently been reviewed by Osterud (44). Primarily, coagulation is activated whereby coagulation factors and platelets are consumed. Impaired liver synthesis and activated protease-mediated destruction of coagulation factors also contribute to the consumption of coagulation factors. Thereafter, there is an increased bleeding tendency due to depletion of coagulation factors. A physiological activation of other proteolytic enzyme systems, producing e.g. elastase and plasmin, which degrade coagulation factors, also contributes to the bleeding tendency. The fibrinolytic system is transiently increased due to release of plasminogen activators. This early response is however followed by an abrupt decline in fibrinolysis activity due to a TNFα-mediated decrease of t-PA and increase in PAI-1 (95). Prevention of coagulation activation during human and primate endotoxemia

does not influence the activation of fibrinolysis (96-98), giving proof for multiple and independent mechanisms of fibrinolysis regulation during DIC.

After endotoxin injection to healthy volunteers, the endothelium is activated as indicated by increased production of t-PA and von Willebrand factor (99). There is also a massive inflammatory response with release of host proteases, cytokines and hormones from various inflammatory and vascular cell types, leading to an extensive damage to the endothelium and loss of tight junctions between endothelial cells. As a result of endothelial dysfunction, the protein C system, TFPI and antithrombin anticoagulant systems are malfunctioning, which contributes to procoagulant changes.

Cytokines in sepsis and DIC

Proinflammatory cytokines, mainly TNFα and IL-6, contribute to the pathogenesis of DIC through several mechanisms. In experimental endotoxemia models, TNFα is the first cytokine to appear in the circulation, reaching peak levels after about 1.5 hours, shortly followed by other proinflammatory cytokines, e.g. IL-1β, IL-6, IL-8 and IL-10. Therefore, TNFα has been suggested to be the cytokine primarily responsible for enhancing other proinflammatory cytokines and inducing activation of coagulation. Endotoxin injection combined with anti-TNFα treatment attenuates the increase in circulating IL-1β, IL-6, IL-10 and other cytokines (100-102). However, although administration of exogenous TNFα elicits activation of coagulation in vivo and induces TF expression in vitro, endogenous TNFα is not required for a coagulation response during endotoxemia or gram negative sepsis (95).

TNFα is the principal cytokine inhibiting fibrinolysis (95) stimulating the endothelial cell production and release of PAI-1, and reducing the endothelial cell t-PA (103). Additionally, treatment with an anti-TNFα antibody completely prevents activation of the antifibrinolytic system after endotoxin challenge to chimpanzees (102). Another function of TNFα is mediating inhibition of the protein C system, by downregulating endothelial cell thrombomodulin (104, 105).

In 1994, van der Poll et al elegantly showed in a chimpanzee model, that blocking of IL-6 inhibited activation of coagulation, but did not influence the induction of other cytokines (106).

It has also been shown that recombinant IL-6 injection induces thrombin formation, without affecting fibrinolysis (107). Thus, IL-6 rather than TNFα mediates the procoagulant response in DIC.

Cytokines in relation to symptoms

Injection of recombinant IL-6 in humans however only induces mild clinical symptoms, e.g. chills and fever, and is not as toxic as recombinant TNF or IL-1β (108, 109). Neither are hemodynamic signs of sepsis seen in dogs after high dose IL-6 injection (110). IL-6 is not essential for development of tissue damage during endotoxemia, as IL-6 gene deficient mice are as sensitive to the endotoxin effects as are wild type mice (111). Inhibition of TNFα or IL-1β with neutralizing antibodies administered before a lethal dose of endotoxin or living bacteria prevents lethality. Thus, although IL-6 is important

for endotoxin induced activation of coagulation, the role of IL-6 in the organ failure associated with endotoxemia is limited (112).

Activation of coagulation

In all experimental models, thrombin generation can be detected 3-5 hours after endotoxin injection (99, 113). The thrombin generation is TF induced, as monoclonal antibodies inhibiting TF or factor VIIa activity resulted in complete inhibition of thrombin generation in endotoxin challenged chimpanzees, and prevents DIC and mortality in baboons injected with E Coli (94, 96, 97, 114). Several proinflammatory cytokines have been shown to induce TF expression on monocytes, macrophages and endothelial cells in vitro (44). The in vivo importance however remains to be elucidated, and endotoxin remains the only known stimulus of monocyte TF expression in vivo. Activation of the contact system in patients with severe sepsis has been described (115). The importance of this can be discussed, since blocking of the contact pathway by an antibody to factor XIIa does not affect E Coli induced DIC in baboons (15). The XIIa antibody however prevented against development of irreversible hypotension. An association between severe hypotension and contact activation has been described in human septic shock (14, 116) giving the contact pathway a possible role in blood pressure regulation in sepsis.

Crosstalk between coagulation and inflammation

Evolutionary there are biochemical links between coagulation and inflammation, as TF structurally belongs to the cytokine receptor superfamily. In vitro, several cytokines have been shown to induce and downregulate TF expression on various cell types. TF in turn may participate in cellular interactions promoting leukocyte adhesion and transendothelial migration (117). TF has also been described to induce IL-6 and IL-8 in isolated monocytes as well as in whole blood (118).

In porcine and baboon models given lethal doses E Coli, administration of recombinant TFPI decreases circulating levels of IL-6 and IL-8 (119, 120). TFPI also diminishes endotoxin induced proliferation in rabbit perivascular cell infiltration (121). On the other hand, after injection of purified endotoxin and recombinant TFPI in healthy volunteers, activation of coagulation was totally blocked, but TFPI did not affect fibrinolysis or the cytokine response (98). These differences in results may be due to species differences, and indicate the difficulties of understanding the pathogenesis of DIC.

Another link between coagulation and inflammation is thrombin, the key coagulation enzyme converting fibrinogen to fibrin. Thrombin is a multifunctional enzyme with procoagulant, anticoagulant, inflammatory and mitogenic properties.

Several parameters of both inflammation and coagulation correlate to prognosis and outcome of patients with sepsis and septic shock, and increased levels of circulating cytokines have been demonstrated in septic shock (85-88). NFκβ extracted from peripheral blood mononuclear cells is a predictor of outcome in septic patients (122). Recently, sepsis patients have been shown to express high levels of TF and CD11b on circulating monocytes (34). Endothelial cells play a central role in the

crosstalk between coagulation and inflammation, reviewed in (123). There is thus a significant body of evidence of crosstalk between coagulation and inflammation at several levels, as illustrated in Figure 4.

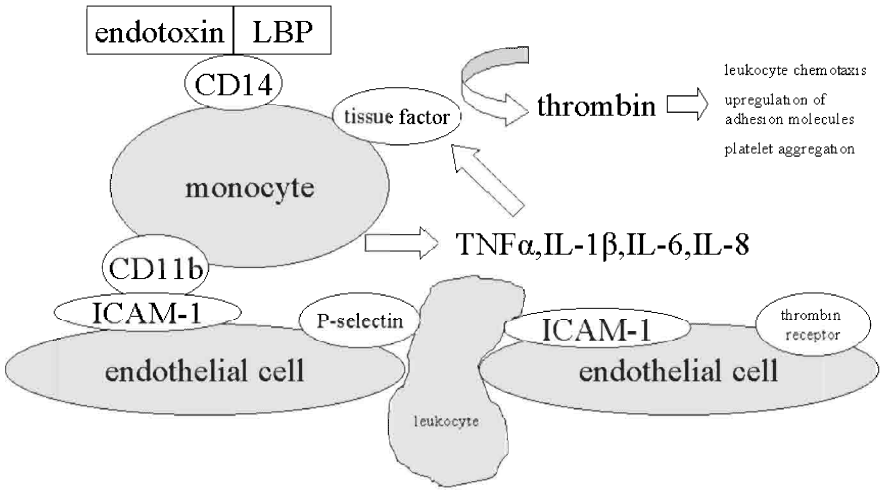


Figure 4. Pathways of crosstalk between coagulation and inflammation.

