The Mechanism of Activation of the Contact System and Fibrinolytic System

Significance for protein homeostasis and protein misfolding diseases

Het Mechanisme van Activatie van het Contact Systeem en het Fibrinolytisch Systeem

Betekenis voor eiwithomeostase en eiwitmisvouwingsziekten
(met een samenvatting in het Nederlands)

Proefschrift

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Coen Maas

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te 's Hertogenbosch

Promotoren:

Prof. dr. Ph.G. de Groot
Prof. dr. B.N. Bouma

Co-promotor:
Dr. M.F.B.G. Gebbink

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Chapter 1

General Introduction

Roles for the contact system and fibrinolytic system beyond haemostasis?

The contact system and the fibrinolytic system are two enzymatic cascades with distinct roles in blood. The contact system is responsible for coagulation *in vitro*, but its physiological role is unknown. The fibrinolytic system is known for its role in the degradation of blood clots. Activation of both systems is seen under a remarkable number of (patho)physiological conditions, that are unrelated to haemostasis. This indicates additional roles for these systems. We here set out to investigate the activation mechanism of these systems in order to help explain what their activation signifies in physiology, as well as in pathology.

1.1 Haemostasis and coagulation

Haemostasis is the prevention of blood loss as a result of injury, requires a tightly regulated mechanism. Upon vascular injury, two events prevent excessive blood loss. Platelets adhere to the subendothelial matrix and become activated, leading to their aggregation. Simultaneously, exposure of plasma to the subendothelium results in coagulation. Together this results in the formation of a thrombus. Coagulation results from the activation of a cascade of enzymes[1], that activate each other in a sequential order, eventually leading to the formation of the enzyme thrombin. Thrombin cleaves fibrinogen to yield monomeric fibrin, which assembles into insoluble fibrillar meshworks to support platelet aggregates. Defects in the production (or functioning) of coagulation enzymes or in platelets are associated with bleeding episodes, underlining the importance of both these systems for haemostasis.

In the injured vessel, coagulation is induced by the transmembrane protein tissue-factor (TF), which is expressed by various cells in the subendothelium. The plasma protein coagulation factor VII (FVII) binds to TF and becomes susceptible for activation by a number of proteases, but most notably thrombin. Activated FVII (FVIIa) in complex with TF subsequently activates factor X into factor Xa, which, with factor Va as a cofactor, can cleave the zymogen prothrombin into thrombin. This process of TF-mediated coagulation is known as the extrinsic pathway of coagulation, since not all required components (i.e. TF) are present in blood.

Besides this TF-driven coagulation cascade, there is a second coagulation cascade. It has been named the contact system, because it is responsible for the clotting of blood when it contacts a surface material such as plastic or glass (Figure 1). Activation of coagulation by the contact system is known as the intrinsic pathway of coagulation, because all protein components required for coagulation are present in blood.

Next to glass, numerous other materials can induce coagulation of plasma, such as the clay-like materials kaolin and celite, metals such as aluminium or titanium[2], the polysulfated sugar dextran sulfate, or the plant-derived compound ellagic acid. More physiologically relevant "surfaces" are amongst others endothelial cell-associated glycosaminoglycans[3], certain nucleic acids such as extracellular RNA[4], platelet-derived polyphosphates[5], aggregated amyloid β peptide[6] or sulfatide[7;8], produced by oligodendrocytes in the central nervous system. However, for what reasons and by what mechanisms the contact system responds to this wide variety of materials is unclear.

1.2 The proteins of the contact system

The initiating enzyme of the contact system is factor XII (FXII). After adhering to a surface, FXII becomes FXIIa and can subsequently activate factor XI (FXI; Figure 1) to FXIa. FXIa in turn can activate a cascade containing factor IX (with factor VIII as cofactor), factor X (with factor Va as cofactor) and prothrombin, finally leading to the formation of active thrombin and fibrin polymers.

Besides the activation of FXI, FXIIa can also activate the zymogen prekallikrein (PK) into

kallikrein. Although both FXI and prekallikrein are very similar and are thought to have evolutionarily diverged "recently" about 124 million years ago by gene duplication (around the time mammals developed[9]), their functions and substrate specificities are strikingly different. Kallikrein has several functions, but most notably, it cleaves the potent vasoactive proinflammatory nonapeptide bradykinin from high-molecular weight kininogen (HK). Hence, this pathway has been named the kallikrein-kinin system. Kallikrein can cleave FXII(a). A first cleavage causes (surface-bound) FXII to become FXIIa, the second cleavage causes dissociation of the light-chain of FXII, containing the protease domain. This fragment of FXII, known as β-FXIIa (or FXIIf), can activate the kallikrein-kinin system, but not coagulation, without a surface present[10] (Figure 1). Thus, two distinct enzymatic systems are triggered by active FXII; one leading to coagulation, the other to an inflammatory response.

Figure 1. Current model of surface-induced activation of the contact system.
When plasma is exposed to a surface such as glass, FXII adsorbs to this surface becomes activated. With HK as a cofactor, both FXI and PK are activated by FXIIa. On the one hand, FXIa propagates the intrinsic pathway of coagulation. On the other hand, the formed kallikrein can cleave the vasoactive- and inflammatory peptide bradykinin from HK and propagate FXIIa formation by cleaving surface-bound FXII once. A second cleavage in surface-bound FXIIa results in the formation of β-FXIIa, a soluble fragment that can only take part in activation of the kallikrein-kinin system, but not in the activation of FXI.

1.3 The paradoxal role of FXII and the contact system in haemostasis
In *vitro*, FXII is essential for coagulation as its absence results in a remarkable delay in clot formation. However, absence of FXII *in vivo* (first described in 1955 in Mr. Hageman) does not lead to a bleeding tendency, as is usually the case in coagulation factor deficiencies[11]. At present, this discrepancy is still not understood.
The surprising observation that deficiency in neither FXII, PK nor HK leads to any form of bleeding disorder suggests that the intrinsic pathway of coagulation is not important for physiological haemostasis. However, deficiency in FXI or any of the underlying proteins of the intrinsic pathway (e.g. FVIII) does lead to bleeding episodes, and are therefore considered important

for physiological haemostasis. How is it possible that only FXI, but not its activator FXII, is important for haemostasis?

Thrombin was identified as a second activator of FXI; when thrombin is generated via the extrinsic (TF-induced) pathway, coagulation is reinforced via a positive feedback loop through activation of FXI[12-14]. Although these findings have been subject to debate for a long time, this is still considered as the most plausible explanation for the observed differences in bleeding tendencies between FXI and FXII[15-17]. As a consequence, the primary contact factors FXII, PK and HK are not considered important for physiological haemostasis. An important question therefore is what the physiological significance of these proteins could be, and by what they become activated *in vivo* to exert their non-haemostatic function.

1.4 Lessons from factor XII-deficient mice

In 2005, a number of experiments were published using FXII-deficient mice[18]. The plasma of these mice was comparable to that of a FXII-deficient person, since it demonstrated little- to no capacity for surface-induced coagulation. However, these mice did not seem to suffer from bleeding episodes or any other obvious defect (like a FXII-deficient person), thus FXII remained unimportant for physiological haemostasis. Upon induction of vessel injury, by $FeCl_3$, FXII- and FXI-deficient mice displayed a less extensive thrombotic response than wild-type control mice. Further analyses pointed out that platelet adhesion did take place, but that thrombus stability was compromised, leading to the conclusion that the FXII-FXI axis plays a role during pathological thrombus formation. This hypothesis was reinforced by similar findings in one mouse model of ischemic stroke (by occlusion of the middle cerebral artery[19]). Further *in vitro* investigations suggested that the FXII-activating surface was present on (or associated with) activated platelets[20], and the most recent reports suggest that this can be attributed to polyphosphate release from dense granules[21]. Similarly, extracellular RNA has been shown to play a role in the activation of FXII during pathological thrombus formation *in vivo*, using a murine thrombosis mode[14]. Since the contact system is specifically involved in thrombosis, but not in physiological haemostasis, it was proposed that FXII may be an attractive therapeutic target for intervention in cardiovascular disease[18;21].

1.5 Lessons from population studies: FXII and the risk of human thrombosis

The above findings at least support a role for FXII and contact surfaces in pathological coagulation, but not in haemostasis. This suggests that a higher plasma level of FXII (or FXI) results in a higher risk for developing thrombosis. However, epidemiological studies suggest that this is not the case: the risk for myocardial infarction (MI) increased with elevated FXI levels as expected, but decreased with increasing FXII levels[22]. These results indicate that either high FXII levels reduce the risk on MI or low levels increase the risk for it. Secondly, the risk for MI in this study was highest in persons with low FXII and high FXI, suggesting that FXII has a different role than the activation of FXI with respect to the risk of developing cardiovascular disease. Another study that investigated the contribution of FXII to the risk of cardiovascular disease, as a part of all-cause mortality, also found that disease risk increases with lower FXII levels[23]. Intriguingly, below 10% of FXII antigen the risk reverted to the risk of the reference group with normal FXII levels. These data may correspond to earlier reports on lowered FXII levels in patients with coronary heart disease[24]. The unexpectedly inverse relationship of FXII levels with cardiovascular disease give rise to the idea that there may be a role for FXII-dependent plasmin

generation *in vivo*, which was reported *in vitro*[25;26].

The most commonly occurring polymorphism in the FXII gene is on nucleotide 46, in a Kozak consensus sequence, where the normally occurring cytosine (C) can also be a thymine (T)[27]. As a result, the 46T allele can not be translated as efficiently as its 46C counterpart, resulting in lower FXII and FXIIa levels. The prevalence of the wildtype 46CC is reported at 57%, the heterozygous 46CT genotype at 38% and the homozygous 46TT genotype at 5% [28], but this may vary with the population that is being studied[27;29]. Although it is to be expected that persons with the 46TT genotype are at risk for developing cardiovascular disease since they have lower FXII levels, no such associations were found, although in the same studies lowered FXII levels did correlate with disease risk[28]. This suggests that reduced FXII levels are a consequence of (cardiovascular) disease rather than a cause.

1.6 Activities of the kallikrein-kinin system

Kallikrein and bradykinin have a number of roles, *in vivo* unrelated to haemostasis. First, receptors for the short-lived peptide bradykinin are present on a large number of cell types, most notably endothelial cells (EC). There are two G-protein coupled receptors for bradykinin: the bradykinin B1 and –B2 receptor. The bradykinin B1 receptor is expressed in response to tissue injury and infection, and has a role in recruitment of leukocytes[30-33]. Also, this receptor is implicated in the mechanism of pain. In contrast to the bradykinin B1 receptor, the bradykinin B2 receptor is constitutively expressed by many cell-types. This receptor has a role in the regulation of blood pressure, since it can induce release of nitric oxide (NO) and prostacyclin (both of which induce vasodilation). Additionally, the bradykinin B2 receptor can associate with angiotensin converting enzyme (ACE), which is able to inactivate bradykinin by degradation. Part of the blood pressure-lowering effects of pharmacological ACE inhibition in cardiovascular patients is attributed to reduced bradykinin degradation. Besides its vasoactive properties, bradykinin is involved by activating mast cells and fibroblasts[34-36], which in turn secrete a number of proinflammatory and chemotactic cytokines.

Secondly, there is interplay between the fibrinolytic- and kallikrein-kinin system. Besides NO and prostacyclin, also tissue-type plasminogen activator (tPA) is released by bradykinin-stimulated EC. Both FXIIa and kallikrein can convert the zymogen plasminogen into the fibrinolytic protease plasmin[25;26]. Kallikrein is also able to enhance the activity of urokinase plasminogen activator (uPA) by a single cleavage step[37], stimulating fibrinolysis. At the moment, the importance of fibrinolytic activation by the kallikrein-kinin system for *in vivo* haemostasis is unclear, but has alternatively been proposed to play a role in wound healing and adipocyte differentiation[38;39].

Thirdly, the contact system can interact with the complement system: FXIIa can directly activate C1 esterase, while β-FXIIa is able to activate the complement system via activation of C1 component[40]. These interactions stimulate activation of the classical complement pathway, possibly facilitated by assembly of the contact system on the C1q receptor on endothelial cells[41]. Also, kallikrein activates C3 convertase[42] and induces C5a formation[43].

Lastly, the kallikrein-kinin system has been suggested to influence the adaptive immune system and is implicated in autoimmune diseases[44].

Although most of the known roles of the kallikrein-kinin system are unrelated to coagulation, both genetic deletion of the kininogen gene and the bradykinin B2 receptor gene in mice protects against thrombosis[45;46]. From these studies, it has become apparent that the kallikrein-kinin system can have harmful effects during thrombosis, which are unrelated to coagulation. However, patient studies show that levels of kallikrein and bradykinin increase after myocardial in-

farction, which correlates to their survival rates[47]. Also, kallikrein confers neuroprotection in rat models of ischemic stroke[48;49], and bradykinin receptor B2 knockout mice display exacerbated postischemic brain injury in a similar model[50]. Obviously, the activities of the kallikrein-kinin system are diverse and its role may protective or detrimental depending on the circumstances via poorly understood mechanisms.

1.7 Involvement of the contact system in disease

Since FXII and the contact system have mainly been studied with respect to its role in surface-induced coagulation, there is special attention for their role in thrombotic disease. However, there are a number of other diseases, in which this system has been implicated, independent of its role in coagulation.

As discussed earlier, the kallikrein-kinin system has protective properties in myocardial infarction and heart failure. The protective effects can partly be attributed to the vasoactive properties of bradykinin, but several other functions have been reported. In response to myocardial infarction, both cardiomyocytes and fibroblast proliferate, leading to ventricular hypertrophy and progression of heart failure (reviewed by Costa-Neto[51]). The interaction of bradykinin with its B2 receptor have been found to confer protective and anti-hypertrophic effects in this setting[51;52].

Renal diseases are characterized by a compromised filtration barrier, amongst other causing loss of protein via the urine (proteinuria). The disease progress is hallmarked by inflammation and fibrosis of the kidney, leading to loss of renal function, and is often a consequence of hypertension. Similarly to myocardial infarction, the kallikrein-kinin system has been reported to exert protective effects in this disease: a polymorphism in the bradykinin B2 receptor is associated with the development of chronic renal failure[53]. The antihypertensive effect of bradykinin is considered important in renal protection, and ACE-inhibition in chronic renal disease contributes to this effect. Also during ischemic acute renal failure, bradykinin (and ACE-inhibition) are protective[54]. However, independent of its blood-pressure regulating properties the kallikrein-kinin system can confer renal protection by reducing inflammation, oxidative stress, apoptosis and fibrosis. This antifibrotic role of bradykinin in renal disease has been elucidated by Schanstra et al.[55], who reported a profibrotic phenotype of bradykinin B2 receptor knock-out mice in a model for renal fibrosis. The antifibrotic effects of bradykinin were attributed to a mechanism, in which bradykinin induces elevation of plasminogen activators and the matrix metalloprotease MMP-2, leading to clearance of fibrotic protein deposits.

Diabetic retinopathy is a commonly occurring complication of diabetes mellitus, leading to loss of vision. As a consequence of increased vascular permeability, macular edema can occur and extravasation of plasma proteins can lead to macular degeneration. Analysis of the vitreous proteome showed that FXII and the contact system are upregulated in proliferative diabetic retinopathy[56]. A recent study showed that the activity of the enzyme carbonic anhydrase I is increased in the diabetic eye, leading to alkalinization of the vitreous[57]. For unknown reasons, this induces activation of FXII and the kallikrein-kinin system, explaining the pathologically increased vascular permeability.

Mutations that cause deficiency or dysfunction of C1-inh lead to the disease hereditary angioedema (HAE) type I and II, respectively. This life-threatening affliction is caused by uncontrolled generation of bradykinin, leading to episodes of swelling in various tissues. The episodes can be triggered by allergy attacks and tissue trauma, but often the cause of an episode remains unknown. An interesting study has shown that the induction of blisters in the skin of HAE patients, but not controls, is accompanied by generation of kallikrein in the blister fluid[58]. It has

been reported that patients receiving ACE-inhibitors are at risk for developing swelling episodes, most likely because of the effects of ACE on bradykinin levels. A third type of hereditary angioedema (type III) is caused by one of two known rare mutations in the FXII gene, leading to increased FXII activity and resulting in the same pathology[59;60]. The commonly described treatment for HAE is injection of recombinantly produced C1-inh, but clinical trials are underway for registration of new kallikrein-specific inhibitors, such as DX-88[61]. Thus, FXII-dependent kallikrein generation is the underlying cause for the bradykinin-induced edema in HAE patients. Paradoxally, this is not accompanied by elevated thrombotic risk, which suggests that the results of the transgenic mouse models on the role of FXII in coagulation and kallikrein formation might be separated *in vivo*.

Besides the pathological activation of the kallikrein-kinin system in diabetic retinopathy and HAE, also exogenous factors can induce similar pathology. A recent report demonstrated that a batch of heparin that was distributed in the United States and Germany gave severe anaphalactoid reactions. The heparin contained a contamination that activated FXII-dependent generation of bradykinin, as well as FXII-dependent complement activation[62]. This contamination was identified as oversulfated chondroitin sulfate, a molecule that resembles the classical *in vitro* FXII-activator dextran sulfate[63]. This publication demonstrated the strength of the inflammatory response that could result from the activation of FXII *in vivo*. However, the activation of the coagulation system was not investigated in these studies since the absence of thrombotic complications by FXIIa could be attributed to the heparin infusion.

In conclusion, FXII and the kallikrein-kinin system have protective roles in a number of diseases. However, (over)activity of this system can also have pathological consequences. The roles of FXII and the contact system in other pathologies, such as Alzheimer's disease (AD)[64;65], inflammatory diseases such as vasculitis[66], and arthritis[67;68], allergy and anaphalaxis, autoimmune disease[44], infection[69] and recurrent pregnancy loss[70-72] are therefore difficult to interpret.

An intriguing question that arises is why and how the contact system becomes activated in all these conditions, since (except for the heparin contamination) no contact-activating surfaces have been reported to be present. Hence, the identification of physiological activators is of immense importance to understand the activation of FXII *in vivo* and to explain why the kallikrein-kinin system activation is often not corresponded by coagulation.

1.8 The fibrinolytic system

The fibrinolytic system is present in plasma with the degradation of fibrin polymers in blood clots. Similar to the contact system, it is a proteolytic mechanism that results in the formation of one main effector enzyme, plasmin, which cleaves fibrin (Figure 2). Plasmin is formed via activation of its liver-produced zymogen plasminogen by either tissue-type plasminogen activator (tPA) or urokinase plasminogen activator (uPA). tPA, which is structurally homologous to FXII, is released by activated endothelial cells in the close vicinity of a thrombus, and can bind directly to the fibrin[73]. Fibrin-bound tPA becomes activated and can in turn cleave plasminogen to yield plasmin. The cleavage of fibrin by plasmin takes place C-terminally from lysine or arginine residues; these residues form additional binding sites for tPA and plasmin, thereby promoting fibrinolysis. Plasmin also cleaves tPA, which is produced as a single-chain protein, to generate the more active two-chain tPA. In contrast to tPA, uPA, does not directly interact with fibrin. Upon activation of (endothelial) cells, uPA is released and the uPA-receptor (uPAR) is externalized. Receptor-bound uPA subsequently activates plasminogen on the cell-surface. Like tPA, single-chain uPA can be cleaved by plasmin to yield a more active two-chain uPA, generating another

positive feedback loop for fibrinolysis.

Although both these plasminogen activators can operate independently of each other, both can be inhibited by serine-protease inhibitors (serpins), such as PAI-1 (plasminogen activator inhibitor 1) or PAI-2. Since PAI-2 is only expressed in the placenta, PAI-1 is the most important plasma inhibitor of the plasminogen activators. In the brain, tPA activity can also be inhibited by neuroserpin. Similarly, the activity of plasmin is mainly inhibited by α2-antiplasmin.
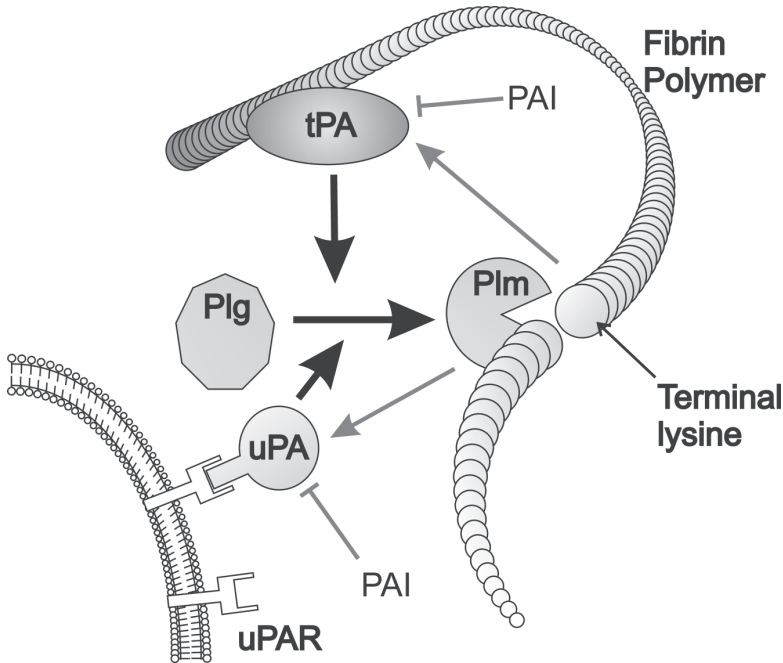

Figure 2. The fibrinolytic system. After the formation of a blood clot, endothelial cells will release tissue-type plasminogen activator (tPA), which can adhere to the fibrin polymer. Subsequently, tPA activates and begins to cleave plasminogen (Plg) which has been incorporated in the fibrin clot. The active enzyme plasmin (Plm) then starts cleaving c-terminal to lysine or arginine residues in the fibin polymer, thereby enhancing fibrinolysis by improving the binding of tPA and Plm via their kringle-domains. Additionally, plasmin can cleave urokinase plasminogen activator (uPA) to enhance its activity and generate a positive feedback loop. Both tPA and uPA can be inhibited by serpins, such as PAI-1. Similarly, Plm activity can be terminated by the inhibitor α2-antiplasmin (not shown).

1.9 Additional roles for plasminogen and its activators.

Active plasmin can cleave fibrin polymers to effectively clear blood clots and reduced fibrinolysis has therefore been identified as a risk factor for cardiovascular disease[74;75]. Conversely, hyperfi-brinolysis can result in pathological bleeding[76]. However, like the contact system, the activity of plasmin and its activators is not limited to haemostasis alone.

Besides fibrin, plasmin is able to cleave a large number of other proteins, such as e.g. von Willebrand factor[77], coagulation factor VIII[78], fibronectin, thrombospondin[79], collagenases, gelatinases, laminin, fibrinogen and β2-glycoprotein I[80]. Several of these cleavages have conse-quences for the functioning of these proteins; for instance, plasmin-cleaved β2-Glycoprotein I (but not the native molecule) can inhibit angiogenesis[81] and is a negative regulator of fibrinoly-

sis[82] that is associated with cerebral infarction. This apparent lack of substrate specificity suggests that plasmin to be involved in a large number of processes besides haemostasis.

tPA is expressed by neurons, where it is stored in synaptic vesicles. It influences long-term memory (long-term hippocampal potentiation) by enhancing synapse formation[83]. tPA exerts this function is through the activation of plasminogen, resulting in activation (by cleavage) of pro-brain-derived neurotrophic factor (pro-BDNF), a key factor for synapse formation, which is also released from synaptic vesicles[84]. Besides its role in memory, the fibrinolytic system has been reported to play a role in neurotoxicity, independent of fibrin cleavage[85;86]. Apparently, tPA-dependent plasmin activation has a pathological role by contributing to the induced cell-death, but neither its activator, nor substrate is fibrin.

Plasmin and its activators are involved in the regulation of angiogenesis via various mechanisms. Extracellular matrix (ECM) remodeling is a key step in angiogenesis, which amongst others results in the cleavage of collagen type XVIII. Plasmin can also degrade pro-angiogenic factors, such as ECM-bound CCN1[87], as well as the key protein vascular endothelial growth factor-A[88]. The fibrinolytic system is involved in wound regeneration and tissue repair. It protects against fibrotic lung disease, in a fibrin-independent manner[89] and recovery of various organs such as the pancreas or the eardrum after perforation, is dependent on the presence of plasminogen[90;91].

Besides its role in tissue repair, plasmin and its activators are also implicated in inflammation and innate immunity. Active plasmin controls macrophage function[92;93], amongst others via activation of matrix-metalloproteases. Also, toll-like receptor 4 signaling is potentiated by plasmin[94], which is important for host defense. During certain infections, the fibrinolytic system induces clearance pathogens, as well as necrotic tissue, via activation of neutrophils and macrophages[95-97].

Although the processes described above can be interpreted as being protective, several studies demonstrated that fibrinolytic activity can have both beneficial and degenerative features in pathological situations, as for example is the case in mouse models of multiple sclerosis[98]. Additionally, interpretation of the various functions of plasmin and its activators is complicated since many pathological inflammatory conditions are corresponded by an upregulation of PAI-1 levels[99]. Taken together, these reports suggest that plasmin and its activators are involved in physiological processes that surpass the breakdown of blood clots.

1.10 A hypothesis to explain the diverse roles of the contact system and fibrinolytic system

Both the contact system and fibrinolytic system play roles in situations where the formation- or breakdown of fibrin polymers does not take part. Moreover, the endogenous activators of both systems under these conditions and the exact reasons for their activation remain to be clarified.

1.11 Protein folding, misfolding and amyloid formation

Globular proteins are synthesized in such way that, in aqueous solutions, their hydrophobic parts reside within the protein molecule, whereas the hydrophilic parts are generally exposed to the environment and allowed to interact with polar water molecules. This folding process can be assisted intracellularly by chaperone-proteins. As a consequence of this folding process, native globular proteins are often soluble in a watery environment and can perform diverse tasks such as enzymatic catalysis because of their specific conformation.

When a protein is denatured *in vitro*, it has a tendency to aggregate, which sometimes even leads to the formation of visible protein precipitates. Changes in temperature, pH, osmolarity, enzymatic cleavage and adsorption to a surface can lead to protein misfolding. However, also *in*

vivo, a protein will misfold as a consequence of its environment and stability, which can lead to aggregation[100]. The aggregation of denatured proteins is a consequence of the solvent-exposure of hydrophobic residues, which normally reside within the protein molecule.

A troublesome form of protein aggregation is seen in about 25 diseases where proteins or peptides with misfolded properties assemble into elongated amyloid fibrils that can not be sufficiently cleared. These diseases are known as the amyloidoses. In these diseases, amyloid fibrils are deposited in tissues and organs that gradually become compromised in their function. Although different proteins are responsible for the individual pathogeneses, the fibrillar structures that they form show structural similarity. The common structural denominator that they display is called the amyloid cross-β structure, which is characterized by an organized stacking of β-sheets in the fibrillar protein polymer[101]. The β-sheets are stacked with a 4.7 Å distance between the amino-acid strands that run perpendicular to the fibril axis, hence the name cross-β structure. This name, as well as the term amyloid, refers specifically to the three-dimensional structural properties of protein fibrils that are commonly known for their involvement in disease. Since the presence of cross-β structure in a protein aggregate can only be confirmed by X-ray diffraction, it is currently not possible to appreciate the presence of amyloid cross-β structure *in vivo*. This problem is partly solved by the use of certain histopathological dyes that show affinity for amyloid deposits in tissue sections. However, it has become clear that these dyes already develop affinity for protein aggregates without fibrillar structure and it can therefore be said that misfolded protein aggregates adopt "amyloid-like" properties.

A number of reports have described that the unfolding of globular proteins leads to the generation of an oligomeric protein species that is toxic to cells[102;103]. Indeed, a number of diseases are hallmarked by the generation of such species, which later on assemble into amyloid fibrils. Examples of such diseases can be neurodegenerative in nature, such as Alzheimer's disease (AD), but also other tissues can become affected, as is the case in diabetes type II or the systemic amyloidoses. Although these diseases are multifactorial in nature, it was commonly accepted that the amyloid plaques caused the pathological tissue degeneration, but at present it is becoming increasingly clear that their oligomeric intermediates may be the perpetrators of disease[104-106]. Interestingly, this intrinsic property of proteins does not seem to be related to proteins that are present in classical protein misfolding diseases such as Alzheimer's disease. Apparently, also disease-unrelated proteins can form toxic oligomers upon their unfolding, giving rise to the idea that it may be an intrinsic property of globular proteins[102]. In line with this idea, novel diseases are identified that are (in part) mediated by misfolded proteins[107;108].

The generic cytotoxic property of misfolded proteins gives rise to the idea that there might be a structural characteristic that they share. Many proteins are able to adopt properties, reminiscent of amyloid structure, under denaturing conditions. It has therefore been postulated that adoption of amyloid-like properties is the common feature of various misfolded proteins[102;103;109;110]. However, if nearly all protein can form toxic species upon their unfolding, with common structural non-native characteristics, their recognition and clearance *in vivo* is warranted.

We hypothesized that both the contact system and fibrinolytic system are involved in the initial recognition of misfolded proteins via several lines of evidence. First, both systems are activated in Alzheimer's disease; both FXII and tPA are present and active in the direct surroundings of amyloid plaques in AD[65;111;112]. These interactions lead to bradykinin formation and complement activation113, as well as plasmin formation[114]. Secondly, it has been demonstrated that the activity of plasmin aids in the clearance of amyloid β peptide[115;116] and lowered plasmin activity has been suggested as a risk factor for AD[117]. Thirdly, both tPA and FXII activation are directly

induced by amyloid β peptide[118;119]. Interestingly, also other misfolded proteins can stimulate tPA-dependent plasmin formation, such as denatured forms of albumin[120], prothrombin[121], fibrinogen[122;123], apoferritin, antithrombin, alpha1-protease inhibitor, α2-macroglobulin[124], as well as aggregates of endostatin[125] and it was demonstrated that tPA is activated by a common structural element in misfolded proteins[109]. However, no such reports are present on FXII, which to date has an elusive role *in vivo*.

In summary:

Protein misfolding is a generic propensity of proteins and amyloid fibrils can be formed by a myriad of proteins in vitro. However, only a small fraction of the proteins that can form amyloid structure, actually have been found to do so in vivo, resulting in amyloid disease[126]. This suggests that most of the other proteins present in the human body either do not form actual amyloid structure upon unfolding, or do not have a chance to form it due to the presence of a mechanism for the recognition and clearance of unfolded proteins. However, not much is known about the molecular mechanisms that underlie the structure-based clearance of proteins. We hypothesized that the contact system and fibrinolytic system recognize misfolded proteins via their initiating enzymes FXII and tPA, leading to their activation.

1.12 Aim of this thesis

Elucidation of the activation mechanism of FXII and tPA.

In chapter 2, we investigated the mechanism of activation of FXII and the contact system. We hypothesized that FXII, like tPA, would be activated by misfolded proteins, leading to activation of the contact system. We also hypothesized that well-known contact surfaces required a misfolded protein intermediate for activation of the contact system. From our studies, it became apparent that FXII is indeed activated by a wide variety of misfolded proteins *in vitro*, but also *in vivo*. Surprisingly, this led to unilateral activation of the kallikrein-kinin system *in vitro* and *in vivo*. These findings indicate the presence of a novel class of activators of FXII and point towards a revised mechanism of contact system activation. Additionally, these findings may help to explain the paradoxal existence of FXII and the enigmatic activation of the kallikrein-kinin system in various pathologies.

In chapter 3, we aimed to identify the binding site of tPA and homologous proteins for misfolded proteins. We found that the fibronectin type I domain is required for binding to misfolded protein aggregates, with amyloid properties. Interestingly, this domain is responsible for the initial binding of tPA to fibrin[73] and implicated in the interaction of FXII with contact surfaces[127].

In chapter 4, we investigated the activation of the fibrinlytic system in systemic amyloidoses, a disease hallmarked by circulating amyloidogenic proteins. We found activation of the fibrinolytic system in the majority of patients with this disease, as measured by the presence of plasmin-α2-antiplasmin complexes. These findings suggest that hyperfibrinolysis may contribute to the haemorrhagic episodes that are seen in systemic amyloidoses.

In chapter 5, we subsequently considered that the interaction tPA with fibrin may not be mechanistically different from the interaction of tPA with misfolded proteins. We therefore hypothesized that fibrin possess amyloid-like properties, as are present in misfolded protein aggregates. From structural determinations, including X-ray fiber diffraction experiments, we conclude that fibrin polymers have amyloid-like properties (using histochemical techniques) and possess amyloid cross-β structure.

In chapter 6, we explored the possibility that the activation of tPA could be used *in vitro*

to detect potentially hazardous misfolded proteins in biopharmaceutical preparations, which are susceptible to protein stability and –aggregation issues due to production and storage. From our studies, it became clear that the amount of misfolded protein in a biopharmaceutical preparation correlated with the incidence and strength of immune responses against it. Our results indicate that breaking of tolerance against biopharmaceuticals, which can be a problematic issue in medicine, is dependent on the structural quality of a biopharmaceutical and suggest that also the immune system can differentiate between native and misfolded proteins.

In chapter 7, we report that immune responses against β2-glycoprotein I (β2-GPI), an autoantigen involved in the antiphospholipid syndrome (APS), are dependent on the structure of this protein. Upon phospholipid binding, β2-GPI underwent significant structural changes, as observed by electron microscopy. Immunization studies with β2-GPI showed that the protein became immunogenic, when co-injected with phopholipid, as well as in absence of phospholipids, but with an artificially modified structure. Further studies will have to be performed to investigate whether the structure-related immune responses against β2-GPI also lead to the pathological antibodies that cause APS.

In chapter 8, we will discuss the implications of our findings and will elaborate on potential opportunities for future research.

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Chapter 2

Misfolded Proteins Activate Factor XII in Humans, Leading to Kallikrein Formation Without Initiating Coagulation
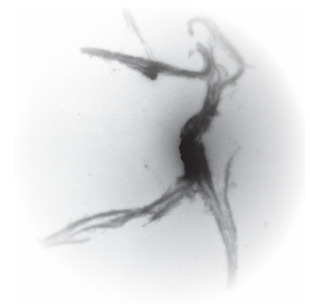
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Coen Maas,[1] José W.P. Govers-Riemslag,[2] Barend Bouma,[1,3] Bettina Schiks,[1,3] Bouke P.C. Hazenberg,[4] Henk M. Lokhorst,[1] Per Hammarström,[5] Hugo ten Cate,[2] Philip G. de Groot,[1] Bonno N. Bouma,[6] and Martijn F.B.G. Gebbink[1,3]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands. [2]Laboratory for Clinical Thrombosis and Haemostasis, Department of Internal Medicine and Cardiovascular Research Institute Maastricht, Maastricht University, Maastricht, The Netherlands. [3]Crossbeta Biosciences, Utrecht, The Netherlands. [4]Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, Groningen, The Netherlands. [5]IFM-Department of Chemistry, Linköping University, Linköping, Sweden. [6]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA.

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Abstract

When blood is exposed to negatively charged surface materials such as glass, an enzymatic cascade known as the contact system becomes activated. This cascade is initiated by autoactivation of Factor XII and leads to both coagulation (via Factor XI) and an inflammatory response (via the kallikrein-kinin system). However, while Factor XII is important for coagulation in vitro, it is not important for physiological haemostasis, so the physiological role of the contact system remains elusive. Using patient blood samples and isolated proteins, we identified a novel class of Factor XII activators. Factor XII was activated by misfolded protein aggregates that formed by denaturation or by surface adsorption, which specifically led to the activation of the kallikrein-kinin system without inducing coagulation. Consistent with this, we found that Factor XII, but not Factor XI, was activated and kallikrein was formed in blood from patients with systemic amyloidosis, a disease marked by the accumulation and deposition of misfolded plasma proteins. These results show that the kallikrein-kinin system can be activated by Factor XII, in a process separate from the coagulation cascade, and point to a protective role for Factor XII following activation by misfolded protein aggregates.

Introduction

The contact system is an enzymatic cascade in blood that becomes activated when blood contacts surface materials. For example, this occurs during blood sampling in a glass or plastic vial. The system consists of the zymogens Factor XII (FXII), FXI, and prekallikrein (PK) and the non-enzymatic cofactor high-molecular-weight kininogen (HK). After activation of FXII into activated FXII (FXIIa), 2 strikingly different events can be triggered: (a) propagation of the intrinsic pathway of coagulation by activation of FXI; and (b) activation of the kallikrein-kinin system by activation of PK. Although the contact system is activated by a large number of mainly non-physiological surfaces *in vitro*, the role of this system is still elusive.

Blood coagulation proceeds via extrinsic and intrinsic pathways; activation of the extrinsic pathway occurs via exposition of tissue factor at a site of injury and plays a critical role in physiological haemostasis. The intrinsic pathway of coagulation also has the for formation of fibrin as its final consequence, and deficiencies of factors in this cascade (such as FVIII, FIX, and FXI) lead to bleeding disorders. This shows that the intrinsic pathway of coagulation contributes to physiological haemostasis. Paradoxically, deficiencies in the primary contact factors FXII, PK, or HK, which activate the intrinsic pathway of coagulation *in vitro*, are not associated with a bleeding phenotype. This suggests that these 3 factors do not contribute to the physiological role of the intrinsic pathway of coagulation. This contradiction was explained after the identification of an alternative route for the activation of FXI: thrombin, generated by the extrinsic pathway (resulting from exposure of tissue factor to plasma) activates FXI even in the absence of a negatively charged surface[1–3]. The discovery that FXI is activated in an FXII-independent manner adds to our understanding of the intrinsic pathway of coagulation in haemostasis, but still leaves FXII without a physiological function.

Activation of the kallikrein-kinin system by FXIIa leads to release of the peptide hormone bradykinin (BK) from HK, which regulates inflammation, blood pressure control, and pain. The importance of this route is illustrated *in vivo* by observations in patients with hereditary angioedema (HAE). These patients experience painful swelling episodes in various tissues, in the absence of thrombosis. HAE can be caused by mutations in the C1 inhibitor gene, which encodes for the primary plasma inhibitor of a component of the complement system (C1) and the contact system components FXII[4], FXI[4], and kallikrein[5]. An important role for the kallikrein-kinin system in HAE was revealed when it was found that fluids from blisters that were induced in HAE patients contained large amounts of kallikrein, whereas similar blister fluids obtained

from control individuals did not[6]. Additionally, a phenotype similar to HAE is seen with rare mutations in the FXII gene[7], which indicates a role for FXII in inflammatory processes (without thrombosis) *in vivo*.

Thus, although both the intrinsic pathway of coagulation and the kallikrein-kinin system are under the control of FXII and both are activated during surface-induced clotting, it is unclear how the 2 systems are activated *in vivo* and whether this occurs simultaneously.

Our aim here was to gain insight into the mechanism of FXII activation and delineate the routes to either coagulation (FXIa) and/or inflammation (kallikrein). Based on 3 lines of evidence, we hypothesized that FXII can be activated by misfolded proteins. First, FXII is activated by aggregated amyloid β peptide (Aβ) *in vitro*, and increased levels of activated FXII are observed in patients with Alzheimer's disease (AD)[8–11]. Second, it has become apparent over recent years that misfolded proteins, in contrast to native proteins, have structural and functional properties similar to those of amyloid[12]. Third, adsorption of proteins to non-physiological surfaces leads to structural perturbations and misfolding[13–20].

