The Biology of Non-Native Proteins

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The Biology of Non-Native Proteins

Relatie tussen structuur en biologische activiteit van misvouwde eiwitten
(met een samenvatting in het Nederlands)

Kóros fehérjék szerkezet és funkció kapcsolata
(magyar nyelvű összefoglalóval)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, Prof. dr. J.C. Stoof, in gevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 27 november des namiddags om 2.30 uur

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Just play. Have fun. Enjoy the game.
Michael Jordan

Anyának és Annácskának

Contents

Glossary of terms

Conformation: Three-dimensional shape of the polypeptide chain.

Native conformation: The intended conformation of a molecule in order to fulfil its specific biological function.

Protein folding: Packaging of polypeptide chains into their unique native conformation.

Denaturation: Disruption of native protein conformation.

Unfolding: Process leading to loss of the native protein conformation, with loss of function.

Misfolded protein: (Partially) unfolded protein with non-native conformation. Misfolded proteins have lost their unique function and acquired new structural and functional characteristics.

Aggregated protein: Clustering of misfolded proteins. Aggregated proteins are heterogeneous and contain defined common structural and functional characteristics.

Amyloid: Extracellular, fibrillar protein deposit exhibiting cross-β structure.

Cross-β structure: A quaternary structural motif of stacked β-sheets with β-strands at a distance of 4.7Å that run perpendicular to the length of an amyloid fibril.

Amyloid-β (Aβ or Abeta): A 40-42 amino acid long peptide described as a main constituent of protein deposits found in brain of patients with Alzheimer's disease.

Amyloidogenic protein: Protein with propensity to misfold, aggregate and form amyloid.

Glycation: Non-enzymatic covalent modification of a protein with carbohydrate moieties. Glycated proteins accumulate in affected tissue. They are reliable markers of diabetes mellitus. Glycation can result in protein misfolding.

Protein oxidation: Variation of modifications of protein side groups by oxygen free radicals including direct oxidation of amino acid residues of the protein backbone. Oxidation can lead to conformational changes through the rearrangement of the secondary and tertiary structure of proteins and is the characteristic for many pathological conditions such as atherosclerosis, neurodegenerative diseases and diabetes mellitus.

Platelet aggregation: Clustering of platelets through a cascade of events including shape change, secretion of platelet granules, thromboxane formation and crosslinking cell surface receptor GPIIbIIIa by fibrinogen.

Platelet agglutination: Clumping of platelets passively through the binding of protein components to platelet receptor GPIbα.

Chapter one

Non-native Proteins and Disease

Proteins are essential elements for life. They are building blocks of all organisms and the operators of cellular functions. Humans produce a repertoire of about 30.000 different proteins, each with a different role. Each protein has its own unique sequence and shape (native conformation) in order to fulfil its specific function. The appearance of incorrectly shaped (misfolded) proteins is associated with various, often highly debilitating diseases for which no sufficient cure is available yet. Here I will illustrate the structural aspects of proteins and the current knowledge underlying the forces that lead proteins to lose their native structure and the consequences of protein misfolding in disease. Finally, I will describe recent efforts that have been made to develop treatments for misfolding diseases.

1. The appearance of proteins

1.1. The structure of proteins

Proteins are molecules composed of an amino acid chain, in which each amino acid is connected to the next one by a peptide-bond. Proteins are very diverse and vary in size from small peptides to large multimers. The common element of proteins is the peptide-backbone formed by peptide bonds that link the amino acids. The variation among peptides relies upon the sequence of the amino acids and their side groups. There are 20 different amino acids and they have either acidic, basic, neutral or hydrophobic side chains. The order of the amino acids determines the *primary* structure of the protein by creating a unique polypeptide chain, which is relatively flexible (Figure 1). Polypeptides can fold into three main *secondary* elements, the α-helix and the β-sheet, that determine the three-dimensional structure of the protein; and the random coil, which has less ordered inter-chain amino acid side-chain interactions, leaving free rotation around each bond. The *tertiary* structure refers to the distribution of the α-helices and β-sheets and random coils in the protein, where these elements are folded into a compact conformation stabilized by hydrogen bonds or ionic interactions. The term *quaternary* structure is used for the description of multimeric proteins, in which the different polypeptide chains are connected (Figure 1).

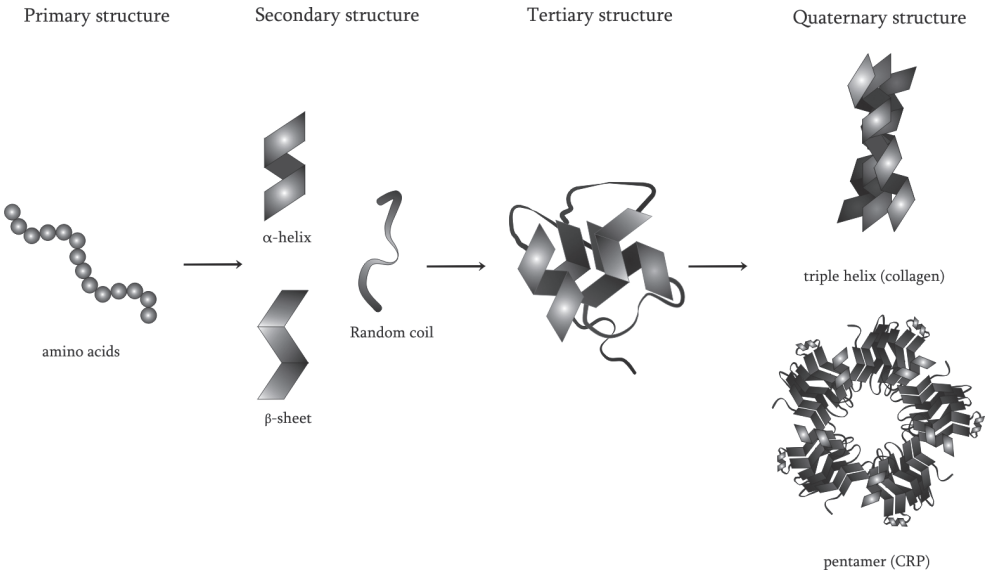

Figure 1. Schematic representation of the structural components of proteins. The order of the amino acids determines the primary structure of the protein, which can fold into secondary elements, α-helix, β-sheet or random coil. The distribution of secondary elements ascertains the *tertiary* structure that is stabilized by hydrogen bonds or ionic interactions. Finally the *quaternary* structure describes the connection of polypeptide subunits in multimeric proteins.

1.2. The correct protein folding

Protein folding is the process by which the newly synthesized protein molecule folds into its unique, three-dimensional structure. The primary product of the protein synthesis is the linear amino acid chain, which lacks any three-dimensional structure. To become functional, the protein has to be packed into its particular *native* conformation. In the cell, a variety of proteins named chaperones assist the newly synthesized polypeptide to attain its native conformation. Some chaperones are highly specific in their function; others are very general and can be of assistance to most globular proteins. Protein folding is a very complex process and the molecular mechanisms responsible for protein assembly are of the most elemental open question in biochemistry. The energy landscape theory that was first proposed by Joseph Bryngelson and José N. Onuchic (2;3), states that folding of a protein does not follow a singular, specific pathway; it is in fact a rather complex self-organizing process that generally does not occur through an obligate series of intermediates, but through routes down a folding funnel (Figure 2)(4). The energy of the different conformations decreases with the development of organized, native-like properties. On the highest energy level, proteins do not comprise ordered structures. As the proteins fold more into the native conformation and the secondary structural arrangements appear at certain positions of the polypeptide

chain, they shift to a lower energy phase. At the end of the correct folding procedure, as the proteins obtain their correctly packed native conformations with a unique set of α-helical and β-sheet motifs, they find their energy minimum. The drive for the energy minimum makes the correct protein folding highly efficient and very rapid. Thermodynamically, the folding process is described as an energy funnel (5), where the unfolded states are characterized by a higher degree of conformational entropy (S) and free energy (G) than the native (6). The facade of this folding funnel is unique for a specific polypeptide sequence under a particular set of conditions. By definition, entropy is a measure of chaos, the amount of all different conformational states that the protein can attain. Free energy stands for the amount of thermodynamic energy in a system which can be converted into work. The unfolded state is associated with more chaos, higher entropy and free energy, which leads to the unstableness of the three dimensional structure. Therefore, as folding proceeds, the narrowing of the funnel represents a decreased number of conformational states as well as lower free energy (Figure 2).

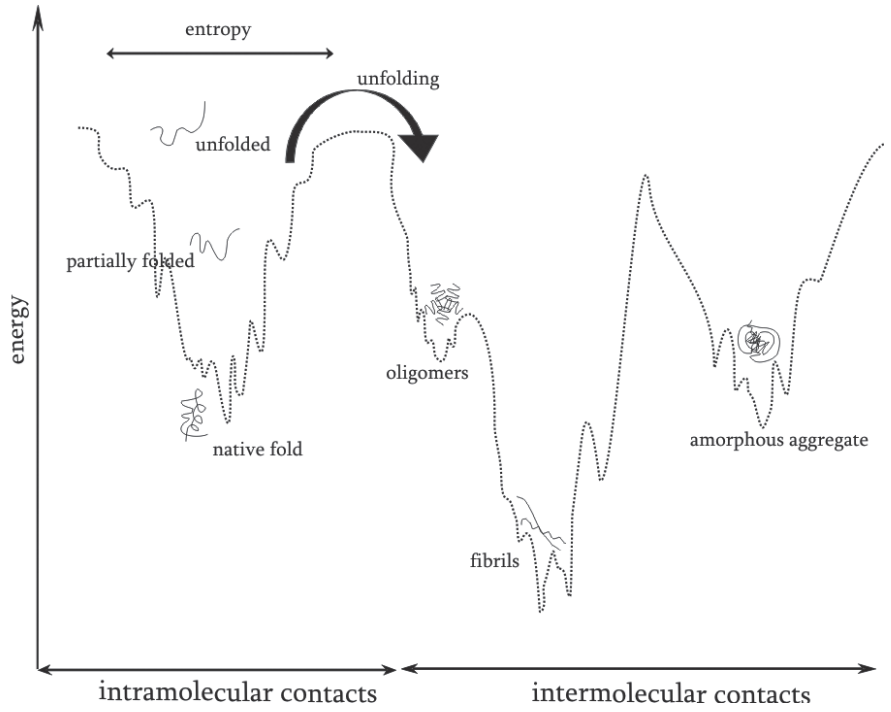

Figure 2. Energy state of protein folding under physiological and misfolding conditions (1). *The shape of the graph shows energy state of the protein conformations moving towards its native or misfolded condition through multiple inter- and intramolecular contact arrangements.*

At the bottom of the funnel, which is also known as the global minimum, the folding alternatives are reduced to a single conformation. Although the free energy funnel is generally described with one global minimum that corresponds to the native conformation; proteins can have a whole set of different native conformations, which are important for fulfilling its biological function. The very rapid and efficient search for the native state is encoded by a network of interactions between 'key residues' in the structure, forming a folding nucleus that establishes the native topology in the transition state ensemble (the folding transition bottleneck) (7).

1.3. Protein misfolding, aggregation and amyloid formation

Protein misfolding is a common and intrinsic propensity of proteins that occurs continuously. Misfolding is influenced by the amino acid composition and certain mutations are known to accelerate the process. Moreover, it also depends on environmental conditions, since once they are exposed to specific environmental changes such as increased temperature, high or low pH, agitation, elevated glucose or oxidative agents (Table 1), proteins can lose their native conformation more rapidly. The process, wherein the native state is disrupted is called denaturation and it generally results in the unfolding of the proteins. Due to the lack of arrangement, unfolded proteins are non-functional. Importantly, the unfolded state is thermodynamically unfavourable and unstable (Figure 2). Seeking for lower energy levels and more stability, unfolded proteins have a tendency to aggregate.

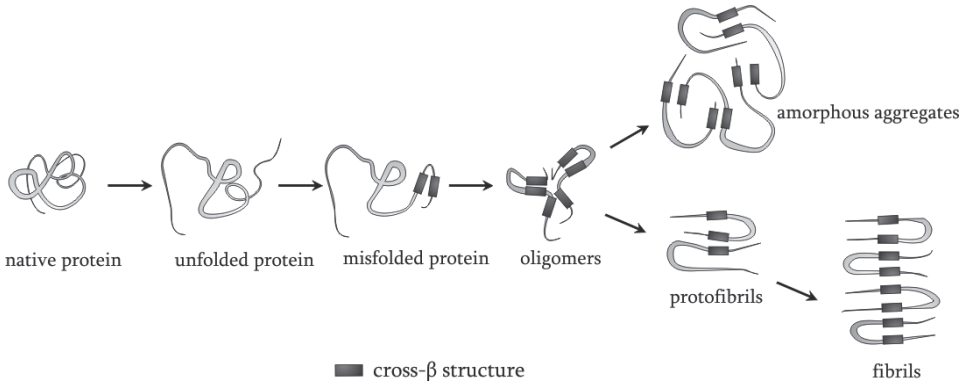

Figure 3. Protein misfolding and aggregation. Under certain circumstances such as pH or temperature change, mechanical stress, glycation or oxidation; proteins undergo conformational changes that results in unfolding and partial misfolding that is associated with the tendency to aggregate. During aggregation, proteins can obtain a range of different structural appearances, which are generally enriched in cross-β structure, including intermediates varying from unordered amorphous aggregates to ordered fibrils that are called amyloid.