Markers of DIC

As previously described, DIC develops in different stages. It is important for treatment and interpretation of tests to know in which stage of activation and consumption of coagulation factors the patient is. An overview of the available parameters for determination of activation of coagulation is outlined in Figure 5.

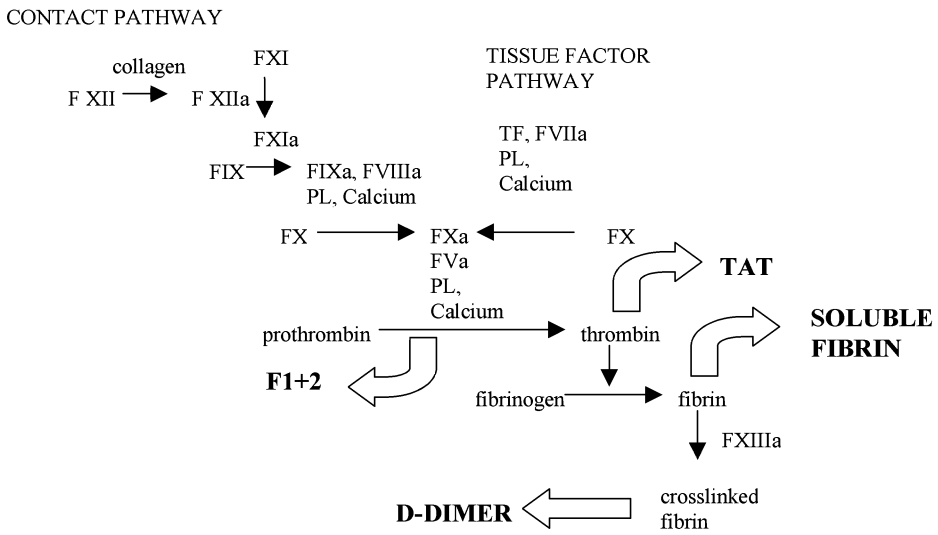


Figure 5. Markers of DIC. F1+2 and TAT are markers of thrombin formation, whereas soluble fibrin and D-dimer are markers or fibrin formation. Additionally, fibrinogen, antithrombin, aPTT and PT (INR) are often measured.

In the primary phase, or "non overt DIC" the coagulation system is activated with intravascular fibrin formation. Routine laboratory tests, e.g. antithrombin, D-dimer and soluble fibrin, are still within reference interval. This is an early, compensated phase of DIC.

An intermediate phase ensues, with consumption of coagulation factors, inhibitors and platelets. Antithrombin is normal or slightly low. D-dimer and soluble fibrin begin to increase. Thereafter "overt DIC" or non compensated DIC follows. Antithrombin is low, and D-dimer and soluble fibrin high. Microthromboses are found in various organs, as well as microhemorrhages, with risk of developing multiple organ failure.

DIC develops from a variety of underlying diseases and the syndrome varies in severity. This has lead to difficulties in defining the syndrome and developing a scoring system for diagnosis of DIC. Recently, the ISTH Scientific and Standardization Committee on DIC suggested laboratory criteria and a scoring system for DIC (93). An important aim of these criteria however were to reach laboratories all over the world, therefore using simple tests. The scoring system is based on platelet

count, D-dimer, prothrombin time and fibrinogen level for overt DIC. For the scoring system of non-overt DIC, antithrombin, protein C and TAT were suggested in addition. These parameters are widely used, but often difficult to interpret. F1+2 is a more accurate parameter, however expensive and not in routine use. F1+2 therefore is mostly used for research and for evaluating therapeutic effects retrospectively.

Platelet count, prothrombin time and aPTT have long been used as screening analyses. Normal levels of these tests however do not exclude non overt DIC. The recommendation has therefore been to repeat tests after a few hours.

Analyses of the current coagulation activity markers are time consuming and often difficult to interpret. There is a need of a quick, whole blood method for bedside assessment of the degree of coagulation activity in patients at risk of developing DIC.

Therapy

There is currently no specific efficient therapy for DIC. Primary treatment is focused on organ support and general resuscitation with intensive care in parallel with treatment of the underlying disease, e.g. antibiotics in the case of sepsis. Based on in vitro results, therapy with antiinflammatory cytokines can be proposed (57, 58). Treatment with antibodies to TF successfully used in mice and primate models of DIC, and anti endotoxin antibodies can also be used, however only prophylactically (94, 124, 125). This kind of treatment strategies are however elaborate and difficult to administer.

Recently, two interesting randomized, placebo controlled, double-blind multicenter trials have been conducted, assessing the beneficial effect of two coagulation inhibitors, antithrombin (126) and APC (127), on mortality from severe sepsis. Antithrombin is an important endogenous anticoagulant and has also been demonstrated to have antiinflammatory effects (128), giving a rationale for its use in the therapy of sepsis. The effect of antithrombin has been investigated previously in several studies with various results. In 2001, a study of 2312 patients assessed the overall 28 day mortality in patients with severe sepsis and septic shock, receiving high dose antithrombin or placebo (126). There was no significant difference in mortality between the groups, however in the subgroup not receiving concomitant heparin treatment the mortality was somewhat lower than in the placebo group. Bleeding was significantly increased in the antithrombin group.

The second study investigated recombinant human APC (rAPC) in severe sepsis, in 1690 patients randomized to rAPC or placebo (127). APC is also an endogenous anticoagulant that promotes fibrinolysis and inhibits inflammation (129). Treatment with rAPC reduced the relative risk of death by19.4%. Although treatment with rAPC significantly increased the incidence of serious bleeding, this study shows promising results regarding future treatment of severe sepsis and DIC.

Nicotinamide

The vitamin B derivative nicotinamide has a variety of anti-inflammatory properties, including inhibition of endotoxin induced TNFα (130, 131). All antiinflammatory effects have been suggested to stem from the ability of nicotinamide to inhibit poly (ADPribose) polymerase (PARP) (131). PARP is a nuclear DNA binding enzyme involved in DNA repair in response to genotoxic stress (132, 133). Activation of PARP, which has been shown to occur upon endotoxin administration (132), depletes intracellular NAD+, slowing down the rate of glycolysis, electron transport and ATP formation, which can result in cell dysfunction and cell death. PARP deficient mice are defective in NFκβ-dependent transcription activation, and PARP deficient mice are extremely resistant to endotoxin induced shock (134). Proinflammatory cytokines as well as TF are transcriptionally regulated via NFκβ. There seems to be multiple mechanisms through which nicotinamide may act beneficially as a drug in conditions associated with activation of coagulation and inflammation, e.g. in sepsis and DIC.