To test this hypothesis, we first investigated whether FXII was activated in patients affected by systemic amyloidosis, a disease in which misfolded plasma proteins of varying origin accumulate into widespread amyloid deposits. We next determined the capacity of misfolded proteins to stimulate FXII-dependent kallikrein generation with both purified proteins and plasma. Subsequently, we determined whether a misfolded protein intermediate is also required for kallikrein formation during contact activation by surfaces. We tested the procoagulant properties of misfolded proteins and determined their capacity to stimulate FXII-dependent FXIa generation. From these experiments, it became apparent that misfolded proteins predominantly stimulated FXII-dependent kallikrein generation, without generating FXIa, and this was verified in plasma of patients with systemic amyloidosis.

Results

Elevated FXIIa levels in systemic amyloidosis.

The cerebrospinal fluid (CSF) of subjects with AD has been reported to contain elevated FXII activity[8;9]. FXII also colocalizes with amyloid plaques in the AD brain[11] and is activated by Aβ *in vitro*[10]. Since we hypothesized that FXII is activated by misfolded proteins in general, we investigated whether elevated activation of FXII was limited to AD or also marked other protein misfolding diseases. Systemic amyloidosis is characterized by deposition of amyloid deposits in various sites of the body. These amyloid fibrils consist not of Aβ peptide, as is the case in AD, but of other amyloidogenic proteins, depending on the specific type of systemic amyloidosis. We found that the plasma levels of FXIIa, determined by enzyme-linked immunosorbent assay (ELISA), were significantly elevated in 25 subjects suffering from systemic amyloidosis (3.36 ± 2.18 ng/mL; expressed as mean ± SD) as compared with 40 age- and sex-matched healthy control subjects (2.07 ± 0.71 ng/mL; P = 0.0079; Figure 1). The significance of this finding was not influenced by the 2 extreme values in the patient population; omitting these values from the data set resulted in a P value of 0.0085 (data not shown). This finding indicates that FXII activation is not limited to the CSF of AD patients, but that it can also occur in plasma during another protein misfolding disease.

Misfolded protein aggregates, but not amyloid fibrils, induce FXII-dependent kallikrein generation in vitro.

Based on these findings in patients, we next investigated whether misfolded proteins other than Aβ are capable of inducing activation of FXII *in vitro*. To investigate this, we used an assay in which activation of FXII was indirectly detected via the capacity of FXIIa to convert PK into
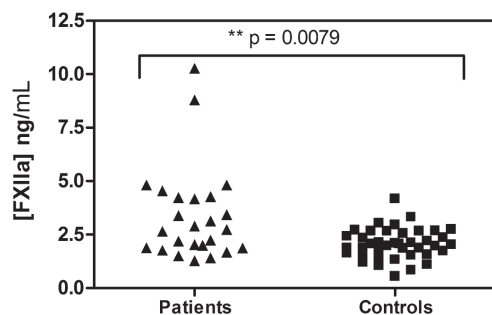
Figure 1. Elevated FXIIa levels in systemic amyloidosis. Plasma samples from 25 patients with systemic amyloidosis (average age, 52 ± 11 years, 36% male) and of 40 healthy controls (average age, 49.4 ± 7.3 years, 37.5% male) were tested for levels of FXIIa by ELISA. Patients with systemic amyloidosis had significantly elevated levels of FXIIa, as compared by 2-tailed Student's t test.

kallikrein. The generation of kallikrein was monitored via cleavage of the chromogenic substrate Chromozym PK. This assay was termed a FXII-dependent kallikrein generation assay, since omission of FXII led to complete loss of kallikrein generation in all our experiments (data not shown), indicating the critical role for FXII.

Misfolded proteins can occur in a variety of forms, such as amorphous aggregates and large amyloid fibrils[21;22]. Since both these forms are present in systemic amyloidosis patients, we also wanted to know which form of a misfolded protein induces FXII activation. The structural properties of protein preparations can be studied by their capacity to enhance fluorescence of the dyes thioflavin T (ThT) and Congo red (CR). Although ThT and CR are primarily known by their affinity for amyloid fibrils, these dyes are also known to display increased fluorescence in the presence of non-fibrillar misfolded protein aggregates (compared with native protein), which can therefore be said to have adopted amyloid-like properties[12;23]. To distinguish between fibrillar and non-fibrillar protein aggregates, protein preparations were also subjected to analysis by transmission electron microscopy (TEM).

We first prepared amyloid fibrils of Aβ (residues 1–42) and tested the ability of this preparation to stimulate FXII-dependent kallikrein generation, using an *in vitro* chromogenic assay that monitors the generation of active kallikrein from PK by FXIIa.

We found that amyloid fibrils of Aβ 1–42 had no capacity to induce FXII autoactivation (Figure 2A), whereas freshly dissolved Aβ 1–42 strongly induced FXII-dependent kallikrein generation (Figure 2B). Analysis by TEM revealed that freshly dissolved Aβ 1–42 contains a large amount of amorphous prefibrillar aggregates, which showed only limited fluorescence of ThT and CR (Supplemental Table 1). The results of this experiment are in line with earlier reports on this topic[8;10] (although no data on fibrillar Aβ was published at that time) and suggest that the FXII-activating properties of Aβ are lost upon fibril formation.

In addition to Aβ, we found that FXII-dependent kallikrein generation was also potently stimulated by misfolded aggregates of transthyretin (TTR; Figure 2C), which forms deposits in both senile and hereditary systemic amyloidosis, but not by the native protein (Figure 2D). Also, amyloid fibrils of the amyloid core fragment of TTR (TTR11; residues 105–115) were completely incapable of inducing FXII-dependent kallikrein generation (Figure 2E). Furthermore, in patients with primary systemic amyloidosis (AL), there is overproduction of immunoglobulin light chains that circulate in plasma and can be purified from urine as non-fibrillar aggregates (called Bence-Jones protein [BJP]). In line with our earlier findings, BJP aggregates were capable of activating FXII-dependent kallikrein generation (Figure 2F; 1 representative patient sample). These experiments show that FXII-dependent kallikrein generation can be initiated by pathogenic proteins in a misfolded aggregated state but not by those that have adopted an amyloid fibril-
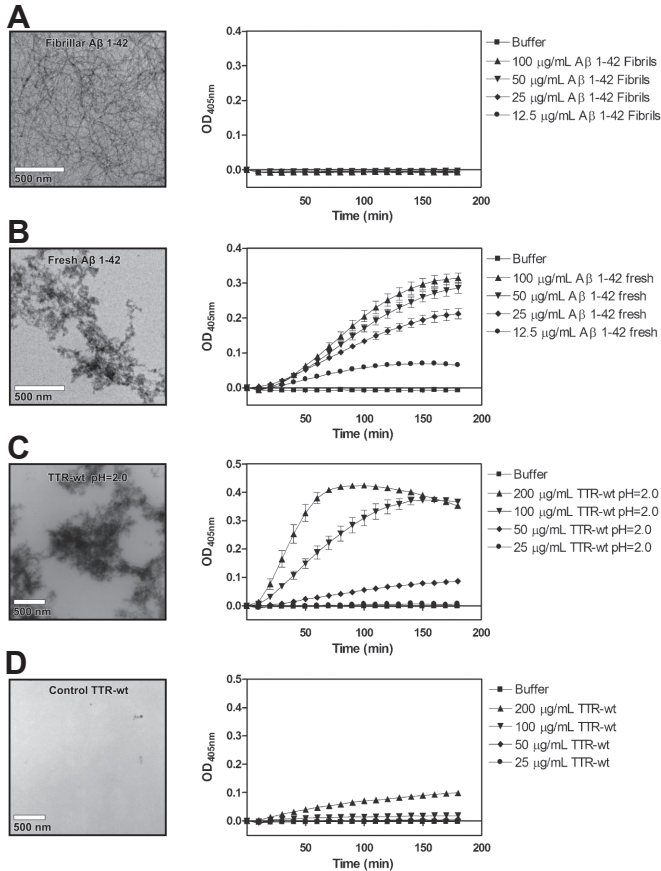
Figure 2. FXII-dependent kallikrein generation is induced by misfolded protein aggregates, but not by amyloid fibrils, *in vitro*. FXII-dependent kallikrein generation was measured *in vitro* by a chromogenic assay. The conversion of 0.3 mM Chromozym PK in the presence of 7.7 nM PK was determined in the presence and absence of 0.97 nM FXII. None of the protein preparations tested was capable of converting Chromozym PK in the presence of PK without FXII present (data not shown). Amyloid fibrils of Aβ 1–42 did not stimulate FXII-dependent kallikrein generation (A), whereas amorphous aggregates of the freshly dissolved peptide did (B). Similarly, only amorphous aggregates of TTR (C), but not native TTR (D) could induce FXII-dependent kallikrein generation. (Figure continues on the next page)

lar structure. We then studied this more extensively using various other proteins.

Glycation of albumin has been reported to lead to formation of aggregated advanced glycation end products (AGE's) with amyloid-like properties[24;25], and glycated albumin has been reported to be present in atherosclerotic plaques[26]. Amorphous aggregates of glycated BSA (BSA-AGE) potently triggered FXII-dependent kallikrein generation, whereas freshly dissolved control BSA did not (Figure 2, G and H, respectively). We also prepared amorphous aggregates of glycated hemoglobin (Hb-AGE), fibrin peptide 13 (FP13), and denatured OVA (dOVA) and endostatin (dEndo), all of which had adopted properties of misfolded aggregates (Supplemental Table 1 and Supplemental Figure 1). All of them, but not their native control preparations, were capable of inducing FXII-dependent kallikrein generation (Supplemental Figure 2, A–H, respectively). Additionally, amyloid fibrils of islet amyloid polypeptide (IAPP; associated with type 2 diabetes) and glucagon were completely inert (Supplemental Figure 2, I and J).

Collectively, these experiments indicate that FXII interacts with a common binding site present in non-fibrillar misfolded protein aggregates but not in amyloid fibrils and that this interaction results in kallikrein generation.

FXII-dependent kallikrein activation by surfaces is modulated by cofactor proteins.

FXII autoactivates in plasma in the presence of surfaces such as kaolin, ellagic acid (EA), and dextran sulfate with an average Mr of 500 kDa (DXS-500k), leading to both coagulation and generation of kallikrein. It has been known for a long time that surfaces such as glass attract and
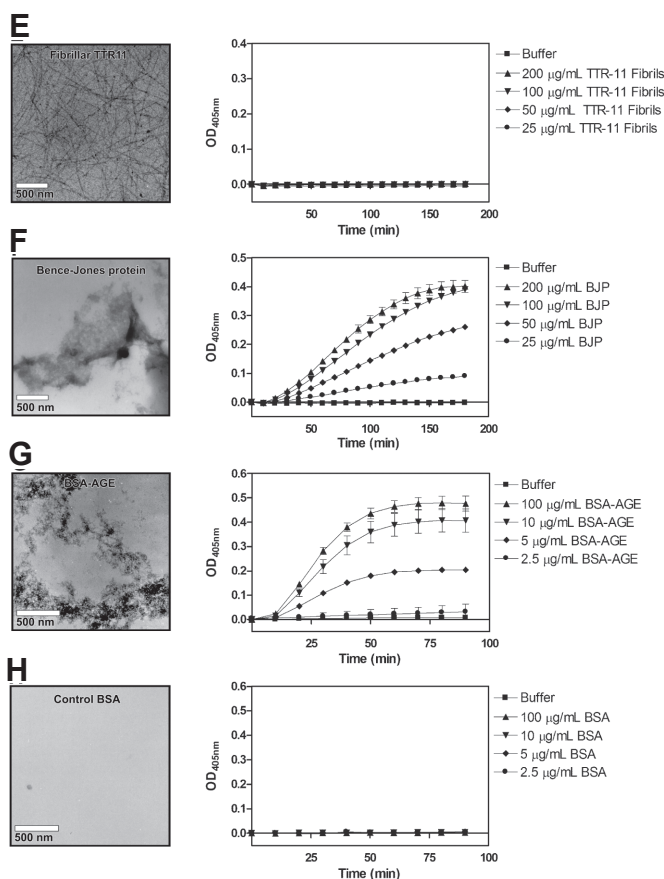
E

F

G

H

Figure 2, continued. FXII-dependent kallikrein generation is induced by misfolded protein aggregates, but not by amyloid fibrils, *in vitro*. Amyloid fibrils of TTR11 (E) could not induce FXII-dependent kallikrein generation. Also, amorphous aggregates of Bence-Jones protein (BJP) (F) and BSA-AGE (G), but not freshly dissolved control BSA (H), could induce FXII-dependent kallikrein generation. The values in the graphs represent the mean ± SEM of duplicate determinations performed within 1 representative experiment of at least 3.

adsorb a number of plasma proteins besides those of the contact system, most notably fibrinogen (Fg) and albumin[13;18;27]. Given our earlier findings, we investigated whether kallikrein generation on surfaces could be mediated via generation of misfolded proteins. To test this hypothesis, we first empirically determined the surface concentrations at which the surfaces were unable to induce FXII-dependent kallikrein generation. Next, we preincubated the surfaces with freshly dissolved BSA, endostatin, and Fg in protein concentrations that gave little or no FXII-dependent kallikrein generation by themselves. In all experiments, FXII-dependent kallikrein generation drastically increased when surfaces were preincubated with either of these proteins.

As shown in Figure 3A, BSA in the presence of kaolin potently induced kallikrein generation. Very similar effects were seen with DXS-500k and EA (Supplemental Figure 3, A and B). As shown in Figure 3B, endostatin in the presence of EA became a strong kallikrein generator. Again, addition of kaolin or DXS-500k to endostatin resulted in similar effects (Supplemental Figure 3, C and D). The combination of Fg and DXS-500k, compared with the individual components alone, also led to synergistic enhancement of kallikrein generation (Figure 3C), which was in agreement with our findings with the 2 other surfaces (Supplemental Figure 3, E and F). As before, no kallikrein generation was observed in any of the experiments in the absence of FXII (data not shown). Although the addition of HK was not necessary for activation of PK in the experiments discussed above (which were performed without HK), addition of HK accelerated the reactions (Supplemental Figure 4). In conclusion, all combinations of 3 contact activating surfaces with 3 proteins that are known to adsorb to negatively charged surfaces led to powerful induction of FXII-dependent kallikrein generation.
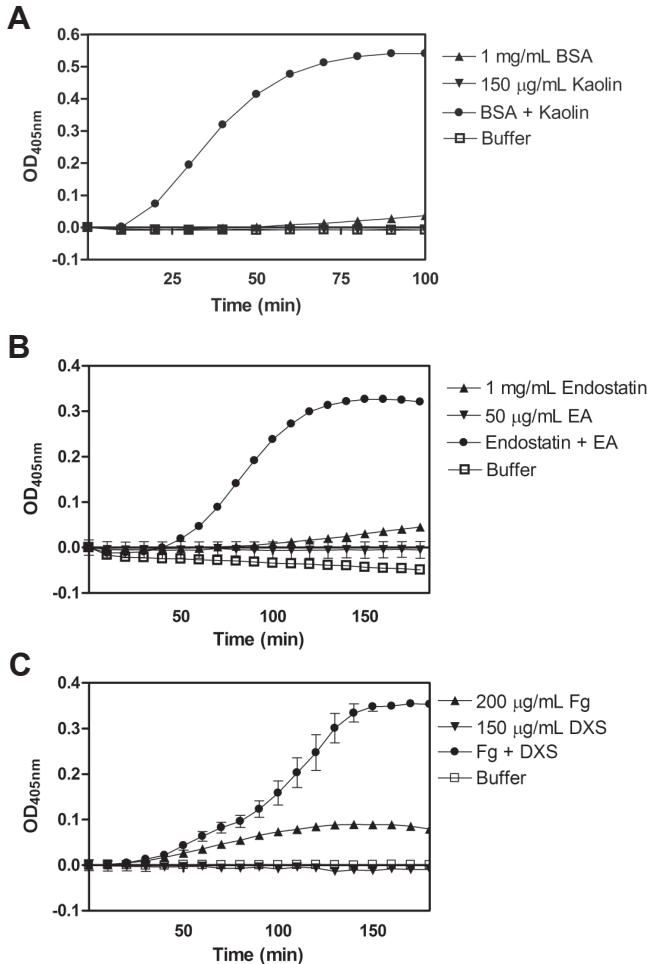
Figure 3. FXII-dependent kallikrein generation by surfaces is modulated by cofactor proteins. Surfaces were incubated for 5 minutes at 37°C with 3 different proteins, BSA (A), endostatin (B) and Fg (C) and analyzed for their ability to induce FXII-dependent kallikrein generation. It was found that addition of these proteins was required for FXII activation by kaolin, EA, and DXS-500k. All other possible combinations of the above proteins with surfaces gave the same results (Supplemental Figure 3). The values in the graphs represent the mean ± SEM of duplicate determinations performed within 1 representative experiment of at least 3.

Proteins undergo structural perturbations in the presence of contact surfaces.

Since we had found that both misfolded protein aggregates alone and combinations of contact surfaces with cofactor proteins result in stimulation of FXII-dependent kallikrein generation, we hypothesized that proteins are being misfolded upon adsorption to contact surfaces. We therefore studied the structural effects that surfaces had on BSA using circular dichroism (CD) spectroscopy, a technique that can give insight into secondary and tertiary protein structure by making use of the optical behavior of (adsorbed) proteins under polarized light. These experiments revealed that immediate changes occur in the tertiary, but not in the secondary, structure of BSA when it binds to a surface material such as kaolin, suggesting that these changes are the minimal requirement for FXII autoactivation (Supplemental Figure 5). To substantiate this suggestion, we searched for and found proteins that did not meet these requirements (Supplemental Figure 6). Gelatin bound to kaolin without undergoing a conformational change and, as a consequence, did not activate FXII-dependent kallikrein generation. Peptides that failed to bind to kaolin (e.g., Roche Blocking Reagent) also failed to mediate FXII-dependent kallikrein generation on kaolin. It is important to note that proteins that have an altered secondary structure also induced FXII-dependent kallikrein generation (e.g., BSA-AGE; Figure 2G and Supplemental Figure 1A). However, alterations in secondary structure are always accompanied by changes in tertiary structure, thereby meeting the requirements for inducing activation of FXII.

Misfolded proteins induce kallikrein generation in plasma but are not procoagulant.

During our studies on activation of the contact system, we used a system of purified proteins

to monitor FXII-dependent kallikrein generation. Based on the fact that the activation of PK is mediated by FXIIa, we first studied the possibility that the generation of FXIIa by misfolded proteins also resulted in a procoagulant effect in plasma. Although kaolin initiated a normal clotting time in an activated partial thromboplastin time–based (aPTT-based) clotting assay, no procoagulant activity of our misfolded proteins was detected under any circumstances (data not shown).

This observation raised the question of whether the FXII-dependent kallikrein generation observed earlier in our system using purified proteins was also abrogated in a plasma environment. We added misfolded or control proteins (selected by efficacy under *in vitro* conditions) to 1:10 diluted plasma and measured generation of kallikrein by conversion of Chromozym PK at 37°C chromogenically. These experiments showed that BSA-AGE, Hb-AGE, and FP13, but not their native controls (structural data are shown in Supplemental Figure 1), were capable of inducing kallikrein generation in a plasma environment (Supplemental Figure 7). In plasma that was deficient in FXII, no kallikrein generation could be observed, again indicating the critical role of FXII. These data showed that FXII-dependent kallikrein generation takes place when plasma is exposed to misfolded protein aggregates, but apparently this is not associated with coagulation. In an effort to explain the observed lack of procoagulant activity of our misfolded protein aggregates, we next studied the FXII-dependent activation of FXI. In brief, FXII that binds directly to a surface becomes activated, and this surface-bound FXIIa converts FXI into FXIa. The generation of FXIa can be monitored via cleavage of the chromogenic substrate Pefachrome XIa3371. We termed this assay a FXII-de-
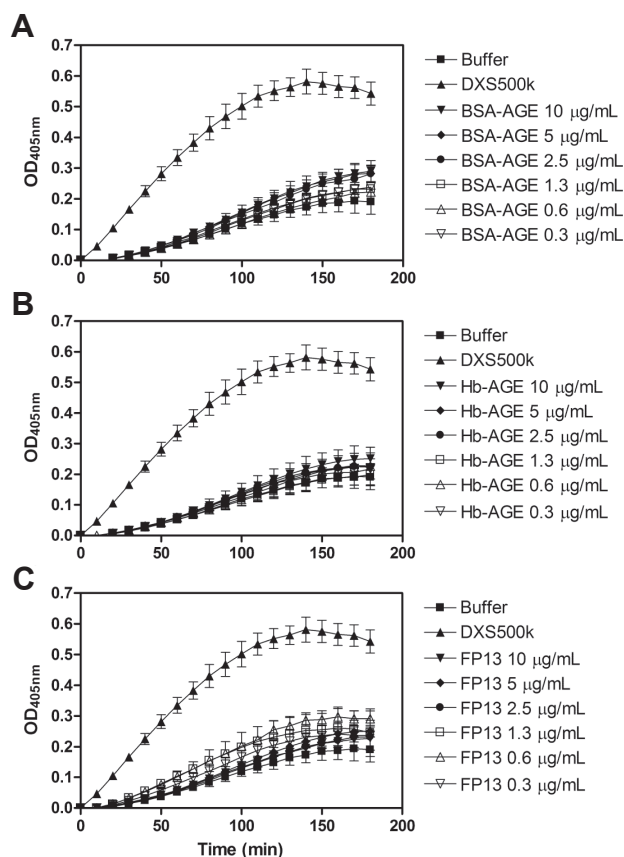

Figure 4. FXII-dependent activation of FXI *in vitro*, is not stimulated by misfolded protein aggregates. Conversion of the chromogenic substrate Pefachrome XIa3371 in the presence of 1 nM FXII, 10 nM FXI, and 30 nM HK was measured. As a control, either FXII or FXI was omitted from the experiments. Although 2.5 µg/mL DXS-500k induced activation of FXIa in an FXII-dependent manner, as expected, misfolded protein aggregates of BSA-AGE (A), Hb-AGE (B), or FP13 (C) could not induce significant activation of FXI above buffer background at any concentration tested. Concentrations of these proteins up to 500 µg/mL were tested and found inactive (data not shown). The values in the graphs represent the mean ± SEM of duplicate determinations performed within 1 representative experiment of at least 3.

pendent FXIa generation assay, since omission of FXII led to complete loss of FXIa generation in all our experiments (data not shown), indicating the critical role for FXII. Although this assay is very similar to the kallikrein generation assay described earlier, we found it to be highly dependent on the presence of HK, whereas kallikrein generation was not. In this setup, we found that whereas DXS-500k was capable of generating FXIa in a FXII-dependent manner, none of our misfolded protein preparations had the same ability at any concentration tested (Figure 4).

This experiment suggested that during exposure of plasma to misfolded proteins, kallikrein is activated by FXIIa, but this does not result in concurrent activation of FXI. This apparent discrepancy was investigated in further detail in plasma. Although determination of the activity of kallikrein and several other proteases in plasma can be performed using chromogenic substrates, amidolytic assays of FXIa activity in plasma are difficult, because of lack of sensitivity. However, activation of FXI and PK in plasma can also be measured indirectly by determination of the amount of enzyme that has been inactivated by serpins such as C1 esterase inhibitor (C1inh)[28]. We looked at the amounts of both (FXIa-C1inh and kallikrein-C1inh) complexes that had been formed in citrated normal pooled plasma after it had been exposed to either kaolin or misfolded protein aggregates. Figure 5 shows that plasma that had been exposed to a contact surface, such as kaolin, contained elevated levels of both kallikrein-C1inh (Figure 5A) and FXIa-C1inh (Figure 5B) complexes, revealing activation of both coagulation and the kallikrein-kinin system. The bars in Figure 5, A and B, represent the maximal amounts of complexes that can be formed by an activator, determined by nonlinear regression of the time curves of complex formation shown in the insets. However, plasma that had been exposed to misfolded protein aggregates acted completely differently. Formation of kallikrein-C1inh complexes was observed when plasma was incubated with all 3 preparations (Figure 5A), whereas few or no FXIa-C1inh complexes were formed (Figure 5B). The generation of these complexes took place in a dose-dependent manner (Supplemental Figure 8). Together with the findings presented in Figure 4, this may explain the absence of procoagulant activity induced by misfolded proteins as measured in an aPTT-based assay and suggests that PK and FXI can be activated by FXIIa in different ways.

Kallikrein formation, but not coagulation, is activated in protein misfolding disease.

Systemic amyloidosis is marked by a variety of hemostatic aberrances that contribute to its morbid pathology. Both thrombotic events and bleeding episodes have been described, but their origin has been poorly delineated. Since we had found that FXIIa levels are elevated in this disease (Figure 1), and FXI and kallikrein do not have to be activated simultaneously in plasma (Figure 5, A and B), we investigated the downstream effects of misfolded proteins on the activation of FXI and PK *in vivo*. In agreement with our *in vitro* studies (Figure 5A), elevated kallikrein-C1inh complex levels could be detected in 9 of 27 patients with systemic amyloidosis (0.83 ± 1.40 ng/mL; expressed as mean $\pm$ SD), compared with healthy control subjects (0.20 ± 0.04 ng/mL). This difference was statistically significant (P = 0.0173; Figure 5C). However, only 2 of 27 patients had elevated FXIa-C1inh complexes (0.94 ± 2.79 ng/mL), compared with controls (0.24 ± 0.15 ng/mL), and as a consequence, no significant difference in these complex levels could be demonstrated (P = 0.2011; Figure 5D).

Discussion

FXII was discovered in 1955[29], when plasma from an asymptomatic individual, John Hageman (FXII is also known as Hageman factor), was found to have a profoundly prolonged clotting time *in vitro* due to a deficiency in this protease. After the discovery of FXII, the components and functions of the contact system were elucidated, mostly using *in vitro* studies. The absence of a
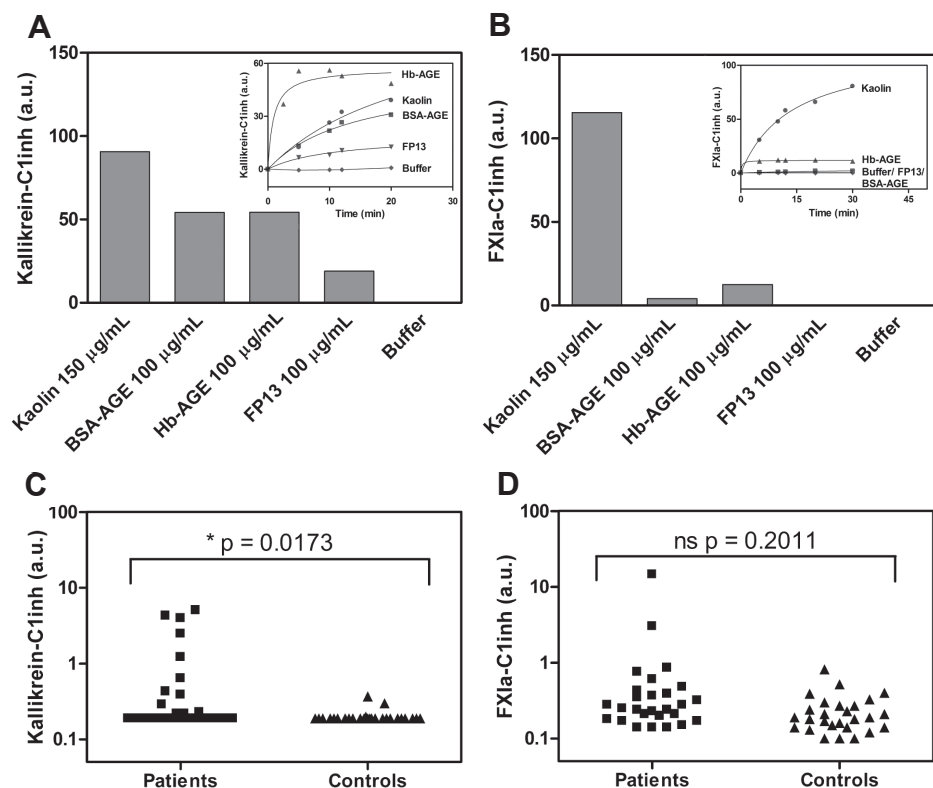
Figure 5. Misfolded proteins induce formation of kallikrein-C1inh complexes, but not FXIa-C1inh complexes, in plasma. (A) The kallikrein-kinin system is activated *in vitro* when plasma is incubated with both contact surfaces and misfolded protein aggregates, as measured by levels of kallikrein-C1inh complexes. (B) Activation of coagulation, measured by plasma levels of FXIa-C1inh complexes, could only be determined in plasma that had been incubated with contact surfaces such as kaolin. The bars represent the maximal amounts of complexes that can be formed by an activator, as determined by nonlinear regression of the time curves of complex formation, shown in the insets. Determinations of these complexes in patients with systemic amyloidosis (average age, 51 ± 10 years, 40% male) show that *in vivo* kallikrein-C1inh complexes (C), but not FXIa-C1inh complexes (D), were significantly elevated as compared by 2-tailed Student's t test with those in controls (average age, 49 ± 8 years, 40% male), indicating that circulating misfolded protein induces preferential activation of the kallikrein-kinin system via FXII.

bleeding diathesis in FXII-, HK- and PK deficiencies contradicts the key role of these proteins in coagulation via the intrinsic pathway *in vitro*, and this has left the physiological significance of the contact system unclear.

Here, we demonstrate that FXII-dependent kallikrein generation is initiated by misfolded protein aggregates, which unexpectedly is not associated with FXIa generation, indicating that the misfolded proteins are not procoagulant (see schematic illustration, Figure 6). These observations were confirmed *in vivo* by the detection of elevated activation of both FXII and PK, but not FXI, in systemic amyloidosis patients. This finding implies that the kallikrein-kinin system and the intrinsic pathway of coagulation can be differentially regulated by FXII and can be activated independently of each other. However, it is in contrast to the simultaneous generation of kallikrein and FXIa when plasma is exposed to negatively charged surfaces such as kaolin that are commonly used in aPTT-based clotting assays. We show that adsorption of native proteins to surfaces such as kaolin leads to a conformational change (Supplemental Figure 5) that cor-

responds to the observed induction of FXII-dependent kallikrein generation (Figure 3), just as misfolded protein aggregates (in the absence of a surface) do (Figure 2). We propose that the kallikrein generation that is observed during aPTT-based clotting assays is the result of a misfolded protein cofactor; indeed, misfolded proteins in absence of a surface induce activation of PK in plasma (Figure 5 and Supplemental Figure 7). However, no such misfolded protein cofactor is required for or capable of inducing FXII-dependent FXIa formation (Figure 4).

We have shown here that proteins adsorbed to a negatively charged surface induce FXII-dependent kallikrein generation and propose that this can also occur when plasma contacts a surface material. Adsorption of plasma proteins is of a transient nature, and changes can occur within a matter of seconds - a phenomenon, referred to as the Vroman effect[27;30]. The sequence of adsorption and desorption events by different proteins on a surface is explained by the relative abundance and (in)stability of proteins, as has been postulated by Vroman and coworkers. The sequence is albumin, IgG, Fg/fibronectin, and HK/FXII. Based on our findings, we propose now that these transiently adsorbed proteins, such as albumin and Fg used in our studies, are responsible for the kallikrein generation that is seen within the time frame of surface-induced kallikrein formation, but not for clotting, whereas the direct adsorption of contact system proteins is responsible for the clotting response to a foreign surface.

Our findings demonstrate that FXII is able to activate either the intrinsic pathway of coagulation or the kallikrein-kinin system, based on the type activator that is present. But how can 1 protease activate these 2 proteolytic pathways separately? So far, 2 forms of activated FXII have been identified, called α-FXIIa and β-FXIIa. A recent publication elegantly demonstrates that adsorption of FXII to surfaces leads to changes in orientation and ordering of the molecules, which controls its activation[13]. This activation of FXII leads to the formation of α-FXIIa, retaining its full-length molecular weight (80 kDa). In plasma, α-FXIIa can undergo a single cleavage by kallikrein, but this form remains surface bound and can activate FXI (resulting in clotting). A second form of FXIIa, called β-FXIIa, is formed when α-FXIIa is cleaved a second time by kallikrein. This 28-kDa fragment is not surface bound and has lost its capacity to induce FXIa formation but has a strong kallikrein-generating potential[31]. Generation of different molecular forms of FXIIa could explain why FXII can control 2 distinct pathways in plasma, but this needs to be elucidated further.

What do our findings contribute to the understanding of the role of FXII in haemostasis? An upsurge in interest in the contact system and FXII took place recently when it was demonstrated that FXII has a role in pathological arterial thrombus formation in experimental mouse models[32], which is suggested to occur via direct interaction with platelets[33]. This role of FXII in pathological thrombus formation is attributed to FXII-dependent generation of FXIa, since FXII- and FXI-knockout mice have the same phenotype[32]. However, there also seems to be a role for HK in arterial thrombosis, since both kininogen[34] and BK receptor B2–deficient mice[35] are protected from thrombosis. Unfortunately, the role of plasma PK has not been elucidated in a similar fashion. Taken together, these reports suggest that there is still no clear role for the contact system (i.e., FXII, PK, and HK) in normal haemostasis. Our findings here do confirm, however, that the procoagulant role for the contact system lies in its direct interaction with a surface (at least, with respect to intrinsic coagulation), and although this system may not be physiologically relevant, it seems to play a role in thrombosis.

What are the implications of these findings? We propose that FXII-dependent activation of PK by misfolded proteins reflects a conserved protective response that is meant to perceive and clear damaged proteins in the extracellular compartment.
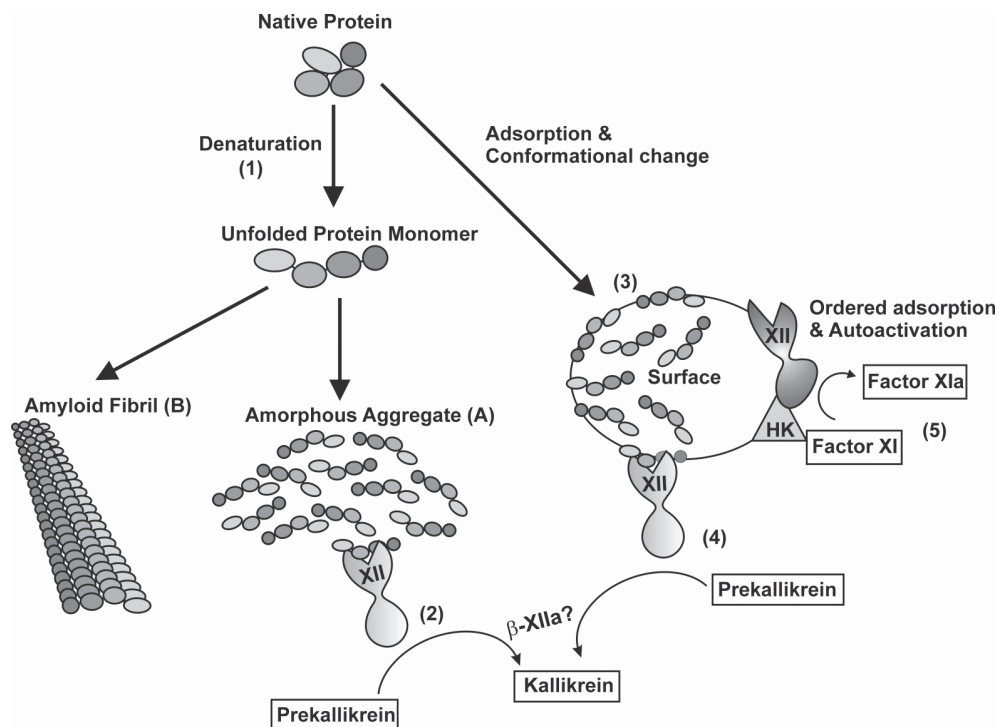
Figure 6. Activation of FXII by misfolded protein aggregates leads to specific activation of the kallikrein-kinin system. A protein can lose its conformation in a number of ways, most notably by denaturation (a) (e.g., due to mechanical stress). Loss of conformation can lead to exposure of sites that are normally hidden within the protein molecule, e.g., due to electrostatic forces in an aqueous environment. As a result, the unfolded protein molecules will either aggregate into amorphous aggregates that can activate FXII-dependent kallikrein generation in the absence of a surface (b) or, in some cases, assemble into amyloid fibrils that do not activate this system independently. Apparently, amyloid fibrils have lost the epitope(s) required for activation of FXII. During adsorption of proteins to classical surface materials such as kaolin, several proteins also lose their native conformation (c), as was shown for BSA, Fg, and endostatin. These adsorbed proteins are able to stimulate FXII-dependent kallikrein generation (d). Misfolded proteins are not capable of inducing clotting, since they are unable to initiate FXII-dependent FXIa generation (e).

Misfolded proteins are generated during injury and are detected by FXII, which leads to kallikrein and BK formation. Several downstream events, which include activation of EC's, are put in motion. First, BK is a potent stimulator of EC prostacyclin synthesis, NO formation, and smooth muscle hyperpolarization factor formation[36]. These factors together make for a fast vasoactive and proinflammatory response. Second, the fibrinolytic system is activated by BK, which induces tissue-type plasminogen activator (tPA) release from ECs[36], while kallikrein activates both urokinase-type plasminogen activator (uPA) and plasminogen. We reported earlier that tPA-dependent plasmin formation is also stimulated by misfolded proteins[23;37] and occurs in systemic amyloidosis[38]. Activation of the fibrinolytic system has various consequences besides removal of fibrin polymers: active plasmin plays a role in inflammation[39] and wound regeneration[40]. In addition, it has been reported that the crosstalk between the fibrinolytic and the kallikrein system (kallikrein-mediated plasmin formation) seems to be of importance for wound repair[41]. Last, more long term cellular events are triggered through recruitment and activation of several cell types, including macrophages as well as cells involved in adaptive immunity[42].

We here describe that misfolded protein aggregates, but not native, monomeric proteins, acti-

vate FXII, resulting in kallikrein formation. Considering the relatively wide variety of unrelated proteins that possess the capacity to trigger this system once they have adopted this aggregated structure, it is conceivable that they share a common conformation-dependent feature. Such features may resemble those of prefibrillar, oligomeric protein species that are currently held responsible for the toxicity seen in protein misfolding disease, for example, as has been described by Kayed et al.[43]. It is therefore tempting to speculate that FXII may play a protective role by recognizing these potentially harmful protein species, thereby aiding in their clearance. Once proteins have assembled into amyloid fibrils, they have lost the ability to activate FXII-dependent kallikrein generation. This in turn may reflect the inability of the human body to cope with this pathological structure, which is seen in many protein misfolding diseases.

Excessive FXII-dependent kallikrein generation may have adverse consequences. A recent paper by Gao et al. elegantly demonstrates that diabetic retinopathy is caused by alkalinization of the vitreous, which in turn results in activation of the contact system[44]. Generation of kallikrein and BK in turn leads to increased vascular permeability, followed by retinopathy. In similar fashion, lactic acidosis (which leads to low pH) has been described to induce activation of FXII and hypotension[45;46]. It is attractive to postulate that changes in pH lead to misfolding of proteins in vitreous, which would explain the consequent FXII and/or kallikrein generation and retinopathy. From a similar point of view, it would be of interest to investigate whether protein misfolding, FXII, and the kallikrein-kinin system are involved in the (unknown) etiology of inflammatory diseases. An example would be inflammatory bowel disease, which is in part mediated by BK and can be experimentally induced by oral administration of DXS to mice[47]. Additionally, 2 recent publications have reported that the presence of the contaminant oversulfated chondroitin sulfate in certain preparations of heparin is responsible for serious adverse clinical events that are specifically attributed to activation of FXII and PK[48;49]. Our data suggests that the anaphylactoid reactions are possibly mediated via the generation of misfolded protein intermediates on this negatively charged molecule that resembles the DXS used in our study, but this remains to be elucidated.