Subsequent to protein unfolding, aggregation starts which consists of two parts. The first one is the nucleation, when proteins reversibly attach to a growing core. When the nucleus crosses the threshold of a critical mass, the second part begins, wherein further protein molecules attach irreversibly to the core developing a large aggregate. Studies with small fibre-forming peptides have shown that during aggregation, the free energy change depends on the concentration of monomer. At low concentrations the monomeric, at high concentrations the aggregated state is favoured because of the large barrier that needs to be overcome to resolve the aggregates (8), which makes the aggregate highly stable. The aggregation procedure is thought to be initiated by protein segments with hydrophobic amino acid residues, β-sheet predisposition and low net charge (9). These segments are the precursor cores, which can facilitate further aggregation. Depending on the protein, there are various alternatives concerning how the precursor is generated from native proteins. These are incomplete or incorrect proteolysis (10) or introduction of a misfolded variant (11;12) that undergoes partial misfolding introducing a precursor pool from which they rapidly aggregate (13). Protein aggregation can result in various different structural appearances with intermediates (oligomers) varying from unordered amorphous aggregates to highly ordered fibrils that are called amyloid (Figure 3). They are generally enriched in cross-β structure (14), yet fluctuate in sequence, time and conditions (15;16). One specific form of the aggregated proteins is amyloid conformation. Amyloid consists of linear, unbranched protein or peptide fibrils. Contrary to the well-known fibrillar proteins such as collagen triple helix or keratin helices, amyloid is exceptional amongst other, naturally occurring biological fibrils (17). The amyloid fibrils bind the fluorescent probes Congo red and Thioflavin derivatives (18;19) and they share a common secondary composition, the cross-β structure (20). The cross-β structure was initially identified by X-ray analysis, which demonstrated a strict arrangement, where the β-strand direction is perpendicular to the fibril axis (Figure 4) (21;22). However, since recent progress in biophysical techniques allows researchers to gain a more detailed view on fibril structure, it is becoming more obvious that there is no universal tertiary and quaternary structure for amyloid fibrils. They can attain parallel (23;24) or antiparallel β-sheet conformation (Figure 4B)(25;26) and other structural elements (20).

Thus, besides their common characteristics, amyloids present conformational plasticity, the ability to adopt more than one stable tertiary fold that accounts for conformational differences within fibrils formed by one single polypeptide. These structural variations raise the question, what the precise composition of cross-β structure is. The work of David Eisenberg and colleagues offers answers to fundamental questions concerning the structural characteristics for amyloid proteins (27). Using X-ray microcrystallography, they investigated

small peptides that developed both microcrystals and fibrils, which consisted of a pair of β-sheet and each sheet was formed by β-strands. One β-strand corresponds to a single peptide and these β-strand peptides compose β-sheets, which is therefore termed steric zippers. These steric zippers (β-sheet pairs) repeated all along the entire needle-shaped crystals. Eisenberg extended his observations to the structure of various amyloid proteins and found divergence between the basic arrangements of steric zippers in amyloid fibrils. He categorized the amyloid proteins into eight classes depending on three structural principles. First, whether their β-strands were antiparallel or parallel; second, whether the β-sheets were packed "face-to-face" or "face-to-back" and third, whether the orientation of the β-sheets were "up-up" or "up-down" with respect to each other (Figure 4C) (28). Most of the eight described variations of steric zippers have been observed in amyloid proteins such as tau, prion, amyloid-β and insulin that are associated with protein misfolding diseases.

Table 1. Summary of protein modifying conditions and their affected proteins or peptides

Modifying conditions	Affected proteins	References
mutations	α-synuclein, amyloid-β	(29), (30)
increased temperature	streptokinase, β-lactoglobulin	(31), (32)
high pH	PABP2, concanavalin A	(33), (34)
low pH	fibroblast growth factor 20, vesicular stomatitis virus glycoprotein G	(35), (36)
increased hydrostatic pressure	lysozyme, insulin	(37), (38)
high shear rate	β-lactoglobulin	(39)
high ionic strength	amyloid-β, melittin	(40), (41)
agitation	β_2-microglobulin, glucagons	(42), (43)
negatively charged surfaces	Ig light chain fragments	(44)
air-water interface	amyloid-β, ovalbumin	(45), (46)
phosphatidylserine	lysozyme, insulin, myoglobin, G3PD, transthyretin, cytochrome C, histone, α-lactalbumin, endostatin	(47), (48)
glycation	APP, albumin, IAPP	(49), (50)
oxidation	Superoxide dismutase, LDL, ApoC-II	(51), (52), (53)

Abbreviations in Table 1: PABP2, poly(A) binding protein 2; G3PD, Glyceraldehyde 3-phosphate dehydrogenase; APP, amyloid precursor protein; IAPP, islet amyloid polypeptide; LDL, low density lipoprotein; ApoC-II, Apolipoprotein C-II

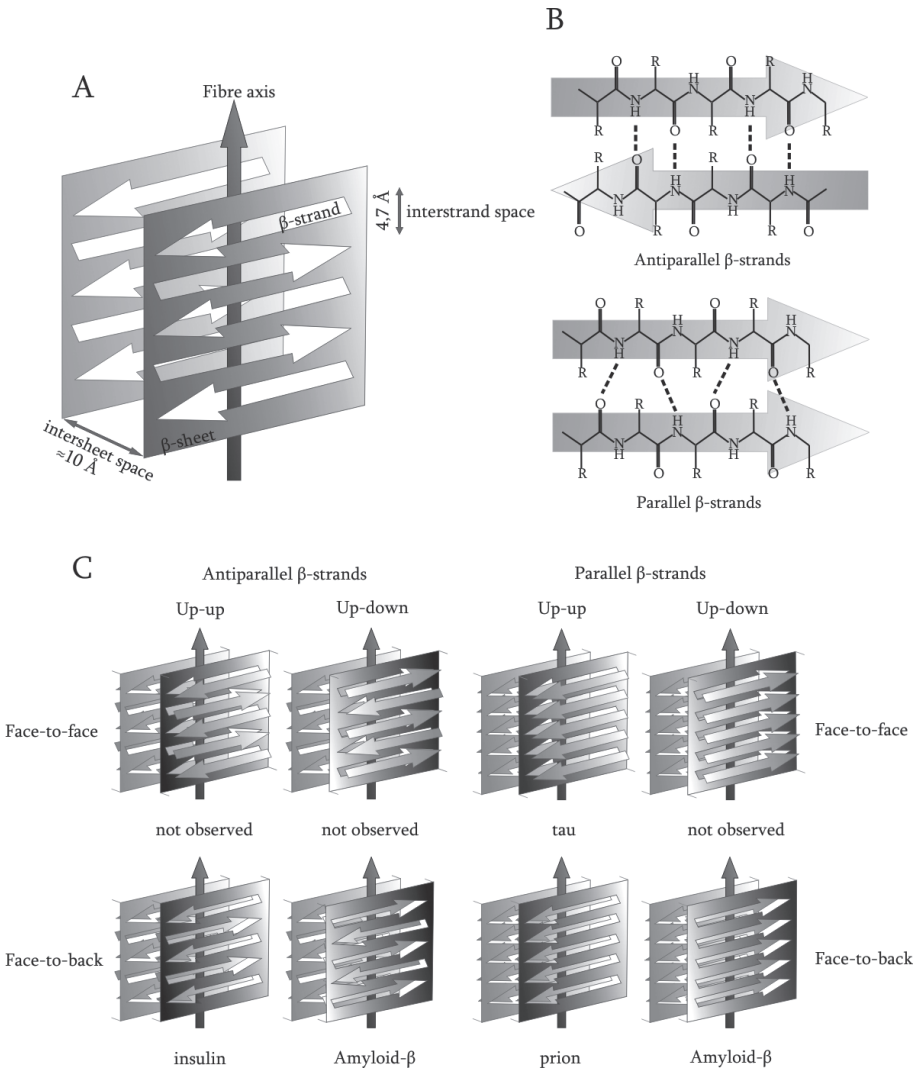

Figure 4. Composition of amyloid cross-β structure.
A: Proteins with amyloid cross-β structure are characterized by 4.7Å interstrand and approximately 10Å intersheet space. B: The arrangement of two β-strands can be antiparallel or parallel depending on their relative direction. C: Based on the position of the β-strands (antiparallel or parallel) and β-sheets (face-to-face or face-to-back and up-up or up-down), there are eight structural categories of amyloid proteins. Two represents amyloid-β protein conformations, whereas tau, insulin and prion have one single structural appearance.

2. Protein misfolding diseases

Protein misfolding diseases represent a group of disorders that have protein aggregation and plaque formation in common. This section provides an overview on the current understanding of protein misfolding diseases. It describes the most studied diseases of the main categories (Table 2) and illustrates the common mechanisms of protein misfolding in protein misfolding diseases.

2.1. Amyloidosis

Amyloidosis is a group of protein misfolding diseases, in which protein aggregates accumulate either systemically or locally in certain organs or tissues (Table 2). Amyloidosis can be either hereditary or acquired (54). The hereditary forms are caused by mutations in the genes of (mostly) plasma proteins. Little is known about the precise conditions that lead to the development of the other, acquired forms. In amyloidosis patients, various symptoms can arise depending on which protein is involved and at what site the aggregation occurs. Most patients have gastrointestinal abnormalities, kidney insufficiencies or heart problems and the affected organs are characteristically enlarged, rubbery and firm.

The most common form of amyloidosis is the primary systemic amyloidosis, in which immunoglobulin light chains or fragments thereof that are produced by plasma cell clones form amyloid. The disease typically develops in individuals after the age of 40 and can affect both genders. The amyloid deposits occur in basically all organs of the body, inducing various complications including congestive cardiac and renal failure; hepatosplenomegaly, neuropathy and skin lesions (55). The diagnosis of the disorder is rather difficult; it requires histological analysis, definition of the correct amyloidosis, and the characterization of the underlying plasma cell clone (56). Unfortunately, efficient therapy is not available for most of the amyloidosis forms. The current treatment aims to reduce the supply of the monoclonal light-chain by suppressing the underlying plasma clone, by autologous stem cell transplantation and chemotherapy. This is an aspecific approach with high potential risk (57).

2.2. Alzheimer's disease

Neurodegenerative diseases were the first described protein misfolding diseases. Alzheimer's disease is one of the most studied conformational diseases. The small 40-42 amino acid long amyloid-β peptide aggregates, accumulates and forms senile plaques in the brain of Alzheimer's disease patients. Amyloid-β is cleaved off from its amyloid precursor protein (APP) by β- and γ-secretases (Figure 5). When the cleavage occurs in the endoplasmatic

reticulum, the γ-secretase generates 42 amino acid long Aβ1-42. However, if it is in the trans-Golgi network, a shorter 40 amino acid long Aβ1-40 is formed. APP is expressed on a wide variety of cell types including neurons, leukocytes, platelets and epithelial cells. APP homologs have been described in evolutionarily distant organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*. APP contains a Kunitz-type protease inhibitor (KPI) (58) and the soluble form of APP is found to be a growth factor-like molecule for the proliferation of neuronal stem cells (59), epithelial cells (60) and keratinocytes (61). According to the amyloid cascade hypothesis (ACH), which was first introduced by John Hardy and David Allsop in 1991, the cleaved and unstable amyloid-β peptide starts to aggregate into large protein assemblies, which subsequently deposit into plaques in the brain and these large aggregates cause neurotoxicity (62). However, it has been described that the soluble oligomers, which are the intermediate products of the fibril formation process are actually more harmful for neuronal cells than the fibrillar end products (63) (the molecular basis and cellular mechanisms for cytotoxicity will be further discussed in section 3.).

Table 2: Summary of protein misfolding diseases and the related proteins and peptides in human.

Diseases	Associated proteins	Affected tissues	References
Amyloidosis – systemic			
Primary systemic amyloidosis (AL)	Ig light chain	most tissues	(54)
Immunoglobulin heavy-chain-associated amyloidosis (AH)	Ig heavy chain	most tissues	(64)
Secondary (reactive) systemic amyloidosis (AA)	SAA	most tissues	(65)
Senile systemic amyloidosis (SAA)	transthyretin	microvasculature	(13)
Hemodialysis-related amyloidosis	β_2-microglobulin	osteoarticular tissues	(66)
Hereditary systemic ApoAI amyloidosis	Apolipoprotein A-I	liver, kidney, heart	(67), (68)
Hereditary systemic ApoAII amyloidosis	Apolipoprotein A-II	kidney, heart	(69), (68)
Finnish hereditary amyloidosis	gelsolin	most tissues	(70)
Hereditary lysozyme amyloidosis	lysozyme	kidney, liver	(71)
Hereditary cystatin C amyloid angiopathy (HCCAA)	cystatin C	most tissues	(72)
Amyloidosis – localized			
Injection-localized amyloidosis	insulin	skin, muscles	(73)
Hereditary renal amyloidosis	fibrinogen	kidney	(74)
Senile seminal vesicle amyloid (SSVA)	lactoferrin, semenogelin	seminal vesicles	(75), (76)
Familial subepithelial corneal amyloidosis (FSCA)	lactoferrin	cornea	(77)
cataract	crystallin	eye	(78)
Medullary thyroid carcinoma (MTC)	calcitonin	thyroid tissues	(79)

Table 2: Summary of protein misfolding diseases and the related proteins and peptides in human.