AIM

The general aim of this thesis was to investigate the mechanisms involved in endotoxin induced activation of coagulation and inflammation, and to develop a bedside monitoring method for coagulation activity. The specific aims were:

To develop an assay based on free oscillating rheometry, suitable for bedside monitoring of coagulation activity.
To study the clotting properties of blood and plasma after recalcification of citrated blood and plasma samples, by measuring clotting onset time, COT.
To establish and validate the COT method and its ability to detect hypo- and hypercoagulation, in whole blood as well as in plasma.
To use the COT method, monitoring activation of coagulation in healthy volunteers after endotoxin injection and in neurotrauma patients.

To establish a whole blood endotoxemia model for studies of monocyte TF and CD11b expression, COT and proinflammatory cytokines.
To thereafter apply the model studying the effects of nicotinamide on endotoxin induced inflammation and coagulation, and to investigate the mechanism behind this response.

METHODS AND PATIENTS

Blood collection

In study I, III and V, peripheral blood was drawn by clean venipuncture from an antecubital vein, after resting 5 minutes, by 19 or 21-gauge syringes, using Vacutainer™ tubes. Minimal or no stasis was applied, and if used released directly after venipuncture. In study II, patients at the neurointensive care were sampled from stationary peripheral venous catheters. In all studies, the first tube was always discarded.

For COT analyses, citrated blood (paper I) or plasma (paper I, II, V) was used. In paper IV, where healthy volunteers were injected with endotoxin, samples were taken at three occasions, in the arm contralateral to the injection site. To avoid activation of coagulation, no stasis was applied, and a 19-gauge syringe was used. Sterile vacutainer compatible polypropylene blood collection tubes (GHI, Linköping, Sweden), that could be directly inserted into the FOR instrument, were used for COT determinations. Tubes were preloaded with thromboplastin isolated from rabbit brain. Before each venipuncture, EMLA™ creme (prilocain and lidocain) was applied to minimize discomfort.

Preparation of leukocytes

In study III, peripheral blood from healthy volunteers was collected into heparinized tubes. After stimulation with endotoxin for two hours, leukocytes were prepared by lymphoprep gradient centrifugation.

In paper V, peripheral blood from healthy volunteers was collected into EDTA tubes. Erythrocytes were hemolyzed by adding 4 ml ice cold NH_4Cl-EDTA lysing solution (0.15 mol/l NH_4Cl, 10 mmol/l $KHCO_3$, 0.1 mmol/l EDTA, pH 7.2) to 200 µl blood and incubating samples for 10 minutes at +15 °C, centrifuged at 300 *x*g for 5 minutes at +4 °C, and washed twice in TBS.

Endotoxin stimulation of whole blood and cell suspensions

Lipopolysaccharide endotoxin E Coli O26B6 (Difco, Detroit, MI, USA) was diluted in TBS to reach a final concentration of 1 ng/ml (paper III) and 10 ng/ml in whole blood experiments in paper V. Endotoxin at 10 ng/ml diluted in RPMI supplemented with fetal bovine serum was used to stimulate cell suspensions in paper V.

In all studies, endotoxin was added 5 minutes after other additives, whereafter tubes were carefully inverted and incubated at 37 °C, 5% CO_2 for 2 or 4 hours.

Endotoxin contamination

The TBS buffer used in paper V was tested to be endotoxin free, i.e. containing <0.125 IU/ml endotoxin. RPMI medium was endotoxin free. The heat inactivated fetal bovine serum used for cell

suspension experiments in study V however may have contained endotoxin particles, explaining the endogenous activation seen in the control sample.

Cell viability

Cell viability was measured in paper III and V by Trypan blue exclusion, and always exceeded 99%.

Endotoxin injection in healthy volunteers

Experiments were conducted at the Intensive Care Unit, Huddinge University Hospital. Nine healthy volunteers rested in the supine position for 30 minutes. Thereafter baseline blood samples were drawn, and intravenous injection of 2ng/ kg endotoxin was administered through a dorsal hand vein. Blood samples were drawn at baseline and after 3 and 6 hours.
Symptoms and physiological examinations of blood pressure, heart rate and body temperature were recorded throughout the experiment.

Free oscillating rheometry (FOR)

FOR measurements are conducted using ReoRox4 instrument, invented by Leif Bohlin (135). The principle of FOR is letting the sample oscillate at about 11 Hz, nearly free of friction. This becomes possible as the sample cup and its holder are connected to the base of the instrument only by a torsion wire, a unit that is kept in place only by a magnetic field (ultralow friction bearings). Oscillations are initiated every 2.5 seconds, and the frequency (Fq) and damping (D) of the oscillations are registered. These variables are related to the changes in viscosity and elasticity of the sample, respectively. During the coagulation process (and thus the gelation of the sample), the viscosity increase will cause a decrease in frequency of oscillations. To some extent, the elasticity will also be altered. The process is followed by measurement of both parameters and the combined effect, ΔC, is calculated as

$$\Delta C = \sqrt{(\Delta D^2 + \Delta Fq^2)}$$

When ΔC exceeds a preset numerical deviation from initial oscillation parameters, the onset of coagulation, COT, is assumed. A schematic picture of the FOR instrument is presented in Figure 6.

The clotting time registered by COT coincides with visual inspection of clotting (M Rånby and S Ramström, personal communication). The FOR instrument was used to develop a monitoring method measuring COT.

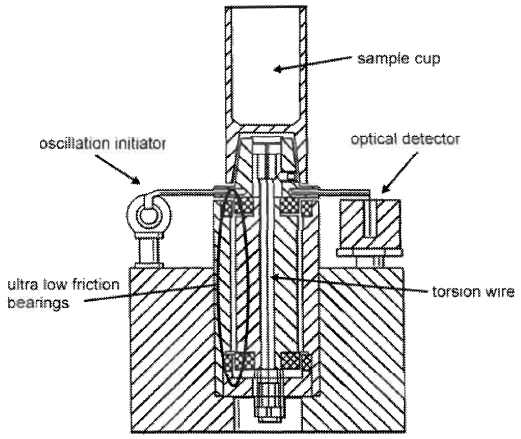


Figure 6. Schematic view of the FOR instrument. The figure is reprinted from GHI AB with permission.

Flow cytometry

Principle of flow cytometry

The principle of flow cytometry is that different cell types are distinguished based on their different laser light-scattering properties. Forward scatter (FS) reflects cell size and side scatter (SS) reflects the complexity or granularity. Cells can also be identified by labeling with specific antibodies to cell surface antigens. These antibodies are conjugated to fluorochromes, which emit light of different wavelength. Up to four different antigens can be detected in each sample using different fluorochrome conjugated antibodies. We measured mean fluorescence intensity (MFI), an arbitrary unit used to obtain quantitative measurement of the amount of antigen. The flow cytometer was calibrated daily for optical and fluorescence alignment as well as for fluorescence intensity with standardized fluorospheres Flow-Check™ and Flow Set™ (Beckman Coulter).

After stimulation, the cell populations can be difficult to identify merely by their FS and SS properties, as their properties may change due to the stimulus. We therefore used antibodies to the leukocyte marker CD45 and the specific monocyte marker CD14 to identify the monocyte population, which was then further analyzed for TF and CD11b expression (Figure 7).