Despite a number of recent reports on the role of FXII in (thrombotic) pathology, a number of epidemiological studies on the contact system show that FXII has a protective role *in vivo*[28;50–52], which seems contradictory at first glance. In most of these studies, there seems to be a non-linear relationship between FXII levels (or FXIIa-C1inh complexes) and disease risk. Such a phenomenon suggests that FXII fulfills more than one role *in vivo*: one is at risk for developing pathological problems when there is a low concentration of FXII (suggesting a protective role) but also when there is too much FXII (suggesting a pathological role). Since the pathological role of FXII has been elucidated by its crucial contribution to thrombosis[32], but not to normal haemostasis, we hereby propose that the protective role of FXII lies in its selective control over the kallikrein-kinin system. In this role, FXII recognizes misfolded proteins during tissue damage and infection and responds by activation of the kallikrein-kinin system.

In conclusion, FXII and the underlying kallikrein-kinin system are activated by misfolded proteins without induction of coagulation, which finally offers an explanation for the paradoxical role of FXII and the contact system.

Methods

Protein and sample preparations. BSA (MP Biomedicals), endostatin (EntreMed), Fg (FIB3L; Enzyme Research Laboratories), gelatin (Merck; EMD), or Roche Blocking Reagent (Blocking Reagent for ELISA; Roche Diagnostics) were dissolved or diluted to 6 mg/mL in 10 mM HEPES,

137 mM NaCl, 4 mM KCl, pH 7.4 (HEPES-buffered saline [HBS]). The proteins were incubated for 15 minutes at 37°C before use. Kaolin (GenFarma), EA (Sigma-Aldrich), and DXS-500k (Amersham Biosciences; GE Healthcare) were dissolved in HBS to concentrations of 900, 300, and 900 µg/mL, respectively; all solutions had a neutral pH. In order to get a fine solution of EA, the suspension was incubated in a 15-mL polypropylene tube under constant rotation for 96 hours. OVA (Sigma-Aldrich) was dissolved at 1 mg/mL in HBS and incubated at 37°C for 15 minutes. Native OVA was freshly prepared for each experiment. Aggregates of misfolded OVA (dOVA) were prepared by gradual heating from 30°C to 85°C over 12 minutes, as previously published[23]. Endostatin was aggregated, as previously published[53], by dialysis for 5 hours at 4°C against a 500-fold excess volume of 8 M urea in citrate phosphate buffer (17 mM citric acid, 66 mM Na_2HPO_4, 59 mM NaCl, pH 6.2) and dialysis against distilled H_2O (3 times over 12 hours; ×2,500 excess volume). Consequently, the endostatin was dialyzed against citrate phosphate buffer twice. Due to this treatment, endostatin becomes a fine white solid that dissolves after brief incubation at 37°C. BSA was incubated at 100 mg/mL for 21 weeks in the presence of 1 M d-glucose-6-phosphate disodium salt hydrate and 0.05% NaN_3. This treatment results in the formation of misfolded aggregates of BSA with amyloid-like properties (BSA-AGE)[24]. After incubation, BSA-AGE was extensively dialyzed against distilled H_2O. In identical fashion, 10 mg/mL of human hemoglobin (Sigma-Aldrich) was glycated for 38 weeks to yield misfolded hemoglobin with amyloid-like properties[25]. Before use of Hb-AGE, all visible precipitates were removed by centrifugation at 16,000 g for 3 minutes. Misfolded protein aggregates were prepared from the fibrin-derived peptide FP13 K157D (KRLEVDIDIDIRS; synthesized by Pepscan Systems) by dissolving the peptide to 1 mg/mL in distilled water, with a similar peptide, FP10 (KRLEVDIDIK), functioning as control. Amyloid fibrils were prepared from residues 105–115 of human TTR (TTR11; YTIAALLSPYS, produced by the Peptide Synthesis Facility of the Netherlands Cancer Institute [NKI]), as previously published[54]. Aβ ("Dutch type" E22Q, residues 1–42; DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA; produced by the Peptide Synthesis Facility of the NKI) was dissolved in DMSO at a concentration of 5 mM, aliquotted, and stored at –80°C. Aggregates of Aβ 1–42 were directly obtained by diluting the Aβ to 400 µg/mL in PBS. Amyloid fibrils were prepared by incubating Aβ 1–42 at 100 µM for 96 hours at pH 2.0 (adjusted with HCl) at 37°C. The solution was neutralized by dilution to 400 µg/mL in PBS. Amyloid fibrils of IAPP 1–37 were prepared as previously published[55] by incubating 1 mM IAPP, monomerized in a small volume of 1,1,1,3,3,3-hexafluoro-isopropanol (HFIP) prior to use, in HBS at 37°C for 24 hours. Amyloid fibrils of glucagon (Glucagen; Novo Nordisk) were prepared by incubation at 5 mg/mL at 37°C in 0.01 M HCl for 48 hours, as previously published[56]. Recombinant human WT TTR (TTR-WT) was unfolded by dialysis versus 10 mM HCl in distilled H_2O (TTR-WT pH 2.0) at 4°C for 96 hours. Aggregation was induced by addition of 25 µL 4 M NaCl to a 1,000-µL sample, giving a final NaCl concentration of 100 mM. Incubation of this sample for 24 hours results in protofibril formation[57]. The aggregated TTR-WT pH 2.0 was dialyzed 3 times against HBS at 4°C.

Free immunoglobulin light chains, isolated from the urine of patients with myeloma-related amyloidosis, were kindly provided by B.J.E.G. Bast (University Medical Center, Utrecht, The Netherlands) and F.A.M. Redegeld (Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Utrecht, The Netherlands). To exclude possible effects of LPS[58], protein preparations were routinely tested for endotoxin by Endosafe kit (Charles River Laboratories), according to the manufacturer's instructions.

Chromogenic FXII-dependent kallikrein generation assay. The conversion of the chromogenic sub-

strate Chromozym PK (Roche Diagnostics) by kallikrein was measured at 405 nm and 37°C in a SpectraMax 340 microplate reader (Molecular Devices), in Costar 2595 microtiter plates. All measurements were performed in duplicate and repeated at least 3 times. The plates were blocked with 2% BSA in HBS 0.1% Tween 20 for 1 hour at room temperature where indicated. In all experiments, omission of FXII resulted in abrogated kallikrein generation. In the absence of PK, no conversion of the chromogenic substrate could be observed. Experiments contained 0.3 mM Chromozym PK and 5.8 μM $ZnCl_2$ in the presence of 7.7 nM PK (Calbiochem; EMD Biosciences) and 0.97 nM FXII (Calbiochem; EMD Biosciences), buffered by HBS. The positive control was 100 μg/mL BSA-AGE, and the negative control was buffer alone.

Chromogenic FXII-dependent FXIa generation assay. As in the kallikrein generation assay, conversion of the chromogenic substrate Pefachrome XIa3371 (Pentapharm) in the presence of 1 nM FXII, 10 nM FXI, and 30 nM HK was measured in Costar 2595 microtiter plates that were blocked with 2% BSA in HBS 0.1% Tween 20 for 1 hour at room temperature. As a control, either FXII or FXI were omitted from the experiments.

Plasma clotting assay. aPTT-based clotting assays were performed in a KC-10 coagulometer (Amelung) by incubating citrated normal pooled plasma with proteins of interest (or a final concentration of 150 μg/mL kaolin) and 10 μM phospholipid vesicles for 3 minutes, after which we added 8.3 mM $CaCl_2$ to initiate clotting. Kallikrein generation in plasma. The generation of plasma kallikrein was determined in triplicate in a 1:10 final dilution normal pooled plasma and FXII-deficient plasma (American Diagnostica Inc.) by chromogenic assay. All proteins and contact surfaces as well as plasma were prepared or diluted in HBS. Kallikrein generation, measured at 405 nm and 37°C in a SpectraMax 340 microplate reader, was determined by conversion of 0.36 mM Chromozym PK.

Plasma FXIIa ELISA. Systemic amyloidosis patients were recruited at Groningen University Medical Center and Utrecht University Medical Center as described previously[38;59;60]. The Institutional Review Boards of Groningen University Medical Center and Utrecht University Medical Center approved this study, and informed consent was obtained from all patients. Our patients included 11 individuals with primary systemic amyloidosis, 10 with secondary systemic amyloidosis (AA), and 4 with the hereditary form (ATTR). Plasma levels of FXIIa were determined in duplicate in citrated plasma by a commercially available ELISA kit (Axis-Shield).

Kallikrein-C1inh and FXIa-C1inh complexes in plasma. Complexes of kallikrein and FXIa with C1inh were measured by ELISA in both pretreated normal pooled plasma and plasma from systemic amyloidosis patients, as previously published[28]. In brief, C1inh was captured with a coated anti-C1inh antibody; subsequently, complexes were detected using antibodies against kallikrein or FXI. In experiments where plasma was incubated with contact surfaces or misfolded protein aggregates, a 1:1 dilution of plasma in HBS (containing the activator) was incubated in Eppendorf cups at 37°C. Samples were taken over time (2 volumes) and added to 3 volumes of PBS containing 100 μg/mL soybean trypsin inhibitor (Sigma-Aldrich) and 0.05% (w/v) Polybrene (Sigma-Aldrich). Data were expressed in arbitrary units as a percentage of positive control plasma and plotted in GraphPad Prism 4 for Windows; maximal complex formation by experimental activators was determined by nonlinear regression.

Statistics. Statistical analysis of data was performed using Graphpad Instat 3.0. Normality of distributions was assessed by the Kolmogorov-Smirnov (KS) test, after which normally distributed data sets (FXIIa levels and FXIa-C1inh complex levels) were compared using unpaired 2-tailed t test with Welch correction and abnormally distributed data sets (kallikrein-C1inh complex levels) using a nonparametric 2-tailed t test (Mann-Whitney). Levels were considered elevated when

they were greater than the sum of the mean value of age- and sex-matched healthy controls and 3 standard deviations. Data were plotted in GraphPad Prism 4 for Windows.

ThT and CR fluorescence assay. Fluorescence of ThT (Sigma-Aldrich) and CR (Sigma-Aldrich) was measured on a Fluoroskan Ascent 2.5 Microplate Fluorimeter (Thermo Fisher Scientific) in black microtiter plates (Greiner Bio-One). The excitation wavelengths were 435 nm and 550 nm, whereas emission wavelengths were 485 nm and 595 nm, for ThT and CR respectively. Fluorescence of 25 µM ThT or 25 µM CR was measured in triplicate at a protein concentration of 100 µg/mL HBS. Background fluorescence of both protein in buffer and dye solution were subtracted from the total fluorescence signal. Fibrillar Aβ ("Dutch type" E22Q, residues 1–40; DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV; produced by the Peptide Synthesis Facility of the NKI) was used as a positive control in all fluorescence assays.

TEM. Formvar/carbon-coated 100-mesh copper grids were placed on top of 5-µL drops of protein solution for 5 minutes. All amyloid fibrillar preparations were analyzed at stock solution concentrations (described above in Protein and sample preparations). For comparisons between native and treated protein solutions, TEM analyses were performed with grids that were prepared from solutions with equal protein concentrations. Unless described otherwise, this was done by diluting samples to 1 mg/mL in HBS. The grids were washed by placing them on a 100-µL drop of PBS and 3 drops of H2O, at each step incubating for 2 minutes. The grids were stained with 2% (mass/vol) methylcellulose with 0.4% uranyl acetate pH 4.0 for 2 minutes. Afterward, analyses were performed on a JEOL 1200 EX Transmission Electron Microscope, and electron micrographs were made at a ×10,000 enlargement. Control grids were made that had been incubated with buffer alone.

CD. Far-UV CD was used to monitor changes in secondary structure induced by contact surfaces. Spectra were obtained using a Jasco J-810 Spectropolarimeter connected to a Jasco Peltier CDF-426S. The ellipticity was measured in a quartz cuvette with a pathway length of 1 mm (Hellma) between 190 and 260 nm in 1-nm steps (100 nm per minute), with a 1-second response time at standard sensitivity. To monitor changes in tertiary structure, near-UV CD was determined in a 1-cm quartz cuvette between 270 and 310 nm (Hellma) under the same conditions as used for far-UV CD. The experiments were performed by mixing 6 mg/mL of protein in a 1:1 ratio with 900 µg/mL of kaolin or DXS-500k or 300 µg/mL of EA in HBS. After mixing, samples were diluted in distilled water. For far-UV CD, a BSA and gelatin concentration of 250 µg/mL was used. For near-UV CD, 1.5 mg/mL of BSA or 6 mg/mL of gelatin was used. Spectra were recorded at ambient temperature for BSA and 37°C for gelatin. Before analysis of the spectra, a blank, containing all components except protein, was subtracted from the corresponding samples. Kaolin binding experiment. Kaolin was incubated with solutions of BSA, endostatin, Fg, gelatin, or Roche Blocking Reagent in Eppendorf cups for 5 minutes under conditions that support protein cofactor activity of albumin and Fg on kaolin (3 mg/mL protein, 450 µg/mL kaolin, buffered by HBS at 37°C). After incubation, kaolin was separated from the protein solution by centrifugation (3 minutes maximal speed), and the supernatant was removed by pipetting. Next, the kaolin pellets were washed by resuspension in 700 µL HBS, followed by centrifugation. This step was repeated 2 more times, after which the pellets were analyzed for protein binding by protein assay according to the manufacturer's instructions (Cytoskeleton).

Acknowledgments

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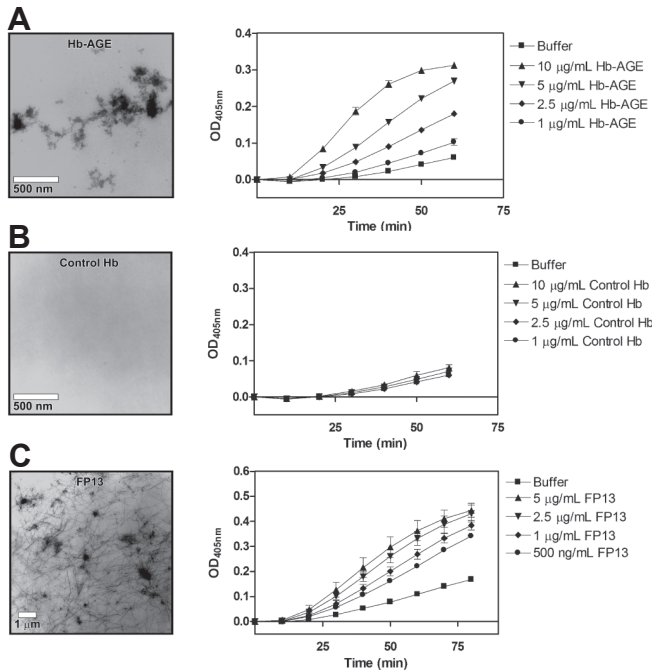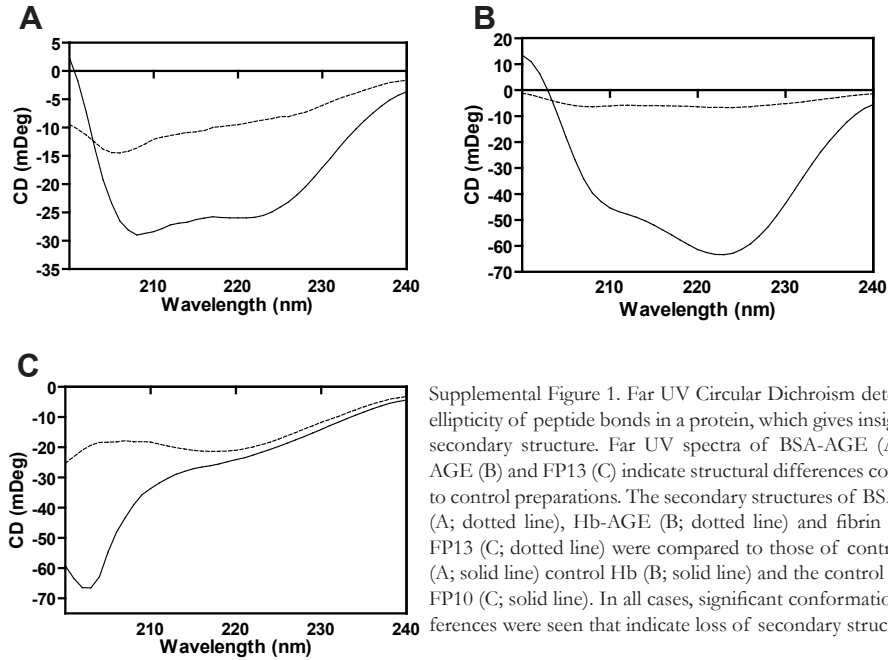23. Maas, C., Hermeling, S., Bouma, B., Jiskoot, W., and Gebbink, M.F. 2007. A role for protein misfolding in

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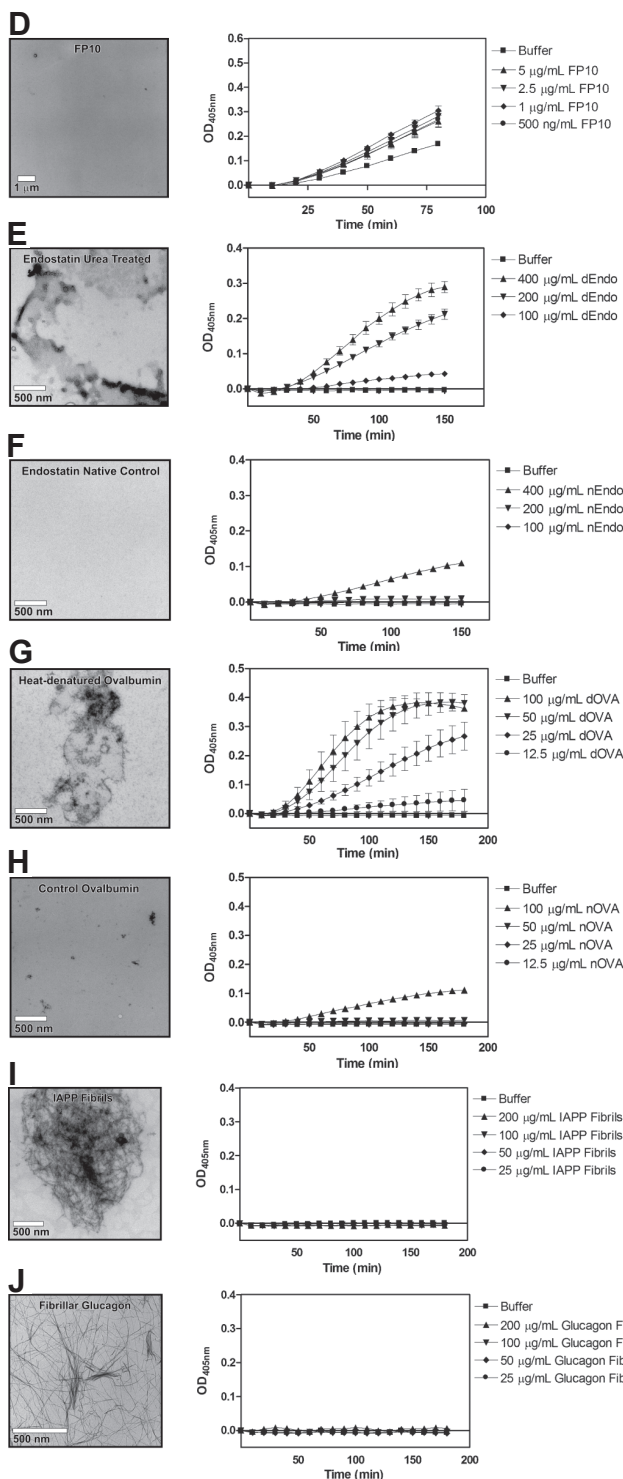
Supplemental Data

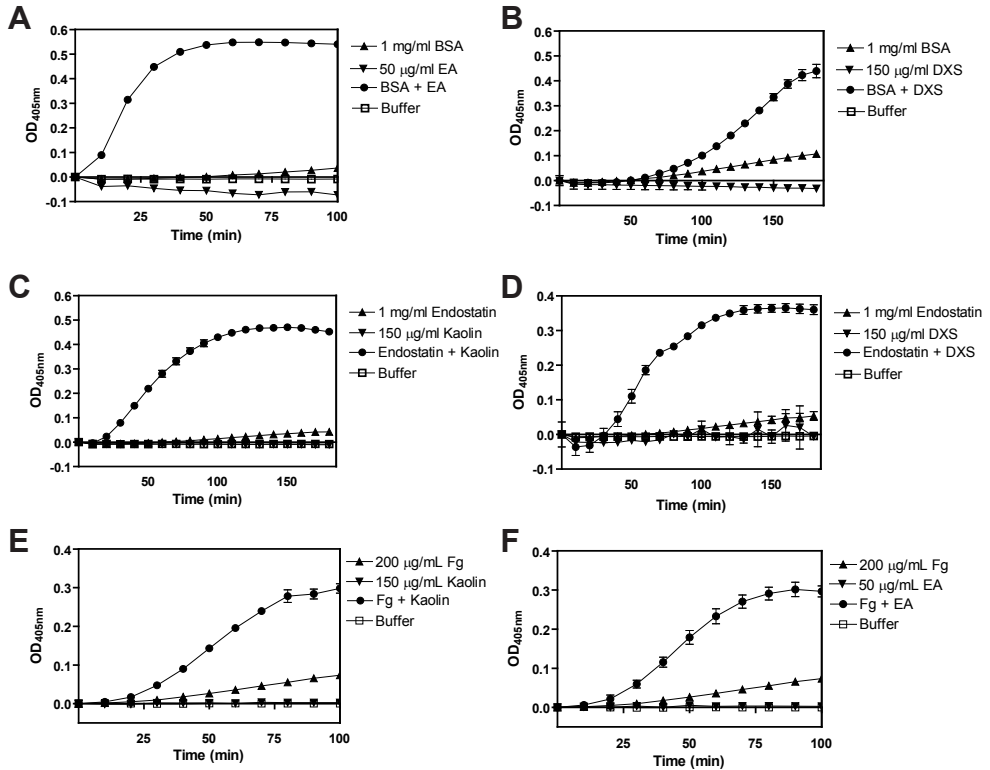| Protein /peptide	Thioflavin T	Congo Red
Aβ (1-42) Fresh	1.13 +/- 0.09	0.18 +/- 0.01
Aβ (1-42) Fibrils	4.16 +/- 0.14	0.94 +/- 0.00
TTR11 Fibrils	0.71 +/- 0.00	0.03 +/- 0.02
TTR-wt pH=2.0	1.61 +/- 0.03	ND
TTR-wt	0.20 +/- 0.11	ND
Bence-Jones protein	0.7 +/- 0.03	ND
dOVA	2.11 +/- 0.04	1.38 +/- 0.01
nOVA	0.24 +/- 0.01	0.02 +/- 0.03
Endostatin Urea	0.38 +/- 0.00	1.22 +/- 0.02
Endostatin Native	0.11 +/- 0.00	0.43 +/- 0.01
BSA-AGE	ND	ND
BSA Fresh	0.02 +/- 0.01	0.00 +/- 0.02
FP13	0.15 +/- 0.06	0.42 +/- 0.01
FP10	0.03 +/- 0.01	0.01 +/- 0.01
Hb-AGE	ND	ND
Hb Fresh	ND	ND
IAPP (1-37) Fibrils	2.10 +/- 0.12	0.64 +/- 0.12

Supplemental Table 1. Fluorescence of Thioflavin T and Congo red by various proteins is indicative for protein misfolding. Fluorescence of Thioflavin T and Congo red was assayed in the presence of 100 μg/mL of protein. ND = not determined. Fibril formation of Aβ (1-42) led to a large increase in both assays, indicative of amyloid formation. Similar increases in ThT and CR fluorescence with lower absolute values were seen after treatment of native ovalbumin (nOVA), native Endostatin (nEndo) and transthyretin, that caused them to form aggregates. No ThT and CR fluorescence could be determined with BSA-AGE and Hb-AGE since it displayed autofluorescence at the wavelengths of both assays. Differences in protein structure were therefore determined by far UV circular dichroism, as is displayed in Supplemental Figure 1.

A

B

C

Supplemental Figure 1. Far UV Circular Dichroism detects the ellipticity of peptide bonds in a protein, which gives insight into secondary structure. Far UV spectra of BSA-AGE (A), Hb-AGE (B) and FP13 (C) indicate structural differences compared to control preparations. The secondary structures of BSA-AGE (A; dotted line), Hb-AGE (B; dotted line) and fibrin peptide FP13 (C; dotted line) were compared to those of control BSA (A; solid line) control Hb (B; solid line) and the control peptide FP10 (C; solid line). In all cases, significant conformational differences were seen that indicate loss of secondary structure.
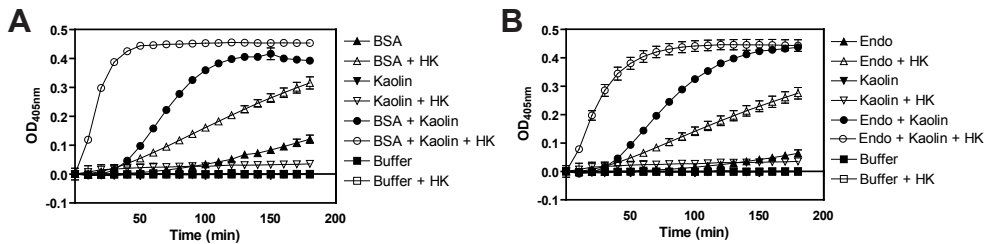
A

B

C

Supplemental Figure 2. FXII is activated by misfolded protein aggregates, but not by amyloid fibrils, *in vitro*. Activation of FXII was measured *in vitro* by a chromogenic assay for FXII dependent kallikrein generation. None of the protein preparations tested was capable of converting the substrate in the presence of PK without FXII present (not shown). In agreement with the data of Figure 2, amorphous aggregates of glycated hemoglobin (Hb-AGE), but not native hemoglobin, activated FXII-dependent kallikrein generation (A, B; experiment performed in blocked wells). (Continues on the next page)

D

FP10

1 μm

Graph (D): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 5 μg/mL FP10
- 2.5 μg/mL FP10
- 1 μg/mL FP10
- 500 ng/mL FP10

E

Endostatin Urea Treated

500 nm

Graph (E): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 400 μg/mL dEndo
- 200 μg/mL dEndo
- 100 μg/mL dEndo

F

Endostatin Native Control

500 nm

Graph (F): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 400 μg/mL nEndo
- 200 μg/mL nEndo
- 100 μg/mL nEndo

G

Heat-denatured Ovalbumin

500 nm

Graph (G): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 100 μg/mL dOVA
- 50 μg/mL dOVA
- 25 μg/mL dOVA
- 12.5 μg/mL dOVA

H

Control Ovalbumin

500 nm

Graph (H): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 100 μg/mL nOVA
- 50 μg/mL nOVA
- 25 μg/mL nOVA
- 12.5 μg/mL nOVA

I

IAPP Fibrils

500 nm

Graph (I): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 200 μg/mL IAPP Fibrils
- 100 μg/mL IAPP Fibrils
- 50 μg/mL IAPP Fibrils
- 25 μg/mL IAPP Fibrils

J

Fibrillar Glucagon

500 nm

Graph (J): OD$_{405nm}$ vs Time (min)
Legend:
- Buffer
- 200 μg/mL Glucagon Fibrils
- 100 μg/mL Glucagon Fibrils
- 50 μg/mL Glucagon Fibrils
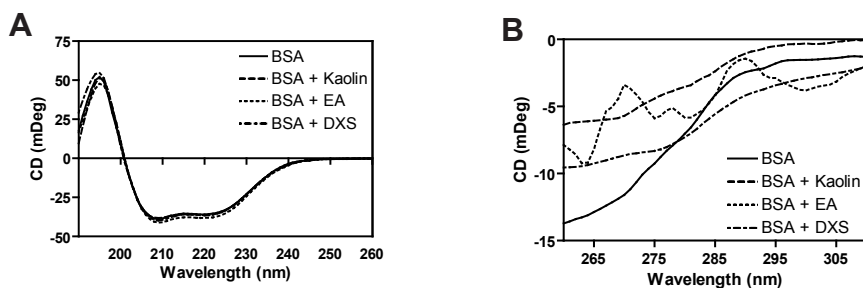- 25 μg/mL Glucagon Fibrils

Supplemental Figure 2, continued. The fibrin peptide FP13, but not FP10, had formed a mixture of both small fibrils and dense amorphous aggregates and activated FXII-dependent kallikrein generation (C, D; experiment performed in blocked wells). Indeed, both aggregates of Endostatin and ovalbumin that had formed during artificial denaturation (E, G, respectively) induced kallikrein generation, whereas their native counterparts could not (F, H). In addition to findings with fibrillar Aβ and TTR11 peptides, amyloid fibrils of IAPP and glucagon were incapable of inducing kallikrein generation (I, J).
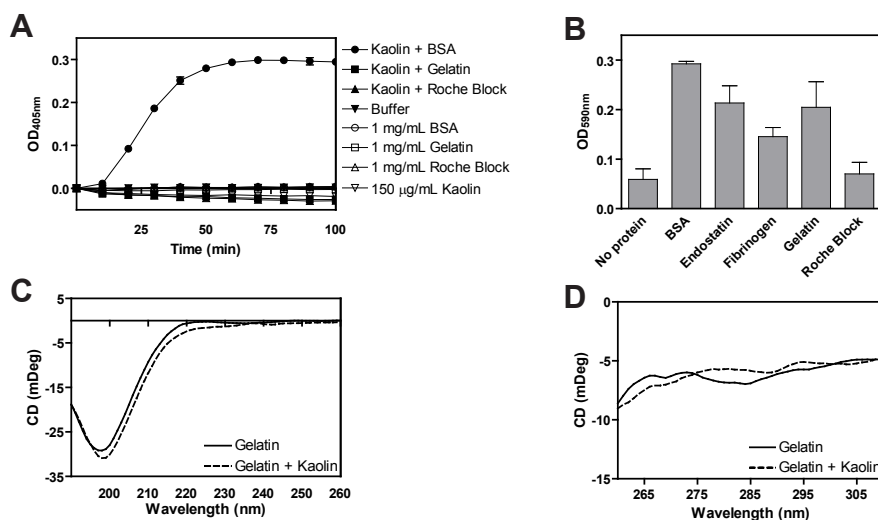
Supplemental Figure 3. FXII-dependent kallikrein generation by surfaces is modulated by cofactor proteins. Surfaces were incubated for 5 minutes at 37 °C with three different proteins, BSA, Endostatin and Fibrinogen (Fg) and analyzed for their ability to induce FXII-dependent kallikrein generation. It was found that addition of either of these proteins was required for FXII activation by kaolin, ellagic acid (EA) and dextran sulfate (500 kDa; DXS).
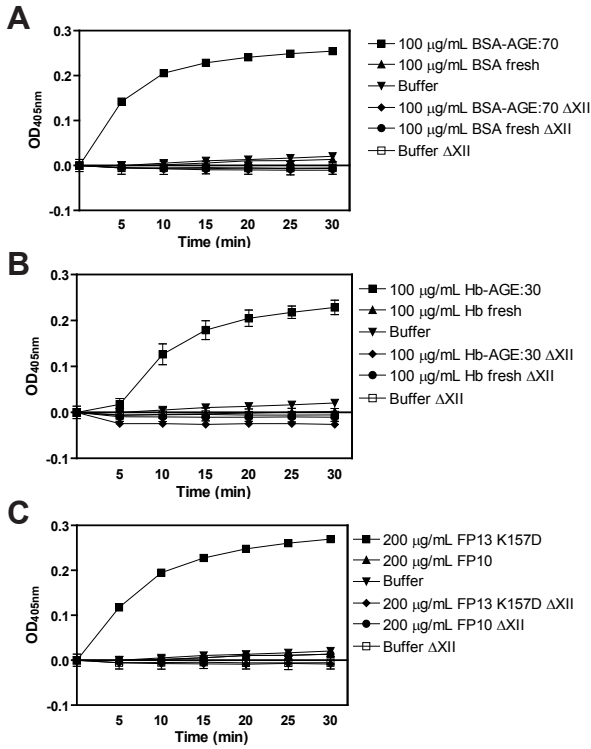

Supplemental Figure 4. HK accelerates FXII-dependent kallikrein generation by a protein cofactor. As described earlier, kallikrein generation was investigated by monitoring the conversion of 0.3 mM Chromozym PK in the presence of 7.7 nM PK and 0.97 nM FXII, with the presence and absence of 5.9 nM HK (Calbiochem). Absence of FXII resulted in no activation under all conditions tested. As described earlier, activation of FXII by 150 μg/mL of kaolin was dependent on the presence of 1 mg/mL of BSA (A) or Endostatin (B). HK enhanced FXII-dependent kallikrein generation by BSA and by BSA mixed with kaolin without enhancing the effect of kaolin alone.
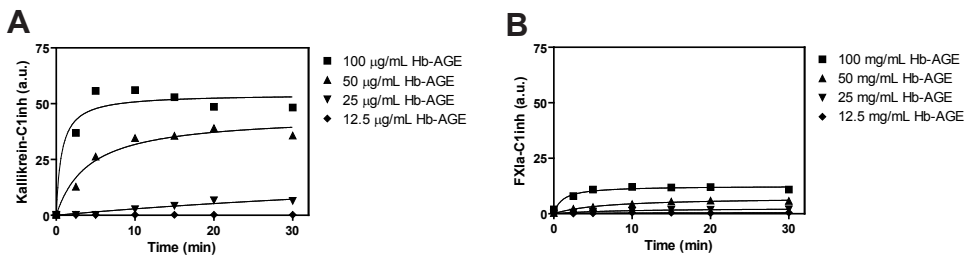
A

B

Supplemental Figure 5. Structure determinations by circular dichroism indicate changes in tertiary, but not in secondary structure of BSA. Far UV Circular Dichroism detects the ellipticity of peptide bonds in a protein, which gives insight into secondary structure. However, near UV spectra are used to give insight into changes in tertiary structure by monitoring ellipticity of aromatic amino acids in a protein. Far and near UV spectra were recorded of BSA in the presence and absence of kaolin, DXS or EA, but no significant changes were observed in secondary structure (A). Near UV spectra of BSA were significantly changed after addition of kaolin, DXS or EA, indicating structural perturbations leading to loss of tertiary structure (B). These findings are in good agreement with those found by others who studied structural changes that occur during protein adsorption[14].

A

B

C

D

Supplemental Figure 6. Gelatin and Roche Blocking Reagent do not support FXII-dependent kallikrein generation in the presence of kaolin. In a chromogenic FXII-dependent kallikrein generation assay, BSA, but not gelatin or Roche Blocking Reagent could function as a cofactor for kallikrein generation in the presence of kaolin (A). In a pull-down experiment, gelatin, but not Roche Blocking Reagent bound to kaolin, explaining the obvious lack cofactor function of this peptide mixture (B). Gelatin was analyzed for its structural properties by circular dichroism spectroscopy when bound to kaolin and was found not to undergo an appreciable conformational change in the presence of this surface; its secondary structure had a large amount of random coil, which did not alter significantly when kaolin-bound (C). Additionally, the near UV CD spectrum (and thus the tertiary structure) of gelatin was not significantly altered in the presence of kaolin (D), which is most likely required for induction of FXII-dependent kallikrein generation.

Supplemental Figure 7. Misfolded protein aggregates stimulate kallikrein generation in plasma, in a FXII-dependent manner. Addition of misfolded protein aggregates of BSA-AGE (A), Hb-AGE (B) or FP13 (C) to 1:10 diluted plasma resulted in conversion of Chromozym PK, whereas native control preparations did not. This process was found to be completely dependent on the presence of FXII in plasma, since plasma deficient in FXII (ΔXII) did not respond to the presence of the aggregates.
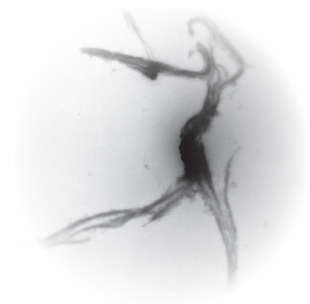

Supplemental Figure 8. Dose-dependency of kallikrein-C1inh complex formation (A) and FXIa-C1inh complex formation (B), in plasma by Hb-AGE.

Chapter 3

Identification of Fibronectin type I Domains as Amyloid-binding Modules on Tissue-type Plasminogen Activator and Three Homologs

Coen Maas,[1] Bettina Schiks,[1,2] Remo D. Strangi,[3] Tilman M. Hackeng,[3] Bonno N. Bouma,[4] Martijn F. B. G. Gebbink,[1,2] and Barend Bouma[1,2]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht and the Institute for Biomembranes, Utrecht, The Netherlands. [2]Crossbeta Biosciences BV, Utrecht, The Netherlands. [3]Department of Biochemistry, Cardiovascular Research Institute Maastricht, University of Maastricht, Maastricht, The Netherlands. [4]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, USA.

Abstract

The serine protease tissue-type plasminogen activator (tPA), a key enzyme in hemostasis, is activated by protein aggregates with amyloid-like properties. tPA is implicated in various pathologies, including amyloidoses. A major task is to further elucidate the mechanisms of amyloid pathology. We here show that the fibronectin type I domain of tPA mediates the interaction with amyloid protein aggregates. We found that in contrast to full-length tPA, a deletion-mutant of tPA, lacking the first three N-terminal domains (including the fibronectin type I domain), fails to activate in response to amyloid protein aggregates. Using recombinantly produced domains of tPA in direct binding assays, we subsequently mapped the amyloid-binding region to the fibronectin type I domain. This domain co-localized with congophylic plaques in brain sections from patients with Alzheimer's disease. Fibronectin type I domains from homologous proteases factor XII, hepatocyte growth factor activator and from the extracellular matrix protein fibronectin also bound to aggregated amyloidogenic peptides. Finally, we demonstrated that the isolated fibronectin type I domain inhibits amyloid-induced aggregation of blood platelets. The identification of the fibronectin type I domain as an amyloid-binding module provides new insights into the (patho)physiological role of tPA and the homologous proteins which may offer new targets for intervention in amyloid pathology.

Introduction

Tissue-type plasminogen activator (tPA) is a serine protease which has a key role in hemostasis. It initiates the destruction of blood clots via activation of the fibrinolytic system. The fibrinolytic system functions by proteolysis (fibrinolysis) of fibrin polymers, which are the main constituents of blood clots. Upon binding to fibrin, tPA is activated and converts the zymogen plasminogen into plasmin, which efficiently cleaves the fibrin polymers. Besides its role in fibrinolysis, roles for tPA and plasmin have been described in many and diverse processes, such as chemoattraction[1], cellular activation[2], remodeling of extracellular matrix[3], tissue repair[4], angiogenesis[5] and inflammation[6]. Surprisingly, these processes are not necessarily related to the presence of fibrin. Many groups have reported that, in addition to fibrin, tPA is activated by numerous unrelated proteins, including denatured forms of albumin, fibrinogen and endostatin and other proteins[7–12]. We showed that tPA is activated by a common structural denominator that is present in amyloid fibrils containing cross-β structure and misfolded protein aggregates with amyloid-like properties[13;14], observations which help to explain how tPA can bind such diverse ligands. Amyloids are well known from a wide variety of protein misfolding diseases, in which tissues and organs become affected by cytotoxic protein deposits. Examples of these diseases are Alzheimer's disease, diabetes type II, systemic amyloidoses and Creutzfeldt–Jakob's disease. Recent observations also suggest that protein misfolding and consequent amyloid formation may contribute to the etiology of atherosclerosis[15;16]. A role for activation of tPA has been implicated in Alzheimer's disease as well as in other misfolding diseases and cardiovascular disease on various occasions[17–23]. In this study, we aimed to identify the binding site through which tPA interacts with proteins that display amyloid properties. We found that the fibronectin type I domain of tPA, mediated binding to proteins with amyloid-like properties. The individual fibronectin type I domain of tPA, as well as those of homologous domains in factor XII (FXII), hepatocyte growth factor activator (HGFA) and fibronectin (Fn) all bound to aggregated amyloidogenic peptides.