Diseases	Associated proteins	Affected tissues	References
Neurodegenerative diseases			
Alzheimer's Disease	amyloid-β, tau	brain	(80) (81)
Parkinson's Disease	α-synuclein	brain	(82)
Lewy-body dementia	α-synuclein	brain	(83)
Huntington's Disease	huntingtin	brain	(84)
Spongiform encephalopathies	prion	brain, periferial nervous system	(85), (86)
Hereditary cerebral haemorrhage with amyloidosis	cystatin C	cerebral vasculature	(87), (88)
Amyotrophic lateral sclerosis	superoxide dismutase 1	brain	(89)
familial British dementia	Abri	brain	(90)
familial Danish dementia	ADan, amyloid-β	brain	(91)
familial amyloidotic polyneuropathy	transthyretin	periferial nervous system	(92)
fronto-temporal dementias	tau	brain	(93)
Other diseases			
diabetes mellitus	IAPP, amylin	pancreas (islet)	(94)
atherosclerosis	modified LDL	arteries	(95)
sickle cell anemia	hemoglobin	erythrocytes	(96), (97)

Abbreviations in Table 2: Ig, Immunolglobulin; SAA, Serum amyloid A; LDL, low density lipoprotein; IAPP, Islet amyloid polypeptide; ABri, British amyloid peptide; ADan, Danish amyloid peptide

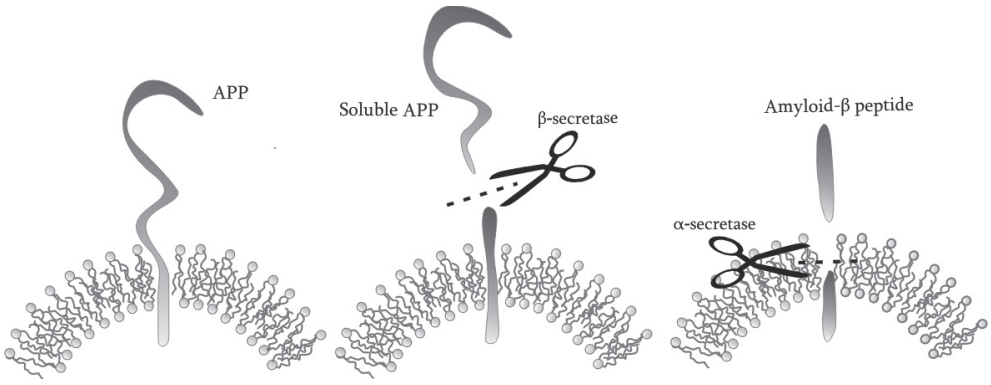

Figure 5. Cleavage of APP by secretases results in release of soluble APP and amyloid-β.

2.3. Vascular inflammation and atherosclerosis

1.1.1. Vascular inflammation

Inflammation is a vascular response to cellular damage, infection, or other harmful irritations. Inflammation can be acute and chronic. The acute form is a crucial defence mechanism, which occurs at the sites of infection, physical irritation and tissue necrosis. As the first step of the battle, leukocytes, monocytes and neutrophils are mobilized from the blood. Monocytes transmigrate through the endothelial line into the endangered area though a cascade of adhesion molecules (98). This process is directed by the endothelial cells, which follow a strictly timed expression of molecule expression. The first step is the expression of early adhesion molecules such as selectins (E-selectin), which initiate the rolling of the monocytes on the surface of the endothelial cells. The second step is the exposure of intercellular adhesion molecules (ICAM) and vascular cell adhesion molecules (VCAM) by the endothelial cells. ICAM and VCAM support stronger cell-cell interactions and adhesion by binding their counter-receptors on monocytes. Finally, a rapid transmigration of the blood cells takes place through the intercellular endothelial junctions. Chronic inflammation shares the same processes with the acute form, but its duration is prolonged to weeks, years or even indefinite (99).

2.3.2. Atherosclerosis

Atherosclerosis is a disease of the vasculature, that is characterized by chronic inflammation and thickening of the intima layer of arteries (plaque formation). Atherosclerosis can start early, even before the age of 20. Its progress is enhanced in some individuals and lasts throughout life (99). High cholesterol levels and blood pressure, smoking, diabetes mellitus, and mutations in genes related to depositions in the vessel wall matrix, cytokines and lipid metabolism are risk factors for atherosclerosis (100).

The site of atherosclerosis is the atherosclerotic plaque, which is the thickening of the arterial wall. It contains fat, cholesterol, calcium, lipoproteins and cells such as macrophages (foam cells) derived from the blood. In the very first step of the disease, cells start to express adhesion molecules (E-selectin, ICAM, VCAM), and cytokines in the same manner as in acute inflammation. The consequence of the prolonged vascular inflammation is that the plaque expands while the vessel wall loses its elasticity and it narrows the blood stream (stenosis), blocking the blood supply to the tissues. The unpredictable, sudden disruption (rupture or erosion/fissure) of an atherosclerotic plaque and release of its contents to blood leads to platelet activation and thrombus formation, which is called atherothrombosis.

Recently, it has been reported that up to 60% of the atherosclerotic lesions contain fibrillar proteins (101). Of the proteins known to misfold *in vivo*, several members of the apolipoprotein family (apo-B100, apoA-I, apoA-II, apoE, serum amyloid A) as well as amyloid-β and α_1-antitrypsin, are present in atherosclerotic lesions (102). Low density lipoprotein oxidation in plasma and in the arterial wall is a key event that leads to the formation of atherosclerotic lesions. Following its uptake mediated by scavenger receptors, oxLDL induces foam cell formation from macrophages and triggers an array of proatherogenic events (103). Protein modifications such as oxidation or glycation induce protein misfolding (see Table 1). The deposition of misfolded proteins in the artery wall may contribute to reduced elasticity of the blood vessels and the initiation of vascular inflammation and atherosclerosis (102).

The current treatment of atherosclerosis involves thrombin inhibitors (104) and cholesterol lowering medication for instance statins (105). Side effects of use of statins are liver damage (106), and muscle complications (107). Since platelets play a critical role in the thrombotic events of atherosclerosis after plaque rupture, antiplatelet therapy (108) gains special importance.

2.3.3. Platelet activation

Platelets are prominent components of the thrombi that occlude arteries. Platelets are present in blood and their role is to arrest bleeding from wounds. Platelet aggregation is initiated by agonists (thrombin, collagen, ADP, adrenalin, thromboxane A2 (TxA_2), serotonin, vasopressin) and has three subsequent phases: shape change, aggregation and degranulation (109). In the resting state, their shape is discoid; and upon activation they become spiny spheres with pseudopods. Platelet shape change results from a rapid reorganization of the cytoskeleton (110). The storage granule membranes fuse with the plasma membrane resulting in the release of their content to the blood. The release of dense-granule ADP and the secretion of generated TxA_2 results in interaction with platelet surface receptors ending up with a positive feedback mechanism. The ultimate result of platelet aggregation is the opening of GPIIbIIIa (αIIbβIII) receptor on their surface. Fibrinogen can then bind to its receptor, and form bridges between adjacent platelets leading to the formation of a platelet plug (111). The activation of the platelets plays central role in arterial thrombosis.

In disorders that are associated with protein misfolding, platelet activation is observed. In Alzheimer's disease, elevated levels of the platelet activation marker thromboxane B_2 were detected (112). Furthermore, amyloid-β is capable of inducing platelet aggregation *in vitro* (113). Atherosclerotic plaques contain oxidized low density lipoprotein (LDL) which has amyloid properties (114) and activates platelets (115). In diabetes mellitus, high blood glucose

can introduce amyloid properties in albumin (50) that might contribute to the hyperactivity of platelets in these patients (116). Several types of systemic amyloidosis are also known to be associated with thrombosis (117;118).

Interestingly, after activation with various stimuli, platelets express amyloid-β protein (119). Amyloid-β is detected in platelet-derived microparticles in healthy subjects (120) and the level of these microparticles is elevated in patients with atherosclerotic disease (121) and diabetes mellitus (122). These particles are sources of tissue factor (123), which is the prime initiator of blood coagulation. This property, together with the capacity of amyloid to activate platelets (124) makes these particles potent triggers for a combined activation of the coagulation cascade and formation of a platelet thrombus. The platelet-derived amyloid accumulates in the brain parenchyma and cerebrovasculature and may contribute to the development of Alzheimer's disease (125).

2.4. Links between protein misfolding diseases

A variety of diseases share the pathological feature of protein deposits, which form plaques causing altered organ function or failure (Table 2). These protein misfolding diseases share similar pathological aspects including hypertension (126), oxidative stress (127) or hyperglycaemia (128), which are associated with aging and protein misfolding (129;130). The co-occurrence of unrelated symptoms with protein deposition in common supports the importance of protein misfolding in the progress of diseases. Atherosclerosis is associated with an increased risk of dementia and Alzheimer's disease (131;132) and share pathophysiological elements such as elevated cholesterol, inflammatory processes and mutations in gens such as CD36 and Apoliporprotein E (133;134). Alzheimer's disease can also be developed as a secondary disorder during the progression of diabetes mellitus (135;136). Secondary amyloidosis was reported to arise in patients suffering from amyloidosis (137).

3. Molecular basis of protein misfolding diseases

Protein misfolding diseases comprise a group of disorders that have one central aspect in common, the appearance of non-native protein structure, which is accompanied by increased aggregation and deposition of proteins. The group contains diseases with dissimilar symptoms and the affected organs and tissues can be drastically different. This section describes the causes of protein misfolding diseases and attempts to describe the mechanisms that lead to toxicity. In addition, a summary of the misfolded protein binding molecules is provided and their role is discussed.

3.1. The causative agents of protein misfolding diseases

The toxic nature of misfolded proteins is obvious, but there is disagreement about the properties of misfolded proteins that cause toxicity. The presence of protein aggregates and fibrils in patients suffering from protein misfolding diseases lead the researchers to the conclusion that the highly organized protein deposits are the primary cause of the pathological symptoms of the disease (138). According the amyloid cascade hypothesis (ACH), the large fibrillar protein deposits are the main cause of neuronal damage. The *in vitro* observations that the fibrillar amyloid-β being toxic to cultured neuronal cells (139) by initiating membrane depolarization and action potential frequency alterations (140) and the *in vivo* data that demonstrates neuronal failure and microglial activation upon injection of fibrillar amyloid-β preparation into the cerebral cortex of aged rhesus monkeys (141) support the ACH theory (142).

However, recent studies suggest that prefibrillar aggregates, called micelles, protofibrils, or ADDLs (beta-amyloid-derived diffusible ligands) rather than fibrils are most potent mediators of cell damage, cyto- and neurotoxicity. These soluble oligomers are temporally unstable and rapidly changing into more mature, and eventually fibrillar forms This is supported by the finding that the severity of cognitive impairment in protein misfolding diseases correlates with the levels small oligomeric aggregates and not with the large fibrillar species (143-146).

3.2. Cellular mechanisms of toxicity

Depending on the particular protein, the environment and the conditions of precursor core generation, protein aggregation has different dynamics. It is difficult to define a general impact of the different appearances of aggregated proteins on cells. The machinery by which they disturb cellular functions is not entirely understood. The identified mechanisms that might be responsible for the cellular damage are initiated by mainly soluble, oligomeric protein aggregates, which disrupt cell membranes by inserting into membranes, disturbe normal ion gradients (147); inactivating normally folded, functional proteins and obstructing proteasome components or chaperone proteins (148). Oligomeric species of amyloid-β, amylin and α-synuclein form amphiphilic, micelle-like aggregates in solution (149) and annular pores in lipid bilayers including cell membranes (150;151) that initiate membrane permeabilization, a common component of misfolded protein toxicity (152). Subsequently, numerous downstream pathological events are stimulated in the cells, such Ca^{2+} immobilization (153), induction of endoplasmic reticulum (ER) stress (154), generation of reactive oxygen species (ROS) (155;156) and finally, cell death (157). Possibly, cell death

induced by misfolded and aggregated proteins is a consequence of stimulation of apoptotic responses (158). Apoptosis proceeds through ROS and Ca^{2+} increase that is followed by the activation of caspases (159). In addition, mitogen-activated protein kinase (MAPK) pathway activation has been observed in protein misfolding diseases (160;161). The MAPK pathway contributes to a number of reactions when the cell responds to various stressful conditions such as oxidative stress, cytokines and heat shock (162-165).

In addition to direct toxic effects, misfolded proteins promote inflammatory responses (133). In the brain of Alzheimer's disease patients, neuroinflammation is characterized by local stimulation of the complement system, an acute phase response, increased expression of C-reactive protein (CRP), activation of inducible nitric oxide synthase (iNOS) and prostanoids generating cyclooxygenase-2 (COX-2) (166;167). It has been found that amyloid-β activates microglia cells (168), which then release a variety of proinflammatory mediators including cytokines, reactive oxigen species, complement factors, secretory products, free radical species and NO, which all contribute to chronic inflammation and neuronal cell death (169). Chronic inflammation is not restricted to Alzheimer's disease; similar processes have been observed in most protein misfolding diseases (13;170-172). For example, microglia cells have a similar role in neuroinflammation than macrophages that are key players in the pathology of atherosclerosis and diabetes mellitus (173). They share the same progenitors, are able to phagocytose debris and produce cytokines, ROS and other secretory products (174). Macrophages express pattern recognition receptors, including scavenger receptors and toll-like receptors (175). These receptors, which participate in the removal of foreign subtances and debris (see 3.3) compose an important part of the innate immune system (176).

3.3. Misfolded protein binding molecules

There are various molecules, cell surface receptors and soluble components that associate or colocalize with misfolded proteins at the sites of the deposits (Table 3). Most of them are cell surface receptors, others are soluble proteins. The most common misfolded protein binding receptors are the scavenger receptors. In contradiction with the one receptor-one ligand classical theory, pattern recognition receptors, such as the scavenger receptors are exceptional since they recognize multiple ligands. The exact nature of the ligand-receptor interaction is not known. Hence, one important task is to determine a common ligand structure in order to understand how such diverse proteins can interact with the same receptor.