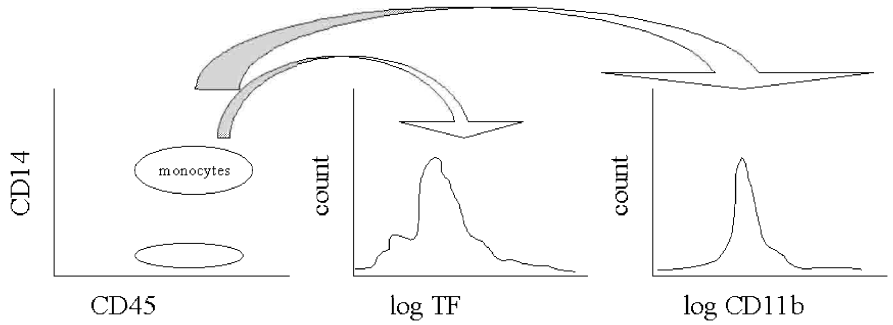


Figure 7. Identification and further analysis of the monocyte population.

Thus, four channel flow cytometry was used, with the following directly conjugated monoclonal antibodies: anti CD14 ^{ECD}, anti CD45 ^{PC5}, anti CD11b ^{PE}, with isotype controls Mouse IgG1^{FITC} and mouse IgG1^{PE} (all from Beckman Coulter, Marseille, France), and anti human TF ^{FITC} (American Diagnostica, Greenwich, CT, USA). At least 2000 CD14 positive events were collected. As a negative control to anti-TF and anti-CD11b one part of the sample was incubated with the isotype controls for FITC and PE together with the anti CD14 ^{ECD} and CD45 ^{PC5} antibodies.

Flow cytometry experiments in whole blood and cell suspensions (paper V)

Fresh or incubated whole blood was kept on ice for 5 minutes, whereafter leukocytes were prepared by incubation 10 minutes at +15°C with 4ml ice cold NH_4Cl lysing solution (0.15mol/l NH_4Cl, 10mmol/l $KHCO_3$, 0.1mmol/l EDTA, pH 7.2), centrifugation and washing. Leukocytes were then fixed in paraformaldehyde, washed and diluted in PBS.

For cell suspension experiments, incubated or non incubated cell suspensions were kept on ice for 5 minutes, whereafter cells were washed and fixed in paraformaldehyde. Subsequent to fixation, cells were washed and diluted in PBS.

For flow cytometry measurements, each sample was incubated for 10 min at room temperature with 10 µl of each antibody described above. Thereafter samples were washed twice in PBS and analyzed on an EPICS XL flow cytometer (Coulter Inc, Hialeah FL USA).

Patients and healthy volunteers

Oral and or written consent was obtained from each patient or volunteer before enrolment in the study. The local ethics committee approved all five studies. A summary of all methods used is presented in Table 2.

Method	Described in paper
Clotting onset time, COT	I,II,IV,V
Flow cytometry	V
In vitro endotoxin stimulation	III,V
In vivo endotoxin injection	IV
PT	I,II
aPTT	I,IV
PARP activity (^{32}P-NAD)	III
Cytokine analyses (IL-1β,IL-6, IL-8, TNFα) Immulite instrument	III,V
F1+2 (ELISA)	IV
TAT (ELISA)	II
Antithrombin (chromogenic substrate)	II
Soluble fibrin (chromogenic substrate)	II
Fibrin D-dimer (ELISA)	II
Fibrinogen (Clauss method)	II
TF antigen (ELISA)	V

Table 2. A summary of the methods used in this thesis

Paper I

28 consecutive patients on warfarin treatment, attending the hospital for their weekly control of PT (INR) levels, 2 healthy volunteers, and 3 patients who were not undergoing warfarin treatment, participated in the study.

Paper II

Plasma samples from patients with isolated head trauma Intensive Care unit at Karolinska hospital, Stockholm. The patients were included in a prospective, randomized, open, controlled pilot study with antithrombin concentrate treatment (ATenativ™, Pharmacia & Upjohn) (REF Grenander). Inclusion criteria were: patients aged 14- 70 years, having traumatic brain injury as the sole, major trauma, and not suffering from any known bleeding disorder or being subjected to anticoagulant therapy or dialysis. Seventeen patients were included in our study. All patients received standard treatment, and eight were randomized to antithrombin supplementation as well (AT group). No placebo drug was used in the non AT group. The antithrombin concentrate was given after first blood sample collection, as a 60 IU/ kg bolus dose followed by two booster doses of 20 IU/ kg after 8 and 16 hours, respectively, in order to achieve supranormal levels of antithrombin. Samples were drawn at nine occasions during the first 5days after hospital admission. The clinical parameter Glasgow Coma Scale was evaluated at admission and Glasgow Outcome Scale after three months.

Paper III, V

In these studies, blood samples were drawn from volunteers, who were healthy according to medical history and routine hemostatic and biochemical screening.

Paper IV

Nine healthy male volunteers, mean age 24 (range 21-31) years, were admitted to Huddinge University Hospital after documentation of good health by detailed family history of thrombotic disease, physical examination, hematological and biochemical screening.

None of the volunteers or controls took any medication during the month preceding the study. From 12 hours before study onset and throughout the experiment, no caffeine containing beverages or tobacco was permitted. Subjects were fasting and lying in bed throughout the experiment. To study the normal variation of laboratory parameters over a day, four healthy male volunteers, not subjected to endotoxin, served as controls.

Statistics

In paper I, ANOVA corrected for within-day variation (136) was used for estimation of CV. The between-days CV was calculated from measurements of samples from one healthy volunteers, where five samples were measured daily for five days.

Wilcoxon matched pair test has been used for within group analyses for dependent samples in papers II, III, IV and V, and Mann-Whitney U test for between group analyses in paper II, using Statistica software (StaSoft Inc., Tulsa, OK) was used for non parametric statistical analyses.

Correlation was assessed with Spearman Rank in paper II, where all 153 data samples from 17 patients were used as patients were used as independent. In paper V, correlation coefficient r^2 was calculated using MSN excel®.

Significance was expressed with p values on the level of 1% ($p<0.01$) or 5% ($p<0.05$).

RESULTS AND DISCUSSION

COT measurements using FOR

A simple method for activation of coagulation, suitable for bedside monitoring in the intensive care should be quick, easy to perform and to interpret. We chose to work with a free oscillating rheometry instrument (ReoRox4, GHI, Linköping, Sweden), using a technique with simple recalcification of citrated blood or plasma measuring COT. The method has been proven to be quick, reliable and easily performed, detecting hypo- and hypercoagulation (137) with acceptable coefficient of variation (CV) both for plasma (between-day CV 2.0 %, within day 5.2 %) and blood measurements (3.4 % and 10.3 %, respectively). The assay endpoint of clotting onset time is determined by a sudden increase in viscoelastic properties of an oscillating sample, allowing identification of the onset of coagulation rather than the later steps of coagulation, e.g. thrombin formation, that are monitored with conventional techniques. In this thesis, COT measurements are conducted in three ways,

1. by recalcification of citrated blood or plasma (papers I and II)
2. by addition of endotoxin stimulated or non stimulated leukocytes to autologous recalcified citrated plasma (paper V)
3. in vacutainer compatible tubes preloaded with thromboplastin (paper IV)

COT induced by calcium repletion (papers I, II)

In order to develop a system that mimics the in vivo situation as much as possible, we chose to initiate the COT monitoring method with simple Ca^2+ repletion, in both citrated plasma and blood, adding no exogenous activators or inhibitors. Initial titrations were carried out to find the optimal $CaCl_2$ concentration. It is a known but unexplained phenomenon that the $CaCl_2$ titration curve has an inflexion point, after which increasing $CaCl_2$ levels increase, in stead of decrease COT. The inflexion point of the curve was found to be at 8 mmol/L $CaCl_2$ in whole blood, and 14.4 mmol/l in plasma.