Methods

Amyloid peptides and proteins. Human amyloid-β (1–40) Dutch type (Aβ, DAEFRHDSGYEVH-HQKLVFFAQDVGSNKGAIIGLMVGGVV), islet amyloid polypeptide (IAPP, KCN-TATCATQRLANFLVHSSNNFGAILSSTNVGSNTY), amyloid fragment of transthyretin (TTR11, YTIAALLSPYS)[24], laminin α1-chain (2097–2108) amyloid core peptide (LAM12,

AASIKVAVSADR)[25], mouse non-amyloidogenic IAPP (20–29) core (mIAPP, SNNLGPVLPP), non-amyloid fragment FP10 of human fibrin α-chain (148–157) (KRLEVDIDIK) [13] and human fibrin α-chain (148–160) amyloid fragment with Lys157Ala mutation (FP13, KRLEV-DIDIAIRS)[13;26] were synthesized by the Netherlands Cancer Institute NKI (Amsterdam, The Netherlands) or Pepscan Systems Inc. (Lelystad, The Netherlands). For pull-down experiments, Aβ (1–40) and IAPP were dissolved in phosphate-buffered saline (PBS) pH 7.2, at 1 mg/mL and kept at room temperature for at least 3 weeks. Alternatively, amyloid Aβ, IAPP, FP13 and LAM12 were disaggregated in a 1:1 (v/v) mixture of 1,1,1,3,3,3-hexafluoro-2-isopropyl alcohol and trifluoroacetic acid, air-dried and dissolved in H_2O (Aβ, IAPP, LAM12: 10 mg/mL; FP13: 1 mg/mL). After 3 days at 37°C, peptides were kept at room temperature for 2 weeks, before storage at 4°C. Freshly dissolved Aβ (10 mg/mL) in 1,1,1,3,3,3- hexafluoro-2-isopropyl alcohol and trifluoroacetic acid was diluted in H_2O prior to immobilization on an enzyme-linked im-munosorbent assay (ELISA) plates. TTR11 (15 mg/mL) was dissolved in 10% (v/v) acetonitrile in water, at pH 2 (HCl), and kept at 37°C for 3 days and subsequently at room temperature for 2 weeks. mIAPP and FP10 were dissolved at a concentration of 1 mg/mL in H_2O and stored at 4°C. For preparation of advanced glycation end products of bovine serum albumin (BSA-AGE), 100 mg/mL bovine serum albumin (BSA) was incubated for 70 weeks in the dark at 37°C with 1M D-glucose-6-phosphate disodium salt hydrate and 0.05% NaN_3 for 70 weeks. The solu-tion was extensively dialyzed against distilled water to remove remaining free glucose and NaN_3. The preparation was free of endotoxin as tested by Endosafe kit (Charles Rivers Laboratories, Margate, UK), according to the manufacturer's instructions. Modification of albumin by glyca-tion (BSA-AGE) leads to formation of soluble aggregates with amyloid-like properties. All pep-tide and protein solutions were tested for the presence of amyloid conformation by thioflavin-T (ThT) or Congo red fluorescence as described earlier[14]. By the treatment described above, ThT and Congo red fluorescence was enhanced for amyloid peptides, and not for non-amyloid mI-APP, FP10 or freshly dissolved Aβ and BSA (not shown).

Recombinant and synthesized proteins. Cloning of human tPA, factor XII, HGFA and fibronectin do-mains was performed using polymerase chain reactions (PCR) and standard recombinant DNA techniques. Each construct was designed with a carboxy-terminal GST-tag (GST). Factor XII, HGFA and fibronectin domains are preceded by two amino acids (GA), following the C-terminus of the tPA propeptide. All constructs, except fibronectin type I domains 10–12 of fibronectin, are followed by the (G)RP sequence derived from the original pMT2-GST vector. PCR reactions were performed using the following oligonucleotides (in which relevant restriction sites have been underlined): tPA F (1) 5' AAAA<u>GTCGAC</u>AGCCGCCACCATGGATGCAATGAAGAGA and (2) 3'AAAA<u>GCGGCCGC</u>CCACTTTTGACAGGCACTGAG; tPA FE: (3) 3'AAAA<u>GCGGC-CGC</u>GTGGCCCTGGTATCTATTTC and (1); tPA E: (4) 5'AAA<u>AGAGATCT</u>GTGCCTGT-CAAAAGTTGC and (3); tPA K1: (5) 5'AAAAG<u>AGATCT</u>GATACCAGGGCCACGTGCTAC and (6) 3'AAAA<u>GCGGCCGC</u>CCGTCACTGTTTCCCTCAGAGCA; tPA FEK1: (1) and (6); GST tag: (7) AAAA<u>GCGGCCGC</u>CTGGCTCCTCTTCTGAATC and (1); fibronectin F4–5: (8) 5'TGCA<u>AGATCT</u>ATAGCTGAGAAGTGTTTTGAT and (9) 3'TGAT<u>GCGGCCGC</u>CCCA-CAGAGGTGTGCCTCTC; fibronectin F10–12: (10) 5'AAAAA<u>AGATCT</u>AACCAACCTACG-GATGACTC and (11) 3'AAAAAA<u>GGTACC</u>GACTGGGTTCACCCCCAGGT; Factor XII F: (12) 5'GAAACA<u>AGATCT</u>CAGAAAGAGAAGTGCTTTGA and (13) 3'ACGG<u>GCGGC-CGC</u>CCGGCCTGGCTGGCCAGCCGCT; HGFA F: (14) 5'GCAAGA<u>AGATCT</u>GGCACA-GAGAAATGCTTTGA and (15) 3'AAGG<u>GCGGCCGC</u>CCAGCTGTATGTCGGGTGC-CTT. The tPA fibronectin type I domain (F, finger domain) and the Finger-EGF (FE) region,

together with the tPA propeptide, were amplified using 1 ng vector Zpl7 containing tPA[27], the product was digested with SalI and NotI and cloned into pMT2SM-GST[28]. As a result Schistosoma japonicum glutathione-S-transferase (GST) is fused to the C-terminus of the expressed constructs. The constructs were subsequently ligated with SalI and EcoRI in pGEM3Zf(7) (Promega, Madison, WI, USA). The resulting plasmid was used as a cloning cassette for preparation of tPA kringle1 (K1), FEK1, E, as well as HGFA F, factor XII F, and fibronectin F4–5 and F10–12 constructs. The selection of fibronectin type I domains of fibronectin was based on the following reasoning: tPA binds to fibrin with its fibronectin type I domain[29] and competes with fibronectin for fibrin binding[30]. The fibrin binding-sites of fibronectin are enclosed in its fibronectin type I 4–5 and 10–12 domains[31]. We show here that the fibronectin type I domain of tPA mediates binding to amyloid. This suggests that also the fibrin-binding fibronectin type I domains of fibronectin can bind to amyloid. All domains were cloned after the tPA propeptide using a BglII restriction site that is present between the tPA propeptide region and the F domain[27], and the NotI or KpnI site that is present in front of the thrombin cleavage site[28]. Subsequently, constructs were ligated HindIII and EcoRI in the pcDNA3.1 expression vector (Invitrogen, The Netherlands). In addition, the GST tag alone, preceded by the tPA propeptide, was cloned into pcDNA3.1. The separate GST-tag has five additional residues at the N-terminus (GARRP). tPA cDNA was a kind gift of M. Johannessen (NOVO Research Institute, Bagsvaerd, Denmark). The cDNA encoding for factor XII was a kind gift of F. Citarella (University of Rome "La Sapienza", Italy). K. Miyazawa (University of Tokyo, Japan), S. A. Newman (New York Medical College, Valhalla, USA) and A. Muro (ICGEB, Trieste, Italy) kindly provided the cDNA encoding for HGFA, for an N-terminal fragment of human fibronectin, comprising fibronectin type I domains 4–5, and for a C-terminal fibronectin fragment, comprising fibronectin type I domains 10–12, respectively. For protein expression, baby hamster kidney (BHK) cells were transfected and stable cell lines were selected. GST-tagged constructs, as well as the free GST tag, were purified from cell culture medium using a 5-m glutathione Sepharose 4B column (Pharmacia LKB, Uppsala, Sweden). Alternatively, a biotinylated finger domain of tPA (residue 39–81: tPA F-biotin) was prepared by chemical synthesis. In brief, two polypeptides (CH_3CONH-Val[39]-Tyr[68]-COSR and NH_2-Cys[69]-Val[81]-Lys-NH_2 (residues numbered according to SwissProt entry for tPA) were prepared by solidphase peptide synthesis[32]. NH_2-Cys[69]-Val[81]-Lys-NH_2 was biotinylated at the side chain of the non-native C-terminal lysine. The two peptides were joined by native chemical ligation[33;34], folded, HPLC purified, lyophilized and frozen until use.

Enzyme-linked immunosorbent assay. Immobilizer plates (Exiqon, Dahlbaek, Denmark), or Microlon high-binding plates (Greiner, catalogue number 655092, Frickenhausen, Germany) were used. Peptides were coated at a concentration of 5 µg/mL in coat buffer (100 mM $NaHCO_3$, pH 9.6, 0.05% (m/v) NaN_3) for 1 h at room temperature. Immobilizer plates were blocked with 1% (v/v) Tween20 in PBS, Microlon plates were blocked with blocking reagent (Roche Diagnostics, Almere, The Netherlands). Binding studies with full-length tPA (Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, The Netherlands) or a truncated form of tPA (K2P-tPA; Reteplase; Rapilysin, Roche Diagnostics GmbH, Mannheim, Germany), were performed in buffer containing PBS, 0.1% v/v Tween20 and 10 mM ε-amino caproic acid (εACA). Binding studies with GST or GST-fusion constructs to immobilized peptides were performed using purified proteins in PBS. tPA was detected with monoclonal antibody 374b or polyclonal antibody 385R (American Diagnostica, Instrumentation Laboratory, Breda, The Netherlands), factor XII with polyclonal antibody 233504 (Calbiochem, Schwalbach, Germany), fibronectin with polyclonal antibody

A0245 (Dako Diagnostics, Glostrup, Denmark), GST-tagged constructs with polyclonal antibody Z-5 (Santa Cruz Biotechnology, CA, USA), and negative control for amyloid binding, human hemoglobin, with polyclonal antibody A0118 (Dako Diagnostics, Glostrup, Denmark). Antibody 3700, directed against the FE domains of tPA was purchased from American Diagnostica. Binding of soluble BSA-AGE aggregates to tPA F-biotin was assayed by coating 5 µg/mL tPA F-biotin in immobilizer plates (Exiqon, Dahlbaek, Denmark), followed by incubation with BSA-AGE, which was detected using a monoclonal antibody against advanced glycation end-products (4B5)[35]. Primary antibodies were detected using monoclonal peroxidase-labeled rabbit anti-mouse or swine anti-rabbit antibody (Dako Diagnostics, Glostrup, Denmark). Subsequently, the plates were stained with 100 µL/well of tetramethyl-benzidine (TMB; Biosource Europe, Nivelles, Belgium) or O-phenylenediamine (OPD; Sigma, St Louis, MO, USA), prepared according to the manufacturer's instructions. The reaction was terminated with 50 µL/well of 2 M H_2SO_4 and substrate conversion was read at 450 nm (TMB) or 490 nm (OPD) on a Spectramax340 microplate reader.

Amyloid pull-down assay. Aβ or IAPP aggregates were incubated with conditioned medium obtained from stably transfected BHK cells. Since the aggregates are insoluble, unbound proteins can be removed from the pelleted peptides following centrifugation and washing. Starting material, bound protein and unbound protein in the supernatant were analyzed by SDS-PAGE followed by immunoblotting. Blots were analyzed with anti-GST antibody Z-5. Control binding studies were performed with 4 µg/mL full-length human tPA (Actilyse, Boehringer-Ingelheim, Germany), 0.8 µg/mL full-length human factor XII (Calbiochem, catalogue no. 233490), 0.5 µg/mL human fibronectin (Harbor Bio-Products, Tebu-bio, Heerhugowaard, The Netherlands, catalogue no. 2003) and endogenous HGFA in 200 times diluted human serum (BioWhittaker, Boehringer Ingelheim, Verviers, Belgium).tPA, factor XII, fibronectin and HGFA were detected on Western blot with antibodies 374b, 233504 (Calbiochem), A0245 (Dako Diagnostics) and HGFA-L (Santa Cruz Biotechnology), respectively. tPA-dependent plasmin generation assay Exiqon peptide immobilizer plates were blocked for 1 h with PBS, 1% Tween 20 and rinsed twice with distilled water. The conversion of the chromogenic substrate S-2251 (Chromogenix, Italy) by plasmin was kinetically measured at 37°C on a Spectramax 340 microplate reader at a wavelength of 405 nm.The assay mixture contained 200 pM tPA, 1.1 mM plasminogen (purified from human plasma), and 415 mM S-2251 in HEPES-buffered saline (10 mM HEPES, 137 mM NaCl, 4 mM KCl, pH 7.4; HBS). A control, containing all components except for tPA was used to detect any tPA-independent plasmin formation. Denatured γ-globulins (100 µg/mL) with amyloid-like structure were used as reference and positive control. Lyophilized human γ-globulins (Sigma) were dissolved in a 1:1 volume ratio of 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic acid and subsequently dried under air. Dried γ-globulins were dissolved in H_2O to a final concentration of 1 mg/mL and kept at room temperature for at least 3 days and subsequently stored at -20°C.

Fluorescence microscopy. Paraffin brain sections of a patient that had deceased from Alzheimer's disease were de-paraffinized and treated with formic acid. Sections were exposed for 2 h with 7 nM purified tPA FE-GST or GST tag, and subsequently overlaid with 200 ng/mL anti-GST antibody Z-5. Powervision (Immunologic, Duiven, The Netherlands, catalogue no. DPVR-55AP) was applied and sections were stained with 3,3'-diamino benzidine (Sigma-Aldrich, catalogue no. D-5905) and hematoxylin. Finally, sections were incubated with Congo red, according to the manufacturer's recommendations (Sigma Diagnostics).

Platelet aggregation assay. Freshly drawn venous blood from healthy volunteers with informed con-

sent was collected into 0.1 volume 130 mmol/L trisodium citrate. The donors stated they had not taken any medication during 2 weeks prior to blood collection. After centrifugation of the whole blood (15 min, 150 x g, 20°C), the platelet-rich plasma (PRP) was carefully removed and the pH was lowered to 6.5 by adding 10% ACD buffer (2.5% trisodium citrate, 2% D-glucose and 1.5% citric acid) to avoid platelet activation. Following centrifugation (15 min, 300 x g, 20°C), the platelet pellet was resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 10 mmol/L HEPES, 5 mmol/L D-glucose, pH 6.5), 10 ng/mL PGI2 (final concentration) was added and the wash step was repeated. The platelet pellet was resuspended in Hepes-Tyrode buffer pH 7.2 to a final platelet count of 2×10^{11} platelets/L. Before the start of the experiments, the platelets were kept at 37°C for at least 30 min. to achieve a resting state. Platelet aggregation was recorded in an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) at 37°C, at 900 rpm. A volume of 270 µL platelet suspension was activated with 30 µL of 100 µg/mL of BSA-AGE or 30 mM thrombin receptor activating peptide (TRAP) as activator. For inhibition experiments, tPA, K2P-tPA or tPA F-biotin was added to the agonist solution directly prior to testing in the platelet aggregation assay.

Results

tPA contains a lysine-independent amyloid-binding site, located in its N-terminal region

The domain architecture of tPA consists of a fibronectin type I domain (finger) domain (F), an epidermal growth factor like domain (E), two kringle domains (K1, K2) and a serine protease domain (P) (Figure 1). In terms of this domain architecture, tPA is homologous to factor XII (FXII), the initiating enzyme of the intrinsic coagulation cascade[36;37], as well as HGFA, which is involved in tissue repair[38;39] and organ formation[40]. Both these enzymes have a very similar finger-EGF-kringle (FEK) region, but are preceded by a fibronectin type II (II) and E-domain (Figure 1), which are not present in tPA. A fourth homolog is fibronectin (Fn), which contains 12 finger domains, but that lacks kringle or protease domains. To localize a possible binding site for amyloid aggregates in tPA, we first compared full-length tPA (fl-tPA) to a truncated form of tPA (K2P-tPA), that lacks three N-terminal domains, for their capacity to induce plasmin formation in the presence of amyloid protein preparations. For these experiments, we prepared amyloid preparations of Aβ (1–40), a peptide of the amyloid core fragment of transthyretin (TTR11) and an amyloidogenic fibrin peptide (FP13). We found that amyloid Aβ (1–40) was only capable of inducing plasmin generation in the presence of fl-tPA and not in the presence of K2P-tPA (Figure 2A). Similarly, only fl-tPA could induce plasmin generation in the presence of FP13 and TTR11 (Figure 2B, C, respectively). These experiments show that the N-terminal FEK1 region of tPA is required for plasmin formation in the presence of various aggregated amyloid peptides. Although K2P-tPA was not activated by these amyloid proteins, its protease domain was equally functional to that of fl-tPA, as determined by conversion of the chromogenic substrate S2756 (Figure 2D). We next investigated whether this region of tPA was involved in binding of proteins with amyloid structure. Where fl-tPA had significant affinity for immobilized Aβ (1–40), as well as the amyloid peptides FP13 and TTR11, truncated K2P-tPA had lost all binding capacity for these preparations (Figure 2E). This shows that the FEK1 region of tPA supports the binding of tPA to proteins with amyloid features and suggest that this binding is a requirement for tPA activation in the presence of amyloid peptide aggregates. Kringle domains bind to lysine residues, as is the case in the binding of plasminogen and tPA to C-terminal lysines in fibrin. We therefore investigated whether tPA binding to immobilized Aβ (1–40) could be inhibited by addition of the soluble lysine analogue ε-amino caproic acid (εACA) to the binding inter-

Construct	Domain Architecture	Amino-acid sequence
tPA		-
K2P-tPA		-
tPA F-GST		NH2-G33-S85-GRP-tag
tPA FE-GST		NH2-G33-T126 –RP-tag
tPA FEK1-GST		NH2-G33-D214-RP-tag
tPA E-GST		NH2-G33A34-V81-T126-RP-tag
tPA K1-GST		NH2-G33A34-D122-D214-GRP-tag
FXII		-
FXII F-GST		NH2-GA-Q131-A177-GRP-tag
HGFA		-
HGFA F-GST		NH2-GA-G198-A244-GRP-tag
Fn		-
Fn F4-5-GST		NH2-GA-I182-V276-GRP-tag
Fn F10-12-GST		NH2-GA-N2199-S2341-tag
GST (tag)		NH2-GARRP-tag

Figure 1. Domain architectures of tPA, truncated K2P-tPA, FXII, HGFA and Fn, amino acid sequences and illustrations of recombinantly produced domains thereof. Amino acid residue numbering is according to SwissProt entries for the respective human full-length protein. F, fibronectin type I domain; E, EGF-like domain; K, kringle domain; P, serine protease domain; II, fibronectin type II domain; III, fibronectin type III domain; PR, proline-rich region.

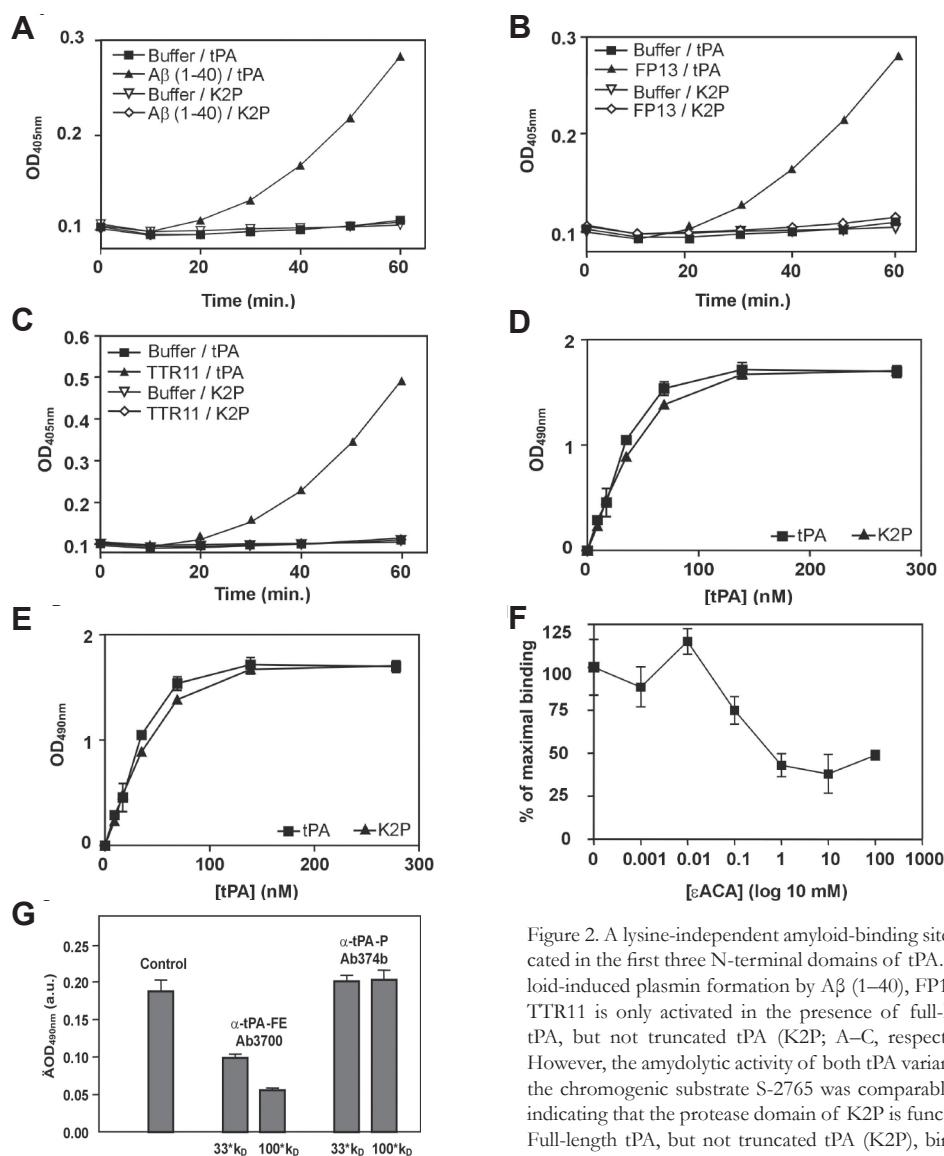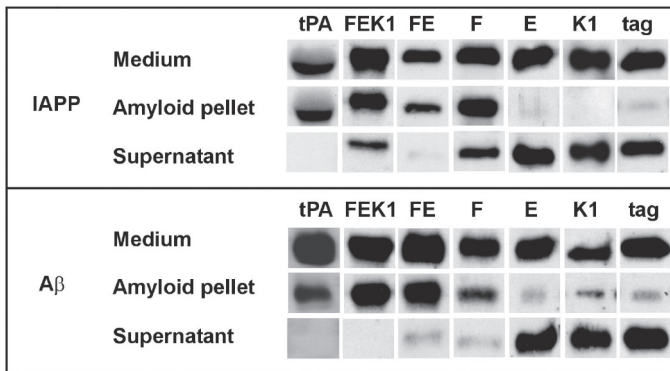Figure 2. A lysine-independent amyloid-binding site is located in the first three N-terminal domains of tPA. Amyloid-induced plasmin formation by Aβ (1–40), FP13 and TTR11 is only activated in the presence of full-length tPA, but not truncated tPA (K2P; A–C, respectively). However, the amydolytic activity of both tPA variants for the chromogenic substrate S-2765 was comparable (D), indicating that the protease domain of K2P is functional. Full-length tPA, but not truncated tPA (K2P), binds to immobilized amyloid preparations of Aβ (1–40), FP13 and TTR11 (E). Binding of tPA to immobilized amyloid Aβ (1–40) can only be inhibited up to 50% by lysine analog εACA, indicative of a lysine-independent binding site for amyloid in tPA (F). tPA binding to immobilized amyloid Aβ (1–40) can be inhibited by a monoclonal antibody directed against the finger-EGF-like domains (a-tPA-FE; Ab3700), but not by a monoclonal antibody directed against the protease domain (α-tPA-P; Ab374b) (G).

action. We found that tPA–Aβ interaction could be inhibited only for approximately 50% with εACA (Figure 2F). This indicates a clear, although partial, functional role for lysine-dependent interactions. These data correspond to earlier findings on amyloid FP13 binding by tPA, which could be inhibited for 40% by εACA[13]. The remaining 50% of the tPA binding to Aβ (1–40) was attributed to lysine-independent binding, which indicates the presence of another type of

amyloid-binding site in tPA. Since kringle domains are involved in lysine binding, amyloid binding most likely occurs through either the finger- or EGF-like (FE) domains. We investigated the role of the FE region by studying the effect of a monoclonal murine antibody (Ab3700) directed against this region of tPA and compared the induced loss of binding with that of a monoclonal murine antibody (Ab374b) directed against the tPA protease domain. tPA binding to Aβ (1–40) was detected with a polyclonal rabbit antibody (Ab385R). Because the affinity constants of both monoclonal α-tPA antibodies were not comparable (Kd for Ab3700 and Ab374b are 700 and 70 ng/mL, respectively), we tested potential inhibition of tPA binding to Aβ (1–40) at 33 and 100 times Kd of the antibodies. This experiment shows that targeting of the FE region of tPA results in significantly reduced binding, whereas a control antibody directed at the protease domain had no effect (Figure 2G). In control experiments, preincubation of immobilized Aβ (1–40) with equal amounts of Ab3700 had no effect on tPA binding (not shown) and binding of the polyclonal rabbit Ab385R was not inhibited by Ab3700 antibody (not shown). Together, these experiments indicate that binding of Ab3700 to the FE region of tPA, inhibits binding of tPA to Aβ. At the highest antibody concentrations, the inhibitory effect of Ab3700 surpassed 50%; we attribute this difference with the lysine-independent binding shown in Figure 2F to an additional sterical effect of the Ab3700 that interferes with the kringle–lysine interactions. These experiments point towards the presence of a lysine-independent binding site for amyloid in one or more of the three N-terminal domains of tPA.

Amyloid binding of tPA requires the finger domain

Since tPA may contain an amyloid-binding site in the FEK1 region (Figure 2), that was likely to be responsible for the multiligand receptor function that we described earlier[13], we investigated the role of these domains in amyloid binding in more detail. Hereto, we recombinantly produced the F, E and K1 domains of tPA, as well as a combined FE and FEK1 constructs in mammalian cells. All constructs were GST-tagged for analytical and purification purposes. Next, condi-
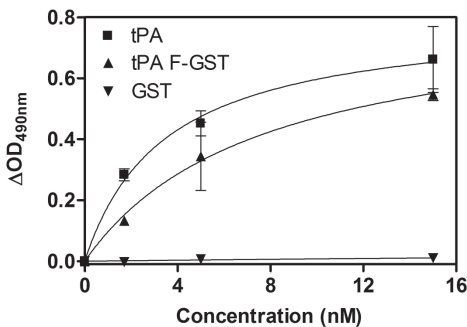
A

B

Figure 3. tPA binds to proteins with amyloid structure by its finger domain. Conditioned media containing either the GST-tagged finger (F), EGF-like (E) or kringle 1 (K1) domain of tPA were incubated with amyloid aggregates of Aβ (1–40) or IAPP. Amyloid binding was assayed by Western blot after centrifugation. The finger domain and all other constructs that contained it could bind to both amyloids, whereas the EGF-like, kringle 1 and tag could not (A). The binding of the finger domain alone was very comparable to that of full-length tPA (B).

tioned media from each transfection were incubated with amyloid aggregates of Aβ (1–40) or IAPP and binding of the produced proteins to the amyloid aggregates was analyzed by a centrifugation pull-down, followed by Western blotting against GST. In this experimental setup, as expected, fl-tPA, as well as the FEK1-GST, bound to both amyloid preparations (Figure 3A). All constructs with the finger domain, including the F-GST construct by itself, were capable of binding to both amyloids, whereas the E-GST and K1-GST or GST-tag alone did not display significant amyloid binding (Figure 3A). We conclude from these experiments that an amyloid-binding site is present in the finger domain of tPA.

In a next series of experiments, we compared the affinity of the purified F-GST construct to that of tPA for various immobilized amyloid proteins (and where possible, their native counterparts) to investigate the multiligand receptor capabilities of the finger domain. We found that both F-GST and tPA bound to amyloid Aβ (1–40) with comparable affinity (Figure 3B). No binding was observed to freshly dissolved Aβ (1–40). In line with this experiment, tPA and F-GST binding to amyloid preparations of IAPP, FP13, TTR11 was comparable (Table I). No binding of tPA or F-GST was detected to murine IAPP (mIAPP), which does not form amyloid structure. Interestingly, no fl-tPA binding was detectable to an amyloid preparation of the laminin peptide LAM12, whereas the F-GST construct bound to it with high affinity. This suggests that there is a finger-binding site present in amyloid LAM12, which may be unavailable to the much larger tPA molecule. Indeed, amyloid LAM12 was only capable of stimulating tPA-dependent plasmin formation with very low capacity (Figure 4). These data suggests that the amyloid-binding capacity of tPA transcends amino acid sequence dependence, since it can bind several unrelated amyloid peptides, and can be mediated by the finger domain alone. Finger-domains of homologous proteins have conserved amyloid-binding capacity. As described above, tPA has several homologs, each of which contains one or more finger domains (Figure 1). These homologs have been suggested to interact with amyloid on various occasions. For example, amyloid structure has been suggested to be a regulatory motive in activation of FXII[41;42]. Since we

Immobilized protein/peptide	tPA		F-GST	
	Affinity constant (nM)	Bmax (OD)	Affinity constant (nM)	Bmax (OD)
Aβ (1-40) Amyloid	3.46	0.80	7.61	0.83
Aβ (1-40) Fresh	-	-	-	-
IAPP	0.55	0.41	0.22	0.44
mIAPP	-	-	-	-
FP13	9.01	0.85	1.19	0.64
TTR11	4.17	0.72	2.06	0.50
Lam12	-	-	0.22	0.98

Table I. Binding of tPA and F-GST is comparable to various amyloids. The binding of full-length tPA (fl-tPA) and tPA F-GST to immobilized proteins was studied by ELISA. Affinity constants and maximal binding were determined by nonlinear regression (one-site binding) in Graphpad Prism 4.00. – indicates no (saturated) binding could be detected. In most cases, the F-GST construct had a slightly higher affinity for the immobilized amyloid proteins than did fl-tPA. In case of LAM12, fl-tPA binding was completely absent. This suggests that in most cases, the binding site for the finger domain on proteins with amyloid structure has better availability to the smaller F-GST construct than to fl-tPA.
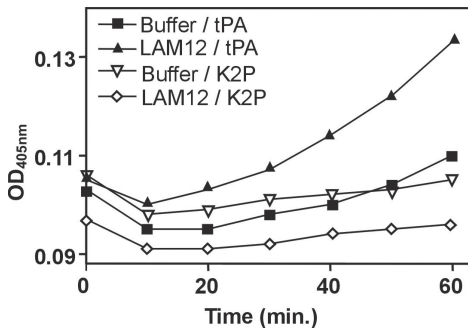
Figure 4. Activation of tPA-dependent plasmin formation by amyloid laminin peptide LAM12 is minimal, which corresponds to lack of tPA binding, determined by ELISA.

had found that the finger domain of tPA is involved in amyloid binding, we next tested whether finger domains of FXII, HGFA and Fn had similar capacities. Hereto, we recombinantly produced F-GST constructs of FXII and HGFA. We also produced GST-tagged constructs of fibronectin containing fingers 4 and 5 (Fn F4–5-GST), and fingers 10 to 12 (Fn F10–12-GST). We next analyzed amyloid binding by pull-down assay. Amyloid aggregates of IAPP associated with full-length FXII, Fn and HGFA in medium and could be retrieved from the amyloid pellets after pull-down (Figure 5). Finger domains of FXII and HGFA, as well as fingers 4–5 and 10–12 of Fn, bound to amyloid IAPP in a similar manner. Amyloid aggregates of Aβ (1–40) bound to coagulation factor XII, as well as its finger domain alone. However, little or no full-length protein was associated with amyloid Aβ for Fn or HGFA, respectively. In the case of full-length Fn, we could not retrieve Fn in the supernatant of the pull-down experiment, indicating full removal of Fn from the medium via the amyloid aggregates. Since the amyloid pellet only contained a small fraction of the initial Fn present, it is likely that the washing procedures used in these experiments had eluted the Fn from the amyloid Aβ. Similar to IAPP aggregates, Aβ aggregates efficiently bound to Fn F4–5 and F10–12. In the case of full-length HGFA, almost no HGFA was bound to the amyloid pellet and nearly all HGFA could be retrieved in the pull-down supernatant. The finger domain of HGFA, however, displayed significant amyloid Aβ binding when tested. Control constructs, containing the GST-tag, failed to associate with the amyloid pellets.
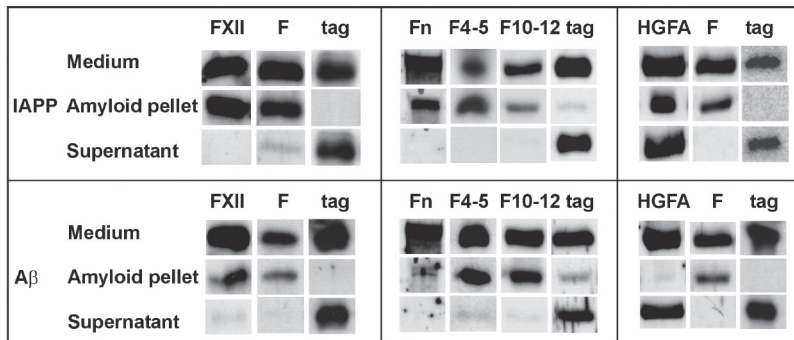

Figure 5. Finger domains of FXII, Fn and HGFA have amyloid-binding capacity, similar to tPA-F. Amyloid binding of full-length FXII, Fn and HGFA was assessed by amyloid pull-down assay. All three bound full length to IAPP aggregates, as well as their finger domains by themselves. Control constructs, containing the GST-tag did not bind to IAPP aggregates. In similar fashion, full-length FXII and its finger domain bound to Aβ aggregates. However, only slight binding of full-length Fn to amyloid Aβ was observed, which could be distinguished by a doublet characteristic for Fn. No Fn could be detected in the supernatant of the pull-down experiment, which suggests that Fn bound to Ab but was lost during washing steps of the amyloid pellets. Both Fn finger domains 4–5 and 10–12 showed significant affinity for Aβ aggregates. Controversially, full-length HGFA did not associate with amyloid Aβ at all, but its finger domain displayed significant amyloid-binding properties. This indicates that there is a binding site on Aβ for the HGFA finger domain, but that this interaction is disturbed in the full-length HGFA.

We conclude from these experiments that amyloid binding by full-length FXII, HGFA or Fn may vary, but their finger domains possess amyloid-binding activity in all cases.

So far, we had investigated the amyloid-binding site of tPA and homologs using aggregated amyloid Aβ and IAPP peptides. From these experiments, it became clear that these interactions required at least the finger domain. Next, we investigated whether a finger domain would recognize pathological amyloid that had formed *in vivo*. We incubated brain sections of a person that had deceased from Alzheimer's disease with the amyloid-specific dye Congo red as well as a construct containing tPA FE-GST, followed by incubation with an anti-GST antibody. The tPA FE-GST construct was selected because it had displayed the highest affinity for amyloid Aβ (1–40) in our earlier experiments (Figure 3A). The tPA FE-GST was visualized with an antibody against the GST-tag, which is displayed as a brown staining on the section. As can be seen in Figure 6A and B, Congophylic plaques present in the section could be stained by the FE-GST construct. Since Congo red and FE-GST apparently co-localize on the plaques, they may have different binding sites for the same amyloid. As a control, GST-tag by itself, followed by incubation with anti-GST antibody, did not stain Congophylic plaques at all (Figure 6C, D). These data indicate that the finger domain of tPA is capable of binding to pathological amyloid that has formed *in vivo*.

In a recent publication by our group, we demonstrated that platelets activate and aggregate in the presence of proteins with amyloid characteristics[15]. Advanced glycation end-products of albumin (BSA-AGE) form soluble amyloid-like aggregates[43] that have been reported to stimulate tPA-dependent plasmin generation[8]. Additionally, advanced glycated proteins are implicated in diabetic vascular dysfunction and nephropathy[35;44]. BSA-AGE, but not native albumin, was found to be one of the most potent inducers of platelet aggregation. It was also shown that fl-tPA could significantly inhibit this process. We used this knowledge to investigate the role of the

Figure 6. Staining of Congophilic amyloid deposits in a postmortem brain section of a patient with Alzheimer's disease by tPA FE-GST. Sections were either stained with tPA FE-GST or GST alone and visualized by subsequent incubation with an anti-GST antibody, resulting in a brown staining. Afterwards, both sections were co-stained by the fluorescent amyloid-specific dye Congo red. Disease-related amyloid deposits could be stained by both Congo red and tPA FE-GST (A and B), whereas the GST control did not stain Congophylic amyloid deposits (C and D).

finger domain. We first compared the binding of fl-tPA and K2P-tPA to immobilized BSA-AGE. As expected from Figure 2E, fl-tPA, but not K2P-tPA could bind BSA-AGE with high affinity (Figure 7A). Next, we evaluated whether immobilized tPA finger domain could bind to soluble BSA-AGE aggregates and observed a high-affinity association (Figure 7B). We next performed BSA-AGE induced platelet aggregation experiments. We observed that tPA, but not K2P-tPA, could inhibit BSA-AGE-induced aggregation (Figure 7C), which is in good agreement with the binding experiments. As a control for specificity of this observation, platelet activation by TRAP, which follows a different platelet activation mechanism by activation of the thrombin receptor PAR-1, was unaffected by both forms of tPA (at a suboptimal concentration of 3 mM; Figure 7E). This shows that the inhibitory activity of tPA remains solely in the tPA FEK region and is specific for platelet activation by BSA-AGE. Thus, we next investigated whether the tPA finger domain was capable of inhibiting BSA-AGE-induced platelet aggregation. Albeit somewhat less efficient compared with tPA, the isolated finger domain could reduce BSA-AGE-induced platelet aggregation with an estimated maximal capacity of 50% compared with fl-tPA (Figure 7D). Again, no effect was seen on TRAP-induced platelet aggregation (Figure 7E). Taken together, these experiments indicate that tPA and its finger domain recognize a binding site on BSA-AGE aggregates that overlaps at least partially with that of the involved platelet receptors and can be used to intervene in platelet activation.