CD36 is a class B scavenger receptor that was first described as a platelet glycoprotein (177). CD36 was thought to be a receptor for thrombospondin, and then it

was recognized as a multiligand scavenger receptor with remarkably diverse ligands such as apoptotic cells, modified LDL, amyloid-β, erythrocytes infected with malaria and anionic phospholipids (for references, see Table 3). Accordingly, there are many biological processes in which CD36 is involved, i.e. cardiovascular diseases, malaria infection, renal failure and cancer.

The receptor for advanced glycation end products (RAGE) is associated with diverse pathological conditions including late diabetic complications, neuropathy amd chronic inflammation (178). In addition to advanced glycation end products, many more ligands have been described for RAGE. Most of them are proinflammatory molecules or particles derived from necrotic cells (see references in Table 3).

The LDL-receptor related protein (LRP) is a large cell surface receptor that recognize various ligands such as lipoproteins, proteinases, extracellular matrix proteins, bacterial toxins, viruses and intracellular proteins (179).

There are various hypotheses about the cellular mechanisms that cause misfolded proteins-triggered inflammation and cytotoxicity. RAGE and LRP are known to have a crucial role in regulation of the amyloid-β levels under physiological conditions in the brain (180) since the transport of amyloid-β through the blood-brain barrier is mediated almost exclusively by the strictly balanced work of the two receptors. While LRP clears amyloid from the brain parenchyma into the cerebral capillaries, RAGE mediates the transcytosis of the protein (180).

The serine protease tissue-type plasminogen activator (tPA) is a key component of blood clot lysis, i.e. fibrinolysis. After the binding of tPA to fibrin, plasminogen is converted by activated tPA to active plasmin, which then chops the fibrin network into small pieces. As a novel and more general function for tPA beyond fibrinolysis, our group described that the appearance of the common cross-β structure in proteins without sequence similarities enables tPA to bind and mediate plasmin formation (181). The role of tPA in Alzheimer's disease is under investigation. In mouse models, injected amyloid-β is cleared by tPA (182). However other reports showed that amyloid-β can lead to excessive tPA activation and toxicity (183). This is in line with other observations showing that elevated tPA is neurotoxic (184).

Serum Amyloid P (SAP) is an acute-phase serum protein and its physiological role is not completely understood. It binds negatively charged carbohydrates and colocalizes with the protein deposits in the brain of Alzheimer's disease patients (185) or Haemodialysis-related amyloidosis (186). When radiolabeled it can be used to visualize amyloid in vivo in humans (187).

Complement component C1q binds to amyloid-β and is colocalized with thioflavine-positive plaques in the brain of Alzheimer's disease patients (188). It forms complexes with β_2-microglobulin in the sera of hemodialysis-related amyloidosis (189). Interestingly, the binding site is localized to the C1q collagen-like residues, a novel interaction site for antibody-independent activation of C1 (190). Furthermore, C1q binds to the modified form of the prion peptide (191).

Clusterin (ApoJ) is a constitutively secreted extracellular chaperone that binds to exposed hydrophobic regions of on non-native proteins (192). Clusterin binding is restricted to oligomeric protein species of lysozyme and amyloid-β that are present at low concentrations during the nucleation phase of the aggregation reaction (193;194).

3.4. Naturally occuring amyloid proteins

Recently, it has been proposed that amyloid formation is not exclusively associated with pathological conditions, but also occurs under physiological conditions. This is a fascinating observation, but whether these amyloids are functional *in vivo* remains to be established.

For example, nonpathogenic amyloid has been detected in mammals in melanosome biogenesis and melanin formation (195). Melanin is a pigment particle that protects the skin against pathogens, toxic small molecules and UV radiation. It is present in most eukaryotic phyla ranging from fungi to insects and humans. Moreover, amyloid fibril formation was identified on the surfaces of fungi and bacteria such as the Curli and Tafi fibrils of *E. coli* and *Salmonella;* or hydrophobins on fungi (196). These bacterial and fungal amyloid derivatives play a crucial role in the invasion of the host.

Table 3. Binding molecules for misfolded proteins

Type	Receptors	Ligands	Diseases or syndroms
Scavenger Receptor Type A	SR-AI and II, MARCO	modified LDL (197), modified albumin (198), lipoteichoic acid (199), Gram negative and positive bacteria (200;201), apoptotic cells (202), advanced glycation end products (203)	atherosclerosis, diabetes mellitus (204), cancer (205)
Scavenger Receptor Type B	CD36, SR-BI, SR-BII	thrombospondin (206), modified LDL (207), apoptotic cells (208), *Plasmodium falciparum* malaria parasitized erythrocytes (209), sickle erythrocytes (210), anionic phospholipids (211), fibrillar amyloid-β (102), HDL (212), anionic phospholipids (211), advanced glycation end products (203)	cardiovascular diseases (213), atherosclerosis (177);(214), renal failure (215)
Scavenger Receptor Type E	LOX-1	oxidized LDL (216), advanced glycation end products (217), aged/apoptotic cells (218), activated platelets (219), bacteria (220)	atherosclerosis (221), diabetes mellitus (216)
Ig superfamily	RAGE	advanced glycation end products (222), HMGB1 (Amphoterin) (223), S100b (224), amyloid-β (225), transthyretin (226)	diabetes mellitus, atherosclerosis (222), amyloidosis, Alzheimer;s disease (227), rheumatoid arthritis (228)l
LDL receptor family	LRP	protease-inhibitor complexes (229);(230), thrombospondin (231), RAP (232), amyloid-β (233), virus (234)	Alzheimer disease (235), viral (rhinoviruses) (234)

Type	Soluble molecules	Ligands	Diseases or syndromes
tPA family	tPA	fibrin (236), tetranectin (237), amyloid proteins (183)	haemorrhages, myocardial infarction, stroke (238)
Pentraxin family	SAP	laminin (239), CRP (240), chromatin (241), amyloid-β (242), Gram-negative bacteria (243), LPS (244)	Familiar British and Danish dementia (245), Alzheimer's disease (246), atherosclerosis (247)
Pentraxin family	C1q	amyloid-β (248), prion peptide (191), β_2-microglobulin (189)	Down syndrome (249), Alzheimer's disease (250) amyloidosis (189), haemodialysis mediated amyloidosis
Clusterin	clusterin (ApoJ)	amyloid-β (251) lysozyme (193), Staphylococcus aureus (252)	diabetes mellitus (253), Alzheimer's disease (254), cancer (255)

Abbreviations in Table 3: LDL, low density lipoprotein; HDL, high density lipoprotein; LOX-1, Lectin-like oxidized LDLreceptor-1; Ig, Immunoglobulin; RAGE, receptor for advanced glycation end products; HMGB1, high-mobility group box 1; LRP, low density lipoprotein receptor-related protein; RAP, low density lipoprotein receptor-associated protein; tPA, tissue-type plasminogen activator; SAP, serum amyloid P component; CRP, C-reactive protein; LPS, lipopolysaccharide

4. Therapeutic approaches to protein misfolding diseases

There is no efficient therapy for inhibiting or reversing protein aggregation and deposition in patients suffering from protein misfolding diseases. In the past decade, our understanding of the underlying mechanisms during development of protein misfolding diseases has much improved, opening new perspectives for designing more efficient medications against these highly devastating disorders. This section highlights recent findings that provide new concepts for inhibition of protein aggregation and its proinflammatory and cytotoxic effects.

Extensive studies on protein misfolding described the inhibition of protein aggregation by small compounds, such as 2,4-dinitrophenol (256), di- and trisubstituted aromatic molecules (257), curcumin, β-cyclodextrin derivatives, hematin, meclocyclin, indomethacin and Congo red (258). The inhibitory effect involves stabilization of the native fold of potentially amyloidogenic proteins or inhibition of their oligomerization or fibrillarization (56;258-260). For example, the fibrillarization of transthyretin peptide that results in its accumulation in organs in human amyloidosis (see Table 2) is blocked when transthyretin complexed with retinol-binding protein (RBP) and thyroxine (261).

One of the most challenging possibilities amongst the potential therapeutic approaches for protein misfolding diseases is the antibody therapy. In animals, monoclonal antibodies have been raised by passive immunization against critical regions of the amyloid-□ that participate in the aggregation process. These antibodies then improved the cognitive function and amyloid pathology after series of injections into transgenic mice that express human APP and serve as a model for Alzheimer's disease model transgenic mice (262;263). Nevertheless, the possibilities of active immunization, when the harmful protein is injected and it stimulates antibody production that can eliminate the aggregated self-proteins or interfere with further aggregation are limited due to the tolerance against self-proteins. Through mechanisms that are incompletely understood, vaccines can overcome tolerance. Immunized transgenic animals showed reduced protein deposition in the brain (264) and mucosal immunizations against prion proteins showed a delay of disease (265). Active and passive immunization experiments in animal models for Alzheimer's and prion diseases offered great perspectives for the immunotherapy against amyloidogenic proteins in the treatment of protein misfolding diseases (266;267). However, after the early success of animal models, the first human trials had to be terminated because of unexpected severe meningoencephalitis in patients (268). Recent studies focus on passive immunizations, i.e. the generation of

conformation-specific antibodies which recognize structural epitopes of misfolded proteins and discriminate between fibrillar (269) and oligomeric (142) conformation. Using new strategies, novel vaccines against Alzheimer's disease are under development or entering clinical trials stimulating immune responses against amyloid-β hopefully without provoking brain inflammation risk (270).

The receptor for advanced glycation end product (RAGE) mediates the uptake of misfolded β_2-microglobulin in dialysis-related amyloidosis patients (271) and contributes to the influx of amyloid-β from the blood into the brain (180). Therefore, RAGE has been in the focus for novel drugs targeting the interaction of RAGE with amyloid-β (Table 4). The elevated levels of the soluble RAGE that lacks the transmembrane domain and therefore circulates in plasma, is associated with reduced risk of coronary artery disease, hypertension, the metabolic syndrome, arthritis and Alzheimer's disease (272). Furthermore, the binding of ligands to RAGE contributes to both development and progression of vascular diseases including atherosclerosis (273). Animal studies show that blockage of the interaction prevents the development of these diseases (274), which stimulated companies to develop drugs to target the interaction of RAGE and amyloid-β (Table 4). The soluble form of RAGE is another promising therapeutic target for inflammation-related disorders including protein misfolding diseases.

In the central nervous system, tPA is implicated in pathological and physiological processes (275). Cerebral tPA activity is decreased during ageing in normal mice and further lowered in mice that express high levels of amyloid-β (276). This supports its role in the clearance of amyloid-β (277). Conversely, tPA is overexpressed in amyloid-rich areas in the brain of Alzheimer's patients (278) and mediates neuronal apoptosis that is precluded by plasmin (279). However, inflammation increases the amount of tPA inhibitors, which prevents the destruction of amyloid-beta proteins by obstructing the action of tPA (280). Taken together, tPA plays a critical role in the clearance of amyloid proteins through tPA activation and subsequent plasmin generation. However, in the absence of plasmin tPA induces toxic effects. Therefore tPA is an interesting target for protein misfolding diseases.

Table 4. Summary of drugs targeting protein aggregation and uptake

Drug	Target / Blockage of	Company, phase of trial
AL-108	amyloid-β aggregation	Allon Therapeutics, Phase II
TTP488	RAGE and amyloid-β interaction; brain amyloid load	TransTech Pharma/Pfizer, Phase IIa
AZD-103	amyloid-β fibrillarization and disassembly of of fibrils	Transition Therapeutics/ELAN, Phase I.
ATG-Z1	β-secretase APP cleaving enzyme	CoMentis, Preclinical
LY450139	γ-secretase APP cleaving enzyme	Eli Lilly, Phase II

5. Aim and outline of the thesis

Protein misfolding diseases, inlcuding Alzheimer's disease, systemic amyloidosis, diabetes mellitus and atherosclerosis are highly debilitating diseases for which no adequate cure is available. The pathology of these diseases share common features, including protein aggregation, cell activation, cytotoxicity and inflammation that are still incompletely understood. The aim of the studies presented in this thesis was to further unravel mechanisms of these diseases with emphasis on the role of misfolded proteins, in order to find molecules that can interfere with misfolded proteins induced cell activation. We addressed the following questions:

1. Do platelets respond to misfolded proteins and which are the pathways mediating the activation? Are misfolded protein binding compounds able to block the interaction between platelets and misfolded proteins? (Chapter two.)
2. Do pooled human immunoglobulins from healthy subjects contain antibodies that bind misfolded proteins and is it possible to raise antibodies against unrelated misfolded proteins? Is the interaction between platelets and misfolded proteins inhibited by these antibodies? (Chapter three.)
3. How do protein aggregation properties influence the capacity to activate platelets? (Chapter four.)
4. Is the conformation of C-reactive protein (CRP) of importance for the ability to bind to and to induce proinflammatory responses of endothelial cells? (Chapter five.)
5. Are structural modifications of protein kinase inhibitors change their cytotoxic properties? (Chapter six.)

Finally, the finding of this thesis and their relevance to current understanding on protein misfolding diseases are discussed. (Chapter seven.)

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Chapter two

Activation of human platelets by misfolded proteins

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Abstract

Protein misfolding diseases result from the deposition of insoluble protein aggregates that often contain fibrils called amyloid. Amyloids are found in Alzheimer's disease, atherosclerosis, diabetes mellitus and systemic amyloidosis, which are diseases where platelet activation might be implicated.