What does the COT method measure?

COT induced by recalcification (papers I and II)

Paper I demonstrates that COT decreases with increasing amounts of added thrombin (Figure 8), indicating that COT measurements are thrombin dependent.

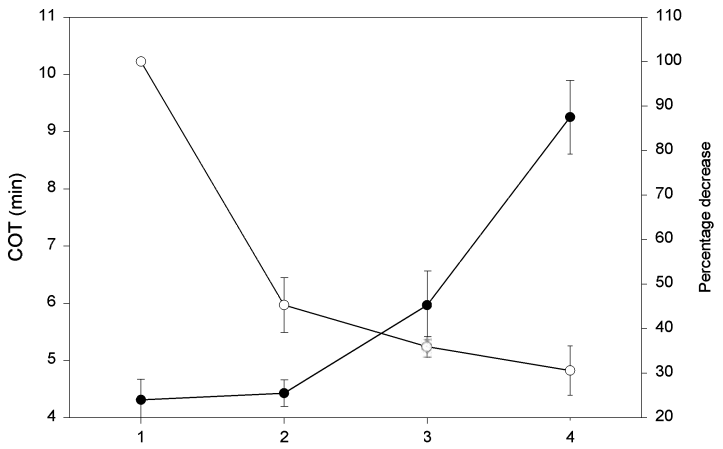


Figure 8. Influence on COT of thrombin and platelet content. Filled circles, left y-axis: plasma was centrifuged at different forces, 1=500xg, 2=1000xg, 3=2000xg, and 4=10000xg. Open circles, right y-axis: COT decreases dose dependently in response to added thrombin. On the x-axis, 1-4 represents addition of 0, 0.01, 0.02 and 0.05 U/ml thrombin, respectively.

In paper I we also show that the COT levels increase with increasing centrifugation velocity, reflecting the degree of platelet depletion.

Recently, circulating TF microparticles, often associated to the membrane of platelets, have been described in healthy volunteers (64). This may explain the above finding of the influence of platelets, as well as why platelets have been shown to enhance the TF activity of monocytes (47, 49). An alternative role of platelets is to provide a negatively charged surface for initiation of the contact pathway. The necessity of platelets in clotting assays is well known, but their exact role remains to be clarified.

CTI, a specific inhibitor of factor XIIa and thus the contact pathway, did not influence COT, which led to the conclusion that the COT method primarily measures TF induced coagulation (138). However, unpublished results demonstrate that addition of a polyclonal antibody inhibiting TF activity (American Diagnostica TF4501 IgG) also fails to alter COT. The overall conclusion from inhibition experiments is therefore that due to the properties of coagulation being a cascade system, it is very difficult to study the influence on coagulation of inhibitory substances, using the COT method with minimally altered blood without exogenous activators.

Using commercial patient plasma (Helena Bio Sciences, Sunderland UK) deficient in factor VII or factor XII, we further investigated the influence on COT measurements of the TF and contact induced pathways of coagulation. Interestingly, in factor XII depleted plasma, no COT was obtained, indicating that at least small amounts of factor XII are needed for COT to be measured. In factor VII depleted plasma, where the TF pathway is not functioning, a long COT was obtained, 24.5 minutes.

The findings in factor VII deficient plasma could be explained by a slow contact activation rendering a long COT. Another interpretation is that the factor deficient plasma is derived from patients, and perhaps contains small amounts of factor VII or VIIa. To further investigate this phenomenon, factor deficient plasma, normal pooled plasma and plasma from two healthy volunteers were incubated with thromboplastin (0, 10, 50 and 100 µl) isolated from rabbit brain (Figure 9).

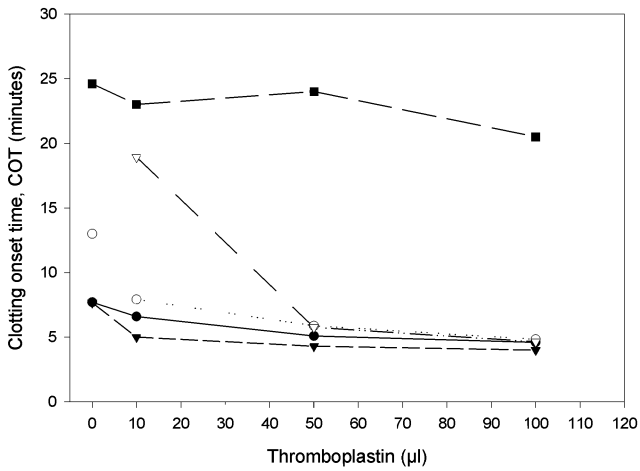


Figure 9. COT measurements after thromboplastin addition. The figure shows the mean of 1-3 measurements in (○) normal pooled plasma, (● and ▼) healthy volunteers, (■) factor VII deficient plasma, (Δ) factor XII deficient plasma, respectively.

There was minimal change of COT in thromboplastin activated factor VII deficient plasma. Therefore we conclude that the long COT result is not due to small amounts of remaining factor VII/VIIa, in which case COT would be shortened by adding thromboplastin. Thus, the late COT is probably due to contact activation. Factor XII deficient plasma on the other hand reacted similar to the normal pooled plasma and plasma from healthy volunteers (Figure 9). This indicates that small amounts of factor XII is needed for COT if there is not a strong stimulus, e.g. exogenous endotoxin or endogenous hypercoagulation, in which case factor XII is not necessary. Thus, COT primarily measures TF induced coagulation, but if this pathway does not work, the contact pathway eventually will take over. This provides strong support of the COT method being a global coagulation assay, adding new information about the coagulation properties of blood and plasma. We have previously shown that blood as well as plasma COT correlate to the routine coagulation marker of the TF pathway marker PT (INR) and to the marker of contact activation, aPTT. Thus, COT shows associations to both tests, supporting our conclusion of COT being a global test, measuring the overall coagulation activity. In

paper I, there was also a correlation between PT and aPTT, which can be explained by the factor VIIa/TF complex activating factor IX as well as factor X (11).

COT in papers IV and V

In paper IV, whole blood COT was assessed in healthy volunteers monitoring the effects of an endotoxin injection. Samples were drawn in vacutainer compatible tubes, specially made for the FOR instrument. Thus, bedside measurements were accomplished without unnecessary pipetting. Native blood clots in about 40 minutes in these tubes, and is very sensitive to the sampling procedure. In paper IV, the tubes were therefore preloaded with thromboplastin; to assure quick monitoring of TF induced coagulation.

In paper V, endotoxin stimulated or non stimulated leukocyte suspensions were washed and added to autologous citrated plasma, whereafter COT was assessed.