Figure 7. Platelet aggregation by BSA-AGE is inhibited by full-length tPA (ft-tPA) and its finger domain, but not by K2P-tPA. Amyloid-like aggregates of BSA-AGE bind to fl-tPA, but not K2P-tPA, with high affinity, indicating a role for the FEK1-region in this interaction (A). Immobilized tPA finger domain (tPA F-biotin) binds to BSA-AGE in solution (B). Washed platelets were activated by a final concentration of 10 μg/ml of BSA-AGE (C, D); freshly dissolved BSA did not activate platelets (not shown). This activation could be inhibited by addition of 50 nM of fl-tPA, but not by addition of 50 nM K2P-tPA (C). Similarly, titration of a construct consisting of tPA biotin-labeled finger domain (tPA F-biotin) inhibited this activation up to 50% (D). As a control, activation of platelets by a final concentration of 3 mM TRAP was unaffected by either fl-tPA, K2P-tPA or F-biotin (E). Experiments shown are representative for at least three individual experiments.

Discussion

The etiology of protein misfolding diseases is an area of intense research as these diseases are generally highly deleterious and of enormous social and economical impact. While much is known about the folding and degradation of proteins within the cell, little is known about the removal of damaged or obsolete proteins in the extracellular environment. Novel insights into the clearance of proteins outside cells can open new avenues in the development of therapeutics for protein misfolding diseases. Earlier work from our group showed that tPA autoactivates in the presence of proteins with amyloid structure, and requires binding for this process[13].

We here demonstrate that this binding capacity resides in the finger domain of this molecule, which can independently support binding to various proteins and peptides with unrelated amino acid sequences. Additionally, isolated finger domains of FXII, HGFA and Fn have similar amyloid-binding capacities. In our studies, binding of fl-tPA to amyloid Aβ (and in an earlier report, FP13[13]) could be inhibited up to 50% with εACA, which indicates a role for kringle-dependent interactions. In this context, it is noteworthy that neither K2P-tPA nor the kringle 1 domain of tPA supported independent binding to amyloid.

Based on these findings, we propose a model in which tPA first binds to proteins with amyloid characteristics via its finger domain, after which kringle-dependent interactions are supported. Interestingly, the same two-step binding model has been proposed for the interaction between tPA and fibrin polymers[29]. The binding site for the finger domain in fibrin has not been clarified after those reports. As several fibrin peptides possess the intrinsic capacity to adopt amyloid structure (when aggregated) it is attractive to think that this structure is a primary target for tPA-mediated clearance of fibrin. In line with these considerations, Raman spectroscopic studies have shown that fibrin polymerization is accompanied by the stacking of β-sheets in the polymers[45], which corresponds to increased tPA-activating potential. Together with our findings here, these data suggest that the interaction between fibrin and tPA may correspond to the interaction between amyloid protein aggregates and tPA.

At this point, the specific details of the interaction between the finger module and proteins with amyloid structure remain to be elucidated. In fact, we only know that finger-binding sites are abundantly present in amyloid proteins, but not in native proteins, which does not directly imply that it is the 4.7 A° cross-β structure that is specifically present in amyloid. Given the number of ligands with unrelated amino acid sequences, the interactions are likely to be mediated by structure over amino acid sequence. The presence of a hydrophobic core in the finger domain[46], as well as the presence of hydrophobic regions in amyloid precursors, suggests that hydrophobic interactions could play a role.

The presence of amyloid proteins *in vivo* has various deleterious effects. The formation of amyloid plaques can cause organ damage or cognitive dysfunction in protein deposition diseases. In the case of Alzheimer's disease, activation of tPA and plasmin has been described on various occasions. The resulting activation of the plasmin system is thought to be directly protective by being responsible for reduced amyloid plaque formation[17;18;21;22;47] and, as was shown in a recent paper, indirectly by decreased fibrin deposition[48]. In contrast to these protective functions of tPA and plasmin, activation of the plasmin system by tPA is suggested to contribute to cerebral hemorrhage in Alzheimer's disease[49], while tPA mediates amyloid cytotoxicity in Alzheimer's disease[50]. In addition to the interaction of tPA with amyloid in Alzheimer's disease, FXII has also been shown to colocalize with amyloid plaques in Alzheimer's disease[51] and is activated by it, both *in vitro* and *in vivo*[42;52;53]. From these reports, it is suggested that the kallikrein–kinin system, which is activated by FXII, may play a role in the inflammatory processes seen in Alzheimer's

disease. Also, fibronectin[54;55] has been found associated with pathological amyloid in Alzheimer's disease and activation of HGFA is reported to take place[56].

In all, all four finger domain-containing proteins are implicated in Alzheimer's disease. However, these proteins are also implicated in other amyloidoses. For example, increased levels of HGF, which is controlled by HGFA, appear to be a good prognostic marker for various types of systemic amyloidosis[57]. An important question that arises is, for what reason the finger domain has evolved as a conserved amyloid-binding module and thus, in what physiological systems it may play its role. It is possible that tPA-dependent plasmin generation may also be activated by non-pathological misfolded proteins *in vivo*, resulting in clearance of these proteins before they have a chance to accumulate. The physiological activators of FXII and HGFA are not known. We therefore hypothesize that FXII and HGFA may have protective roles on sites of injury by their capacity to activate inflammatory processes and tissue repair mechanisms in the presence of misfolded proteins.

An especially interesting role for finger domains is present in Fn, which consists of no less then 12 of these type I domains (Figure 1). Finger domains 1–5, most notably domains 4–5, are involved in the binding of Fn to fibrin[30;58], and play a key role in self-association of Fn[59]. Additionally, Fn F6 and F7–9, are together present in a functional domain that binds to denatured collagen (gelatin), whereas Fn F10–12 form a second stretch of finger domains with affinity for fibrin. For Fn to exert its 'matrix assembly' function, the Fn F1–5 region first has to bind to cells and associate with the first type III domain (III-1) on another Fn molecule, which needs to be unfolded for this binding[60]. Indeed, unfolded type III domains are required for Fn assembly in general[61] and it has been suggested that this occurs by self-assembly of type III-1 domains [62], which can form amyloid-like fibrillar structures when isolated[63]. An interesting model known as 3D domain swapping has been suggested as an explanation for self-association of molecules, which may be applicable in this setting[64;65]. In this model, domains that normally interact with each other within a single protein molecule, interact with equal domains from other molecules in the same microenvironment. However, such a model in which only unfolded III-1 associate with each other does not explain the critical dependence on the Fn F1–5 region, which can inhibit Fn matrix formation on cells. Our findings, as well as findings of others[66], in which proteins with unfolded or amyloid structural properties interact with Fn finger domains may be suggestive for a 3D domain swapping model in which finger modules of Fn interact with unfolded type III domains during self-association.

We show here that the finger module can bind to amyloid plaques and demonstrate that we can inhibit amyloid-induced cell activation. A recent publication elegantly demonstrated the use of a soluble form of low-density lipoprotein (LDL) receptor-related protein 1 (LRP) in clearance of circulating Aβ peptide, thereby reducing the Alzheimer's disease-related pathology *in vivo*[67]. Besides its known ligands (LDL and coagulation FVIII), LRP-1 has been described to bind to Aβ peptide *in vitro*[68;69]. Further studies will decide whether finger domain-containing constructs can be applied to enhance clearance of amyloidogenic peptides from plasma and/or prevent amyloid-related pathology in a similar fashion. In conclusion, we propose that the interaction between finger domains and amyloid proteins reflects a mechanism for recognition and clearance of misfolded proteins from the extracellular compartment. Our findings may offer new targets for therapeutic interventions to conquer protein misfolding diseases.

Acknowledgements

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Chapter 4

Increased Plasmin-α2-antiplasmin Levels Indicate Activation of the Fibrinolytic System in Systemic Amyloidoses

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Coen Maas,[1]* Barend Bouma,[1]* Bouke P.C. Hazenberg,[2]
Henk M. Lokhorst,[3] Martijn F.B.G. Gebbink[1]

[1]Laboratory for Thrombosis and Haemostasis, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht and the Institute for Biomembranes, Utrecht, The Netherlands. [2]Department of Rheumatology and Clinical Immunology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands. [3]Department of Hematology, University Medical Center Utrecht, Utrecht, The Netherlands. * These authors contributed equally to this work

With related commentary by Dr. Per Hammarström:
"The bloody path of amyloids and prions."
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Introduction

Systemic amyloidosis is marked by accumulation of amyloid deposits in various tissues throughout the body. In most cases, impairment of organ function by these deposits leads to morbid and complex pathological conditions. Primary systemic amyloidosis (AL) is caused by accumulation of monoclonal immunoglobulin light-chains (LC), which are overproduced by plasma cells[1]. AL is the most prevalent form of systemic amyloidosis. Secondary systemic amyloidosis (AA) can be recognized by the accumulation of serum amyloid A protein, a process that is thought to be a hazardous side-effect of chronic inflammation[2]. Thirdly, hereditary forms of systemic amyloidosis exist that are caused by mutations in transthyretin (ATTR[3]) or, more rarely, by lysozyme, fibrinogen or other proteins[4–8], causing these proteins to accumulate into amyloid deposits. Patients suffering from systemic amyloidosis are at risk for developing bleeding complications, which may have life-threatening consequences[9–17]. This problem has not yet been fully elucidated, but can partially be explained by decreased coagulation activity[17–22]. Amyloid is defined by the presence of deposits of fibrillar protein aggregates in tissue, which can be stained by dyes such as Congo red. Amyloids of different origin have common structural characteristics, most notably a unique quarternary fold, termed the cross-β structure, with a 4.7 Å inter-sheet regularity. We have identified tissue-type plasminogen activator (tPA), an activator of fibrinolysis, as a multiligand receptor for amyloid cross-β structure *in vitro*[23]. Plasmin is a serine protease that mediates proteolysis of many substrates, including fibrin. Increased plasmin activity has previously been implicated in bleeding[24–30]. Accumulating protein deposits in systemic amyloidosis patients share the structural characteristics that activate plasmin formation and their amyloidogenic progenitors are present in plasma[31;32]. The levels of these circulating proteins are predictive for the clinical outcome of the diseased patients.

From this perspective, we hypothesized that the observed bleeding tendency of amyloidosis patients may be attributed at least in part to constitutive fibrinolysis. To test this hypothesis, we have determined the plasmin-α2-antiplasmin (PAP) complex levels in systemic amyloidosis patients and compared them with age- and sex-matched healthy controls.

Methods

Systemic amyloidosis patients were recruited at Groningen University Medical Center and Utrecht University Medical Center as described earlier[33–35]. Selection was based on positive Congo red biopsies and number and location of sites with amyloid depositions. Thirty-one patients with systemic amyloidosis were included in the study. Five patients suffered from hereditary TTR amyloidosis, nine from AA and seventeen from AL. Blood was drawn by venipuncture and collected in 6-mL Vacutainer tubes (BD, Franklin Lakes, NJ, USA, #388410) with a final concentration of 0.32% sodium citrate. Plasmas were isolated by 10 min centrifugation at 2700 x g at room temperature. Samples were snap-frozen and temporarily stored at –20°C before transport to the laboratory and storage at –80°C. Plasma concentrations of PAP complexes were determined in duplicate within 5 months using a commercially available ELISA (Technoclone GmbH, Vienna, Austria, Cat. No. TC11060), according to the manufacturer's instructions.

Data were drawn and analyzed in GRAPHPAD PRISM 4 for Windows; statistical analysis of differences between individual groups was performed by two-tailed non-parametric t-tests (Mann–Whitney). Overall differences between more than two groups were compared by one-way ANOVA (Kruskal–Wallis).
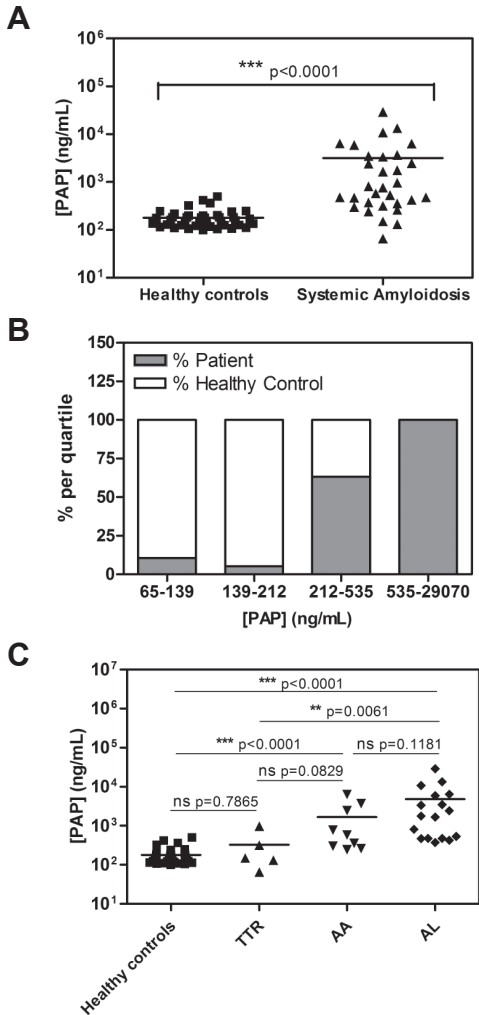
Fig. 1. Activation of the fibrinolytic system in systemic amyloidosis. (A) Plasma plasmin-α2-antiplasmin (PAP) levels were determined by ELISA in 31 patients with systemic amyloidosis (38.7% male, average age 51.8 ± 10.5) and compared with those of 40 healthy controls (37.5% male, average age 49.4 ± 7.3). Data were analyzed by two-tailed non-parametric t-test and a highly significant difference was found between both groups, indicating the profibrinolytic state of the patient population. (B) Division in quartiles demonstrates that all subjects with high fibrinolytic activity are systemic amyloidosis patients. (C) Subanalysis of our patient population showed a significant difference in PAP levels between the three forms of amyloidosis, as determined by one-way ANOVA (***P < 0.0001). Individual two-tailed non-parametric t-tests revealed that high PAP levels were prominent in AA and AL amyloidosis, but not in TTR amyloidosis, as compared to controls.

Results

Plasma levels of PAP complexes were significantly elevated in patients with systemic amyloidosis, compared with healthy control subjects, as shown in Fig. 1A. Elevated PAP levels were found in 71.0% of the patients suffering from systemic amyloidosis, compared with 2.5% of control subjects (elevated PAP levels are defined as being above the sum of the mean value of 40 healthy controls and 3 SD). This is indicative for a shift in the hemostatic balance towards a fibrinolytic state in systemic amyloidosis patients. Division of patients and controls into quartiles is illustrated in Fig. 1B. The first and second quartile together comprises 33 healthy controls (82.5%) and only three systemic amyloidosis patients (9.7%). The third quartile comprises seven control subjects (17.5%) and 11 patients (35.5%). The highest quartile consists only of systemic amyloidosis patients (54.8%). Thus, systemic amyloidosis is often accompanied by an increase in PAP levels.

Next, the question arose of whether the hyperfibrinolytic state seen in systemic amyloidosis was preferentially or equally divided over the three forms of systemic amyloidosis. A two-tailed one-way ANOVA indicated a P-value < 0.0001, indicating significant differences between groups. Individual non-parametric t-tests showed that only AA and AL patients had strongly elevated PAP levels compared with healthy controls, whereas ATTR patients showed no increased PAP levels (Fig. 1C). This suggests that a hyperfibrinolytic state is mainly present in AA and AL patients, with no significant difference between these two groups, although on average AL patients have slightly higher PAP levels compared with AA patients. While PAP levels in ATTR patients are significantly lower than in AL patients, similar differences between AA and ATTR patients did not reach statistical significance, probably because of lack of power.

Generation of plasmin has been associated with inflammation[36]. CRP is clinically used as

a marker for inflammation. Twenty-four out of 31 patients had been routinely tested. We analyzed those values with the same statistical methods used for analyses of PAP complex levels. We found that CRP was significantly elevated in AA, but not in AL or ATTR (not shown). No correlation between PAP and CRP levels could be demonstrated in our patient population, suggesting that elevated PAP levels are not directly related to inflammation in our patients. The elevated CRP levels in AA can be explained by the chronic inflammation that leads to AA amyloidosis[2;31;37]. In addition, albumin levels, routinely measured in 26 out of 31 patients, were equal between ATTR, AA and AL amyloidosis (not shown). This suggests that determination of PAP levels in our study was not influenced by variation in general protein metabolism, which could have been caused by lowered liver function or nephropathy.

Systemic amyloidosis has a poorly understood bleeding phenotype. Previous data have indicated that this bleeding tendency can, in part, be attributed to decreased coagulation.

Both lowered levels or acquired deficiencies of certain coagulation factors (i.e. FX, FV and von Willebrand factor) are reported as being responsible[17;20–22]. In particular, recruitment of FX to amyloid deposits has been considered causative. This explanation, based on activity measurements alone, has been challenged[19]. In a large population of AL patients decreased amounts of active FXa were found, rather than lowered levels of FX. Others have hypothesized that decreased activation of FX might be due to the loss of tissue factor from blood vessels to amyloid deposits and/or inactivation by soluble protein aggregates[38]. Loss of tissue factor may also affect the stability of the clot in another way. Recent data show that tissue factor, via activation of FXI, is involved in stabilizing fibrin. FXIa enhances fibrin generation but also strengthens the clot because it inhibits fibrinolysis through the generation of thrombin-activatable fibrinolysis inhibitor[39].

Taken together it is evident that bleeding induced by amyloidosis can be ascribed to decreased coagulation. We found here that 71.0% of systemic amyloidosis patients have elevated PAP levels, as compared with 2.5% in control subjects, which is indicative for elevated plasmin generation and fibrinolytic activity in systemic amyloidosis. Elevated PAP levels were most pronounced in AA and AL patients, but not in ATTR patients. Our findings indicate that systemic amyloidosis can be accompanied by constitutive plasmin formation, which is reflected in the circulating PAP complexes. Given the previous notion that tPA is activated by misfolded protein aggregates in general, we presume that the fibrinolytic system in these patients is activated directly by the amyloidogenic misfolded proteins. Consequently, this is a physiological response in order to remove these potentially harmful protein structures to alleviate the symptoms. In certain AA and AL patients, this response of the fibrinolytic system may become pathological and lead- or contribute to bleeding. This hypothesis is illustrated by our finding that one AL patient with very high PAP levels in our study (13,455 ng/mL; sample taken in June 2004), was treated and reported for having fibrinolysis-related bleeding symptoms in September 2000[10]. This would suggest a longterm constitutive hyperfibrinolytic state in amyloidosis patients. This patient had widespread vascular amyloid deposits (H.M. Lokhorst, personal communication), which might explain the systemic character of the observed bleeding symptoms. Elevated fibrinolytic activity has been reported as being of importance in individual cases of systemic amyloidosis patients suffering from bleeding episodes[10;40]. Interestingly, hemorrhaging has also been described in ATTR patients, which can be ascribed to ruptures of the cranial microvasculature caused by localized amyloid deposits in vessel walls[11–13;41]. Similarly, reports state that amyloid deposits in small vessels can cause splenic rupture in AA[42]. Apparently, mechanic vessel rupture by localized accumulating amyloid causes bleeding. However, the bleeding tendency seen in most patients is

not only related to vessel rupture by amyloid[14–16;19] and lacks a well-defined cause.

Current studies point towards prefibrillar amyloidogenic proteins as *in vitro* activators of fibrinolysis (our unpublished data). The differences in the fibrinolytic status of patients with specific amyloidoses (i.e. ATTR, AA, AL) may be explained by the differences in the presence of these activating species *in vivo*. Indeed, a generally less severe bleeding tendency is observed in ATTR patients than in AA and AL patients (B. P. C. Hazenberg, personal communication). Further studies are required to identify the exact molecular characteristics of amyloidogenic proteins that are able to stimulate fibrinolysis. This may broaden our perspective on the role of the fibrinolytic system. Recent developments have already pointed to more roles for plasmin than mere involvement in fibrinolysis. Most notably, it has been described that plasmin formation mediates inflammatory responses in a number of conditions, for example atherosclerosis[36]. Perhaps, plasmin formation in general, aids in the removal of misfolded protein aggregates, a response that may turn harmful under pathological conditions, such as systemic amyloidoses. Further investigations are required to validate these hypotheses.

In conclusion, we suggest hyperfibrinolysis as a causative factor for bleeding events in certain forms of systemic amyloidosis. Screening for hyperfibrinolysis in idiopathic amyloidosis-related bleeding may therefore prove useful. Amelioration of symptoms may be achieved by administration of antifibrinolytics, such as tranexamic acid or epsilon amino caproic acid, which has already been reported in individual cases[10;43]. We propose that amyloid deposits inflict physical damage and impairment on vessels and organs and, at the same time, sequester and activate fibrinolysis, resulting in hemorrhage. A better understanding of this mechanism may offer new targets and more effective treatment of systemic amyloidosis and/or amyloidosis-related hemorrhaging.

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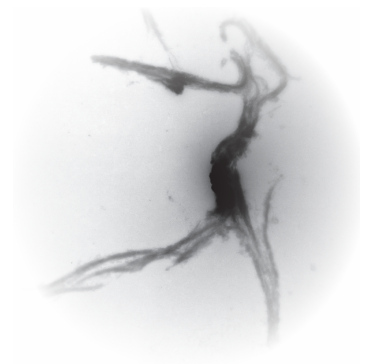32. Palladini G, Lavatelli F, Russo P, Perlini S, Perfetti V, Bosoni T, Obici

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Chapter 5

Fibrin in Blood Clots Forms Natural Amyloid
Submitted

Barend Bouma,[1] Coen Maas,[2] Loes M.J. Kroon-Batenburg,[3]
Onno Kranenburg,[4] Ya Ping Wu,[1] Martijn F.B.G. Gebbink[1,2]

[1]Crossbeta Biosciences BV, Utrecht, The Netherlands. [2]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands. [3]Department of Crystal and Structural Chemistry, Bijvoet Center for Biomolecular Research, Utrecht University, Utrecht, The Netherlands. [4]Department of Medical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands.

Amyloids are fibrillar protein aggregates found in plaques that are associated with the pathogenesis of degenerative diseases such as Alzheimer's disease and Creutzfeld-Jakob's disease, commonly referred to as amyloidoses. Amyloid fibrils are composed of stacked β-sheets with a 4.7 Å distance between the amino-acid strands that run perpendicular to the fibril axis (Figure 1A). This structure has been named cross-β structure and can arise irrespective of a specific amino-acid sequence[1].

Fibrin polymers are formed in response to vascular damage and act as a wound sealant in blood clots to prevent excessive blood loss. During wound healing, the fibrin polymers are enzymatically degraded by the serine protease plasmin, in a process called fibrinolysis. The fibrin polymer initiates this process, by binding to- and activation of tissue-type plasminogen activator (tPA), which converts the zymogen plasminogen into plasmin. However, the structural requirements for the activation of fibrinolysis by fibrin are unknown[2].

Besides its role in clot lysis, fibrinolysis has been reported in various amyloidoses[3] and it can be directly induced by the pathological protein aggregates[4]. Recently, we have observed that the interaction of tPA with both fibrin and amyloid is mediated by the same domain[5].

Thus, strikingly both amyloid plaques and fibrin clots consist of elongated fibrils and both evoke fibrinolysis in the same way. This leads to the question, if there is a common feature in both fibrin and amyloid fibrils.

We hypothesized that fibrin polymers have amyloid structure and tested this hypothesis using various established methods for the detection of amyloid structure. We prepared fibrin clots by incubation of purified fibrinogen with the enzyme thrombin and incubated them with two histopathological dyes for amyloid; namely Thioflavin T (ThT) and Congo Red (CR). As can be seen in the insets of Figure 1B and 1C, the clots became stained by these dyes, despite extensive washing. A second, more specific, feature of these dyes is their optical behavior in the presence of amyloid fibrils. Thioflavin T displays specific fluorescence when bound to amyloid, and we found that it behaves similarly in the presence of fibrin; ThT fluorescence increased over time as fibrin was formed (Fig. 1B). Additionally, amyloid-bound Congo Red displays apple green birefringence when exposed to polarized light in the presence of amyloid fibrils and was found to behave the same in the presence of fibrin polymers (Figure 1 C). Finally, we analyzed fibrin fibrils by X-ray fiber diffraction. As can be seen in Figure 1D, there is a broad peak at 4.7 Å (representing the distance between the strands that make up a β-sheet) and one at 9.7 Å (representing the inter-sheet distance). These structural features are typically characteristic of fibrils with amyloid cross-β structure.

Serum amyloid P (SAP) is a protein that is associated with amyloid plaques in various amyloidoses and is used as a diagnostic tool. We observed that SAP interacts similarly with fibrin (Figure 1E).

In all, these data show that fibrin forms amyloid cross-β structure. This is the first report on a naturally occurring extracellular protein with amyloid structure. The presence of the same structure in fibrin and pathological amyloid suggests a similar function. We propose that this structure, which can be formed by a myriad of proteins, including the fibrin precursor fibrinogen[6], triggers fibrinolysis, contributing to removal of obsolete proteins such as fibrin in blood clots.

In amyloid disease, attempts to remove of pathological amyloid fibrils with the same structure may be beneficial (as shown in mouse models for Alzheimer's disease), but eventually prove futile. In the search for therapeutics targeting amyloid diseases, these new findings should be considered and further identification of proteins involved in the recognition of obsolete proteins is warranted.
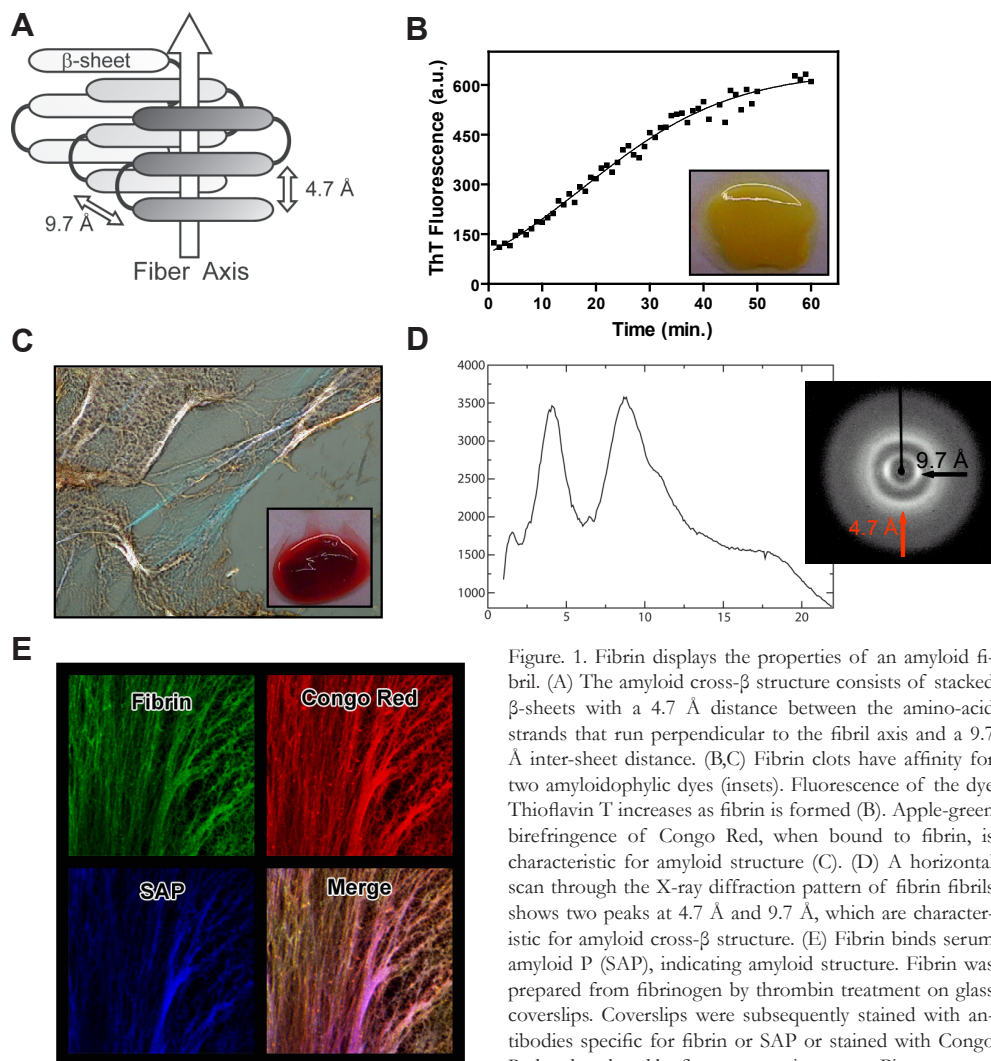
Figure. 1. Fibrin displays the properties of an amyloid fibril. (A) The amyloid cross-β structure consists of stacked β-sheets with a 4.7 Å distance between the amino-acid strands that run perpendicular to the fibril axis and a 9.7 Å inter-sheet distance. (B,C) Fibrin clots have affinity for two amyloidophylic dyes (insets). Fluorescence of the dye Thioflavin T increases as fibrin is formed (B). Apple-green birefringence of Congo Red, when bound to fibrin, is characteristic for amyloid structure (C). (D) A horizontal scan through the X-ray diffraction pattern of fibrin fibrils shows two peaks at 4.7 Å and 9.7 Å, which are characteristic for amyloid cross-β structure. (E) Fibrin binds serum amyloid P (SAP), indicating amyloid structure. Fibrin was prepared from fibrinogen by thrombin treatment on glass coverslips. Coverslips were subsequently stained with antibodies specific for fibrin or SAP or stained with Congo Red and analyzed by fluorescent microscopy. Picture merge demonstrates that all three methods stain the same amyloid regions in fibrin.

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Chapter 6

A Role for Protein Misfolding in Immunogenicity of Biopharmaceuticals

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Coen Maas,[1] Suzanne Hermeling,[2,3] Barend Bouma,[1]
Wim Jiskoot[2] and Martijn F. B. G. Gebbink[1]

[1]Laboratory for Thrombosis and Haemostasis, Department of Clinical Chemistry and Haematology, University Medical Center Utrecht and the Institute for Biomembranes, Utrecht, The Netherlands. [2]Department of Pharmaceutics, Utrecht Institute for Pharmaceutical Sciences (UIPS), Utrecht, The Netherlands. [3]Central Laboratory Animal Institute, Utrecht University, Utrecht, The Netherlands

Abstract

For largely unknown reasons, biopharmaceuticals evoke potentially harmful antibody formation. Such antibodies can inhibit drug efficacy and, when directed against endogenous proteins, cause life-threatening complications. Insight into the mechanisms by which biopharmaceuticals break tolerance and induce an immune response will contribute to finding solutions to prevent this adverse effect. Using a transgenic mouse model, we here demonstrate that protein misfolding, detected with the use of tissue-type plasminogen activator and thioflavin T, markers of amyloid-like properties, results in breaking of tolerance. In wild-type mice, misfolding enhances protein immunogenicity. Several commercially available biopharmaceutical products were found to contain misfolded proteins. In some cases, the level of misfolded protein was found to increase upon storage under conditions prescribed by the manufacturer. Our results indicate that misfolding of therapeutic proteins is an immunogenic signal and a risk factor for immunogenicity. These findings offer novel possibilities to detect immunogenic protein entities with tPA and reduce immunogenicity of biopharmaceuticals.

Introduction

Over the past decades, the use of therapeutic proteins has become common practice in medicine and as their use is very promising, many more biopharmaceuticals are under development[1;2]. Unfortunately, a major drawback of protein therapeutics is the risk of antibody formation[3-7]. These immunogenicity problems are of concern regarding therapeutic efficacy and patient safety[5;8]. For example, drug-induced neutralizing antibodies to erythropoietin (EPO)[3] result in pure red cell aplasia[9], whereas drug-induced acquired anti-factor VIII (FVIII) antibodies worsen the pathology associated with hemophilia[10]. As more and more recombinant therapeutic proteins become licensed for marketing, the incidence of immunogenicity problems is expected to rise. Initially, when mainly proteins from animal origin were used for therapy, it was thought that their foreign (non-self) nature was the main cause of immunogenicity. Unexpectedly, however, both human plasma derived as well as recombinant human protein therapeutics such as EPO[11] and FVIII[12] also elicit immune responses. This suggests that the molecular characteristic evoking antibody responses is at least more complex than being self or non-self to the human immune system.

Several additional factors contributing to immunogenicity have been proposed, including contaminants or impurities, protein aggregation[13], chemical degradation and protein modification, such as differences in glycosylation or oxidation[14;15] to explain the induction of antibodies. Protein misfolding is an intrinsic and problematic property of proteins, which underlies a variety of degenerative diseases, such as Alzheimer disease. These diseases are characterized by the occurrence of fibrillar deposits, classically termed amyloid, containing aggregates of misfolded proteins. Whereas the term amyloid is classically used to classify these fibrillar deposits, aggregation of proteins, irrespective of amino acid sequence, results in formation of amyloid-like properties with similar common features[16]. Amyloid can be defined histochemically by affinity for amyloid-specific dyes, but also morphologically when 6–10-nm filaments are seen by microscopy. X-ray diffraction experiments can confirm the presence of cross-β structure, a structural element characteristic for amyloid. We have reported that amyloid proteins, positive for the amyloid markers described above, also are able to bind and activate tissue-type plasminogen activator (tPA) *in vitro*[17;18]. Concomitantly, it has been reported that denatured protein aggregates have the same capacity, whereas native proteins do not bind tPA[19;20]. Therefore, tPA can serve as a fast novel tool for detection of misfolded protein with amyloid-like properties[17;21]. Protein misfolding can be accelerated by a number of environmental factors, including protein modifications such as glycation, deamidation, or oxidation[17;22;23], interaction of proteins with surfaces, such as mica[24] or negatively charged phospholipids[25], or conditions such as heating[26], lyophilization[27], sonication[28],

packaging materials[26], and others[29]. Amyloids and proteins with amyloid-like properties activate the immune system, which can lead to the formation of (auto-) antibodies[30;31].

Because protein misfolding is an intrinsic problematic propensity of proteins we hypothesized that protein misfolding processes in general could underlie immunogenicity of biopharmaceuticals. We investigated whether the common structural features of misfolded therapeutic proteins are detectable by markers for amyloid formation. We exposed biopharmaceuticals to denaturing conditions and investigated markers for amyloid formation in the preparations. Moreover, we analyzed several purchased biopharmaceuticals for the presence of misfolded proteins using these common markers for amyloid properties. Both experiments pointed out that biopharmaceuticals, like any other protein, are amyloidogenic and that misfolding, detected by amyloid markers, takes place in several preparations. These markers, however, are not necessarily specific for fibrillar amyloid, but also for smaller misfolded protein species.

Next, we tested the main hypothesis that misfolded protein, detectable by these amyloid markers, is causative for increased risk of having immune reactions against biopharmaceuticals.

We compared the immunogenicity of experimentally misfolded protein solutions to control solutions, containing native protein. The titer of antibody responses against the native protein was taken as a measure for immunogenicity. Although further study is required to identify the exact immunogenic misfolded species that mediates this mechanism, our experiments confirmed the hypothesis that misfolding leads to biopharmaceutical immunogenicity.

Experimental Procedures

Thioflavin T and Congo Red Fluorescence Measurements. Fluorescence of Thioflavin T (ThT; Sigma, T-3516) and Congo Red (Aldrich Chemical Company Inc.) was measured on a Hitachi F-4500 spectrophotometer at an excitation wavelength of 435 nm and emission wavelength of 485 nm for ThT and an excitation wavelength of 550 nm and emission wavelength of 595 nm. Five μL of the various protein solutions were diluted in either 1 mL of 25M ThT in 50mM glycine buffer, pH 9.0, or 1 mL of 25 M Congo Red in phosphate-buffered saline (PBS), pH 7.2, and incubated for 30 min at room temperature. Fluorescence was measured in triplicate with an integration time of 5 s per reading. Background fluorescence of both protein in buffer and dye solution were subtracted from the total fluorescence signal. Five μg/mL of aggregated amyloid Aβ "dutch type" E22Q, residues 1–40; DAEFRHDSGYEVHHQKLVFFAEDVG-SNKGAIIGLMVGGVV, produced by Peptide Synthesis Facility of the Dutch Cancer Institute NKI) was used as a positive control in all fluorescence assays. This positive control was prepared by dissolving lyophilized Aβ peptide at 50 mg/mL in a 1:1 mixture of trifluoroacetic acid and 1,1,1,3,3,3-hexafluoro-2-propanol. After evaporating the solvent, the pellet was resuspended at 10 mg/mL in H_2O and incubated at 37°C for 72 h.

tPA Binding Assay. Nunc Immobilizer plates (Nalge Nunc, number 436013) were coated with 50 μL containing 5 μg/mL of sample protein (unless indicated otherwise) in 100 mM $NaHCO_3$, pH 9.6, 0.05% (m/v) NaN_3 for 1 h at room temperature. Plates were washed twice with Tris-buffered saline, pH 7.2, containing 0.1% Tween 20 (TBST) and blocked with PBS containing 1% Tween 20 for 1 h at room temperature. Plates were washed twice with TBST and incubated, in duplicate, with a concentration series of either tPA (Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, The Netherlands) or a truncated form of tPA (Reteplase; Rapilysin, Roche Diagnostics GmbH, Mannheim Germany), lacking the amyloid binding domain, diluted in PBS containing 0.1% Tween 20 (PBST). Incubations were performed for 1 h at room temperature in the presence of 10 mM ε-amino caproic acid. ε-Amino caproic acid is a lysine analogue and is used to

avoid potential binding of tPA to lysine-containing ligands via its kringle2 domain. Plates were washed five times with TBST and incubated with antibody 374b -tPA (American Diagnostica, Instrumentation Laboratory, Breda, The Netherlands) diluted 1:1000 in PBST for 1 h at room temperature. Plates were washed five times with TBST and incubated with peroxidase-labeled anti-mouse immunoglobulins (RAMPO; DAKOCytomation, Glostrup, Denmark) diluted 1:3000 in PBST for 30 min at room temperature. Plates were washed five times with PBS, 0.1% Tween 20, and stained with 100 μL/well of tetramethylbenzidine substrate (Biosource Europe, Nivelles, Belgium). The reaction was terminated with 50 μL/well of 2 M H_2SO_4 and substrate conversion was read at 450 nm on a Spectramax340 microplate reader. Curves were fitted with a one-site binding model (GraphPad Prism version 4.02 for Windows, Graphpad Software) from which Kd and Bmax were determined.

tPA Activation Assay. Exiqon Peptide Immobilizer plates were blocked for 1 h with PBS, 1% Tween 20 and rinsed twice with distilled water. The conversion of the chromogenic substrate S-2251 (Chromogenix, Italy) by plasmin was kinetically measured at 37°C on a Spectramax 340 microplate reader at a wavelength of 405 nm. The assay mixture contained 400 pM tPA, 100 μg/mL plasminogen (purified from human plasma), and 415 μM S-2251 in HEPES-buffered saline, pH 7.4. Denatured γ-globulins (100 μg/mL) with amyloid-like structure was used as reference and positive control. Lyophilized γ-globulins (Sigma) were dissolved in a 1:1 volume ratio of 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoroacetic acid and subsequently dried under air. Dried γ-globulins were dissolved in H_2O to a final concentration of 1 mg/mL and kept at room temperature for at least 3 days and subsequently stored at -20°C. Maximal tPA activating capacity was determined from the linear increase seen in each activation curve and expressed as a percentage of the standardized positive control. To confirm tPA dependence of plasmin generation, all samples were assayed for their ability to convert plasminogen into plasmin in the absence of tPA.