We induced amyloid properties in six unrelated proteins and found that all induced platelet aggregation in contrast to fresh controls. Amyloid-induced platelet aggregation was independent of thromboxane A_2 formation and ADP secretion but enhanced by feed back stimulation through these pathways. Treatments that raised cAMP (iloprost), sequestered Ca^{2+} (BAPTA-AM) or prevented amyloid-platelet interaction (sRAGE, tissue-type plasminogen activator) induced almost complete inhibition. Modulation of the function of CD36 (CD36-/- mice), $p38^{MAPK}$ (SB203580), COX-1 (indomethacin) and glycoprotein Ibα (Nk-protease, 6D1 antibody) induced ~50% inhibition. Interference with fibrinogen binding (RGDS) revealed a major contribution of αIIbβ3-independent aggregation (agglutination).

Protein misfolding resulting in the appearance of amyloid induces platelet aggregation. Amyloid activates platelets through two pathways: one is through CD36, $p38^{MAPK}$, thromboxane A_2 mediated induction of aggregation; the other is through glycoprotein Ibα-mediated aggregation and agglutination. The platelet stimulating properties of amyloid might explain the enhanced platelet activation observed in many diseases accompanied by the appearance of misfolded proteins with amyloid.

Introduction

Proteins typically adopt a well-defined three-dimensional structure. There is now an increasing amount of evidence that abnormalities in this process have far reaching consequences for human health. Certain mutations and post-translational modifications such as glycation and oxidation interfere with proper folding, resulting in protein misfolding, aggregation and ultimately polymerization into insoluble fibrils called amyloid (1;2).

The term amyloidosis defines a group of systemic and localized diseases associated with the deposition of amyloid in different tissues. Alzheimer's disease is caused by abnormal folding of amyloid-β and formation of amyloid-rich plaques that obstruct neurons and microvessels of the brain (3). One has argued that these plaques cause the hyperreactivity of platelets observed in these patients as illustrated by P-selectin positive platelets and increased levels of urinary thromboxane A_2 metabolite (4;5). An environmental risk factor for Alzheimer's disease is Herpes Simplex virus. It contains glycoprotein B, a protein fragment that assembles into fibrils that are ultrastructurally indistinguishable from amyloid-β (6).

Protein misfolding is not restricted to Alzheimer's disease but a common feature in the pathology of atherosclerosis, diabetes mellitus and systemic amyloidosis (7-9). Atherosclerotic plaques contain oxidized low densitiy lipoprotein (LDL) which has amyloid properties (10) and activates platelets (11). Interestingly, Herpes Simplex virus also contributes to initiation and progression of coronary atherosclerosis (12). In diabetes mellitus the high blood glucose glycates haemoglobin and albumin introducing amyloid properties (13) that might contribute to the hyperactivity of platelets in these patients (14). Several types of systemic amyloidosis are also known to be associated with thrombosis(15;16).

Amyloids are filamentous protein structures rich in β-sheets that share a structural motif, the cross-β structure. Amyloids have been defined in a number of different ways: operationally in terms of their capacity to bind dyes like Congo red and thioflavin derivatives, morphologically as 6-10 nm filaments, and structurally as "cross-β structure" fibrils in X-ray diffraction. The term cross-β refers to the stacking of β-sheets perpendicular to the fibril axis. Proteins with amyloid may meet these definitions independent of aminoacid sequence. We recently showed that tissue-type plasminogen activator (tPA) selectively binds to amyloid (17). Structural characterization and biochemical analysis of fibril assembly revealed that amyloid forms through a transition of soluble oligomers into intermediate elements that form the fibrils. Consequently, most amyloids are heterogenous in nature. We here use the term amyloid properties to refer to non-native protein aggregates, containing extended β-sheet arrangement, including oligomeric intermediates, amorphous aggregates and end-stage

fibrils. There is growing consensus that misfolding and formation of amyloid is an intrinsic property of any protein. Amyloid formation can be caused by mutations that affect protein folding, an increase in temperature, changes in pH, oxidation, glycation and contact with a negatively charged surface (10;13;18;19). These conditions enhance the relative β-sheet structure content of proteins that subsequently aggregate and polymerize into fibrils. This may occur within the cells and at extracellular locations and greatly interferes with proper cell function (20).

In the present study we addressed the question whether protein aggregates with amyloid properties have the capacity to activate platelets. To avoid bias by other peptide domains, a number of unrelated proteins was modified and the appearance of amyloid properties was evaluated in platelet activation assays. The results reveal that proteins with amyloid properties have potent and specific platelet activating properties that might underlie the development of atherothrombosis observed in diseases known to be associated with formation of amyloid.

Methods

Reagents - We purchased the following reagents: plasmin-specific chromogenic substrate S-2251 (Chromogenix; Milano, Italy), PGI_2 (prostacyclin, Cayman Chemical; Ann Arbor, MI), PGI_2 analog iloprost (Schering AG; Berlin, Germany), renaissance chemiluminescence western blot reagent (PerkinElmer Life Sciences; Boston, MA), collagen (collagen type I/III fibrils from Horm, Lins, Austria), $p38^{MAPK}$ inhibitor SB203580 (Alexis Biochemicals, San Diego, CA), tissue-type plasminogen activator, tPA (Boehringer-Ingelheim; Alkmaar, The Netherlands), D-glucose-6-phosphate disodium salt hydrate (ICN; Aurora, OH), trifluoro-acetic acid (Pierce Biotechnology, Rockford, IL), 1,1,1,3,3,3-hexafluor-2-propanol, Thioflavin T, Congo red, indomethacin, human haemoglobin (Hb, H7379-1G) and bovine serum albumin (BSA, A-7906) all from Sigma; St.Louis, MO, USA. We purchased polyclonal antibodies against phosphorylated $p38^{MAPK}$ (phosphoplus $p38^{MAPK}$) and horseradish peroxidase-labeled anti-rabbit IgG from New England Biolabs (Beverly, MA), monoclonal anti-actin antibody Clone AC-40 from Sigma (St.Louis, MO, USA) and rabbit anti-mouse IgG from DakoCytomation (Glostrup, Denmark). AR-C69931MX was a kind gift from Astra Zeneca (Loughborough, UK). The soluble extra-cellular fragment of the receptor for advanced glycation end-products (sRAGE) was prepared by cloning human RAGE cDNA (clone IRALp962E1737Q2, RZPD, Berlin, Germany) into a pTT3 vector containing an N-terminal His-tag. sRAGE

was expressed in 293E human embryonic kidney cells and purified from the culture medium using a Hi-trap Ni^{2+}-NTA column. After concentration, the buffer was exchanged for 20 mM Tris-HCl, 200 mM NaCl, pH 8.0. Plasminogen was purified as described.(21) Thrombin receptor (PAR1)-activating peptide (TRAP, SFLLRN) was synthesized with a semi-automatic peptide synthesizer (Labortec AG SP650, Switzerland). Amyloid-β (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV; 1-40) and an amyloidogenic peptide of Herpes simplex virus called glycoprotein-B, (GDVGRAVGKVVMGIVGGVVSA) (22) were synthesized by the Netherlands Cancer Institute NKI (Amsterdam, The Netherlands). To induce amyloid properties, freshly prepared amyloid-β (fresh amyloid-β in short) was first treated with a 1:1 (v/v) mixture of trifluoro-acetic acid and 1,1,1,3,3,3-hexafluor-2-propanol to introduce peptide monomers. After evaporation amyloid-β monomers were dissolved in phosphate buffered saline (PBS) and incubated for 1 week at 37°C to obtain modified amyloid-β (refered to as amyloid-β). Glycoprotein B is a membrane protein required for the cellular entry of herpes simplex virus.(23) Fresh glycoprotein B was dissolved in destilled water and incubated for 48 hours at 37°C to obtain amyloid containing modified glycoprotein B. Glycated haemoglobin (Hb-AGE) and bovine serum albumin (BSA-AGE) were prepared by incubating 10 mg/mL human Hb and 100 mg/ml BSA in the dark at 37°C with 1M D-glucose-6-phosphate disodium salt hydrate and 0.05% NaN_3 for 75 and 70 weeks respectively.(13) Samples were extensively dialyzed against distilled water to remove remaining free glucose. Human fibrin α-chain derived sequences 10 ($_{148}$KRLEVDIDIK$_{157}$), -12 ($_{148}$KRLEVDIDIKIR$_{159}$) and -13 ($_{148}$KRLEVDIDIKIRS$_{160}$)(17) were synthesized by Pepscan Systems (Lelystad, The Netherlands) and incubated for 1 week at 37°C. Fibrin peptides 12 and -13 form β-sheets and amyloid, but fibrin peptide 10 is composed solely of α-helices and does not form amyloid under the present conditions.(17) The presence of amyloid cross-β structure in proteins was demonstrated by binding of Thioflavin T and Congo red (24), tPA binding and activation assays (see below)(17) and fibrillar structures in transmission electron microscopy.(25) Freshly dissolved amyloid-β/glycoprotein B, non-glycated Hb/BSA and fibrin peptide 10 were negative in these analyses and used as amyloid negative controls.

Detection of amyloid proteins by tPA-mediated plasminogen activation - Aliquots of 25 µg/ml amyloid-containing proteins/peptides or amyloid-free controls were mixed with 400 pM tPA, 20 µg/ml plasminogen and 0.42 mM plasmin-specific chromogenic substrate S-2251 in Hepes-buffered saline (20 mM HEPES, 4 mM KCl, 3 mM $CaCl_2$, pH 7.4). The conversion of plasminogen to plasmin was inferred from the liberation of p-nitroaniline at 405 nm at 1 minute intervals for 3 hours at 37°C in 96-well plates (Corning Incorporation, NY).(17)

Platelet isolation - Freshly drawn venous blood from healthy volunteers with informed

consent was collected into 0.1 volume 130 mmol/l trisodium citrate. The donors claimed not to have taken any medication during 2 weeks prior to blood collection. After centrifugation (15 minutes, 150*g*, 20°C), the platelet rich plasma (PRP) was 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 minutes, 300*g*, 20°C), the 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 PGI_2 (final concentration) was added and the wash step was repeated. The platelet pellet was then 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 minutes to achieve a resting state. C57Bl/6 mice were obtained from Charles River (Maastricht, The Netherlands). CD36-deficient mice were kindly provided by Dr. M. Febbraio (Department of Medicine, Weill Medical College of Cornell University, New York, USA). Mice had unlimited access to water and regular chow diet, containing 4.3% (w/w) fat with no added cholesterol (RM3, Special Diet Services, Witham, UK). All experimental protocols were approved by the local ethics committees for animal experiments. For the isolation of murine platelets, mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/ml), ketamine (40 mg/l) and atropine (0.05 mg/ml), and blood was subsequently collected into 0.1 volume 130 mmol/l trisodium citrate and 0.1 volume of ACD by heart puncture. PRP was obtained by centrifugation (15 minutes, 87*g*, 20°C). The remainder of the blood was diluted with Hepes-Tyrode buffer pH 6.5 and 0.1 volume of ACD and centrifuged again (15 minutes, 87*g*, 20°C). PRP samples were pooled, and platelets were isolated by centrifugation (15 minutes, 350*g*, 20°C) in the presence of 0.1 volume of ACD buffer and resuspended in Hepes-Tyrode buffer (pH 6.5). PGI_2 was added to a final concentration of 10 ng/ml, and the washing procedure was repeated once. The platelet pellet was resuspended in Hepes-Tyrode buffer (pH 7.2). Platelet count was adjusted to 1×10^{11} platelets/l. For inhibitory experiments, platelets were preincubated with 100 µM RGDS (Arg-Gly-Asp-Ser) peptide (15 minutes, 37°C; Bachem, Bubendorf, Switzerland), 20 µM PD98059 (10 minutes, 37°C; Calbiochem, La Jolla, CA), 10 µM BAPTA-AM (2 minutes, 37°C; Calbiochem, La Jolla, CA), 10 µM PP1 (15 minutes, 37°C; Alexis Biochemicals, San Diego, CA) or 500 µM adenosine-3- phosphate-5'-phosphosulphate (A3P5PS, 2 minutes, 37°C; Sigma, St Louis, MO). To address the role of GPIbα, platelets were preincubated with 5 µg/ml Nk-protease (20 minutes, 37°C) which cleaces two mucin-like substrates within anionic amino acid sequences containing sulfated tyrosines of the aminoterminal region of GPIbα. The metalloprotease Nk was purified from Naja kaouthia cobra venom (Sigma, St Louis, MO) and was a kind gift of Dr. R. K. Andrews

(Monash University, Clayton, Australia). 6D1 is an inhibitory monoclonal antibody directed against GPIbα and was a kind gift of Dr. Barry Collar, (Mount Sinai Hospital, New York, NY). Before experiments, platelets were incubated with a 200 fold diluted 6D1 sample (15 minutes, 37°C). As a positive control for agglutination, purified von Willebrand factor (5 µg/ml, purified from 250 IU Haemate P, Behringwerke, Marburg, Germany) was activated by ristocetin (0.5 mg/ml, DiaMed AG, Cressier, Switzerland). Fixed platelets were prepared by incubating washed platelets with 1% paraformaldehyde. After 1 hour of incubation at 37°C, the platelets were spun down and resuspended in Hepes-Tyrode buffer (pH7.2).