COT in neurotrauma patients (paper II)

The initial stage of neurotrauma is always associated with hemostatic abnormalities, as the brain tissue contains high concentrations of TF. A local thrombotic and hemorrhagic process takes place adjacent to the injured brain tissue, extending the brain trauma and edema. It has been shown that the magnitude of coagulation activation is proportional to the amount of brain tissue affected (139), and the degree of coagulation activation correlates to outcome (140-142). Neurotrauma patients are therefore an interesting population to monitor with the COT method. The COT investigations are based on a prospective pilot study by Grenander et al (143), investigating if administration of high doses of the coagulation inhibitor antithrombin would shorten the time of hypercoagulation, and possibly also have a favorable effect on the secondary brain damage. 17 patients with isolated neurotrauma were randomized to standard treatment plus high dose antithrombin during the first day (8 patients) or standard treatment alone (9 patients). We assessed COT as a monitoring method compared to routine coagulation parameters and clinical outcome. At admission, all patients had hypercoagulation, with no differences between the groups. All coagulation parameters were persistently high throughout the study, or normalized simultaneously in both groups. COT was the only parameter that could discriminate antithrombin treated patients from non treated patients, as COT levels rapidly increased towards normalization in the antithrombin group, but did not increase until day 3 in the non treated group (Figure 10).

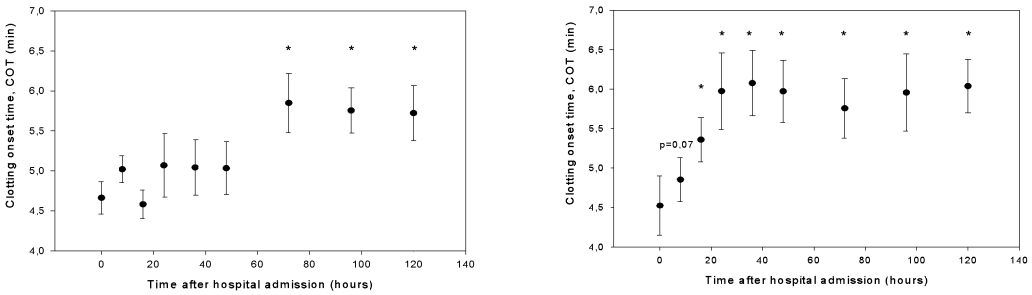


Figure 10. COT levels in the non antithrombin (left) and antithrombin (right) treated patients, during the first 5 days after hospital admission. Whiskers show SEM. * marks significance on the level of p<0.05.

COT levels were compared with routine markers of coagulation, showing a weak but significant correlation to soluble fibrin, leukocyte count, D-dimer, and also antithrombin and TAT (Table 3).

Parameter	Spearman Rank Correlation Factor R	Significance
Soluble fibrin	-0.39	p<0.01
Leukocyte count	-0.49	p<0.01
Platelet count	-0.11	ns
D-dimer	-0.30	p<0.01
Hemoglobin	-0.16	ns
PT	0.17	p<0.05
Fibrinogen	0.12	ns
TAT	-0.32	p<0.01
Antithrombin	-0.27	p<0.01

Table 3. Correlation between COT and other hemostatic parameters, n=153, ns= non significant. All 153 samples from 17 patients were included.

TAT and antithrombin should perhaps not be included in correlation assessment, as antithrombin concentrates were given to some patients, and these concentrates have been shown to contain TAT as well, directly influencing the correlation results. Correlations of COT to TAT and antithrombin in both subgroups analyzed one by one were however similar to the above results, indicating only a minor influence of antithrombin administration on the correlation results. In paper I, PT (INR) correlated to COT in patients undergoing warfarin treatment, whereas in the neurotrauma study, there is no such correlation. PT (INR) levels in warfarin treatment show hypocoagulation as the vitamin K dependent coagulation factors are low, and no rapid changes occur. PT (INR) does not normally increase as a

sign of hypercoagulation in an acute phase of e.g. neurotrauma, but rather decrease as a consequence of coagulation factor depletion, in a later phase. Therefore, in the early phase of neurotrauma described in paper II, COT and PT (INR) should not be expected to correlate.

Patient outcome was assessed after three months with Glasgow outcome scale (GOS), which was equal in both groups. Interestingly, COT levels at hospital admission correlated to GOS, $R=0.51$, $p<0.05$. We conclude that COT was the only parameter able to detect the attenuation of coagulation activation induced by antithrombin treatment. However, after 3-5 days, there was no longer any difference in COT or plasma antithrombin concentration between the groups. Perhaps if the antithrombin administration had been prolonged, there would have been a sustained effect of the treatment. The finding of a correlation between COT at admission and patient outcome needs further investigation.

Endotoxin induced effects measured by COT

Endotoxin injection to healthy volunteers (paper IV)

Monitoring of coagulation is crucial in intensive care, to discover changes in the hemostatic balance before clinical exacerbation of disease and development of DIC and organ failure. COT was measured bedside in nine healthy volunteers subjected to intravenous injection of endotoxin (2 ng/kg), and blood samples were drawn before endotoxin injection (baseline), at 3 and 6 hours. Heart rate and body temperature was measured, as well as F1+2 and aPTT. The endotoxin injection produced the expected clinical signs of acute systemic inflammation with chills and nausea (83, 99, 144, 145). Heart rate and temperature reached maximum levels at 3 hours, whereafter they decreased but were still slightly elevated at the end of the study. Baseline COT was 10.6±3.6 minutes (Figure 11).

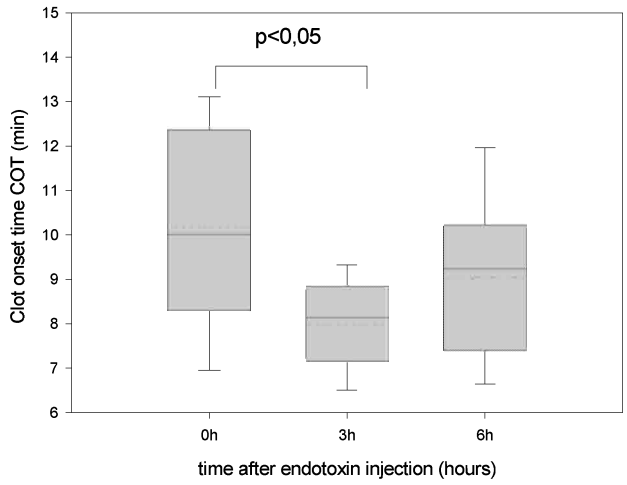


Figure 11. COT after endotoxin injection in healthy volunteers. Boxes with 5th 95th percentiles.

After endotoxin injection, activation of coagulation was seen as a significant decrease of COT at 3 hours. At 6 hours, the COT had recovered towards normalization and did no longer differ from baseline levels. The levels of aPTT were significantly decreased 3 hours after endotoxin injection, however the levels never decreased below reference interval. A significant increase of the F1+2 levels were seen at 3 hours, whereafter F1+2 continued to increase even at 6 hours (Figure 12).