Analyses of Protein Therapeutics. Protein therapeutics were obtained from the local hospital pharmacy and analyzed within the expiry limits as stated by the manufacturers. Fivel of the various protein therapeutics were tested for their ability to enhance both ThT and Congo Red fluorescence. tPA activating capacity of the protein therapeutics was determined in 1:10 diluted samples (unless indicated otherwise). tPA binding ELISAs were performed by coating protein therapeutics 1:10 in 100 mM $NaHCO_3$, pH 9.6, 0.05% (m/v) NaN_3.

Stability Testing. To mimic accelerated stability testing several therapeutics were exposed to denaturing conditions and assayed for amyloid-like properties before and after treatment by tPA activation assay at 100 μg/mL protein and ThT fluorescence enhancement assay at 25 μg/mL protein. For this purpose, 5 mg/mL glucagon (Glucagen; Novo Nordisk Farma B.V., Alphen aan de Rijn, The Netherlands) was incubated at 37 °C in 0.01 M HCl for 48 h. One mg/mL Etanercept (Enbrel; Wyeth Pharmaceuticals B.V., Hoofddorp, The Netherlands) in 67 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0, was gradually heated from 30 to 85 °C over a period of 12 min and afterward cooled to 4 °C for 5 min, this treatment was repeated 4 times. Abciximab (Reopro; Centocor B.V., Leiden, The Netherlands) and Infliximab (Remicade; Schering-Plough B.V., Utrecht, The Netherlands) were incubated at 65 °C for 16 and 72 h, respectively. A detailed description of the composition of these biopharmaceuticals is listed in supplemental Table 1 (http://www.jbc.org/cgi/content/full/M605984200/DC1).

Inducing Protein Misfolding in Solutions of rhIFNα2b and Ovalbumin. Unformulated human recombinant interferon α2b (rhIFNα2b; kindly provided by Alfa Wasserman, Italy) was incubated at 300 μg/mL with 4 mM ascorbic acid, 40 M $CuCl_2$ for 3 h at room temperature, buffered by 10 mM

sodium phosphate buffer, pH 7.2. This method for metal-catalyzed oxidation has been reported to result in oxidation of methionine residues in rhIFNα2b[32]. There are 6 methionines present in IFNα2b. The reaction was stopped with 1mM EDTA and dialyzed overnight against 4 liters of PBS. For testing of dose-dependent immune reactivity toward amyloid-like properties in rhIFNα2b, mixtures of unmodified rhIFNα2b and oxidized rhIFNα2b were prepared containing 0, 25, 50, 75, and 100% oxidized rhIFNα2b (total protein concentration of rhIFNα2b was equal in all samples). A solution of 1 mg/mL ovalbumin (Sigma) in 67 mM sodium phosphate buffer, 100 mM NaCl, pH 7.0, was gradually heated from 30 to 85 °C over a period of 12 min and afterwards cooled on ice.

Immunization Experiments. The animal experiments were approved by the Institutional Ethical Committee. Wild type BALB/c and FVB/N mice were obtained from Charles River Laboratories (Wilmington, MA) and housed at the Central Laboratory Animal Institute (Utrecht University, The Netherlands). Food (Hope Farms, Woerden, The Netherlands) and water (acidified) were available ad libitum. Human IFNα2b transgenic mice were bred from a wildtype FVB/N strain. Groups of 5 mice received 10g of the different rhIFNα2b preparations subcutaneously on days 0–4, 7–11, and 14–18. Groups of 5 wild-type female BALB/c mice (7–9 weeks old) received 10 µg of the different ovalbumin preparations subcutaneously on days 0–4, 7–11, and 14–18. Blood was taken from the vena saphena on days 0, 7, and 14 just before injection of the interferon or ovalbumin preparations, and on day 21. The blood samples were incubated on ice for 2 h. Sera were collected after centrifugation, stored at 20 °C, and analyzed later for the presence of anti-rhIFNα2b or anti-ovalbumin antibodies by ELISA.

Antibody Titer Determinations - rhIFNα2b. Sera were analyzed for IgG antibodies against native rhIFNα2b, coated in microtiter plates. Microlon high-binding 96-well plates were coated with 100 µL of native rhIFNα2b (2 µg/mL in PBS) per well for 1 h. The wells were drained and washed 4 times with 300 µL of PBST. After washing, the wells were carefully tapped dry on a tissue. Wells were blocked by incubating with 200 µL of 2% bovine serum albumin in PBS for 1 h. The wells were drained and washed 2 times with 300 µL of PBST. After the last wash, wells were carefully tapped dry on a tissue. Sera (diluted 100-fold with 2% bovine serum albumin in PBS) were added to the wells and the plates were incubated for 1 h. The plates were washed 4 times with 300 µL of PBST. After the last wash, wells were carefully tapped dry on a tissue. Because most immunogenicity problems of biopharmaceuticals are mediated by neutralizing the IgG, we chose to measure IgG titers against rhIFNα2b. Peroxidase-labeled antimouse IgG (Sigma) was added to the wells and the plates were incubated for 1 h. Plates were drained and washed 4 times with 300 µL of PBST and twice with 300 µL of PBS. After the last wash, wells were carefully tapped dry on a tissue. ABTS (2,2- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) substrate(Roche) was added and the absorbance was recorded after 30 min on a Novapath microplate reader (Bio-Rad) at 415 nm and a reference wavelength of 490 nm. All incubations were in covered plates at room temperature with constant orbital shaking. Sera were defined positive when the absorbance of the 1:100 dilution minus the background was 3 times higher than the average absorbance value of the pretreatment sera minus the background. Antibody titers of the positive sera were determined by adding the sera in 3-fold serial dilutions (starting from 1:10) to plates coated with native rhIFNα2b. The other steps of the ELISA procedure were as described above and the absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows, Graphpad software). The dilution needed to obtain 50% of the maximum absorbance was taken as the titer of the serum.

Ovalbumin. Sera were analyzed for antibodies against native ovalbumin coated in microtiter plates.

Microlon highbinding 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 50 µL of native ovalbumin (5 µg/mL in 100 mM $NaHCO_3$, pH 9.6, 0.05% NaN_3) per well for 1 h. Then the wells were drained and washed 2 times with 300 µL of PBST. After washing, the wells were tapped dry on a tissue. Wells were blocked by incubating with 200 µL of 1x Roche blocking reagent (Roche) in PBS for 1 h. The wells were drained and washed twice with 300l of PBST. After the last wash, wells were tapped dry on a tissue. Antibody titers were determined by adding pooled sera of each experimental group (n = 5) in 3-fold serial dilutions (starting from 1:10, 50 µL/well) to plates coated with native ovalbumin. The plates were washed 4 times with 300 µL of PBST. After the last wash, wells were tapped dry on a tissue. RAMPO, diluted 1:3000 in PBST, was added to the wells and incubated for 1 h. Plates were drained and washed 4 times with 300 µL of PBST and twice with 300 µL of PBS. After the last wash, wells were tapped dry on a tissue. The plates were stained for 5 min using 100 µL/well of tetramethylbenzidine substrate (Biosource Europe, Nivelles, Belgium), the reaction was stopped with 50 µL/well of 2 M H_2SO_4 and read at 450 nm on a Spectramax340 microplate reader. Analysis of obtained data were performed as described above.

Results

Various Biopharmaceuticals Display Amyloid-like Properties upon Exposure to Conditions of Stress, Indicating Protein Misfolding

During manufacturing and storage, biopharmaceuticals may also become exposed to various conditions of stress that can potentially underlie protein misfolding and the formation of amyloid-like properties. To mimic stability testing we examined whether exposure of biopharmaceuticals to conditions of stress, such as low pH or heat, induced amyloid-like properties.

Fig. 1 shows that amyloid-like properties are adopted by Etanercept, Glucagon, Abciximab, and Infliximab upon exposure to these harsh denaturing conditions. Thus, like any protein, biopharmaceuticals can adopt common amyloid-like properties during misfolding and this phenomenon can be enhanced upon storage or under conditions of stress.

The Presence of Misfolded Protein with Amyloid-like Properties in Various Biopharmaceuticals

Next, we examined whether proteins with amyloid-like properties are present in marketed biopharmaceuticals. As indicators for amyloid-like properties we measured the fluorescence of ThT, Congo Red, and binding and activation of tPA, all qualitative measures for the presence of amyloid cross-β structure conformation in proteins in solution[17;18].

Figure 1. Various biopharmaceuticals display amyloid-like properties upon exposure to conditions of stress, indicating protein misfolding. N, native; D, denatured. Etanercept, Glucagon, Abciximab, and Infliximab were exposed to denaturing conditions (see "Experimental Procedures") and subsequently analyzed for the presence of amyloid-like properties, using ThT fluorescence (A) and tPA activation assay (B); expressed as percentage of standardized positive control).

As shown in Table 1, several biopharmaceuticals showed significant potential to enhance fluorescence of thioflavin T and/or Congo Red, indicating the presence of amyloid-like structure. These biopharmaceuticals also bound tPA with high affinity and activated tPA-mediated plasminogen activation. These findings indicate that misfolded proteins with amyloid-like properties are present in various marketed therapeutic proteins.

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Therapeutic protein	Fluorescence (a.u. +/- SD)		tPA Binding		tPA activation
	ThT	CR	Bmax (OD450nm)	Kd (nM)	Max. Activation (%)
Albumin[a]	1970 +/- 5	978 +/- 2	1.228	11.22	47.67
Somatropin	1317 +/- 10	429 +/- 2	0.9369	9.048	113.95
Insulin Zinc Suspension	387 +/- 72	79 +/- 6	0.7558	105.4	17.44
Insulin Aspart	172 +/- 3	81 +/- 2	3.617	694.7	70.93
Factor VIII[a]	306 +/- 12	290 +/- 6	0.5398	229.8	4.22
Abciximab	8 +/- 8	25 +/- 1	0.5329	216.3	0
Epoietin Alfa	14 +/- 2	19 +/- 3	ND[b]	ND	0
Etanercept	23 +/- 3	ND	ND	ND	0
Infliximab	19 +/- 1	67 +/- 2	ND	ND	0
Gamma Globulin[a]	25 +/- 2	0 +/- 1	ND	ND	ND
Glucagon	48 +/- 1	ND	ND	ND	11.25

Table I. The presence of misfolded protein with amyloid-like properties in various biopharmaceuticals. Content of protein with amyloid-like properties in biopharmaceuticals was determined by enhancement of ThT and Congo Red fluorescence, binding of tPA and tPA-dependent plasminogen activation (% of standardized positive control). Biopharmaceuticals containing the highest levels of amyloid-like properties are listed at the top. [a] Plasma purified drug products. [b] ND, not determined.

Storage Increases the Level of Misfolded Protein in Biopharmaceuticals
Most protein pharmaceuticals can be stored for prolonged periods of time without losing their bioactivity. However, because proteins have the intrinsic propensity to lose their unique native structure, some fraction of proteins may gradually lose their structure and degrade. We examined the effect of storage on the level of protein with amyloid-like structure in a number of biopharmaceuticals, i.e. insulin, human albumin, and somatropin, proteins previously known to be able to form aggregates or amyloid under certain conditions. Fig. 2 shows that the level of protein with amyloid-like properties increases when these biopharmaceuticals were examined closer to their expiration date. These findings show that protein misfolding is time-dependent in biopharmaceuticals, when measured by elevated markers for amyloid structure.
Immunogenicity of Misfolded IFNα2b
We then examined whether a biopharmaceutical solution, containing misfolded protein, could trigger antibody formation and break tolerance. We chose to use metal-catalyzed oxidized rhIFNα2b as a model protein because it is known that this modification induces unfolding and aggregation of rhIFNα2b, which correlates with its immunogenicity[32;33]. As shown in Fig.
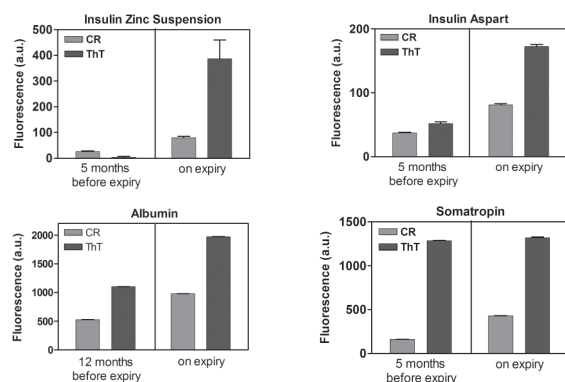
Figure 2. Levels of misfolded protein in biopharmaceuticals increase during storage within expiry limits, under conditions as defined by the manufacturers. Biopharmaceutical preparations were tested (at 25 μg/mL protein) twice over several months for their capacity to enhance ThT and Congo Red (CR) fluorescence. Samples were measured in triplicate at each time point; values are expressed as averages ± S.D.

3, oxidation of rhIFNα2b induces amyloid-like properties as exemplified by the finding that oxidized rhIFNα2b greatly enhances ThT fluorescence, and facilitates the binding and activation of tPA.

Native rhIFNα2b does not enhance ThT fluorescence and hardly binds or activates tPA (Fig. 3, A-C, respectively). These findings indicate that oxidation of rhIFNα2b induces amyloid-like properties, which is indicative for protein misfolding. The generation of IgG antibodies in wild-type mice against a foreign antigen (rhIFNα2b) was found to be significantly accelerated when oxidized rhIFNα2b with amyloid-like

properties was injected, compared with the native protein (Fig. 3D). In contrast, mice transgenic for rhIFNα2b remained tolerant to native protein (Fig. 3E). In hIFNα2b transgenic mice injected with rhIFNα2b with amyloid-like properties, tolerance was broken and a potent IgG response was induced (Fig. 3E).

These findings strongly indicate that protein misfolding, detectable by markers for amyloid, induces an immune response.

Next, we examined whether the immunogenic potential of rhIFNα2b was dependent on the level of misfolded rhIFNα2b with amyloid-like properties. We injected mice transgenic for hIFNα2b with preparations in which native and oxidized rhIFNα2b were mixed to obtain varying doses of protein with amyloid-like properties. ThT and tPA-activating potential of the mixtures was determined and found to be

Figure 3. Immunogenicity of misfolded IFNα2b. Amyloid-like structure, indicative for misfolding, in rhIFNα2b was induced by oxidation and was verified using thioflavin-T fluorescence enhancement assay (A, 5 μg/mL of positive control A reached 2344 units, not shown), tPA binding ELISA (B), and tPA-dependent plasmin generation assay (C). The total binding of anti-interferon antibodies in an ELISA with 1:100 diluted sera was used as a measure for antibody generation after injections of rhIFNα2b with native- or amyloid-like properties in wild-type mice (D) and rhIFNα2b transgenic mice (E).

gradually increased (Fig. 4, A and B). Again, in mice injected with solely unmodified rhIFNα2b no breaking of tolerance was observed (Fig. 4C). In mice injected with increasing doses of rhIFNα2b with amyloid-like properties, both the number of mice in which tolerance was broken rose from 2 to all 5 and their respective antibody titers were increased from a mean IgG titer of 22 to a titer of more than 230 (Fig. 4C).

These observations demonstrate that immune responses against misfolded proteins, with amyloid-like properties, are dependent on the extent of protein misfolding that has occurred in a sample. This concentration effect was found to influence both the magnitude of raised antibody titers and incidence of tolerance breaking. Besides formation of misfolded protein, oxidation of proteins also leads to modification of amino acids. To investigate whether immunogenicity

Figure 4. Breaking of tolerance by misfolded IFNα2b is dose-dependent. Native and oxidized rhIFNα2b were mixed in a ratio of 1:0, 3:1, 1:1, 1:3, and 0:1. This resulted in increasing levels of protein with amyloid-like properties between samples, as confirmed by the enhanced fluorescence of ThT (A) and determination of tPA activating capacity (B; expressed as percentage of standardized positive control). Antibody generation in transgenic mice increased as the level of injected with amyloid-like properties increased (C). The values represent antibody titers of individual mice (5 mice per group) 14 days after the start of the experiment.

Figure 5. Oxidation-independent misfolding of ovalbumin leads to an enhanced immune response. Amyloid-like properties were determined by the thioflavin-T fluorescence enhancement assay (25 µg/mL protein) (A) and tPA-dependent plasmin generation assay (100 µg/mL protein). B, antibody titers in BALB/c mice after 14 days (C), preimmune sera contained no α-OVA antibodies (not shown). nOVA, native ovalbumin; dOVA, heat denatured ovalbumin.

could be induced by a misfolded protein with amyloid-like properties in the absence of amino acid modifications, we used ovalbumin, an immunological model protein, that has been shown to form amyloid-type cross-β structure after gradual heating[35]. As expected, the gradual heating of ovalbumin in solution led to both large increases in the enhancement of ThT fluorescence (Fig. 5) and increased in the capacity to activate tPA (Fig. 5B). Mice immunized with this heat-denatured ovalbumin developed 2 weeks after immunization with a 5-fold higher general antibody titer, compared with mice injected with freshly dissolved ovalbumin (Fig. 5C). This further indicates that immunogenicity is caused by common structural characteristics, i.e. amyloid-like properties, without the need of chemical protein modification.

Discussion

The advent of recombinant technology has enabled the large scale production of biopharmaceuticals, such as FVIII, EPO, IFN, and various monoclonal antibodies. The use of these biopharmaceuticals is very promising and the number of biopharmaceuticals is expected to rise rapidly[1]. Unfortunately, the generation of antibodies against therapeutic proteins has posed a mystifying problem for biopharmaceutical manufacturers, medical practitioners, and scientists. We now show that adoption of generic amyloid-like proper ties, a hallmark for misfolded proteins, is a potential cause of drug-induced immunogenicity. So far, several seemingly unrelated factors have been described to influence biopharmaceutical immunogenicity. For instance, post-translational modifications such as oxidation, deamidation, and aggregation influence protein immunogenicity[14;15]. Strikingly, specific modifications in formulations and packaging have also been correlated to biopharmaceutical immunogenicity[36;37]. Most of these factors may influence the structural properties of a therapeutic protein molecule[23;26]. Metal-catalyzed oxidation of rhIFNα2b was described to lead to increased immunogenicity[32]. We found that metalcatalyzed oxidation of rhIFNα2b resulted in a conformational change accompanied by adoption of amyloid-like properties, indicative for protein misfolding, that was far more immunogenic *in vivo* than its native counterpart and broke immune tolerance. We also found that, in general, biopharmaceuticals can have amyloid-like properties and that these properties can be induced upon storage or conditions of stress. These data indicate that misfolded proteins with amyloid-like properties can be responsible for enhanced immunogenicity of biopharmaceuticals and breaking of tolerance. Based on our results, we propose a unifying mechanism by which individual immunogenic factors, such as oxidation or formulation changes, via the formation of misfolded protein with common amyloid-like properties, ultimately lead to an (enhanced) immune response. Protein misfolding in biopharmaceutical solutions is expected to lead to a heterogeneous distribution of aggregates[39]. Similarly, our preparations of misfolded rhIFNα2b and ovalbumin may very well contain multiple species of aggregates and protein in both native and misfolded conformations. However, once misfolding is induced, markers for amyloid increase and this dose-dependently correlates to the immunogenic potential of the preparation. Additional investigations are required to isolate the exact immunogenic misfolded protein species, but at present time it is difficult to predict and control the stability of fibrils and oligomeric species, especially *in vivo*. Hopefully, assays using antigen-presenting cells will help to answer this question and further elucidate the underlying mechanism. Our results point to a common mechanism by which the immune system perceives misfolded proteins. We hypothesize that this lies in the changed conformation of the protein backbone itself. This implies that the innate immune system may be activated by recognition of the amyloid-like properties of misfolded protein. Indeed, several cellular receptors for the amyloid- like protein fold have been identified: scavenger receptor

A, CD36, receptor for advanced glycation end products, low density lipoprotein receptor-like protein, and scavenger receptor B type I[17]. Moreover, these receptors are expressed on dendritic cells and could initiate an immune response against these proteins[34;38].

Drug formulation is an important factor in the development of biopharmaceuticals. This also affects immunogenicity, which, for example, has been observed with a specific formulation of EPO, that was far more immunogenic than its previous formulation[36;37]. One of the main problems in formulation development is maintaining protein stability, because chemical modification, adsorption, and most importantly, aggregation phenomena are generally difficult to prevent[14;29]. Our findings indicate that various biopharmaceuticals have a tendency to misfold, which may result in the generation of immunogenic proteins with amyloid-like properties in various drug products that are on the market. At this moment, it is difficult to predict those conditions that will preserve the native non-immunogenic fold of a protein pharmaceutical. Identification of conditions, compounds, excipients, and materials that induce amyloid-like properties in therapeutic proteins during production and/or storage may be valuable for improving the quality of biopharmaceuticals by reducing immunogenicity related adverse effects. Monitoring the formation of amyloid-like properties in a biopharmaceutical may even have predictive value toward pre-clinical determination of expiry dates for a drug, because it seems to be a marker for an immunogenic protein entity. Hereto, it is required to pinpoint the exact markers for amyloid-like properties that correlate best with immunogenicity and further develop sensitive and robust tools to detect these misfolded protein species. This may ultimately lead to development of better and safer drugs.

Acknowledgments

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Chapter 7

Immune Responses Against β2-Glycoprotein I are Dependent on Protein Conformation
Preliminary Report

Coen Maas,[1] Miranda E. van Berkel,[1] Bas de Laat,[2] Rolf T. Urbanus,[1] Philip G. de Groot,[1] and Martijn F.B.G. Gebbink[1]

[1]Department of Clinical Chemistry and Haematology, University Medical Center Utrecht, Utrecht, The Netherlands. [2]Department of Plasma Proteins/Blood coagulation at Sanquin Research, Amsterdam, The Netherlands.

Abstract

When immune tolerance breaks against endogenous proteins, the resulting autoantibodies can have pathological consequences. However, the mechanisms that cause autoimmune responses remain poorly understood. The antiphospholipid syndrome (APS) is an autoimmune disease that is characterized by thrombotic events and/ or recurrent pregnancy loss. In the antiphospholipid syndrome, antibodies are generated against a multitude of endogenous proteins, such as prothrombin, tPA and Factor XII, but the clinically most relevant autoantigen of this disease is β2-glycoprotein I (β2-GPI). Although this protein is present in plasma at a concentration of 200 μg/mL, its function is unknown. Like prothrombin, β2-GPI can bind to negatively charged phospholipids. Especially the presence of antibodies that are directed against a cryptic epitope within β2-GPI correlates well to the risk on thrombosis. This epitope is exposed upon phospholipid binding, suggesting that conformational changes take place in this process. This has led us to hypothesize that the conformational change is also required to obtain immune responses against this protein. We reported earlier that misfolded proteins can adopt amyloid-like properties, which correlate to their immunogenicity. In this report, we describe that such structural changes of β2-GPI occur upon incubation with phospholipids and correlate with its immunogenicity in immunization experiments. β2-GPI that was misfolded in vitro also displayed an enhanced immunogenicity. These data indicate that the immunogenicity of this protein is dependent on a structural change. Although some of the mice that had developed antibodies against β2-GPI also displayed lupus anticoagulans in in vitro clotting assays, further studies will have to prove whether the conformation of this protein is of importance for the development of the pro-thrombotic antibodies that mediate APS.

Introduction

The antiphospholipid syndrome (APS) is a non-inflammatory autoimmune disease that is characterized by thrombotic episodes in both the venous- and arterial system. The disease is also associated with recurrent pregnancy loss. APS mainly occurs in women, with only about 20% male cases. A confirmed diagnosis of APS is not only dependent on clinical features, but also on laboratory parameters. The patient has APS when she/he has persistent antibodies against the negatively charged phospholipid cardiolipin (CL) or the plasma protein β2-glycoprotein I (β2-GPI). Additionally, a persistent lupus anticoagulant activity (a phospholipid-dependent prolongation of the clotting time) present in patient plasma is a clinical marker for APS. Currently, the treatment for APS is based on oral anticoagulation, especially since there is no proper way to neutralize or eliminate the pathological antibodies that mediate the disease.

Although the name of APS suggests that the disease is caused by immune reactivity against phospholipids, it has become clear that this is not the case. In fact, immune responses against phospholipid-binding proteins are key to the thrombotic pathology[1]. Many autoantigens have been described in APS, but antibodies against β2-GPI correlate best with the incidence of thrombosis[2].

β2-GPI is a 44 kDa plasma protein with a plasma concentration of approximately 200 μg/mL. It consists of five sushi-domains, each consisting of approximately 60 amino acids. It also has 4 N-linked carbohydrate chains, 11 disulfide bridges and a hydrophobic loop extending from the fifth domain, which is held responsible for phospholipid binding[3]. While it is known that β2-GPI has affinity for negatively charged phospholipids, and contributes to pathological thrombosis when antibodies are present against it, not much is known on the physiological role of β2-GPI.

When β2-GPI binds to negatively charged phospholipids, a number of aspects of the protein change. For instance, it obtains an increased affinity for megalin, a member of the LDL-receptor family[4]. Apoptotic cells and activated platelets expose negatively charged phosphatidylserine (PS) on their surface, as a marker for their clearance. Several receptors are known that are involved

in the recognition of PS, thereby aiding in the clearance of apoptotic cells. β2-GPI binding to PS exposing apoptotic cells is considered an important mediator for their clearance by macrophages[5-7].

Thus, according to these studies, a conformational change alters the functionality of this protein, resulting in its clearance, as well as its ligands. Alternatively, another role for β2-GPI has been hypothesized in host defense during infection. A recent paper describes that the protein is a source of antimicrobial peptides that can be release by neutrophil-associated proteases[8], setting the stage for such a function.

The paradoxal observation that APS patients suffer from thrombosis, while having plasma lupus anticoagulant activity is a puzzling one. The lupus anticoagulant activity (LAC) of pathological anti-β2-GPI antibodies can be explained by their capacity to enhance the affinity of β2-GPI for negatively charged phospholipids. During clotting assays, phospholipid vesicles are added to plasma to provide a surface for phospholipid-dependent clotting factors. When this surface is (partially) covered by β2-GPI-antibody complexes, coagulation will be inhibited.

The easiest and most probable explanation for the paradox between LAC *in vitro* and thrombosis *in vivo* is that a number of haemostatic pathways inhibited by LAC are not involved in *in vitro* clotting assays, but are involved in thrombosis *in vivo*. For instance, the β2-GPI-antibody complexes inhibit the anticoagulant role of activated protein C, but since thrombomodulin is absent in a clotting assay, no APC is formed[9]. Alternatively, there is evidence to suggest that β2-GPI-antibody complexes induce a pro-inflammatory response in EC, resulting in upregulated expression of cell adhesion molecules and tissue-factor[10;11]. Lastly, interaction of these complexes is thought to affect the functioning of platelets, priming them for activation and adhesion[12;13]. Taken together, the anticoagulant nature of antibodies against β2-GPI is overruled by their prothrombotic effects.

APS patients develop antibodies against a number of epitopes on β2-GPI, but only antibodies directed against one epitope have a strong risk for developing thrombosis. This epitope is situated in a region around a glycine residue on position 40 (G40) and an arginine residue on position 43 (R43) within the first sushi domain[14]. Antibodies directed at this epitope are thought to recognize it if a blocking carbohydrate chain is displaced[15]. This occurs when the protein is immobilized on a hydrophilic (negatively charged) surface, such as a microtiter plate or negatively charged phospholipid vesicles. Additionally, enzymatic removal of the carbohydrate chains or crosslinking by glutaraldehyde leads to exposure of the R43 epitope[15]. Apparently, a conformational change, displacing one or more carbohydrate chains, leads to exposure of a cryptic epitope in domain I. Binding of antibodies to this epitope enhances the affinity of β2-GPI for negatively charged phospholipids, which results in LAC *in vitro* and thrombosis *in vivo*.

At this moment, not much is known about the mechanisms that cause the generation of pathological antibodies against β2-GPI. Immunization studies in mice have been helpful by showing that human β2-GPI, incubated with phospholipids becomes immunogenic[16]. Similarly, β2-GPI becomes immunogenic in combination with apoptotic cells[17;18]. Thus, while binding to these negatively charged phospholipids, it is likely that β2-GPI undergoes a conformational change. Earlier work from our group identified protein misfolding as a risk factor for immune responses against biopharmaceuticals. In these studies, altering the conformation of a therapeutic protein led to increased immunogenicity and tolerance breaking in a transgenic mouse model. We hypothesized that the immune responses against β2-GPI are the consequence of a conformational change. Therefore, we investigated the changes in conformation that occurred in β2-GPI when it was incubated with the negatively charged phospholipid cardiolipin. Additionally, we

performed immunization studies to investigate whether misfolded β2-GPI displayed increased immunogenicity in absence of negatively charged phospholipids.

Results

The effects of cardiolipin on the structure of β2-GPI

A protein's conformational change can be monitored in a number of ways. First, biophysical examination of the properties can be informative. Circular Dichroism (CD) studies can provide insight into protein structure by making use of the net optical behavior of a protein with respect to polarized light. Similar to published CD studies on the structure of β2-GPI[16], we found that conformational changes take place in the molecule when it is incubated with cardiolipin vesicles (data not shown).

The misfolding of proteins is often accompanied by the adoption of amyloid-like properties, meaning an increased affinity for small-molecular dye compound that are commonly used to stain pathological amyloid deposits. The dye Thioflavin T (ThT) binds to amyloid fibrils but also misfolded protein aggregates. When bound, it obtains a specific fluorescent characteristic that can be used to track conformational changes. We investigated whether ThT fluorescence was altered when β2-GPI was incubated with cardiolipin vesicles. Figure 1A shows that ThT fluorescence increased as a consequence of cardiolipin binding by β2-GPI. This indicates that a conformational change took place in the protein that resembles either protein misfolding or amyloid formation. However, the nature of these changes cannot be deduced from dye binding since the binding site(s) of ThT are not known.

Like ThT fluorescence, the binding and activation of tissue-type plasminogen activator can be used as a marker for misfolded, potentially immunogenic, protein species[19]. We first investigated whether tPA-dependent plasmin formation was stimulated by β2-GPI and β2-GPI that had been incubated with cardiolipin. However, no stimulation of tPA-dependent plasmin formation was

Figure 1. Cardiolipin induces the refolding and aggregation of β2-GPI. The fluorescence of the dye Thioflavin T increases when β2-GPI is incubated with cardiolipin vesicles (A). Additionally, this treatment led to the exposure of a binding site for tPA (B). Both these experiments are indicative for a conformational change in β2-GPI. Electron microscopic analysis revealed that β2-GPI assembles into polymeric meshworks upon incubation with cardiolipin vesicles (C, right panel; native control is shown in the left panel). The scale bars represent 1μm.

observed by either of these preparations (not shown). This is surprising since most proteins that undergo a conformational change which increases their affinity for ThT also stimulate tPA-dependent plasmin formation[19;20]. One potential reason is that the cardiolipin vesicles influence the enzymatic conversion of the substrate in our assay. Binding experiments showed that tPA was able to bind to β2-GPI that has been incubated with cardiolipin, but not the individual components (Figure 1B). The binding of tPA to this phospholipid-bound β2-GPI was mediated by one of three N-terminal domains of tPA, that are not present in the deletion mutant K2P. This interaction was lysine-independent since these binding experiments were performed in the presence of excess amounts of a soluble lysine-analogue. Thus, tPA binds to cardiolipin-bound β2-glycoprotein I but it is not activated by this binding.

Protein aggregation often takes place when proteins misfold, which often corresponds to the affinity of the protein for ThT. We next investigated whether the structure of β2-GPI changed by electron microscopy. Surprisingly, the interaction of β2-GPI with cardiolipin vesicles lead to its assembly into a meshwork of branching fibrils (Figure 1C, right panel). β2-GPI by itself did not appear as an aggregated protein (Figure 1C, left panel). The fibrils appear flexible and can seemingly self-associate to form thicker fibrils. The fibrils were associated with vesicles that presumably consist of cardiolipin. When β2-GPI was cleaved by exposure to plasmin which leads to

Figure 2. Conformational change in β2-GPI cause immune responses against it. tPA has an increased affinity for misfolded β2-GPI (A). Mice were repeatedly injected with native β2-GPI, β2-GPI in combination with cardiolipin or misfolded β2-GPI. After 36 days, sera were pooled (n=5 per group) and analyzed for antibodies against native and misfolded β2-GPI. In mice injected with β2-GPI in combination with cardiolipin (CL) or misfolded β2-GPI, antibodies were generated that recognized the immobilized native protein. No such antibodies arose when mice were injected with native β2-GPI (B, C). Antibodies against the misfolded β2-GPI were mainly present in the pooled sera of mice that were injected with the same preparation (D, E). This indicates that misfolded β2-GPI has exposed a number of immunogenic epitopes, not present in cardiolipin-bound β2-GPI.

impaired phospholipid binding[21], the polymerization was abrogated (not shown). From these experiments, we conclude that β2-GPI aggregates as a consequence of binding to phospholipid vesicles, which helps to explain how phospholipid vesicles are precipitated by β2-GPI[22].

The influence of β2-GPI structure on its immunogenicity

To investigate the influence of conformational changes in β2-GPI on its immunogenicity, we prepared misfolded β2-GPI. Hereto, the protein was unfolded in urea, the disulfide bridges were reduced by incubation with diothiotreitol and the exposed cysteine residues were alkylated with iodoacetamide to prevent restoration of the disfulide bridges. This misfolded form of β2-GPI had an increased affinity for ThT (Figure 1A) and bound tPA (Figure 2A). In contrast to cardiolipin-bound β2-GPI, tPA-dependent plasmin formation was stimulated by misfolded β2-GPI (not shown). This indicates that misfolded- and cardiolipin-bound β2-GPI, albeit both conformationally different from native β2-GPI are not identical. Whereas misfolded β2-GPI resembles any denatured protein (since it can stimulate tPA activation), cardiolipin-bound β2-GPI does not and can therefore be termed "refolded". Next, mice were exposed to a series of intravenous injections with 15 µg of β2-GPI with or without cardiolipin, or alternatively 15 µg of misfolded β2-GPI.

After four injections over a period of 36 days, mice that had been injected with a solution of β2-GPI together with cardiolipin had developed antibodies against the native protein. Mice injected with native β2-GPI had not developed such antibodies. Similarly, injection of misfolded β2-GPI induced the generation of antibodies against native β2-GPI (Figure 2B, C). However, when the different sera of these mice were tested for antibodies against misfolded β2-GPI, only mice that had been injected with misfolded β2-GPI had developed significant antibody titers, indicating that misfolded β2-GPI exposes a number of immunogenic epitopes other than those present in native β2-GPI. These experiments suggest that the structure of β2-GPI is of importance for its immunogenicity.

The risk on thrombosis in APS patients is related with high-affinity antibodies against a cryptic epitope domain I of β2-GPI[14;23]. We next tested whether the sera contained antibodies against this epitope.

As shown in figure 3, little to no antibodies against the cryptic epitope on domain I had formed in the mice injected with native β2-GPI. The sera of the two other groups however, displayed antibody binding to domain I of β2-GPI that was coated on a hydrophobic plate, exposing the epitope. This suggests that, during immunization of mice with either β2-GPI and cardiolipin or misfolded β2-GPI alone, antibodies are generated that recognize a specific pathology-associated epitope on domain I of β2-GPI.

Figure 3. Sera of mice immunized with β2-GPI and cardiolipin, or misfolded β2-GPI, contain antibodies directed against a cryptic epitope domain I. Pooled sera of the mice in each group were diluted 100x and tested for the binding of domain I of β2-GPI, coated either on a hydrophobic or a hydrophilic microtiter plate. The pathology-associated cryptic epitope is exposed when domain I is coated on a hydrophobic plate, but not when coated on a hydrophilic plate.

Discussion

The antiphospholipid syndrome raises a number of interesting questions. Although there is increasing insight into the mechanisms that cause thrombosis in APS, the etiology of autoimmune responses against β2-GPI remains poorly delineated.

We here investigated whether the immunogenicity of β2-GPI correlated with its structure and acquired some evidence to support such relationship. We earlier showed that immune responses against a therapeutical protein can be a consequence of protein misfolding[19] and we show here that a self protein, such as β2-GPI, becomes immunogenic when misfolded. Antibodies that were generated in mice, injected with misfolded β2-GPI, were directed against both the native protein and the misfolded protein and seemed to recognize a specific pathology-related epitope on domain I. The antibodies that were generated in mice injected with a mixture of β2-GPI and cardiolipin vesicles only recognized the native protein and the epitope on domain I, but hardly recognized misfolded β2-GPI. This indicates that, even though conformational change of this protein is an immunogenic trigger in both cases, misfolded β2-GPI evokes antibody generation against a number of epitopes that are not immunogenic in phospholipid-bound β2-GPI.

Thus, it seems that the artificially misfolded β2-GPI has different immunogenic properties from cardiolipin-bound β2-GPI, leading to the question: What happens to the conformation of β2-GPI when it binds to phospholipids and what are the consequences of these changes? At present, two studies have reported a conformational change in β2-GPI upon binding phospholipid vesicles by examination of its biophysical properties[16;22]. Our experiments confirm that cardiolipin-bound β2-GPI undergoes a conformational change, by adopting an increased affinity for the amyloidophilic dye ThT. Additionally, the protein can assemble into large polymers when incubated with cardiolipin vesicles, forming a continuous meshwork. Our experiments indicate that (under our conditions) β2-GPI has the capacity to aggregate in absence of pathological antibodies, forming multimers larger than dimers. It was recently reported that phospholipid vesicles precipitate *in vitro* when full-length human β2-GPI is present[22], which may be explained by phospholipid-induced polymerization of β2-GPI, as seen in our experiments, Further experiments must show whether β2-GPI behaves similarly in a more physiological environment and on other sources of negatively charged phospholipids, such as apoptotic cells. Additionally, it is interesting to see whether anti-β2-GPI antibodies stabilize these polymeric structures of β2-GPI or prevent them from assembling, for instance by inducing dimer formation. Perhaps, the observed conformational changes and/or polymerizing behavior is related to the elusive physiological role of β2-GPI and alterations in this process may have pathological consequences. It is tempting to speculate that β2-GPI refolds upon contact with negatively charged (phosphor)lipids which are exposed by apoptotic cells or derived from pathogens. As a consequence, refolded β2-GPI is recognized by scavenger receptors on phagocytes that are sensitive to these types of conformational changes.

At present we hypothesize that β2-GPI while binding phospholipids changes its conformation in a specific organized fashion, which (as a gain of function) results in a clearance signal for cellular uptake. If true, excessive exposure of this clearance signal may result in breaking of tolerance and autoimmune disease. Such may happen during infection and sepsis, which are a suggested risk factor for APS[24].

Immune responses against misfolded β2-GPI can be explained by resemblance to a denatured protein, thereby becoming a target for protein clearance and a more general immune response. From this perspective, it is a challenge to stabilize β2-GPI in its phospholipid-bound conformation and identify whether this conformational change is sufficient to induce pathological im-

mune responses against the protein. Additionally, it would be interesting to compare the relative immunogenicity of individual subunits of β2-GPI and try to relate this information to their biophysical properties. Such studies may help to support new insights into the mechanisms that underlie the often observed autoimmune responses against this "self" protein.

There a two more considerations concerning the experiments presented here: our studies were performed with β2-GPI, purified from human plasma, which is about 78% homologous to murine β2-GPI and thus not completely "self" to mice. In order to speak of breaking of tolerance against β2-GPI, future experiments will have to be performed using murine β2-GPI. Finally, although the anti-domain I antibodies suggest the generation of prothrombotic antibodies in mice injected with conformationally changed β2-GPI, we did not establish that these mice had developed the thrombotic phenotype that is characteristic for APS. Although preliminary data suggests that some mice had developed LAC after immunization, additional experiments should confirm this. Furthermore, investigation of the thrombotic character of mice with anti-β2-GPI (or domain I) antibodies in *in vivo* thrombosis models is necessary to confirm that the etiology of APS is dependent on the conformation of β2-GPI.