Platelet aggregation - Platelet aggregation was followed in an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) for 15 minutes at 37°C at 900 rpm. A volume of 270 µl platelet suspension was incubated with 30 µl solution containing samples for analysis at indicated concentrations. For inhibition experiments, 270 µl platelet suspension was incubated with 15 µl protein/peptide solution and 15 µl solution with inhibitors. In experiments with inhibitors, amyloid proteins were pre-incubated at 22°C with sRAGE (100 µg/mL) or tPA (500 nM) for 10 minutes, and platelets were pre-incubated at 37°C with the cyclooxygenase inhibitor indomethacin (30 µM, 30 minutes) and the $P2Y_{12}$-agonist AR-C69931MX (50 nM, 2 minutes). The maximal aggregation was expressed as arbitrary units or as a percentage of the response induced by 8 µM TRAP.

Isolation of soluble amyloid proteins - To analyze the solubility of proteins/peptides, fractions were centrifuged in a Beckman L-80 ultracentrifuge (1 hour at 100,000 x g, 4°C). Supernatants were collected and analyzed in transmission electron microscopy, tPA-mediated plasminogen activation and platelet aggregation.

Transmission Electron Microscopy - For the analysis by transmission electron microscopy, 5 µl solution with amyloid proteins (1 mg/ml) together with appropriate controls was applied on copper-coated grids. After washing with PBS and water, the grids were incubated with 1% methyl-cellulose uranyl pH 4 for 5 minutes at 22°C. The grids were analyzed with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at 10.000 x magnification.

$p38^{MAPK}$ phosphorylation assay - A volume of 75 µl platelet suspension was incubated with 25 µl protein/peptide solution at indicated concentrations for 1 minute at 37°C. Cells were fixed with 3% formaldehyde and transferred to ice for 15 minutes. Suspensions were centrifuged (1 minute, 5600*g*, 20°C) and pellets were resuspended in 60 µl SDS polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and boiled for 5 minutes. Samples were analyzed by SDS-PAGE in 12% polyacrylamide gel, transferred to nitrocellulose membranes using a mini-protean system (Bio-Rad, Richmond, CA, USA), blocked with 4% w/v BSA and Tween 20 (0.1% (v/v)) in phosphate-buffered saline (PBS); membranes were incubated overnight with

polyclonal antibody against phospho-p38^{MAPK} (Thr-180/Tyr182, 1:2000) at 4°C. Membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (1:3000) for 1 hour at 4°C, and protein bands were visualized by enhanced chemiluminescence reaction and analyzed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). Since bands of p38^{MAPK} are not resistant against stripping, lane-loading controls were prepared on stripped membranes (1 hour at 80°C in 2% SDS and 0,1% Tween 20 in PBS) by blocking with 4% w/v BSA and Tween 20 (0.1% (v/v)) in PBS and incubation with monoclonal antibody against actin (1:4000 dilution) and thereafter incubated with rabbit anti-mouse antibody (1:4000) for 1 hour at 4°C. Data were expressed as percentage of the activation induced by 5 μM TRAP.(26) In a few experiments platelets were preincubated with 10 μM SB203580 (30 minutes, 37°C), which inhibits p38^{MAPK} by occupying the ATP binding site (27).

Statistical analysis - Data are means ± SD, with number of observations, n. Statistical analyses were performed by one-way ANOVA with Bonferroni's multiple comparison post test. Values of $P<0.05$ were considered significantly different.

Results

Appearance of amyloid properties in a number of unrelated proteins - Treatment of freshly prepared amyloid-β with a trifluoro-acetic acid/hexafluor propanol mixture to introduce monomers followed by evaporation and prolonged incubation in PBS introduced amyloid as illustrated by the binding of Thioflavin T and Congo red, (28;29) the capacity to activate tPA (17) and a fibrillar structure in electron micrographs (Figure 1). These observations indicate that the modified amyloid-β met the criteria for amyloid containing proteins. Freshly prepared amyloid-β did not show these characteristics illustrating that is was devoid of amyloid. Also Hb-AGE, BSA-AGE, modified glycoprotein B and fibrin peptides 12 and -13 met these requirements wheras Hb, BSA and fibrin peptide 10 were negative in these assays (data not shown). Thus, there was a clear separation between protein samples with and without amyloid properties in these unrelated proteins.

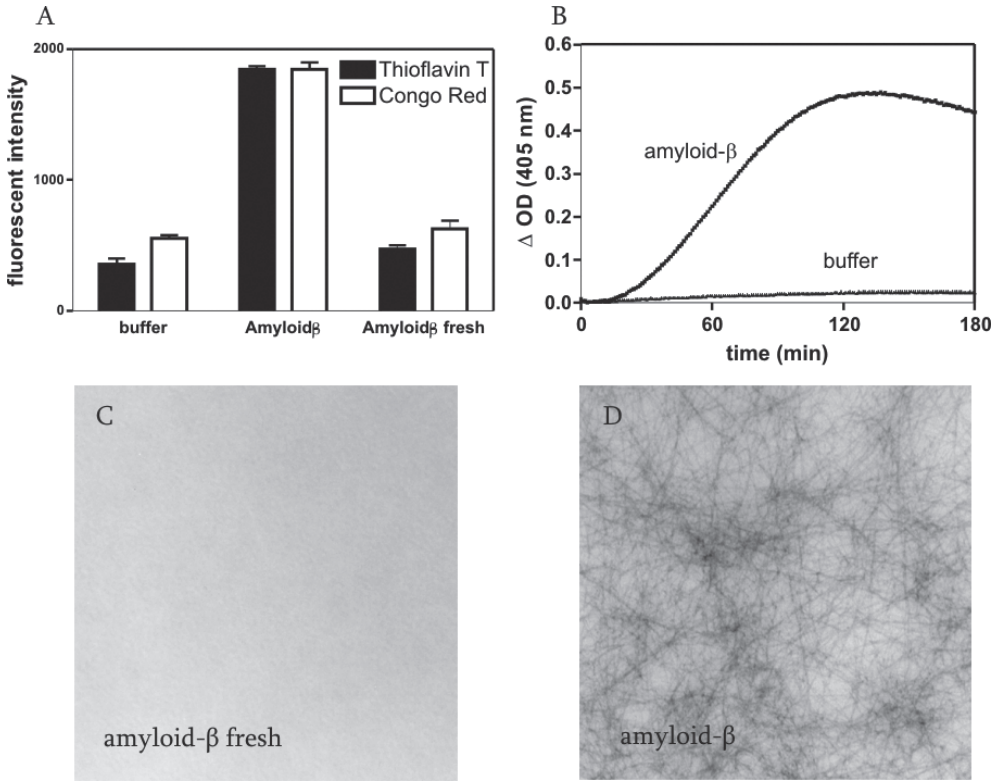

Figure 1. Demonstration of amyloid properties - The presence of amyloid was analyzed by binding of Thioflavin T and Congo red (A), the capacity to activate tPA (B) and the presence of fibrillar structures (C,D). Shown are results for amyloid-containing modified amyloid-β (A,B,D) and the fresh control (C). Similar results were obtained with Hb-AGE, BSA-AGE, glycoprotein B and the fibrin peptides 12 and 13 (not shown).

Amyloid proteins induce platelet aggregation - Amyloid-β induced platelet aggregation (Figure 2). At 12,5 µg/ml amyloid-β there was a slight shape change followed by little aggregation. Higher concentrations induced a dose-dependent increase in aggregation reaching a maximum at 50 - 100 µg/ml amyloid-β.A similar response was induced by glycated haemoglobin (Hb-AGE) although aggregation was slightly weaker than with modified amyloid-β.Also glycated BSA (BSA-AGE) induced aggregation but there was a delay of about 500 s at low and 250 s at high concentration.Aggregation induced by Herpes Simplex glycoprotein-B was rapid but responses were weaker than seen with the other proteins.The fibrin peptides 12 and -13 also induced aggregation but higher concentrations were required to obtain an effect. None of the fresh control proteins/peptides had platelet-activating properties and also fibrin peptide 10 which lacks amyloid failed to induce aggregation (not shown). Using aggregation by a suboptimal concentration of TRAP as an inter-assay reference, studies were

repeated in platelets from five different donors for calculation of dose-response relationships (Figure 2B), maximal aggregation (Figure 2C) and EC_{50} data (Figure 2D). Amyloid-β and Hb-AGE induced the highest aggregation. BSA-AGE, modified glycoprotein B and fibrin peptide 12 were slightly less effective and fibrin peptide 13 induced the lowest response. Hb-AGE and BSA-AGE had the lowest EC_{50} (about 10 µg/ml), amyloid-β, glycoprotein B and fibrin peptide 12 showed an intermediate activity (about 30 µg/ml) and fibrin peptide 13 had the highest EC_{50} (about 100 µg/ml). Thus, different proteins with only amyloid properties in common induce platelet aggregation and the extent of aggregation varies with individual amyloid proteins.

Effect of inhibitors on amyloid-induced platelet aggregation - Since amyloid proteins posses a fibrillar structure and therefore might trap platelets resulting in agglutination rather than aggregation, platelets were treated with the stable prostacyclin analog iloprost to raise cAMP (30). This treatment completely abolished responses induced by modified amyloid-β, Hb-AGE and BSA-AGE as well as the aggregation by TRAP (Figure 3A). Thus the change in light transmission of platelet suspensions stimulated with amyloid proteins depends on undisturbed platelet activating sequences. Aggregation is known to be enhanced by formation of thromboxane A_2, followed by stimulation of TP-receptors and by release of ADP from δ granules followed by stimulation of $P2Y_1$ and $P2Y_{12}$ receptors. Blockade of thromboxane A_2 synthesis with the COX-1 blocker indomethacin inhibited aggregation induced by amyloid-β by 30%. Interference with the $P2Y_{12}$ receptor with AR-C69931MX reduced the response by 40%. When both inhibitors were present there was a further reduction of the aggregation. Similar results were found with Hb-AGE and BSA-AGE (Figure 3B,C).

Thus, depending on the type of amyloid protein, 20-50% of the aggregation was the result of direct platelet activation by these proteins, which was enhanced by feed back stimulation by thromboxane A_2 and ADP. To investigate whether other activating pathways contributed to amyloid-induced aggregation, platelets were treated with different metabolic inhibitors. There was about 50% inhibition by an inhibitor of mitogen activated kinase (PD98059), an upstream regulator of ERK1/2, 80-90% inhibition by an inhibitor of Src family kinases (PP1) and a Ca^{2+} chelator (BAPTA-AM) whereas an inhibitor of the P2Y1 ADP receptor (A3P5PS) induced only 15-30% inhibition (Table 1.).

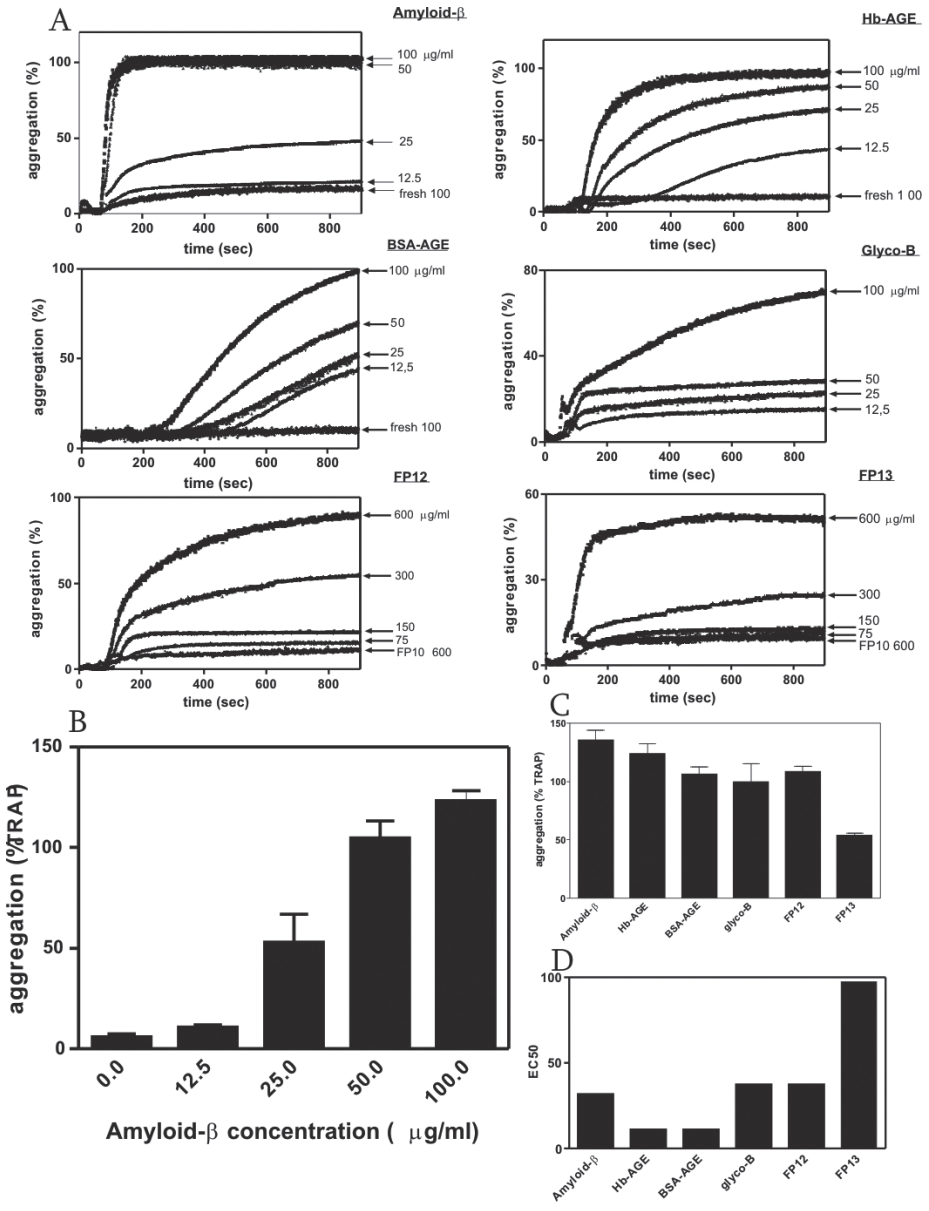

Figure 2. Amyloid-like proteins induce platelet aggregation. - A: Platelets were stimulated with amyloid-β, Hb-AGE, BSA-AGE and glycoprotein B (Glyco-B) at indicated final concentrations. Freshly dissolved amyloid-β, Hb, BSA and amyloid-free fibrin-derived peptide 10 (FP10) served as controls. Note that aggregation scales are different. B: Dose-response relation of amyloid-β induced aggregation expressed as percentage of aggregation induced by 8 µM TRAP. C: Maximal platelet aggregation induced by 100 µg/mL amyloid-β, Hb-AGE, BSA-AGE, Glyco-B and 600 µg/mL fibrin peptide 12 (FP12) and fibrin peptide 13 (FP13). D: EC50 values calculated from dose-response curves of each amyloid protein or peptide. (Means ± SD, n=5)