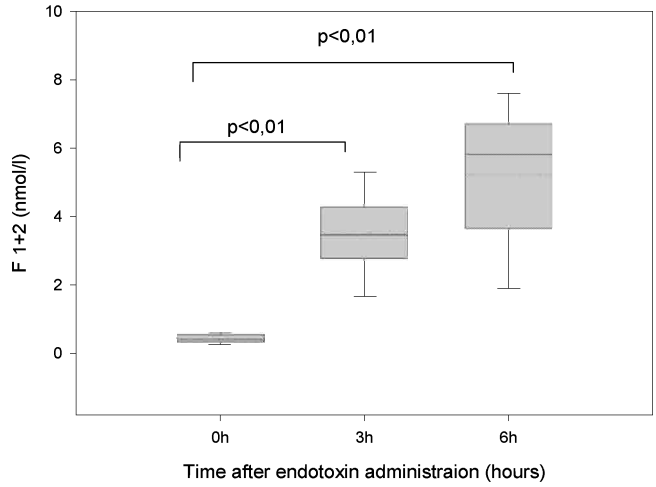


Figure 12. F1+2 levels after endotoxin injection in healthy volunteers. Box plots are given with 5th 95th percentiles.

These findings illustrate that even a mild, transient activation of the coagulation system caused by intravenous endotoxin injection can be monitored with the COT method. COT levels normalized at the end of the experiment, whereas F1+2, considered to be one of the best parameters for DIC evaluation (146, 147), increased throughout the experiment and did not return towards baseline levels at the end of the experiment, which COT and the clinical symptoms did. As COT analyses are carried out in less than 15 minutes, sequential tests may easily be performed allowing studies focusing on the clinical course and monitoring the effect of therapeutical interventions. In summary, COT shows promising results for bedside testing, specifically for repeated measures over time, visualizing the degree of activation of the coagulation system.

Endotoxin induced effects in vitro measured by COT (paper V)

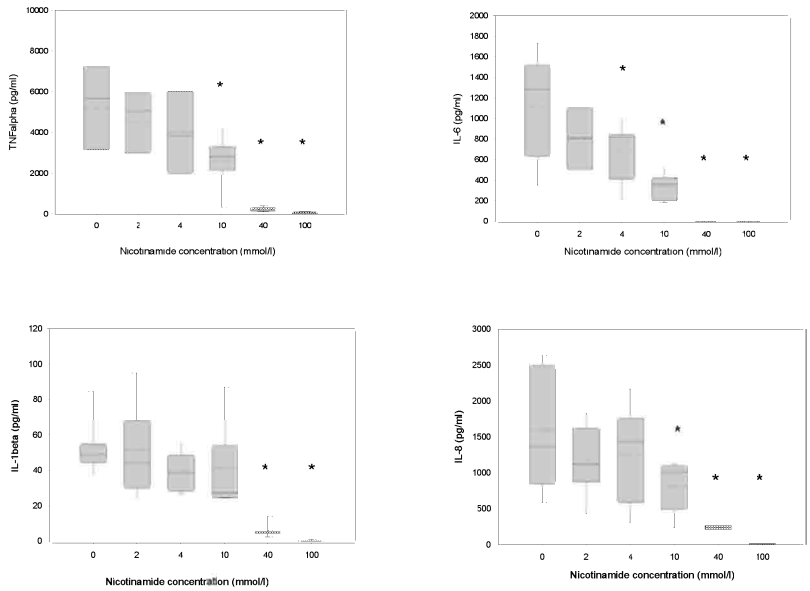
In paper V, peripheral blood leukocytes were incubated with or without endotoxin, in RPMI supplemented with heat inactivated fetal bovine serum. Subsequent to stimulation, cells were washed and 50 µl aliquots containing about 0.5 x10^6 cells, were added to autologous citrated plasma, whereafter COT was assessed by recalcification. Simultaneously, monocyte TF expression was assessed with flow cytometry, measuring the TF mean fluorescence intensity (MFI). The association between COT and monocyte TF expression is demonstrated by a significant correlation, r^2=0.48, between COT and TF (MFI). The correlation is convincing considering that the number of added cells is very low, 0.5 x10^6 leukocytes rendering approximately 10^3 TF expressing cells added to each 0.5 ml

plasma sample. Our conclusion is that COT is a sensitive method, able to detect even small changes in monocyte TF expression.

However, the changes in COT may be partly induced by other procoagulant changes, e.g. COT correlated to the inflammatory marker monocyte surface CD11b levels as well, which may be a parallel phenomenon, or indicate that CD11b indeed contributes to activation of coagulation. CD11b can proteolytically cleave and activate factor X, and is also a receptor for fibrinogen, but whether these findings are of significance in vivo is unknown.

Effects of nicotinamide on proinflammatory cytokines, TF and CD11b

The modulating effects of nicotinamide on the proinflammatory and procoagulant responses to endotoxin were assessed. In paper III, heparinized human blood from healthy volunteers was stimulated with 1ng/ml endotoxin, achieving high levels of proinflammatory cytokines after the 2 hour incubation. When coincubating blood, endotoxin and nicotinamide, all four proinflammatory cytokines measured were inhibited in a dose dependent manner (Figure 13).



Figures 13. Effect of nicotinamide on endotoxin induced proinflammatory cytokines. Box plots are shown with 5th 95th percentiles, *is significance on the level of p<0.05.

Inhibition was seen already at a nicotinamide concentration of 2 mmol/l. These result confirm previous reports of a down-regulator effect on TNFα, but also demonstrates that nicotinamide is a

potent modulator of several other proinflammatory cytokines, and thus has potent immunomodulatory effects in vitro, in a model mimicking human inflammatory disease.

In paper V, the effect of nicotinamide on endotoxin induced monocyte TF and CD11b expression was investigated in peripheral blood leukocyte suspensions and whole blood from healthy volunteers. In response to endotoxin, there was an increase in monocyte TF expression in leukocyte suspensions as well as in whole blood, measured by flow cytometry. Figure 14 shows the TF mean fluorescence intensity (MFI) of endotoxin incubated or non incubated leukocyte suspensions from 8 volunteers.

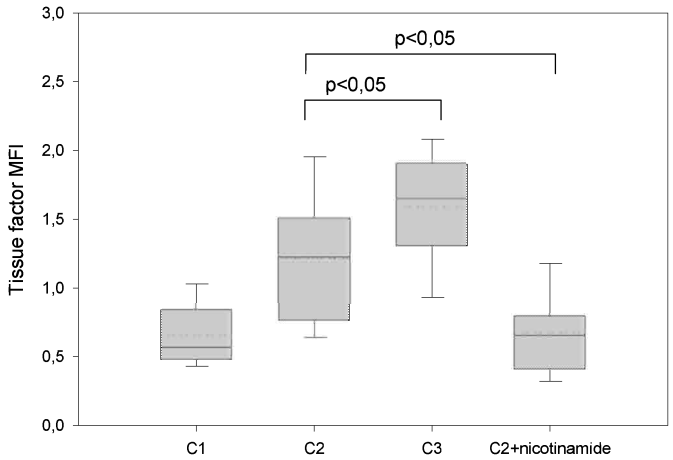


Figure 14. The TF (MFI) in leukocyte suspensions from healthy volunteers, (C1) non incubated, (C2) incubated without endotoxin, (C3) incubated with endotoxin and (C2+nicotinamide) samples are incubated with 100 mmol/l nicotinamide, without endotoxin.

There is some spontaneous activation after 4 hour incubation without endotoxin, likely due to endotoxin contamination in the fetal bovine serum added to the RPMI buffer. Note that the control incubated with only nicotinamide depresses also the spontaneous activation seen in the incubated control. Nicotinamide caused a dose dependent decrease of monocyte TF (MFI) in the endotoxin stimulated leukocyte suspensions (Figure 15). Similar results were obtained in whole blood (results not shown). The decrease in monocyte TF expression was also detected by COT. This describes a previously unknown inhibitory effect of nicotinamide also on the procoagulant changes associated with endotoxemia, and suggests that nicotinamide may have a therapeutic potential in modulating conditions where there is activation of coagulation and inflammation, such as in sepsis and DIC. At nicotinamide doses of 16-40 mmol/l, there was a decrease in monocyte (and granulocyte) CD11b expression as well.