In conclusion, not only pathological interaction of certain antibodies against β2-GPI is related to its conformation. Also the immunogenicity of this protein seems to relate to its structure. Although more work is required to obtain a proper understanding on the mechanisms of antibody generation in APS, such studies may provide information on the role of β2-GPI *in vivo* and provide insights into autoimmune responses in general.

Materials and methods

Purification of β2-glycoprotein I. β2GPI was purified form fresh plasma as described before, with minor modifications[25]. In brief, protease inhibitors were added to the plasma, which was then dialyzed and successively applied to a DEAE sephadex column, SP-sepharose column, protein G-sepharose column and heparin-sepharose column. The eluate was analysed by SDS-PAGE, followed by silver-staining, and contained a single 42 kDA protein band. The identity of the band was confirmed as β2GPI by ELISA, using an in-house produced monoclonal antibody against β2-GPI (8A8) as well as by western blot analysis. The protein preparation was 98% pure; additionally the amount of proteolytically cleaved β2-GPI was 1-2% of the total protein, as detected by specific ELISA using another in-house produced antibody (13A10[26]).

Misfolding of β2-glycoprotein I. β2-GPI was unfolded by reduction in 4M urea and 10 mM dithiothreitol (DTT) in the presence of N2 gas for 3 hours. Subsequently, the cysteine residues were alkylated by incubation with 0,5 mM iodoacetamide. The mixture was dialyzed for 2 hours in H_2O and overnight in phosphate-buffered saline (PBS), pH 7.2 at 4°C. There was no visible protein precipitation as a consequence of this treatment.

Preparation of cardiolipin vesicles. Cardiolipin vesicles were prepared according to the method described by Pengo et al.[27]. In brief, multilamellar cardiolipin in ethanol solution was transferred to a glass tube, the ethanol was evaporated with N2 gas, resuspended in Tris-buffered saline (50 mM Tris, 100 mM NaCl, pH 7.4: TBS) and vortexed thoroughly to obtain vesicles.

Incubation of β2-GPI with cardiolipin vesicles. β2-GPI and cardiolipin were diluted to 300 μg/mL and 50 μM in TBS, respectively. They were mixed by pipetting in a 1:1 ratio and allowed to incubate for 10 minutes prior to analysis.

ThT Fluorescence experiments. Fluorescence of of the amyloidophylic dye Thioflavin T (ThT; Sigma) was measured on a Hitachi F-4500 spectrophotometer at an excitation wavelength of 435 nm and emission wavelength of 485 nm. All samples were prepared in TBS. Fluorescence was

measured in triplicate with an integration time of 5 s per reading. Background fluorescence of both protein in buffer and fluorescence of the dye alone were subtracted from the total fluorescence signal. 100 μg/mL of heat-denatured ovalbumin was used as a positive control.

Tissue-type plasminogen activator enzyme-linked immunosorbent assay (ELISA). Hydrophobic plates (catalog no. 2595; Costar) were coated with 50 μL containing 10 μg/mL of sample protein in 100 mM NaHCO$_3$, pH 9.6, 0.05% (m/v) NaN$_3$ for 1 h at room temperature. Plates were washed twice with Tris-buffered saline, pH 7.2, containing 0.1% Tween 20 (TBST) and blocked with PBS containing 1% Tween20 for 1 h at room temperature. Plates were washed twice with TBST and incubated, in duplicate, with a concentration series of either tPA (Actilyse, Alteplase; Boehringer-Ingelheim, Alkmaar, The Netherlands) or a truncated form of tPA (Reteplase; Rapilysin, Roche Diagnostics GmbH, Mannheim Germany), lacking the amyloid binding domain, diluted in PBS containing 0.1% Tween20 (PBST). Incubations were performed for 1 h at room temperature in the presence of 10 mM ε-amino caproic acid. ε-Amino caproic acid is a lysine analogue and is used to avoid potential binding of tPA to lysine-containing ligands via its kringle2 domain. Plates were washed five times with TBST and incubated with antibody 374b α-tPA (American Diagnostica, Instrumentation Laboratory, Breda, The Netherlands) diluted 1:1000 in PBST for 1 h at room temperature. Plates were washed five times with TBST and incubated with peroxidase-labeled anti-mouse immunoglobulins (RAMPO; DAKOCytomation, Glostrup, Denmark) diluted 1:3000 in PBST for 30 min at room temperature. Plates were washed five times with PBS, 0.1% Tween20, and stained with 100 μL/well of tetramethylbenzidine substrate (Biosource Europe, Nivelles, Belgium). The reaction was terminated with 50 μL/well of 2 M H$_2$SO$_4$ and substrate conversion was read at 450 nm on a Spectramax340 microplate reader. Curves were fitted with a one-site binding model (GraphPad Prism version 4.02 for Windows, Graphpad Software) from which Kd and Bmax were determined.

Transmission Electron Microscopy (TEM). Formvar/carbon-coated 100-mesh copper grids were placed on top of 5 μL drops of protein solution for 5 minutes. The grids were washed by placing them on a 100-μL drop of PBS and 3 drops of H$_2$O, at each step incubating for 2 minutes. The grids were stained with 2% (mass/vol) methylcellulose with 0.4% uranyl acetate pH 4.0 for 2 minutes. Afterward, analyses were performed on a JEOL 1200 EX Transmission Electron Microscope, and electron micrographs were made at a ×10,000 enlargement. Control grids were made that had been incubated with buffer alone. The samples were applied to 100-mesh copper grids with carbon-coated Formvar (Merck) and subsequently washed with PBS and H$_2$O. The grids were applied to droplets of 2% (m/v) methylcellulose with 0.4% (m/v) uranylacetate pH 4. After a 2-min incubation, the grids were dried on a filter. Transmission electron micrographs were recorded at 60,000x magnification at 80 kV on a JEM-1200EX electron microscope (JEOL, Tokyo, Japan).

Immunizations. All animal experiments were performed in compliance with institutional guidelines and were approved by the institutional animal care and ethics committee. Seven- to nine-week-old Balb/C mice (Jackson laboratories) were immunized intravenously in the tail vein on day 1, 5, 15 and 19 with 15 μg of human β2GPI, misfolded β2GPI or β2GPI pre-incubated with cardiolipin. Whole blood was drawn from the vena saphena and collected in Easy Collect tubes with Z serum clot activator (BD Bioscience), serum was collected after centrifugation at 15,000 rpm for 15 minutes. On day 26, either serum or plasma was collected. If plasma was collected, whole blood was extracted and anticoagulated by 3.8% Sodium Citrate. The blood was centrifuged at 15,000 rpm in a tabletop centrifuge for 15 minutes twice, to remove cells and platelets.

Detection of anti-β2GPI antibodies. Sera were analyzed for antibodies against unmodified (immobi-

lized) β2-GPI. Microlon high-binding 96-well plates (Greiner, Alphen aan den Rijn, The Netherlands) were coated with 50 μL native β2-GPI (5 μg/mL in 100 mM NaHCO$_3$, pH 9.6, 0.05% NaN$_3$) per well for 1 hour. Then the wells were drained and washed 2 times with 300 μL PBS, 0,1% Tween20 (PBST). After washing, the wells were tapped dry on a tissue. Wells were blocked by incubating with 200 μL 1x Roche Blocking Reagent (Roche, Almere, The Netherlands) in PBS for 1 hour. The wells were drained and washed twice with 300 μL PBST. After the last washing step, wells were tapped dry on a tissue. Antibody titers were determined by adding pooled sera of each experimental group (n=5) in three-fold serial dilutions (starting from 1:30, 50 μL/well) to plates coated with native β2-GPI. The plates were washed 4 times with 300 μL PBST. After the last wash, the plates were tapped dry on a tissue. RAMPO, diluted 1:3000 in PBST, was added to the wells and incubated for 1 hour. Plates were drained and washed 4 times with 300 μL/well PBST and twice with 300 μL/well PBS. After the last wash, wells were tapped dry on a tissue. The plates were stained for approximately 5 minutes using 100 μL/well of TMB substrate (Biosource Europe, Nivelles, Belgium), the reaction was stopped with 50 μL/well of 2M H$_2$SO$_4$ and read at 450 nm on a Spectramax340 microplate reader. Alternatively, staining was performed with ortho-phenylene diamine (OPD solution; 4 mg/mL OPD diluted in 0.1 M NaH$_2$PO$_4$/0.1 M Na$_2$HPO$_4$). The coloring reaction was stopped with the addition of 1 M H$_2$SO$_4$, and absorbance was measured at 490 nm. The absorbance values were plotted against log dilution. Curves were fitted with a sigmoidal curve (GraphPad Prism version 4.02 for Windows, Graphpad Software, CA, USA). Antibodies against domain I of β2-GPI were determined as published[14].

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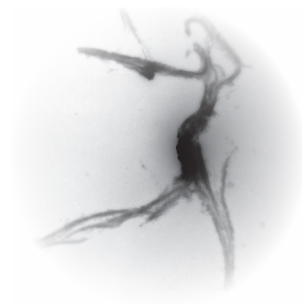1. Galli M, Comfurius P, Maassen C et al. Anticardiolipin antibodies (ACA) directed not to cardiolipin but to a plasma protein cofactor. Lancet 1990;335:1544-1547.

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Chapter 8

General Discussion
How misfolded proteins affect biological systems; new insights and questions

Prior to this thesis, we hypothesized that the enzymes factor XII (FXII) and tissue-type plasminogen activator (tPA) have functional roles in the recognition of misfolded proteins, leading to activation of the contact system and fibrinolytic system. We expected that this could provide insight into the enigmatic role of the contact system *in vivo*, as well as a better understanding about the activities of the fibrinolytic system in diverse haemostasis-unrelated situations. We will here discuss our findings concerning these matters, as well as our findings on the role of misfolded proteins in immune responses.

8.1 The known roles of FXII

The intrinsic pathway of coagulation was first reported in 1955. In those years, the clotting time of whole blood in glass tubes was a routine preoperative determination, taking about 30 minutes. Prolongations of this clotting time normally indicated a clotting defect and consequently a bleeding risk. Enigmatically, three persons were identified that had a tripled whole-blood clotting time, but no apparent bleeding tendency[1]. Moreover, these persons had undergone medical procedures before, without any sign of a haemostatic defect, as well as normal bleeding times. Hereafter, the presence of a "clot-promoting factor" was identified that was of crucial importance for *in vitro* clotting, but not for physiological haemostasis. This factor was named Hageman-factor after the railroad-worker in which the defect was first identified. In the nomenclature of the coagulation cascade, it was later called factor XII (FXII).

Since then, a number of studies identified how FXII promoted coagulation, and revealed the intrinsic pathway of coagulation. Secondly, activation of the kallikrein-kinin system by FXII was described. The major question that remained was why and by what FXII is activated *in vivo*, so that its function may be elucidated.

At the moment, the role of FXII is interpreted as follows; FXII has a role in pathological coagulation, which may be initiated *in vivo* on a negatively charged surface, such as kaolin *in vitro* and phospholipids or extracellular RNA *in vivo*. Activation of FXII leads to simultaneous activation of kallikrein and FXI. However, FXII does not play a role in physiological haemostasis, since deficiency of FXII, prekallikrein (PK) or high molecular-weight kininogen (HK) does not result in a bleeding diathesis. Independent from FXII, the intrinsic pathway of coagulation has an additive role in support of physiological coagulation, due to a feedback loop in the coagulation cascade. Thus, because of this exclusive role of FXII in thrombotic pathology, it may be a therapeutic target. However, several epidemiological studies demonstrate an unexplained protective role for FXII and prekallikrein, but not for factor XI.

8.2 Towards a new role for factor XII *in vivo*

Since kallikrein generation occurs simultaneously with coagulation *in vitro*, it was presumed earlier that both systems are activated by FXIIa in an identical manner *in vivo* (i.e. on a negatively charged surface). The work in chapter 2 of this thesis contributes to understanding the role of FXII *in vivo* by identifying that generation of kallikrein by FXIIa occurs mechanistically different from the activation of FXI.

Our findings indicate that the activation of coagulation by FXII requires the direct interaction of FXII with a surface, whereas a surface is not necessarily required to induce FXII-mediated kallikrein formation. This suggests that the role of FXII with respect to pathological coagulation is mediated in the same way and point towards negatively charged compounds as the responsible procoagulants that contribute to thrombosis. Misfolded protein aggregates, rather than surface materials, induce FXII-dependent kallikrein formation. During surface-induced coagulation,

the kallikrein generation can be attributed to a misfolded protein cofactor that yields a similar epitope as a "free" misfolded protein. Since this mechanism can operate independently of coagulation, and does not require a surface, suggests that a similar mechanism takes place under physiological circumstances. This could help to explain the observed protective role of FXII in (cardiovascular) disease, which we will discuss later.

8.3 An explanation for separate pathways after activation of FXII

The initial step in the activation of FXII is autoactivation which can take place on both hydrophobic and hydrophilic surfaces[2]. The generated FXIIa is able to activate FXI, as well as prekallikrein and has its molecular weight of 78 kDa, despite a single cleavage step by the formed kallikrein. This form of FXIIa is called α-FXIIa. During surface-induced coagulation of plasma, a cleavage product of FXIIa forms, which is a result of a second cleavage step by kallikrein. This 28 kDa cleavage product, called β-FXIIa, has lost the capacity to support coagulation or to bind procoagulant surfaces[3]. Although it was thought that β-FXIIa can form by cleaving FXII that is directly surface-bound, an alternative explanation is that surface-bound FXII can only be cleaved once by kallikrein, whereas misfolded protein-bound FXII can be cleaved twice. As a consequence, the activation of FXII in the presence of misfolded proteins can result in the generation of β-FXIIa and kallikrein, while the activation of FXI is insignificant in absence of a surface.

8.4 The fibrinolytic system and its additional roles

In contrast to the contact system, the fibrinolytic system has a clearly defined function in haemostasis. The activity of plasmin is the main contributor to the clearance of fibrin fibrils that support the integrity of a thrombus. However, the fibrinolytic system is implicated in a wide variety of (patho-)physiological conditions, without a clear link to haemostasis. We here describe a number of findings which add to our understanding of the fibrinolytic system, but at the same time provide a number of new questions.

Mapping the fibronectin type I domain

Tissue-type plasminogen activator (tPA) activation occurs in the presence of denatured proteins[4] and later it was reported that several protein preparations, that had adopted amyloid properties stimulated this process[5]. In chapter 3, we performed a study to investigate the binding site in tPA for misfolded protein preparations with amyloid properties, which led us to conclude that the fibronectin type I domain (Finger-domain) was key to the interaction of tPA with protein that displayed amyloid properties. Also finger-domains of FXII, Hepatocyte Growth Factor activator and fibronectin had similar capabilities. The protein preparations that bound tPA and homologues, as well as their finger-domains, showed amyloid properties. Thus, our study identified a conserved binding site in tPA and its homologues for proteins and peptides that had adopted amyloid properties. However, studies using better characterized preparations are required to investigate the binding site of the finger-domain in misfolded protein aggregates with amyloid properties. Possibly, crystallographic studies using amyloidogenic peptides and finger-domains will reveal new insights in the specifics of this molecular interaction.

Activation of the fibrinolytic system in systemic amyloidosis

Since activation of the fibrinolytic system can be induced by misfolded protein aggregates *in vitro*, we next decided to investigate whether this also takes place *in vivo*. Hereto, we recruited a group of patients suffering from systemic amyloidoses, a selection of diseases hallmarked by circulating amyloidogenic proteins. Depending on the type of amyloidosis, amyloid protein deposits can affect organ functions, induce vascular ruptures, and (as we hypothesized) cause

haemostatic aberrances. In these patients, plasmin α2-antiplasmin (PAP) complexes were significantly elevated as compared to age-and-sex matched controls. These findings indicate that the fibrinolytic system can be induced by non-fibrin activators that are present in patients with systemic amyloidosis. Elevated PAP-complex levels are likely to contribute to bleeding episodes, which are known to occur in this disease.

We hypothesized that elevated PAP-complex levels would be present in systemic amyloidoses because of elevated tPA activation, induced by circulating misfolded proteins. Unexpectedly, we did not detect elevated tPA levels or elevated tPA-PAI1 complex levels. Although both tPA and tPA-PAI1 complexes are cleared very rapidly, their absence suggests that plasmin is activated via a different mechanism in systemic amyloidoses. Potentially, it could proceed via a urokinase plasminogen activator (uPA)-mediated mechanism, as has been described to occur in Alzheimer's disease[6]. Although tPA is implicated in Alzheimer's Disease[7-9], only polymorphisms in the gene for uPA[10-12] are associated with disease risk. The involvement of uPA in our hypothesized protein clearance is a confusing one. uPA does not have a binding site for misfolded protein as tPA does, for a similar reason it is also considered of less importance to fibrinolysis. However, uPA is involved in a myriad of processes like infection[13;14], wound healing[15], allergy[16] and cancer[17]. In these cases, uPA activity, as well as externalization of its receptor, is thought to stand under control of cells. In our mechanism of protein clearance, we need to identify whether cells can discriminate between misfolded and native proteins, in turn potentiating the uPA-dependent activation of plasminogen.

An alternative explanation for the elevated PAP-complex levels in systemic amyloidosis could be that another plasminogen activator (besides tPA and uPA) is responsible. In this case, FXII and kallikrein are interesting candidates, especially since FXIIa levels and kallikrein-C1inh complex levels were elevated in systemic amyloidosis (Chapter 2). Moreover, a subdivision into AL, AA and ATTR amyloidosis gave a distribution of FXIIa levels, identical to the PAP-levels published in chapter 4 (Unpublished data). Additionally, there may be a possibility that the tPA-homologue Hepatocyte Growth Factor activator plays a role in plasmin formation during systemic amyloidoses. However, further experiments are required to reveal the mechanisms behind the observed hyperfibrinolysis in systemic amyloidoses.

Investigations on the structure of fibrin polymers

A third hypothesis on the interaction between tPA and misfolded proteins, considered that the normal interaction between tPA and its natural ligand fibrin is not mechanistically different. This hypothesis is based on several lines of evidence. First, although many things are known about fibrin structure and fibrinolysis, the intrinsic difference between fibrin and fibrinogen that triggers initiation of fibrinolysis is not known[18]. Secondly, denatured fibrinogen is able to activate tPA-dependent plasmin formation[19;20]. Thirdly, there is a form of amyloidosis, caused by a mutation in the Aα-chain of fibrinogen, showing that fibrinogen is an intrinsically unstable protein and has an amyloidogenic tendency[21]. Fourthly, there are two binding sites for fibrin on tPA. The first one is the finger-domain, and the second one is the kringle domain[22]. Although the kringle domain has affinity for lysine residues, the basis of interaction of the finger-domain with fibrin is unknown. However, it is this finger-domain that also has affinity for misfolded proteins with amyloid characteristics (Chapter 3). We next analyzed fibrin fibrils formed *in vitro* for their structural properties, which revealed that fibrin possesses all the properties of an amyloid fibril. This is the first time that a human extracellular protein has been described to exhibit these features, without leading to disease, suggesting that amyloid structure is not necessarily pathological. However, there are two differences between amyloid fibrils and fibrin polymers that are noteworthy: 1)

amyloid fibrils are rigid and unbranched, which does not apply to fibin polymers and 2) to our knowledge, disease-related amyloid polymers are much more resistant to proteolysis then fibrin is. There are several explanations possible for our findings. It may be possible that the formation of amyloid structure is a general feature of protein misfolding, yielding a clearance signal to which tPA responds. Alternatively, since amyloid structure is present in the strongest known biopolymers like spider silk, the amyloid structure in fibrin is meant to give strength to the fibrils, or alternatively aid in their polymerization or elasticity. In medicine, the word coagulation refers to the solidification of blood plasma by fibrin formation. In biochemistry, the same word refers to the denaturation and precipitation of proteins. Our data suggests that, because both fibrin formation and protein denaturation is accompanied by display of amyloid properties, the word coagulation is not necessarily a disambiguation.

At this moment, we propose that the fibrinolytic system is induced by tPA binding to a binding site that is the same in misfolded proteins, as it is in fibrin polymers. It is clear that the lysis of fibrin polymers is a primary task of tPA and the fibrinolytic system. However our data indicate that the system can be induced by another class of activators, structurally similar to fibrin, which may be present in situations of inflammation and tissue damage, explaining the contribution of the fibrinolytic system in these instances.

8.5 Misfolded proteins; an elusive biological entity

The folding of proteins follows a common pathway that is mainly dependent on the presence of water molecules. These water molecules want to associate with hydrophilic- (charged), but not with hydrophobic amino acid residues. Hence, depending on an amino acid sequence, the chain will fold to expose as much hydrophilic residues, while internalizing hydrophobic regions. This process is assisted intracellularly by an intricate system that operates as a "quality control" monitor. However, proteins are not infinitely stable. As a result of changes in local environment, posttranslational modifications or physical stress, proteins can unfold. If the protein remains unfolded, because it has a secondary thermostable state, preventing refolding, it can be called misfolded.

During our studies, we hypothesized that FXII could differentiate between native and misfolded proteins. Hereto, we used proteins solutions in which we chemically or physically induced conformational changes. Moreover, we used synthesized peptides that behave like a denatured protein (i.e. amyloid β peptide). Although some misfolded protein aggregates were more potent in activating FXII-dependent kallikrein formation then others, it is still striking that such a wide variety of proteins and peptides are able to do this. We therefore propose that protein misfolding is accompanied by the adoption of a generic structural feature, which can be recognized by FXII. Denatured proteins that are studied *in vitro*, almost invariantly attain affinity for dyes that are used to stain amyloid plaques. The use of these dyes in fluorescence experiments allowed us to monitor the effects of a denaturing condition on a protein in solution, although the binding sites for these dyes are not known. Unlike amyloidogenic peptides or proteins that assemble into long unbranched fibrils, most proteins aggregated into amorphous aggregates when they were denatured *in vitro*. It is this form of protein (i.e. amyloid-like aggregate) that has been described to be toxic to cells, independent of its underlying amino-acid sequence[23]. Thus, endogenous clearance mechanisms to clear these toxic structures are required for organisms to survive.

What can this common structural denominator be? It has been hypothesized earlier that unfolding of a protein is accompanied by the formation of β-sheets, which were held responsible for amyloid-dye binding as well as cytotoxicity. However, recent work from various groups has

pointed out that amyloid-fibrils, containing high amounts of β-sheets are not necessarily cytotoxic, especially compared to their prefibrillar oligomeric precursors. It has therefore been propose that the β-sheets are responsible for cytotoxicity, but only in small oligomeric structures[24;25].

Following a conversely directed reasoning, those proteins that can form β-sheets upon unfolding and amyloid cross-β structure *in vivo* have a tendency to accumulate in tissues, leading to amyloidoses. This indicates that amyloid formation is accompanied by increased resistance against endogenous protein clearance mechanisms. This suggests that the formation of β-sheets and amyloid structure does not provide an endogenous clearance signal for these proteins. Interestingly, besides the classical amyloidosis, other diseases are hallmarked by deposition of (lipo) proteins, leading to disease. Indeed, oxidized low-density lipoprotein (oxLDL) accumulation is a key step in the formation of atherosclerotic plaques, and an increased body of evidence suggests that oxLDL is amyloidogenic in nature[26;27]. Additionally, in rheumatoid arthritis deposition of immunoglobulins and fibrinogen is observed in the synovium fluid of patients. These deposits are proinflammatory and contribute to disease progression, however immunoglobulin light- and heavy chains, as well as parts of fibrinogen are amyloidogenic *in vitro*, and are each involved in specific forms of hereditary amyloidosis. Therefore, it is attractive to think that the innate charactistic of these molecules to form amyloid structure contributes to their problematic tendency to deposit in tissues. Therefore, determination of the structure of these (lipo)proteins in disease-related deposits may be of interest. Additionally, anti-amyloid strategies may prove to be beneficial in the treatment of amyloidoses, but also in other diseases where protein accumulation plays a part.

A second hypothesis on the existence of a common clearance signal in unfolded proteins originates from an immunological point of view; breaking of immune tolerance against so-called "self" proteins is a largely unexplained field and lies at the basis of a large number of autoimmune pathologies. An exciting model was put forward that proposed that the (innate) immune system is triggered not only by non-self proteins, but also by danger signals that are associated with damage[28]. For example, necrotic cells can act as an adjuvant, potentiating immune responses. This model was later expanded with the notion that immune responses against proteins can arise when they expose hydrophobic sites upon their misfolding[29]. Although hydrophobicity is the driving force of protein folding, it also is a driving force for the formation of protein aggregates. When these aggregates can assemble in an organized way, as is the case in amyloid formation, hydrophobic patches are not always solvent-exposed. However, if the aggregation process occurs by a disorganized association of hydrophobic patches in misfolded proteins, some of these patches are likely to remain solvent-exposed. This hypothesis unifies the cytotoxicity of amorphously aggregated proteins and prefibrillar amyloidogenic peptides by a common structural feature. In line with this, we found in chapter 2 that FXII responds to misfolded protein aggregates, but not with several amyloid fibrils, which is suggestive for the idea that exposed hydrophobic patches may induce FXII-dependent kallikrein formation. Additionally, these hydrophobic regions can become exposed when a protein loses its tertiary structure, which is a structural requirement for induction of FXII-dependent kallikrein formation by bovine serum albumin.

Although we presently use this model to explain our findings concerning the contact- and fibrinolytic system, the danger model was meant to overcome immunological paradoxes: The exposure of hydrophobic regions by a protein (aggregate) can evoke immune responses, which may be undesired in some cases, but are probably meant to clear protein species that pose a threat to viable cells. We propose a similar role for the contact- and fibrinolytic system.

8.5 Immune responses as a consequence of protein misfolding

Immune responses can be dangerous when directed against "self" proteins, but also when directed against proteins that are administrated therapeutically. In line with the danger-model described above, we have investigated whether protein misfolding was of influence on immune responses against biopharmaceutical compounds. In chapter 6, we have shown that immune responses against recombinant human interferon α2b (rhIFNα2b) are dependent on protein structure. By induction of protein misfolding, which was corresponded by aggregate formation and adoption of amyloid-like properties, the immunogenicity of the protein was increased in wildtype mice. In transgenic mice that expressed human IFNα2b, tolerance was broken when they were injected with misfolded rhIFNα2b, but not with the native protein. We could determine the presence of misfolded protein aggregates in several biopharmaceutical preparations, using amyloidophilic dyes, as well as a tPA-dependent plasmin generation assay. The question remains which existing method for detection of protein misfolding correlates best with the generation of immunogenic (or cytotoxic) protein species, since the exact immunogenic trigger within a misfolded protein remains unknown. However, the use of small-compounds to detect exposure of new epitopes upon misfolding shows a promising relationship with immunogenicity of soluble proteins. Additionally, using endogenous receptors for detection of immunogenic protein species theoretically has the maximal potential for optimalization of biopharmaceutical formulations.

In similar fashion with the immune responses against biopharmaceutical compounds, the protein misfolding theory could help to explain the etiology of autoimmune diseases, as was proposed by Seong et al.[29]. In chapter 7 of this thesis, we have investigated this hypothesis by testing whether immune responses against β2-Glycoprotein I (β2-GPI), the major autoantigen in the antiphospholipid syndrome (APS), are dependent on the structure of the protein.

This autoimmune disease is characterized by both venous and arterial thrombosis, and/or recurrent pregnancy loss. In APS, antibodies arise against a large number of "self" proteins, such as β2-GPI, prothrombin (both of which can bind phospholipids), tPA, FXII and other proteins. Antibodies against β2-GPI are related to the risk on thrombosis[30], but thrombosis risk associates best with a special class of proteins that recognize a part of the protein which becomes exposed on phospholipid binding[31]. Thus, it is possible that antibodies against β2-GPI arise as a consequence of conformational changes and especially those thrombosis-related antibodies that recognize a cryptic epitope within the protein. In a preliminary report, we can confirm that this autoantigen undergoes conformational changes when binding to phospholipids, which correlates to elevated immunogenicity. Also, conformationally changed β2-GPI in absence of phospholipids showed enhanced immunogenicity. Further research will have to elucidate what the immunodominant epitopes are in this protein and whether this mechanism of immune induction is corresponded by a thrombotic phenotype, as is the case in APS patients.

8.6 The clearance of misfolded proteins

In this thesis, we have shown that both the contact system and fibrinolytic system react to misfolded protein aggregates of various origin, and demonstrated that this leads to the generation of plasmin and kallikrein, respectively. Although it is likely that both mechanisms are involved in the clearance of redundant and/or cytotoxic misfolded proteins, we did not show this in our studies.

We hypothesize that the clearance of obsolete- or damaged proteins can occur in two ways; either direct uptake takes place via cellular multiligand receptors, or proteolytic degradation is

induced to destroy the potentially harmful misfolded proteins. It is possible that the contact system and fibrinolytic system can interact with cells to promote the uptake of misfolded proteins, e.g. by upregulating scavenger receptor expression, However, it is also possible to think the involvement of both system in the degradation of toxic protein species. For instance, several enzymes are candidate to execute the actual proteolysis of the (aggregated) misfolded proteins. Neutrophil elastase, neprilysin, endothelin-degrading enzyme and insulin-degrading enzyme, are all known for cleavage of various substrates but share the capacity to degrade amyloid β peptide. As a result, these enzymes influence the pathology of Alzheimer's disease, but are therefore also possible candidates for a role in physiological protein clearance. Because of their enzymatic promiscuity, regulatory mechanisms may be required. For instance, it may be possible that after FXII-dependent activation of the kallikrein-kinin system, sufficient amounts of bradykinin and kallikrein have been formed to attract and activate neutrophils. These neutrophils in turn are known to aggregate and degranulate in response to FXIIa/kallikrein activity, releasing neutrophil elastase[32;33]. A similar response of neutrophils in the presence of plasmin has also been described[34], linking both the contact- and fibrinolytic system to neutrophil activation. Finally, in baboon models of lethal septicemia, inhibition of FXII led to decreased activation of the fibrinolytic system, as well as complement activation and neutrophil elastase release, indicating the interplay between these systems[35]. Thus, there may be pathways via which the kallikrein-kinin system and fibrinolytic system can contribute to the enzymatic degradation of misfolded protein aggregates. Our most recent data confirms that neutrophil elastase is indeed able to degrade a number of misfolded protein aggregates *in vitro*, reducing their affinity for the amyloidophylic dye Thioflavin T (unpublished data), but further research will have to confirm whether such mechanisms can operate as a whole and whether they can operate *in vivo*.

8.7 The protective role of factor XII and the kallikrein-kinin system

We proposed earlier that our findings on the activation of the contact system may help to explain the epidemiological findings in this area that have been postulated by a number of groups[36-39]. Although FXII is involved in *in vitro* clotting, it is not a risk factor for thrombosis. Conversely, FXII protects against (cardiovascular) disease, since lowered levels correlate with elevated risk for disease. This relationship is interpreted as a causal relationship: lower levels cause disease. However, it can also be interpreted the other way around; disease causes lowered FXII levels. This would be in line with the observation that the lowermost levels of FXII (between 0 and 10%) do not associate with disease risk, because they are caused by hereditary deficiency, and neither does the C46T polymorphism, leading to lowered FXII levels.

It is possible that this occurs by increased availability of possible ligands of FXII in the circulation of diseased persons, which at the same time constitute classical risk factors for arterial thrombosis. The idea that protein misfolding plays a role in atherosclerosis may be surprising[40]. However, via unknown mechanisms, the apoE ε4 polymorphism is a risk factor for both Alzheimer's disease and for atherosclerosis. Additionally, the apoE deficient mouse is a model system for both those diseases, linking the homeostasis of lipoproteins and one of the most well-known misfolded proteins; amyloid β peptide, together.

From our hypothesis, ligands for FXII in atherosclerosis can comprise oxidized proteins, caused by smoking[41], glycated proteins, or modified lipoproteins such as oxLDL. To support this hypothesis, we confirmed that oxLDL can activate FXII-dependent kallikrein formation *in vitro* (unpublished results), but it is important to investigate whether this also occurs under physiological conditions. To underline the importance of conformational change for our hypothetic FXII

ligands, epidemiological studies did not find an association the contact system with lipoproteins in general[42]. A second possibility in which the contact system protects against disease may lie in the interaction of FXII with proteins of the innate immune system. For instance, serum amyloid A and serum amyloid P, as well as various pentraxins are involved in atherosclerotic plaque formation. It is possible that the contact system exerts its protective role via mediators of innate immunity that deposit on sites where "danger" signals are present and form a ligand for FXII. Future studies will have to prove whether FXII levels become depressed as a consequence of cardiovascular disease; for instance, will an apoE knockout mouse have lowered FXII levels compared to wildtype control mice? Will atherosclerotic plaque formation progress more speedily in a mouse that is also deficient in FXII or prekallikrein? Is FXII present in atherosclerotic plaques, and does the amount of FXII in those plaques associate to their relative (in)stability? Does the presence of FXII reduce the effects of oxidative stress by contributing to the clearance of oxLDL? Additionally, the question remains why FXII deficiency is not directly associated with increased disease risk, suggesting a redundancy in the protective function of FXII *in vivo*, and opening up new avenues for indentification of other receptors involved in the recognition of misfolded proteins.

8.8 Final considerations

This thesis has focused on the behavior of two enzymatic systems in response to misfolded proteins. At present, much is known about the intracellular process of protein folding, which gives us insight into the common mechanism that underlies the biological complexity of life. Equally much is known about the unfolding and aggregation processes that lie at the basis of amyloid diseases.

During the lifetime of a human being, most of the cells are replaced several times, to allow for a longer lifespan. Accordingly, tissues that become injured are usually healed by replacement of the damaged cells. In line with this, it is commonly accepted that all proteins have their own lifespan, after which they should be cleared. However, surprisingly little is known about the extracellular mechanisms that distinguish between proteins that are "in good shape" and those that need to be removed. Apparently, such mechanisms are operational since only 25 out of the approximately 20,000 proteins that are present in the human body can cause amyloid protein deposition diseases. Given the fact that misfolded proteins are both functionally useless and harmful to cells, clearance mechanism are required to sustain life.

We here show that the contact system, fibrinolytic system and immune system can distinguish between native and misfolded proteins and respond to them by activation.

However, a number of important questions require further investigation:

1) What is the actual clearance signal in a misfolded protein (aggregate)? Does it have to be an aggregate or can a monomeric unfolded protein also induce a clearance response? Is the clearance signal already exposed after a change in tertiary structure (as shown for FXII activation in chapter 2) or does it require β-sheet formation and stacking as described by Kranenburg et al. with respect to the activation of tPA?

2) Under what conditions are misfolded proteins and their clearance mechanisms interacting *in vivo*, besides protein misfolding diseases?

So far our studies, as well as those of others have focused on Alzheimer's disease and systemic amyloidoses. However, do similar mechanisms also take place in the development of hypersensitivity reactions, wound healing, transplantation reactions or biocompatibility issues? Moreover,

can we use our understanding on the role of FXII to explain its role in cardiovascular disease? 3) What are the cellular receptors for misfolded proteins, or their degradation products, and what cells are involved? The generation of immune responses against proteins is a complex and well-regulated process. Although our studies show that biological systems can differentiate between protein conformations, important pieces are missing from the puzzle that help to explain the genesis of tolerance breaking, the etiology of amyloidosis-related hyperfibrinolysis and the actual clearance of misfolded proteins that are recognized by FXII.

4) How can we modulate these systems to enhance the clearance of damaging compounds, or to prevent pathological overactivity? Can we expand the current knowledge to eventually prepare safer biopharmaceuticals and adjuvant-free vaccines, induce tolerance against "self" proteins, influence allergy, or inhibit pathological inflammatory processes. Can we use our knowledge to design better fibrinolytic agents, than are currently available?

In conclusion, our studies suggest that well-known biological systems can identify harmful protein structures, most likely via a common structural denominator. Although not always beneficial, these responses are meant to clear toxic protein species and aid in protein homeostasis, which is a necessity for maintenance of mammalian life.
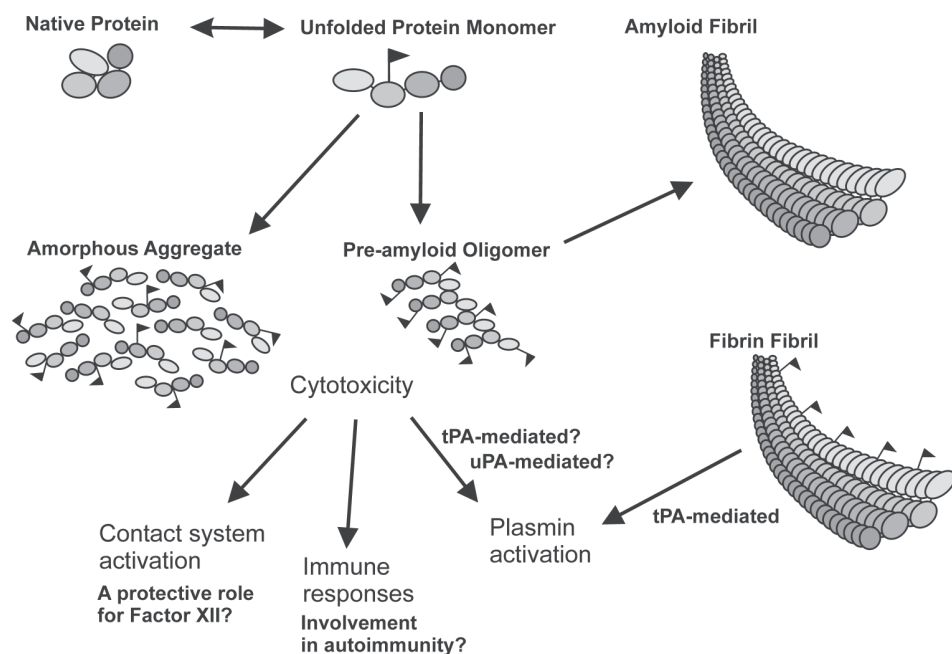

Figure 1. The recognition of misfolded proteins as part of the body's maintenance system. The flags symbolize the epitope in a misfolded protein that activates the contact system and/or fibrinolytic system and/or immune system.
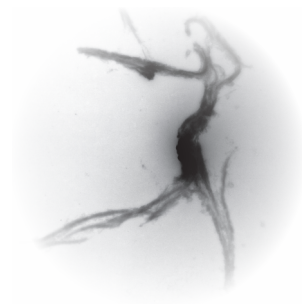
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Chapter 9

Nederlandse samenvatting

Wanneer een eiwit beschadigd raakt en hierdoor zijn originele structuur verliest, kan het zijn functie vaak niet meer vervullen. Deze misvormde eiwitten zijn schadelijk en het opruimen hiervan is nodig om het lichaam te beschermen. In dit proefschrift is beschreven dat Factor XII, bekend uit het bloedstollingsysteem, en tissue-type plasminogen activator (tPA), dat betrokken is in het opruimen van bloedstolsels, beiden reageren op misvormde eiwitten. Hierbij wordt door Factor XII een ontstekingsreactie veroorzaakt, welke onafhankelijk van de bloedstolling kan plaatsvinden. Omdat de rol van Factor XII in het lichaam op paradoxale wijze nog onbekend was, kan dit mechanisme het bestaan van dit enzym verklaren. Activatie van tPA leidt tot de vorming van plasmine, een enzym dat bloedstolsels op kan ruimen. Wij beschrijven hier dat plasminevorming ook plaatsvindt in ziekten waar misvormde eiwitten zich ophopen en dat de structuur van fibrine, een belangrijk onderdeel van een bloedstolsel, lijkt op dat van misvormde eiwitten. Dit suggereert dat het opruimen van een bloedstolsel vergelijkbaar is aan het opruimen van misvormde eiwitten. Ten slotte beschrijven wij dat misvormde eiwitten de vorming van antistoffen kunnen stimuleren; dit zou het ontstaan van ongewenste afweerreacties tegen medicijnen en lichaamseigen eiwitten kunnen verklaren.