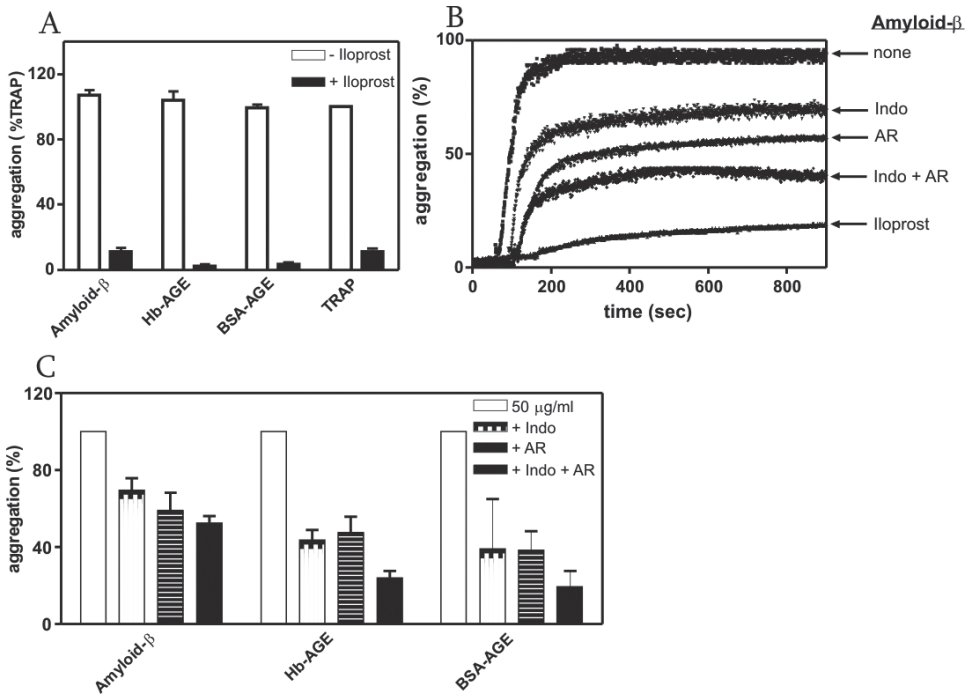

Figure 3. Effect of metabolic inhibitors on amyloid-induced platelet aggregation - A: The PGI2 analogue Iloprost (20 ng/ml, 2 minutes pre-incubation, 37°C) inhibits aggregation induced by 50 µg/ml amyloid-β, 100 µg/ml Hb-AGE, 100 µg/ml BSA-AGE or the positive control 4 µM TRAP. Data are percentages of TRAP-induced aggregation. B, C: Indomethacin (Indo, 30 µM), AR-C69931MX (AR, 50 nM) or the combination inhibits amyloid-β induced aggregation. Data are expressed as arbitrary units (B) and percentage of aggregations without additions (C). (Means ± SD, n=3)

Amyloid-induced platelet aggregation is inhibited by sRAGE and tPA - Amyloids, including amyloid-β and glycated proteins are ligands for the receptor for advanced glycated end products (RAGE) (31-34). The presence of soluble RAGE strongly interfered with platelet aggregation induced by amyloid-β, Hb-AGE and BSA-AGE demonstrating that binding of sRAGE to amyloid-β neutralized its platelet activating properties. In contrast, sRAGE did not interfere with the aggregation induced by TRAP and collagen (Figure 4A,C,E). Also tPA binds with high affinity to proteins with amyloid (17). Again, the platelet activating properties in amyloid-β, Hb-AGE and BSA-AGE were abolished by tPA but TRAP- and collagen-induced aggregation were not affected (Figure 4B,D,F). Taken together, these findings illustrate that amyloid proteins activate platelets through the epitope for sRAGE and tPA binding, which is apparently absent in collagen and TRAP.

Table I. Effect of metabolic inhibitors on amyloid-induced platelet aggregation - Preincubation of platelets with an inhibitor against $p42^{MAPK}$ (ERK1/2, PD98059) induced a partial inhibition of aggregation induced by 50 µg/ml amyloid-β, Hb-AGE and BSA-AGE. More inhibition was seen with an inhibitor of Src-family kinases (PP1) and the calcium chelator (BAPTA-AM). An antagonist against P2Y1 ADP receptor (adenosine-3'-phosphate-5'-phosphosulphate, A3P5PS) induced only a slight inhibition (means ± SD, n=4).

	Amyloid-β	Hb-AGE (%)	BSA-AGE (%)
Control	100	100	100
PD98059	48.3±9.9	40.3±8.6	46.6±8.3
PP1	26.4±6.6	9.5±2.8	17.3±7.8
BAPTA-AM	20.6±5.1	4.0±2.0	12.1±5.2
A3P5PS	67.0±4.9	72.9±10.0	85.7±12.8

Soluble amyloid proteins retain platelet-activating properties - Amyloid proteins show varying degrees of multimerization ranging from oligomers to matured fibrils. To determine the effect of multimerization on the platelet activating properties, preparations of amyloid-β and related proteins were centrifuged at 100,000 *g* to separate soluble and insoluble fractions, which were then compared in electron micrographs, tPA activation tests and aggregation assays. The soluble fractions of amyloid-β, Hb-AGE and BSA-AGE contained fibrils (Figure 5A) and retained the capacity to activate tPA (Figure 5B) and to initiate platelet aggregation (Figure 5C). In contrast, the soluble fraction of fibrin peptide 12 was devoid of fibrils. This fraction had lost about 50% of its tPA-activating properties and failed to initiate platelet aggregation. Thus, the capacity to activate platelets is present in both the soluble and insoluble fibrillar structure of the amyloid proteins.

Amyloid proteins activate platelets in part through $p38^{MAPK}$, COX-1 and CD36 - Since amyloid proteins are known to bind to scavenger receptors (35), which in many cell types are regulators of MAP-kinases (36), we investigated whether amyloid containing proteins and peptides initiated platelet aggregation through $p38^{MAPK}$. Amyloid-induced platelet aggregation was strongly inhibited by the $p38^{MAPK}$ inhibitor SB203580 (Figure 6A). The presence of indomethacin left this inhibition unchanged suggesting that part of the amyloid induced aggregation was the result of thromboxane A_2 formation through $p38^{MAPK}$ and COX-1. Indeed, there was a potent, dose-dependent activation of $p38^{MAPK}$ by modified amyloid-β, Hb-AGE and BSA-AGE but not by the fresh controls (Figure 6B). A major scavenger receptor on platelets is CD36 (glycoprotein IV), which is a class-B receptor. To address the role of CD36 in platelet activation by amyloid, platelets from wild type and CD36-deficient mice were incubated with amyloid-β, Hb-AGE and BSA-AGE and fresh controls and the activation of $p38^{MAPK}$ was measured. Again, amyoid proteins induced a 3-4 fold activation of $p38^{MAPK}$ in wild

type platelets as seen in their human counterparts (Figure 6C). In CD36-deficient platelets $p38^{MAPK}$ activation was strongly impaired and reached the range found after addition of buffer or fresh proteins. Aggregation experiments showed that amyloid-β induced aggregation was about 50% lower in CD36-deficient platelets compared with wild type controls (Figure 6D). Thus, a major part of amyloid induced platelet aggregation is the result of signalling through CD36, $p38^{MAPK}$ and COX-1 which are upstream steps in the formation of thromboxane A_2.

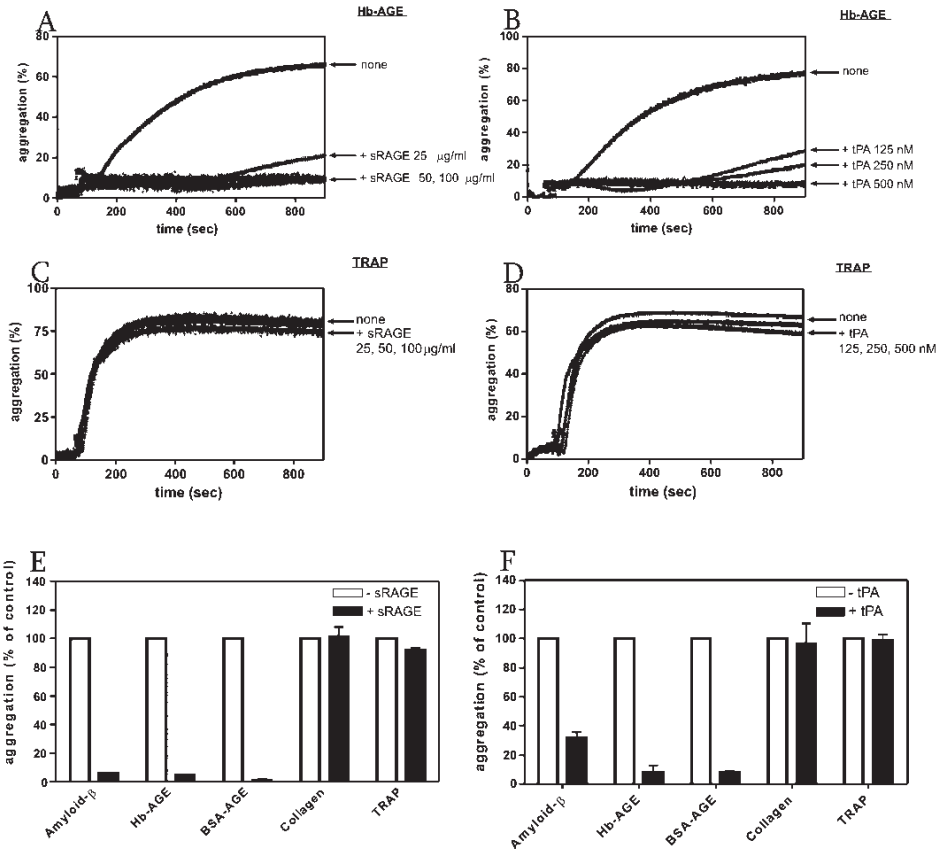

Figure 4. Amyloid-induced platelet aggregation is inhibited by soluble RAGE and tPA. - A-D: Platelet aggregation induced by 25 µg/ml Hb-AGE is dose-dependently inhibited by soluble (s)RAGE (A) and tPA (B), but TRAP (4 µM)-induced aggregation is not disturbed (C,D). E,F: sRAGE (100 µg/mL, E) and tPA (500 nM, F) inhibit aggregation induced by amyloid-β (50 µg/mL), Hb-AGE (25 µg/mL) and BSA-AGE (25 µg/mL), but aggregation by TRAP (4 µM) and collagen (4 µg/mL) is not changed.

Amyloid proteins activate platelets in part through glycoprotein bα. Since inhibition of CD36 signalling blocked only part of the amyloid-induced aggregation, the nature of CD36 independent aggregation was investigated in more detail. An inhibitor of fibrinogen binding

to αIIbβ3 caused 80% inhibition of TRAP-induced aggregation but left about half of amyloid-induced aggregation unchanged (Figure 7A).