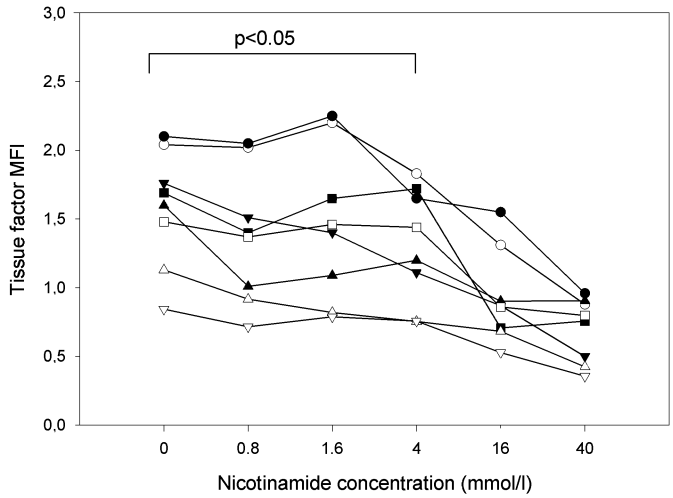


Figure 15. Nicotinamide caused a dose dependent decrease of endotoxin induced monocyte TF (MFI) in leukocyte suspensions, n=8.

Shedded TF

Soluble TF in plasma from whole blood experiments was measured in two healthy volunteers. At 40 mmol/l nicotinamide, an increase from baseline in soluble TF antigen could be detected with ELISA, indicating that the mechanism of the decrease in monocyte TF is at least partly due to shedding from the cell surface.

Mechanism of action of nicotinamide

Nicotinamide is an inhibitor of PARP, giving a rationale for a potential beneficial role of nicotinamide treatment in conditions associated with activation of coagulation and inflammation. PARP is an important mediator of endotoxin responses, as endotoxin administration activates PARP (132), and PARP deficient mice are resistant to endotoxin induced shock (134). Many of the antiinflammatory effects exerted by nicotinamide are thought to be due to nicotinamide inhibiting PARP (131). In paper III, we investigated whether the potent inhibition exerted by nicotinamide on endotoxin induced proinflammatory cytokines was due to PARP inhibition. The endotoxin induced PARP activity was dose dependently decreased by 4-40mmol/l nicotinamide. At 4-100µmol/l 6(5H) phenanthridinone, a specific PARP inhibitor, the same effect on PARP was achieved (148). In these doses sufficient to inhibit PARP activity, however, 6(5H) phenanthridinone was unable to inhibit the proinflammatory cytokines. Nevertheless, the mechanism behind the cytokine inhibition in our model seems not to be due to PARP inhibition. However, we can not exclude a possible PARP dependent inhibition of cytokine protein synthesis in our model, since only cytokine release was measured.

CONCLUSIONS

COT

The coagulation activity monitoring method COT, based on a free oscillating rheometry instrument, proved to be a quick, reliable and easily performed test, able to detect hypo- and hypercoagulation. COT can be assessed in whole blood as well as in plasma. The method allowed identification of the onset of coagulation rather than the later steps of coagulation that is measured with conventional techniques. COT primarily measures thrombin dependent, TF induced coagulation, but is a true global monitoring method as it is influenced also by the contact pathway, and shows correlation to routine coagulation tests.

The COT method showed promising results as a bedside monitoring method, able to detect the mild transient activation of coagulation caused by intravenous injection of endotoxin to healthy volunteers. The COT method is sensitive even to small changes in the amount of endotoxin induced monocyte surface TF. This could partly explain why COT was superior to the well established DIC marker F1+2 in monitoring coagulation activity.
In the neurotrauma study, COT was a predictor of prognosis for the patients. This observation, however, is difficult to interpret and requires further investigation.

Nicotinamide

Nicotinamide was demonstrated to be a potent inhibitor of three major endotoxin induced proinflammatory cytokines, IL-1β, IL-6, and IL-8, in addition to previous results of nicotinamide inhibiting endotoxin induced TNFα. The dose dependent inhibition of endotoxin induced inflammatory responses was shown not to be due to PARP inhibition.

In endotoxin stimulated leukocyte suspensions as well as in whole blood, nicotinamide caused a dose dependent decrease of monocyte TF expression. This describes a previously unknown inhibitory effect of nicotinamide on the procoagulant changes associated with endotoxemia. The decrease of monocyte TF expression is at least partly caused by shedding from the monocyte surface.
Our overall conclusion is that nicotinamide may have a therapeutic potential in modulating conditions associated with activation of coagulation and inflammation, such as in sepsis and DIC.

SAMMANFATTNING PÅ SVENSKA

Blodförgiftning och dess komplikationer är en vanlig dödsorsak bland patienter inom intensivvården. Vid blodförgiftning orsakad av gram negativa bakterier utlöser endotoxin från bakteriernas cellmembran en aktivering av kroppens försvarsmekanismer. Mest betydelsefull är aktiveringen av koagulation och inflammation, och aktivering av dessa två system kan snabbt och dramatiskt försämra patienters kliniska tillstånd. För att upptäcka förändringar innan de ger upphov till kliniska symtom är det viktigt att ofta mäta parametrar som avspeglar aktivering av dessa system.

Vi har utvecklat en övervakningsmetod för koagulationsaktivering, clotting onset time (COT), baserad på viskositetsmätningar. Metoden har visat sig vara snabb och pålitlig för att mäta graden av koagulationsaktivering i plasma eller blod.

COT metoden har även visat lovande resultat som patientnära övervakningsmetod i en studie av endotoxin injektion i friska frivilliga försökspersoner, där COT kunde upptäcka och mäta den övergående koagulationsaktivering som följde efter injektionen.

I en studie med patienter med isolerade skallskador hade COT ett prediktivt värde för patienternas individuella prognos. Det sistnämnda fyndet är dock svårt att tolka och behöver studeras vidare.

Vi har också undersökt vitamin B derivatet nikotinamid och dess effekter på endotoxin inducerad aktivering av koagulations- och inflammationssystemen, i en provrörsmodell av blodförgiftning. I dessa studier har vi kunnat visa att nikotinamid är en potent hämmare av tre viktiga proinflammatoriska cytokiner, IL-β, IL-6 och IL-8. Det är tidigare endast känt att nikotinamid hämmar TNFα. Mekanismen för den hämmande effekten är ännu okänd. I provrörsmodellen av blodförgiftning har vi upptäckt att nikotinamid även har en hämmande effekt på koagulationssystemet, i och med att nikotinamid hämmar monocyters uttryck av tissue factor. Detta har hittills varit okänt. Vi har också visat att hämningen åtminstone delvis beror på så kallad shedding av tissue factor från monocytens yta. Sammanfattningsvis är vår slutsats att nikotinamid är ett möjligt framtida läkemedel vid behandling av sjukdomar där koagulations- och inflammationssystemen är aktiverade, exempelvis vid sepsis och dess komplikationer.

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