9.1 Introductie

De circulatie van bloed is van essentieel belang om alle lichaamsweefsels van zuurstof en voedingsstoffen te voorzien. Op het moment dat een bloedvat beschadigd raakt, moet er dus voorkomen worden dat er teveel bloed ontsnapt. Het lichaam beschikt over een paar oplossingen voor dit probleem, waarmee het in staat is de gaten in de bloedvatwand af te dekken. Het wondgebied wordt herkend door bloedplaatjes die er blijven zitten en samenklonteren. Tegelijkertijd wordt het bloedstollingsysteem aangezet door signalen vanuit het wondgebied; dit leidt tot de vorming van draden, bestaande uit het eiwit fibrine, die het bloedstolsel stevig maken. Na verloop van tijd heeft de bloedvatwand en het onderliggende weefsel zich hersteld, en moet het bloedstolsel weer opgeruimd worden om de bloeddoorstroming niet langer in de weg te staan. Dit gebeurt door het kapotknippen van de draden in het stolsel (fibrinolyse).

De bloedstolling komt op gang wanneer enzymen in het plasma in contact treden met een eiwit in de weefsels buiten de bloedvaten, genaamd tissue-factor (weefselfactor). Het plasma eiwit factor VII bindt hieraan en activeert dan. Hierna kan het factor X activeren, wat op zijn beurt protrombine omzet in trombine. Dit enzym kan fibrinogeen omzetten in fibrine, wat zich rangschikt in netwerken van lange draden. De bloedstolling en fibrinolyse samen vormen het hemostatisch systeem.

Naast blootstelling van het plasma aan tissue-factor, kan stolling ook beginnen wanneer het in aanraking komt met materialen zoals glas of plastic. Dit proces staat ook wel bekend als contact activatie van het bloed. Om deze reden is het noodzakelijk om een antistollingsmiddel toe te dienen aan de bloedbuizen die voor diagnostische bepalingen bestemd zijn. Om bloedstolling op een glazen oppervlak te krijgen moet een apart enzymatisch systeem aangezet worden. Allereerst moet factor XII (FXII) aan het glas binden en activeren. Daarna kan geactiveerd FXII factor XI activeren, wat op zijn beurt via een aantal stappen tot fibrine vorming leidt. Tegelijkertijd word door geactiveerd FXII het eiwit prekallikreine omgezet in het enzym kallikreine. Dit enzym is onder andere betrokken in de vorming van het peptide bradykinine, wat in vasodilatie (het uitzetten van bloedvaten) en ontsteking resulteert. Afwezigheid van FXII in het bloedplasma leidt tot een enorme vertraging in de bloedstolling door een (glazen) oppervlak en afwezigheid van kallikreine vorming.

9.2 Vragen rondom het contact systeem en de fibrinolyse

In 1955 werd voor het eerst een drietal personen beschreven die niet in staat waren om factor

XII te produceren. In tegenstelling tot tekorten van andere stolfactoren, bleek dat de afwezigheid van FXII in het bloedplasma niet gepaard gaat met bloedingen. In muismodellen voor trombose, waarbij door een vaatwandbeschadiging (m.b.v. laser of chemicaliën) een bloedprop wordt geïnduceerd, blijkt dat een muis zonder FXII minder trombose ontwikkeld dan een normale muis. Dus FXII speelt wel een rol in pathologische bloedstolling. De vraag is dus wat het nut van FXII in het bloed is, als het niet nodig is voor de normale bloedstolling, maar wel betrokken lijkt in trombose.

Om een stolsel op te ruimen (fibrinolyse) is het fibrinolytisch systeem nodig. Het enzym dat fibrine draden kapot kan knippen heet plasmine en wordt gevormd door de voorloper plasminogeen te activeren. Activatie van plasminogeen gebeurt door plasminogeen activatoren. Hier bestaan er twee van. Ten eerste is er urokinase-type plasminogeen activator (uPA), dat op het oppervlak van cellen buiten het bloedvat plasminogeen kan omzetten in plasmine. Ten tweede is er tissue-type plasminogeen activator (tPA). Dit molecuul is niet aanwezig in het bloedplasma, maar wordt locaal geproduceerd door (endotheel)cellen in het wondgebied. tPA kan binden aan fibrinedraden, waarna het activeert en dan plasmine vormt uit plasminogeen. Omdat het fibrine kan binden wordt tPA gezien als de belangrijkste plasminogeen activator. Ondanks zijn duidelijke rol in het opruimen van bloedstolsels, is het fibrinolytisch systeem ook betrokken in een aantal condities waar bloedstolsels in betrokken zijn. Zo is het bijvoorbeeld betrokken in ontstekingsreacties, de ziekte van Alzheimer en de aanleg van geheugen. Het is de vraag welke rol het fibrinolytisch systeem vervuld in deze situaties.

9.3 Nieuwe activatiemechanismen

Eiwitten hebben hun unieke structuur, die van essentieel belang is voor hun functie. Na verloop van tijd, of door omstandigheden kan deze structuur verloren gaan. Naast het verlies van functionaliteit, zijn misvouwen eiwitten over het algemeen ook schadelijk voor cellen. Omdat veel verschillende eiwitten in misvouwen toestand schadelijk zijn voor cellen, is er gesuggereerd dat dit gebeurt via een gemeenschappelijk structureel kenmerk. Misvouwen eiwitten hebben een aantal kenmerken verworven die overeenkomen met de pathologische eiwitaggregaten die men tegenkomt in een groep van ongeveer 25 eiwitmisvouwingsziekten die gezamenlijk de amyloidoses genoemd worden. De ziekte van Alzheimer is hier een voorbeeld van, maar elke individuele amyloidose wordt veroorzaakt door een ander eiwit wat zich in bepaalde weefsels als amyloide fibrillen afzet.

In dit proefschrift is bestudeerd hoe contact activatie van het bloed tot stand komt. Wij stelden ons voor dat, net als tPA, FXII geactiveerd kan worden door misvouwen eiwitten. In hoofdstuk 2 hebben wij laten zien dat dit inderdaad het geval is. Een aantal soorten misvouwen eiwitten, maar niet natieve (normale) eiwitten induceerden activatie van FXII. Dit gold ook voor eiwitten die aan een oppervlak bonden, en hierdoor misvouwden. Deze resultaten suggereerden dat FXII interactie aangaat met een structurele overeenkomst die aanwezig is in misvouwen eiwitten. Bovendien leidde deze activatie niet tot stolling, maar de formatie van kallikreine vond echter wel plaats. Dit gaf aan dat stolling en kallikreine vorming in het bloed twee individuele routes volgen.

De activatie van FXII was eerder beschreven in de hersenvloeistof van mensen met de ziekte van Alzheimer. Om er achter te komen of FXII activatie bij eiwitmisvouwingsziekten een algemeen fenomeen was, bepaalden wij de hoeveelheden geactiveerd FXII in het plasma van patiënten met systemische amyloidoses. Hiernaast bepaalden wij of er kallikreine formatie, danwel activatie van de stolling door FXI had plaatsgevonden. Uit onze bleek dat de hoeveelheden geactiveerd FXII

verhoogd waren in de patiënten ten opzichte van een vergelijkbare controlegroep. Bovendien ging dit gepaard met verhoogde vorming van kallikreine, maar niet van geactiveerd FXI.

Op basis van onze resultaten stellen wij voor dat FXII betrokken is in kallikreine-gemedieerde ontstekingsreacties, die getriggerd worden door misvouwen eiwitten. Omdat misvouwen eiwitten schadelijk zijn voor cellen, dienen deze herkent en opgeruimd te worden; wij stellen voor dat dit FXII-gemedieerde mechanisme daar een rol in speelt. Men kan zich voorstellen dat misvouwen eiwitten vrijkomen in beschadigde weefsels, en vormen hiermee een potentiële fysiologische vervanger gevonden voor negatief geladen "oppervlakken" die het contact systeem kunnen stimuleren . Omdat de rol van FXII in kallikreine generatie niet gepaard hoeft te gaan met bloedstolling verklaart het mogelijk de paradoxale afwezigheid van een bloedingsneiging in FXII-deficiënte personen.

Op basis van domein-architectuur zijn tPA en FXII sterk vergelijkbaar. Hiernaast zijn er nog twee eiwitten bekend die er veel op lijken, te weten Hepatocyte growth factor activator (HGFa) en fibronectine (Fn). Deze twee eiwitten zijn betrokken in wondheling (HFGa) en de vorming van extracellulaire matrix (Fn). In hoofdstuk 3 is beschreven dat het in al deze eiwitten voorkomend fibronectine type-I domein, ook wel het Finger-domein genoemd, op zichzelf in staat is om aan eiwitten met amyloide kenmerken te binden. Omdat amyloide kenmerken ook voorkomen in misvouwen eiwitten die niet betrokken zijn in een amyloidose, stellen wij voor dat de herkenning van een misvouwen eiwit door het Finger-domein een fysiologisch mechanisme is, met als doel misvouwen eiwitten te herkennen en een opruimrespons te starten. Hiernaast kan dit type interactie ten grondslag liggen aan een aantal meer bekende fysiologische processen; zo kan dit bijvoorbeeld betekenen dat het Finger-domein van tPA, zoals meer dan twintig jaar geleden is beschreven, aan fibrine kan binden omdat fibrine een aantal kenmerken van een misvouwen eiwit in zich heeft. Dit zou inzicht kunnen geven in de manier waarop tPA fibrine kan onderscheiden van fibrinogeen. In hoofdstuk 5 beschrijven we dat fibrine structurele overeenkomsten vertoont met de amyloide fibrillen die zich afzetten in weefsels als gevolg van eiwitmisvouwingsziekten. Deze bevinding geeft aan dat de amyloide cross-β structuur, die voorheen alleen bekend was door zijn aanwezigheid in pathologische eiwitaggregaten, ook terug te vinden is in belangrijke fysiologische eiwitpolymeren. Dit betekent dat deze structuur niet per definitie schadelijk hoeft te zijn en levert de vraag op wat de functie ervan in fibrine is. Het is mogelijk dat de structuur een natuurlijk opruimsignaal voor tPA oplevert. Het is echter net zo goed mogelijk dat de vorming van de structuur samenhangt met de mechanische eigenschappen, of het polymerisatiegedrag, van fibrine.

Systemische amyloidoses zijn een verzameling van 3 eiwitmisvouwingsziekten waarbij de voorlopereiwitten van de amyloide aggregaten (zgn. amyloidogene eiwitten) in het bloed circuleren. Deze ziekten gaan gepaard met complexe veranderingen in het hemostatisch systeem, en de patiënten ontwikkelen soms trombose, maar vaak ook bloedingen. Uit onze eerdere experimenten was gebleken dat de belangrijke plasminogeen activator tPA activeerde in de aanwezigheid van amyloidogene eiwitpreparaten. Wij hypothetiseerden dat ongewenste plasmine formatie zou kunnen plaatsvinden in het bloed van systemische amyloidose patiënten. De bloedingen die gepaard gaan met systemische amyloidose zouden dus deels verklaard kunnen worden door verhoogde fibrinolytische activiteit, wat de vorming van nieuwe stolsels kan afremmen. Om deze hypothese te toetsen hebben wij in een kleine groep patiënten en vergelijkbare controles de plasma concentraties van plasmine-α2-antiplasmine (PAP) complexen bepaald. Deze complexen vormen zich nadat plasmine in contact komt met een van zijn natuurlijke remmers α2-antiplasmine, en blijven circuleren totdat ze worden opgenomen door cellen. Als gevolg hiervan

zijn verhoogde PAP-complex niveaus in plasma tekenend voor verhoogde plasmine formatie. In hoofdstuk 4 beschrijven wij dat systemische amyloidoses in twee van de drie varianten (AL en AA amyloidose) gepaard gaat met verhoogde PAP-levels. Dit suggereert dat in deze twee amyloidoses plasmine formatie plaatsvindt als gevolg van circulerend amyloidogeen eiwit en dat een soortgelijke activator ontbreekt in de derde variant (erfelijke ATTR amyloidose). Als gevolg hiervan stellen wij voor dat verhoogde fibrinolytische activiteit een risico vormt voor bloedingen in patiënten met systemische amyloidose.

9.4 Misvouwen eiwitten en het immuunsysteem

Het immuunsysteem is bedoeld als verdedigingsmechanisme tegen infectie door pathogene microorganismen en parasieten. De generatie van antistoffen die delen van deze organismen herkennen is hierbij van essentieel belang. Ondanks de beschermende functie van het immuunsysteem ontstaan er in een aantal gevallen ongewenste immuunresponsen tegen voedingsbestanddelen, medicijnen of lichaamseigen eiwitten (autoimmuunziekten).

Voor nagenoeg elk eiwitmedicijn dat op de markt is, is beschreven dat een deel van de patiënten er antistoffen hebben ontwikkeld. Dit kan leiden tot een verminderde werkzaamheid van het medicijn, en omdat dit soort medicijnen vaak gegeven word om lichaamseigen eiwit aan te vullen kan soms ook het lichaamseigen eiwit doelwit van antistoffen worden, met potentieel dodelijke consequenties. In hoofdstuk 6 hebben wij onderzocht of de structuur van een eiwitmedicijn van invloed is op zijn immunogeniciteit (de capaciteit tot het oproepen van een immuunrespons). Uit onze experimenten bleek dat de misvouwing van een eiwitmedicijn, wat gepaard gaat met het vertonen van amyloide kenmerken, een verhoogde immunogeniciteit van het preparaat veroorzaakt. In een transgeen diermodel voor het humane eiwit interferon α2b leidde injectie met misvouwen interferon α2b tot het breken van de tolerantie. Ook immuunresponsen tegen een niet-medicinaal modeleiwit bleken afhankelijk van de structuur van het eiwit. Bovendien waren er in een aantal commercieel verkrijgbare eiwitmedicijnen eiwitten met amyloide kenmerken terug te vinden. Deze resultaten geven aan dat eiwitmisvouwing kan plaatsvinden in eiwitmedicijnen, wat kan bijdragen aan ongewenste immuunresponsen.

In hoofdstuk 7 beschrijven wij een aantal experimenten die, in het verlengde van hoofdstuk 6, bedoeld zijn om te onderzoeken of eiwitmisvouwing een rol zou kunnen spelen in het ontstaan van immuunresponsen tegen eiwitten die betrokken zijn in autoimmuunziekten.
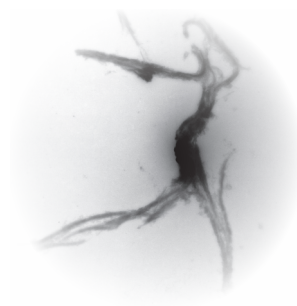
Het antifosfolipiden syndroom (APS) is een autoimmuunziekte waarin antistoffen ontstaan tegen β2-Glycoprotein I (β2-GPI), welke verantwoordelijk gehouden worden voor de trombose die de ziekte kenmerkt. In een fosfolipiden gebonden toestand is β2-GPI in staat pathologische protrombotische antistoffen te binden. Dit suggereert dat er een conformationele verandering plaatsheeft in het molecuul wanneer het aan fosfolipiden bindt, waardoor een verborgen trombose-gerelateerd epitoop beschikbaar komt. Wij hypothetiseerden dat deze conformationele verandering ook verantwoordelijk zou kunnen zijn voor immuunresponsen tegen β2-GPI. In een kort verslag beschrijven wij dat β2-GPI, tijdens het binden aan fosfolipiden polymeriseert en tegelijkertijd amyloide-achtige kenmerken ontwikkelt. Injectie van fosfolipiden-gebonden β2-GPI in muizen leidde tot immuunresponsen tegen dit eiwit, bovendien ontstonden er antistoffen tegen het epitoop dat betrokken is in trombose. Om te onderzoeken of de conformationele verandering van β2-GPI verantwoordelijk is voor immuunresponsen ertegen, maakten wij een misvouwen variant van β2-GPI die na injectie in muizen antilichamen opwekten tegen β2-GPI, het trombose-gerelateerde verborgen epitoop en tegen een aantal epitopen die niet beschikbaar zijn in fosfolipiden-gebonden β2-GPI. Deze experimenten wijzen erop dat de immuunrespon-

sen tegen β2-GPI het gevolg zijn van een conformationele verandering in het molecuul, maar dat deze conformationele verandering specifieker lijkt dan algehele misvouwing van het eiwit. Het is mogelijk dat deze conformationele verandering (polymerisatie) van belang is voor de nog onbekende functie van β2-GPI.

9.5 Conclusies

In dit proefschrift is de activatie van een aantal biologische systemen door misvouwen eiwitten onderzocht. Wij stellen voor dat de geobserveerde activatie van het contact systeem, het fibrin-olytisch systeem en het immuunsysteem facetten vormen van een opruimsysteem voor bescha-digde eiwitten, dat nodig is om de schadelijke effecten van de aanwezigheid van deze eiwitten tegen te gaan.

Dankwoord, Curriculum Vitae en Publicatielijst

En nu voor het meest-gelezen gedeelte van dit proefschrift; de dankbetuigingen! De afgelopen vier jaar heb ik in een flits voorbij zien gaan, en dat is helemaal niet erg. Net zoals met de meeste dingen, zou ik het nog wel een keertje opnieuw willen proberen want er is zoveel om me heen gebeurd dat ik de helft heb gemist. In elk geval ben enorm blij dat ik in aanraking ben gekomen met een grote hoeveelheid mensen met verschillende achtergronden, leeftijden en interesses, die ondanks komen en gaan toch een kleine familie vormden in de labs waar ik rondliep.

Dr. Gebbink, beste Martijn. Vanaf mijn allereerste gesprekken voor een stage bij de Medische Oncologie heb ik met jou te maken gehad. Ondanks het feit dat ik eigenlijk een ander pad wilde gaan volgen (in het bedrijfsleven, nota bene!), heb ik tijdens die eerste stage de smaak voor wetenschap te pakken gekregen – waarvoor enorm bedankt! Het oprichten van een bedrijf is een stap die niet veel academici maken, maar in een toenemend economisch gemotiveerde wereld wel logisch lijkt te zijn. Zoals we beiden hebben gezien, is het definiëren van de scheidslijn tussen de academische geest en bedrijfbelang een belangrijk onderwerp geweest in de afgelopen jaren van mijn promotie; wat een leerzame situatie heeft opgeleverd. Het schrijven en discussiëren met jou is een groot plezier geweest en ik ben blij dat ik nooit een blad voor de mond heb hoeven houden. Ik kan alleen maar zeggen dat ik enorm veel heb geleerd over dit soort dingen maar ook over mezelf. Hierdoor zou ik het zonder twijfel zo nog een keer over doen als ik moest kiezen. Bedankt.

Prof. dr. De Groot, beste Flip. Wetenschap is geen van negen-tot-vijf baan, heb ik je vaak horen zeggen, en loopt ook niet van maandag-tot-vrijdag. Misschien is het dus wel niet echt een baan, maar eerder een uit de hand gelopen hobby, of serieuzer gesproken, een roeping. Wat dat betreft ben je een enorm goed voorbeeld en ik vind het mooi dat jij deze gedachte ook in stand houdt in de vakgroep. Het blijft een uitdaging om (wetenschappelijke) discussie in groepsverband te stimuleren en ik hoop hierin ook in de toekomst een bijdrage aan te kunnen leveren.

Prof. dr. Bouma, beste Bonno. Voor mij is het slechts zes jaar geleden dat ik voor het eerst in aanraking kwam met factor XII en het contact systeem. Het is een bijzondere ervaring geweest om mijn ballonnetjes op te laten tijdens een van onze besprekingen, waarbij jouw uitgebreide kennis op dit gebied én de rust die je uitstraalt mij elke keer opnieuw met opgeladen batterij het lab weer indreven. Ik ben de afgelopen periode alleen maar meer geïntrigeerd geraakt door dit systeem en zijn rol in de (patho)fysiologie en hoop dat ik ook in de toekomst op je mag rekenen voor advies.

Dr. Roest, beste Mark. Er zijn maar weinig mensen die (bijna) altijd lachen, zulke mensen zijn enorm belangrijk voor de stemming in een groep. Het is goed om te zien dat je ondanks verant-woordelijkheden, toch je eigen mens kunt blijven. Ik hoop dat je het niet erg vindt als ik in de toekomst een hoop domme vragen kom stellen over de epidemiologie, want ik heb er zelf (nog) weinig verstand van. In elk geval, bedankt voor al die keren dat we een bakkie hebben gedaan en hebben geluncht, ik hoop het nog vaak te herhalen.

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belangrijk zijn. Ik hoop nog altijd een keer écht goed met je te discussiëren over de definitie van zogenaamde "cross-β's".

Harry, Tuna en Richard. Beste heren, jullie expertise op verschillende gebieden doet mij inzien dat persoonlijke focus en enthousiasme nodig is in de wetenschappelijke wereld. Ik zou zeggen dat het belangrijk is om een onderwerp uit te kiezen en daar voor te gaan; nu moet ik er alleen nog achter komen hoe en wat dat precies voor mij is. Hopelijk kunnen jullie me daarbij nog helpen.

Sandra. Toen jij voor het eerst op het lab verscheen (als studente bij Marjolein), moest ik je verdunningen nog narekenen; ze klopten eigenlijk (bijna) altijd. Nu hoeft niemand je nog iets te vertellen en kan ik aan jou soortgelijke dingen vragen. Door jou heb ik altijd veel plezier gehad op lab 3, veel plagerijtjes, biggetjes stelen en goed uitkijken als je een boze bui had. Dankjewel, je hebt Uddel positief op de kaart gezet en daarom heb je nog een Flugeltje of twee van me tegoed ;)

Arnold. Mijn huidige labbuurman, een echte pipetboy (5 proeven tegelijk) en een zachtaardig mens. Ik vind het mooi dat we soms (het liefst met een biertje erbij) gesprekken kunnen houden over lastige onderwerpen. Wederzijds respect is enorm belangrijk, vliegende Hollander.

Suzanne Kroon. Je enthousiasme voor het organiseren van extracurriculaire activiteiten is opvallend. Blijkbaar ben je een team-speler in optima forma en dat is precies wat we nodig hebben!

Dianne. Mijn buurvrouw in de AIO-kamer en opvolgster van Dr. Badlou. Jouw grappen hebben mij vaker dan een keer tot tranen gedreven (Haha! goede grap Bassie!). Andersom heb ik jou ook vaker dan één keer de gordijnen in gejaagd. Je schrikreacties zijn enorm heftig! Ik ken niet veel meisjes die met gesloten vuist meppen! Nu nog afleren om mij te meppen als iemand anders je beledigd. Heel veel succes, maar vooral plezier in de rest van je promotie.

Suzanne Korporaal. Suus. Het is altijd fijn om je weer te zien en gelukkig gebeurt dat ook regelmatig. Ondanks je "vertrek" naar Leiden, hebben we vaak de kans om even bij te kletsen over papers, verdelingen van auteurschappen, beursaanvragen, maar ook leuke dingen. Blijf langskomen! Dat maakt het hier een stukje gezelliger. Doe de groeten voor me aan Wouter en Jelle (Ik ben He-Man kwijtgeraakt, dus ik moet een nieuwe regelen).

Arjan "Sjaak" Barendrecht. Het viel me op dat ik sinds ik jou ken steeds meer zwarte T-shirts ben gaan dragen en ook naar gitaarmuziek ben gaan luisteren. Ik vraag me af of ik over een tijdje ook van uitslapen hou en een lange staart heb laten groeien. Het is me altijd een plezier om er eentje met je te pakken bij de Primus.

Erik. Dokter Erik, altijd fijn om een arts in de buurt te hebben voor noodgevallen. Goed dat je je niet al te snel door mensen (i.e. mij) op de kast laat jagen. Volgens mij ben je van (bijna) alle markten thuis, dus maak daar optimaal gebruik van.

Anja. Dinsdag Anjadag; altijd weer een succes! Ondanks je duidelijke voorkeur voor Amsterdam kom je toch elke dag opnieuw naar Utrecht, hoe zit dat nou? In elk geval moet ik ten overstaan van iedereen rechtzetten dat Anja *niet* van sherry houdt, voordat ze nog meer flessen van vertrekkende studenten cadeau krijgt. In elk geval, altijd leuk om je in de buurt te hebben en te kletsen over van alles (insuline) en nog wat (diabetes).

Mieke. Het was enorm fijn om je in de eerste periode van mijn AIO-tijd in de buurt te hebben. Ik vind het nog steeds zonde dat je bent vertrokken, maar ik begrijp het natuurlijk helemaal. Hoe is het met jullie kleine meid?

Bettina. Ik heb gehoord dat ook jij grootse plannen hebt voor de toekomst. Veel succes en plezier toegewenst!

Nicole. Nu is het mijn beurt om te promoveren; ik zou ook wel zo'n mooie bootreis willen

maken, zoals jij er zelf een gemaakt hebt na je promotie. Ik verwacht een uitnodiging voor je eerstvolgende optreden, zoals we hebben afgesproken.

Cor. De man met kladblok en pen. Al jaren ben je onverstoorbaar (op werkbesprekingen na), altijd met een vrolijk humeur; hoe doe je dat? Bovendien is aan je hoeveelheid mappen en lab-journaals duidelijk te zien dat je georganiseerd met informatie omgaat. Deze dingen ga ik van je proberen over te nemen, net als "Cor's Magische Blotjes-Wasprotocol".

Miranda. Een echt Duracel-konijntje; high energy! Dat werkt enorm aanstekelijk (voor mij in ieder geval). Zal vast wel iets met je Brabantse afkomst te maken hebben. Leuk dat je je doch-tertje voor het eerst mee naar de kroeg hebt genomen terwijl Remo en ik er zaten; een heugelijk moment. Laat af en toe wat van je horen, als je weer terug bent onder de rivieren.

Silvie. Mijn kleine reislustige, plaatjesdraaiende surf-vriendinnetje. Ik ben er blij mee dat je zo goed op me past, zonder jouw scheve blikken en aanmaningen zou ik er waarschijnlijk minder goed aan toe zijn. Bovendien hebben we al een hoop leuke dingen gedaan en ik hoop dat we nog meer gaan ondernemen. Van mij mag je altijd frietjes stelen.

Evelien en Martine. Een professionele werkhouding en goed kunnen samenwerken zijn belangrijke eigenschappen voor een onderzoeker (in opleiding). Hierin kan ik nog heel wat van jullie leren.

Brigit. Ik ken jou als een vriendelijke levensgenietster, wat blijkbaar toch te combineren valt met grondige kennis van de enzymkinetiek (wat is een Kcat ook alweer?). Heel veel plezier in Indo-nesië; drink een Bir Bintang voor me, dat is lekkerder dan Bali Hai.

Annet. Jou ken ik al vanaf mijn eerste stage. Oncologie zat om de hoek. Altijd in voor een kletspraatje.

Ya-Ping. Dr.Wu, it has been my pleasure to hear, but especially see, presentations of your work. Did you know that pictures like the ones you can make are sometimes displayed in musea? Obviously (and not surprisingly), biology is art.

Martin. Ken ik niet; weggooien! Martin, jij bent de spil in de organisatie van onze labs. Via jou heb ik kennis mogen maken met zaken die er voor nodig zijn om de hele operatie draaiende te houden; daar komt dus nog heel wat bij kijken. Bedankt voor je hulp de afgelopen jaren.

Joukje. Als we jou niet hadden, zou alles in het honderd lopen. Ik kan me niet eens voorstellen wat een geduld je moet hebben om te kunnen zorgen voor een berg warhoofdige wetenschap-pers. Veel respect!

Rosmina. Selamat Siang! Ik spreek nog steeds alleen een paar losse woorden Bahasa Indonesia, we moeten dus nog verder oefenen. Terimah Kasih! Mas Coen.

Floor. Dit is dus het boekje waar ik het met je over had. We zijn onder de indruk van de grondig-heid waarmee je je werk aanpakt; zo zouden we het allemaal moeten doen!

Arno. Jij bent de man die van alles geregeld krijgt. Je no nonsense – instelling is erg belangrijk om te maken dat wij ons werk goed kunnen doen. Ik hou nog wel een rondje uit je beertender tegoed (ik ga voor Murphy's geloof ik), denk maar niet dat ik het vergeet!

Maarten Pennings. Als een labrador door het promoveren in een pitbull kan veranderen, dan vraag ik me af wat ik ondertussen ben geworden (geen antwoord geven!). In elk geval, op som-mige momenten zou ik willen dat ik wat van jouw ontspannen houding zou kunnen overnemen. Hang loose.

Robbert. Harde werker en kritisch wetenschapper. Grappig om te zien hoe dingen na onze gezamenlijke opleiding uiteindelijk zijn gelopen. Wie had ooit kunnen denken dat wij samen een publicatieborrel zouden geven! Als je met je korte broeken in de winter een fashion-statement wil maken, denk ik dat je dat gelukt is!

Teun. Goed om te zien dat je een gemotiveerde wetenschapper bent; dat motiveert anderen (mij) ook. Alleen proeven doen tijdens een vrijdagmiddagborrel is, wat mij betreft, slechte planning.

Maarten Emmelot. Als er een kerel vanaf 30 meter afstand dagelijks naar me loopt te zwaaien zou ik me zorgen kunnen gaan maken. In jouw geval maak ik een uitzondering, maar dan wil ik wel die introductiecursus gepast FACS-gebruik bij je volgen.

De laatste periode zijn er weer een hoop nieuwe gezichten verschenen in de AIO-kamer. Gwen, Chetin, Esther, Vivian – zet hem op! Ik hoop een mooi proefschrift te ontvangen wanneer het jullie beurt is om te promoveren!

Eszter. Dr.Herczenik, I had a great time with you in, as well as outside the lab. I have also experienced as being part of a small family; obviously, this is how people behave when times are a little tense. I hope/know you are having a great time in Amsterdam and continue to keep singing. I am a great fan! I am also a great fan of your cooking; especially your vanilla/brioche dessert from the oven. Either you give me the recipe, or keep on inviting me to come over for dinner. I personally prefer the second option!

Lorena. The whining Buitre aka Pancho Sanchez (I know it's Sancho Panza in the book). I still need to come and visit you in Philadelphia, but I have a flat tire. I'll let you know when I fixed it. Anyway, I guess you won't be at my promotion party, which is a good thing since I do not enjoy to see such a small girl consume more beer then I do, and survive! I'm very glad we met and I'm missing your south-american accent, as well as the salsa-lessons (although I hate salsa-dancing).

Ronan. Dr. Pendu, aka mr. Disco-Inferno. Two sides of the same coin, but different in appearance. Perhaps I am leading a somewhat similar life. Anyway, we have had good times, man! I hope we will have more in the future.

Caro. The first time we met, you taught me two ways to toast beers in French, I forgot one of them. Therefore, I need a rematch! However, you are an inspiration for having an active and social life. Hope to see you soon!

Rolf. Ondanks jarenlange ervaring in ons lab ben je nog steeds een labrador (ondanks je messcherpe cynisme en voorliefde voor harde gitaarmuziek). Een blinde-geleide hond om het zo maar te zeggen. Je weet alles te vinden en kan deuren open maken. Veel blinden en slechtzienden (inclusief mijzelf) steunen op je om de weg te vinden; ik probeer dit tot een minimum te beperken. Jij klaagt hier niet (openlijk) over, maar uit zelfbescherming zou het misschien wel slim zijn iets vaker te delegeren en af-en-toe "nee" te zeggen. Volgens mij staat er weinig in de weg tussen jou en een glansrijke carrière in de wetenschap. Je bent sterk in de samenwerking, technische vaardigheden, organisatie en beschikt over een geheugen, waarnaast dat van mij op het geheugen van een goudvis lijkt. In elk geval; je speelt een hoofdrol in ons lab en dat lijkt me niet meer dan terecht!

Cees. Getalenteerd wetenschapper en bon-vivant. Wie zegt dat het AIO-schap een bittere tijd is, speelt het verkeerd. Verse koffiebonen, grillworst van de keurslager, een astronomisch aantal speciale bieren op de teller en een uitgebreide kennis van bijna alle soorten sport, muziek en films. Dit alles omdat je dat leuk vindt. Zo sta je ook in de wetenschap: het moet leuk zijn. Een hele gezonde mentaliteit, die ik zeker aanhang – proeven moet je doen omdat jij zelf in geïnteresseerd bent in het antwoord. Daar komt echte motivatie vandaan. Wie kan er zowel Ab Normaal, het kalkoen-geluid van Menno, of de tekst van het intro van de Snorkels perfect nadoen zónder te oefenen? Cees. Als ik ooit in een televisie-quiz terecht kom, bel ik jou als hulplijn, als ik een vreemd plan voor een proefje in mijn hoofd heb, doe ik precies hetzelfde.

Tobias. Beste Toby, mijn Duitse vriend. Mijn beleving van de Lange Nieuwstraat was bij lange na niet zo positief geweest als jij er niet was komen wonen, maar mijn slaaptekort was bij lange na niet zo groot geweest als jij niet naar Nederland was verhuisd. Het is een goede zaak voor mij dat je er nog een tijdje bent om zelf te promoveren; dan kunnen we nog een paar leuke dingen doen én één of twee biertjes drinken - Geen smoesjes.

Jeroen (je weet dat ik Grendel wil opschrijven). Eindelijk is het dan zover; heb ik dat boekje afgemaakt. Een van de belangrijkste punten van het bedrijven van wetenschap, maar ook het leiden van een goed leven, is het relativeren van problemen. Mede door jouw capaciteit in deze ben jij een buitengewoon gewaardeerde vriend; onze gesprekken hebben mij veel verder geholpen. Bovendien is het mij een plezier geweest om samen op zaterdagochtenden champignonnetjes te gaan kopen op de markt. Thanks!!!

Mademoiselle Alix, je bent het zonnetje in huis. Jouw optimisme en zorgzaamheid zijn geweldig; misschien dat ik me daarom kind aan huis voel bij jullie (Alhoewel de hete kip mij ook wel motiveert). Mochten jullie tezijnertijd jullie gezamenlijke toekomstplannen willen gaan uitvoeren, dan kunnen jullie op mijn steun rekenen.

Bas. Mijn studievriend en voor een jaartje ook een beetje collega. Ik ben blij om te zien dat je goed op je plek bent terechtgekomen, want dat pipetteren stond je niet (geintje). Ik denk dat je alleen goed kunt pipetteren als je ook goed kunt RA-en (oh nee, want Appie kan ook niet RA-en). Omdat je zo'n goede vriend bent, vergeef ik het je dat je bier drinkt met je pinkje omhoog en haal ik een extra zakje chips in huis. P.S. There is only one true Yuri!

Mark. Bijna altijd de rust zelve en in controle over (bijna) elke situatie, en ook zo goed als niet boos te krijgen (behalve door Bas). Vanaf het eerste jaar van onze studie zijn we samen opgetrokken. We hebben het meer dan eens zo laat gemaakt dat er suikerzakjes aan te pas moesten komen ;). Omdat je zo'n goede vriend bent, vergeef ik je je oosterse accent en zorg ik voor Pizza Hawai, want daar hou je van. P.S. Kermit; bijna altijd goed voor een tweede plaats op de lijst in CTF Coret Facility.

Remo. Wat een vent ben je toch! Je kwam voor een stage en was nagenoeg niet meer weg te branden. Waar de meeste mensen uit passieve motivatie zouden blijven hangen, deed jij dit omdat je iets uit de situatie wilde halen. Nu je uit het biomedisch onderzoek bent gestapt, vermoed ik dat je hebt gekregen waar je voor bent gekomen. Ik hoop het in ieder geval. Je hebt een onstuitbare drang naar het opdoen van nieuwe ervaringen, die ook (lichtelijk) op mij is overgeslagen. Helemaal niet erg, ben ik eigenlijk wel blij mee. P.S. Wist je dat tandenpoetsen met je linkerhand eigenlijk heel erg moeilijk is?

Appie. De überchap en de man die vijf dagen achter elkaar "Dude, where's my car?" kon kijken (Ja, dat heb jij echt gedaan). Ik ben blij dat we rond dezelfde tijd promoveren, precies zoals met elkaar hebben afgesproken. Elke keer als we langs het Academiegebouw liepen, zeiden we tegen elkaar: "Nog even en dan hebben wij hier een afspraak." Alleen daar al om, vind ik het ergens zonde dat het erop zit. Misschien moeten we een nieuwe gezamenlijke uitdaging kiezen. In elk geval, met jouw post-doc baan in Leiden en verhuisplannen wordt het iets lastiger om last-minute te maccen of een check-ronde te doen – blijkbaar levert het afronden van de ene uitdaging, weer een nieuwe uitdaging op. Net wetenschap!

Marcia. Hoi Marsje! Ik moet zeggen dat ik niet zo heel erg veel stress heb gehad tijdens dat hele promoveren en je zou honderd redenen aan kunnen dragen waarom dat niet zo was. Ik zou voornamelijk zeggen dat jij daar voor verantwoordelijk bent geweest. Met jou heb ik me zo vaak helemaal slap gelachen en de tranen op mijn wangen gehad. We hebben gewandeld over het strand, sneeuwballen gegooid, brandoefeningen in een winters Liverpool gehouden, rondgecrossed door een groot deel van Europa, gezwommen in de Dordogne en hebben samen de Borobudur beklommen. Herinneringen die niemand me meer afpakt. Je bent een prachtig natuurfenomeen; honderdduizend keer bedankt voor alles!

Imke. Zusje, ben je een beetje trots op me? Ik zeker op jou!

Peter en Ria. Zo, dat waren dan weer een paar jaar. Weer een beetje gegroeid, maar nog lang niet uitgegroeid. Uiteindelijk ben ik dus een soort van Max Laadvermogen geworden; een kinderdroom wordt werkelijkheid. Ik ben benieuwd wat de toekomst te bieden heeft, maar ik beloof dat ik goed voor mezelf zal zorgen. De mogelijkheid tot zelfontplooiing is een kostbare luxe, en ik wil jullie enorm bedanken voor het bieden van alle mogelijkheden hiervoor in sport, muziek en opleidingen. Heb ik even mazzel gehad met jullie! Enorm bedankt voor alles!

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 6 december 1980 te 's Hertogenbosch. Na het behalen van het Gymnasium diploma aan het St. Janslyceum te 's Hertogenbosch, begon hij met de opleiding Medische Biologie aan de Universiteit Utrecht. Als onderdeel van deze studie werd een onderzoeksstage voltooid bij het Laboratorium voor Medische Oncologie in het Universitair Medisch Centrum Utrecht, onder begeleiding van Dr. B. Bouma, Dr. M.F.B.G. Gebbink en Prof. dr. E.E. Voest. Hierna heeft hij een master-programma in Biomedical Science en Business Administration voltooid, met een stage bij Bioceros B.V., onder begeleiding van Prof. dr. M. de Boer. Van 2004 tot 2008 was hij werkzaam als Assistent in Opleiding bij het Laboratorium voor Klinische Chemie en Haematologie in het Universitair Medisch Centrum Utrecht, onder begeleiding van Dr. M.F.B.G. Gebbink, Prof. dr. B.N. Bouma en Prof. dr. P.H. de Groot.

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