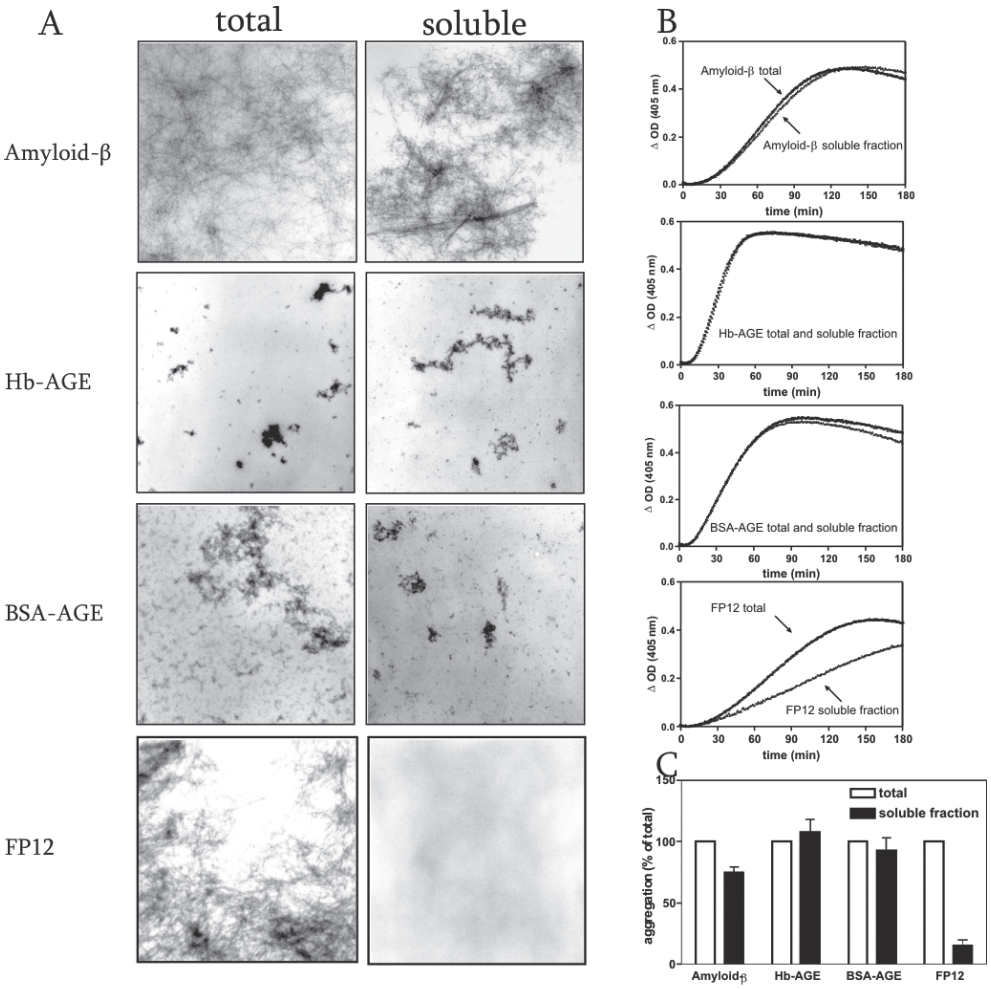

Figure 5. Soluble amyloid proteins retain platelet activating properties - A–C: Amyloid-like proteins (1 mg/ml) were centrifuged (1 hour, 100,000g, 4°C) and supernatants were compared with total fractions by electron microscopy (A), tPA-mediated plasminogen activation (B) and platelet aggregation (C). Supernatants contained fibrils (Amyloid-β and fibrillar aggregates (Hb-AGE, BSA-AGE) and retained the capacity to stimulate plasminogen activation and to induce aggregation. Supernatant of fibrin peptide 12 (FP12) showed no residual structures and had lost tPA- and platelet activating properties.

This αIIbβ3-independent aggregation is generally referred to as agglutination and typically observed when fixed platelets are treated with a von Willebrand factor (vWf)-ristocetin mixture. In contrast to activated vWf, amyloid proteins failed to induce agglutination

of fixed platelets. In intact platelets activated vWf triggers agglutination/aggregation by signal transduction through the vWf receptor glycoprotein (GP) Ibα, which is part of the $GP(Ib)_2V(IX)_2$ complex. Removal of the extracellular part of GPIbα by treatment with Nk-protease reduced aggregation to 40%, the remainder probably reflecting CD36-mediated aggregation (Figure 7B). A similar effect was seen in the presence of anti-GPIbα antibody 6D1 (Figure 7C). RDGS reduced aggregation by Nk-protease treated platelets to the range found with iloprost-treated platelets, suggesting that signalling through CD36 induced αIIbβ3-mediated aggregation (Figure 7B). When platelets were treated with indomethacin to halt CD36-mediated signalling to thromboxane A_2, RDGS inhibited only half of the remaining aggregation. This indicates that signalling through GPIbα induced aggregation as well as agglutination.

Discussion

The present work demonstrates that a number of unrelated proteins, but with common amyloid properties induce platelet aggregation. This capacity is absent in the fresh proteins and neutralized by sRAGE and tPA which are specific, high affinity, blockers of amyloid.

Platelet activation by amyloid containing proteins is mediated through two, mutually independent pathways. One pathway signals through CD36, activation of $p38^{MAPK}$ and COX-1, which are intermediates in thromboxane A_2 formation, and starts a normal, αIIbβ3- mediated aggregation response. The second pathway signals through the vWf receptor GPIbα and triggers aggregation as well as agglutination.

Proteins with amyloid properties initiate a minor shape change response followed by a rather weak aggregation, which is enhanced by thromboxane A_2 formation and ADP secretion. Since CD36-induced aggregation and GPIbα-induced aggregation/agglutination start immediately after platelet contact with amyloid, both pathways signal hand in hand. It is possible that the agglutination induced by GPIbα interferes with the shape change induced by amyloid and reduces the fall in light transmission normally seen with platelet activating agents. Aggregation is inhibited by prostacyclin analog probably reflecting inhibition of $p38^{MAPK}$ signaling since this enzyme is extremely sensitive to elevated cAMP levels (37). Of interest is the observation that inhibition by iloprost is complete, indicating that also the GPIbα-mediated aggregation/agglutination is blocked by high cAMP. Studies with RDGS

reveal that about 50% of the GPIbα-induced response reflects αIIbβ3-mediated aggregation, which is blocked by elevated cAMP. The finding that also agglutination is absent suggests that it is the result of an earlier, cAMP-sensitve platelet aggregation initiated by GPIbα. Also Ca^{2+} sequestration by BAPTA-AM and inhibition of Src-family kinases by PP1 led to almost complete inhibition, confirming the importance of signalling steps in the induction of aggregation and agglutination by amyloid proteins.

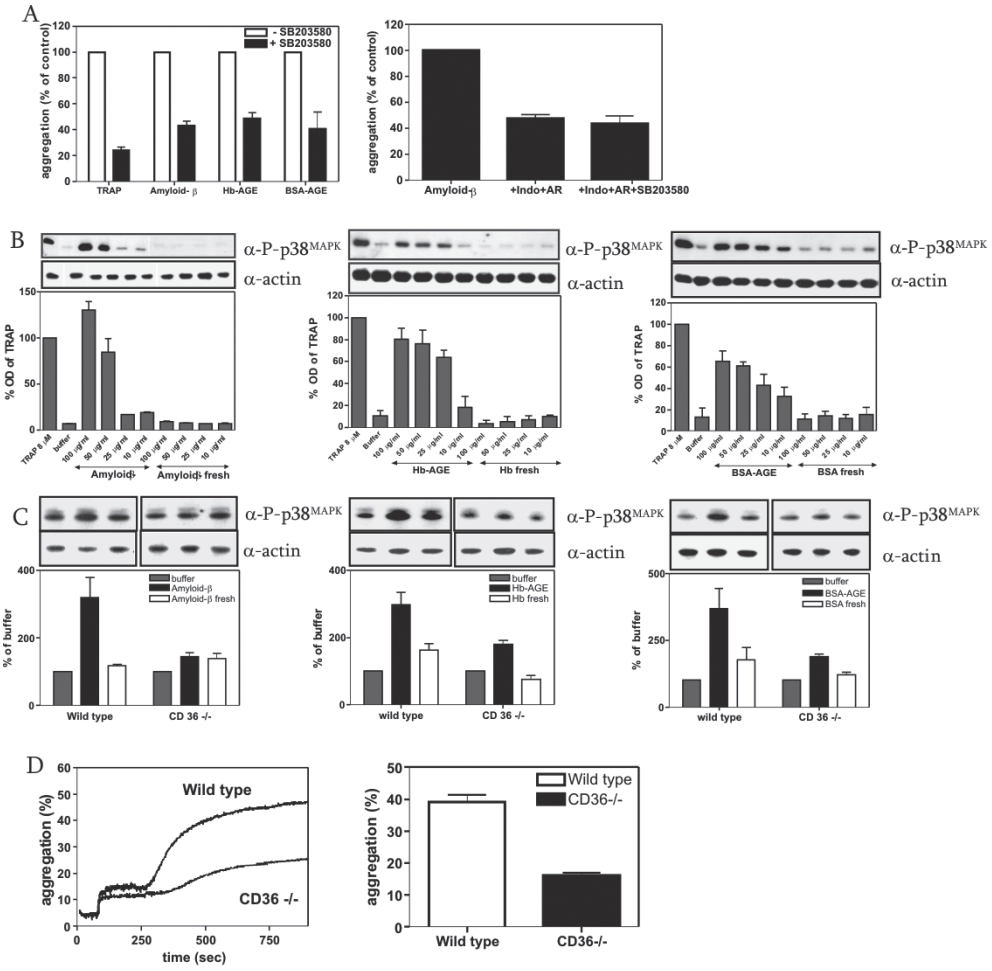

Figure 6. Amyloid proteins activate platelets in part through p38MAPK, COX-1 and CD36 - A: left; Platelet aggregation without and with the p38MAPK inhibitor (SB203580, 10 μM) induced by TRAP (8 μM) and by amyloid-β, Hb-AGE and BSA-AGE (50 μg/ml each). Right; SB203580 does not induce more inhibition in indomethacin-treated platelets. B: Platelets were stimulated with amyloid-β, Hb-AGE and BSA-AGE (1 minute, 37°C). Samples were analyzed by SDS-PAGE and immunoblot with an antibody against phosphorylated P38MAPK. C: Similar experiments in w.t. and CD36 deficient mice. Densities were expressed as a percentage of samples after addition of vehicle (buffer) and are means ± SD, n=4. D: Platelet aggregation induced by amyloid-β (100 μg/ml) in wild type and CD34-deficient mice platelets (n=6).

Whereas these inhibitors of intracellular activation pathways induced complete inhibition of amyloid-induced aggregation/agglutination, the interference by extracellular inhibitors was incomplete. Indomethacin and an inhibitor mimicking the action of the clopidogrel metabolite Act-Met induced a 50-60% reduction, suggesting that platelet activation by amyloid through GPIbα is largely insensitive to interference with $P2Y_{12}$ signalling. This observation might have important clinical implications since it indicates that *in vivo* activation through this pathway is partially unresponsive to aspirin and clopidogrel, which are important drugs against arterial thrombosis. In addition, the agglutination occurs independent of αIIbβ3 and is therefore unresponsive to abcixymab and other antagonists of ligand binding to αIIbβ3.

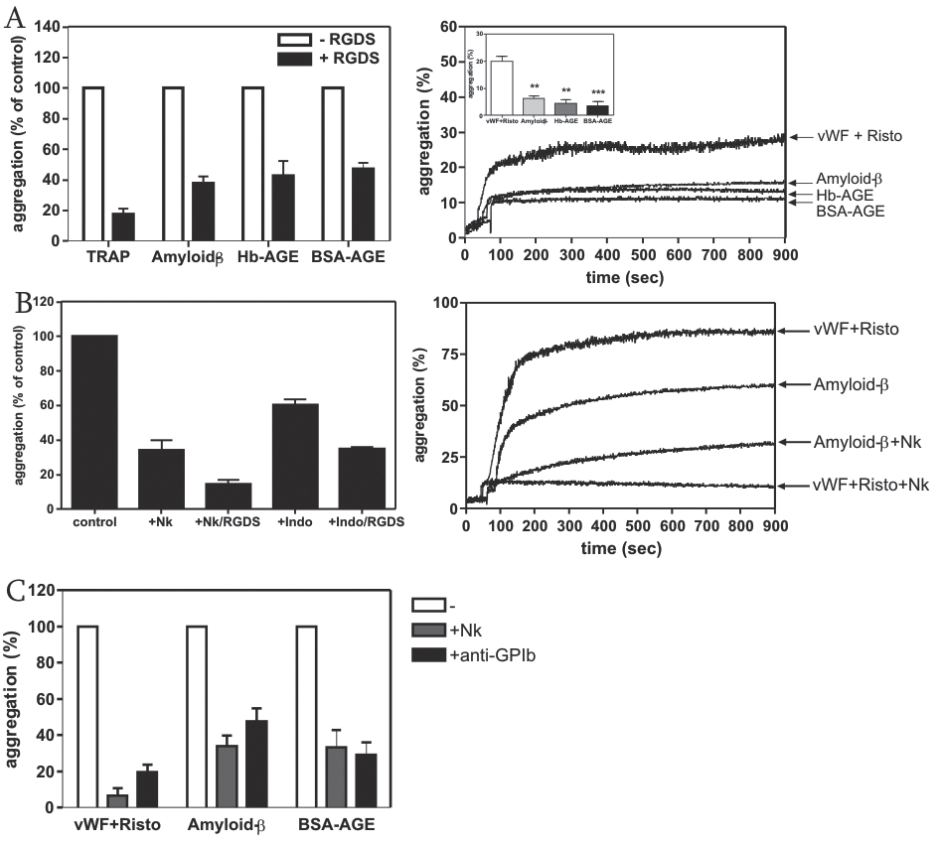

Figure 7. Amyloid proteins activate platelets in part through glycoprotein Ibα - A: left; Effect of αIIbβ3 blockade by RGDS on aggregation induced by TRAP and amyloid proteins. Right; Von Willebrand factor–ristocetin mixture induces agglutination of fixed platelets but amyloid proteins do not. B: left; cleavage of GPIbα by the snake venom Nk-protease reduces amyloid-β (100 µg/ml)–induced aggregation; RDGS induces a further inhibition. Right; Amyloid-β (100 µg/ml) induces aggregation that is inhibited in part by Nk-protease; Von Willebrand factor–ristocetin induced aggregation is fully inhibited by Nk-protease. C: The anti GPIbα antibody 6D1 induces the same inhibition as Nk-protease.

Although each of the amyloid proteins activates platelets, their capacity to do so varies considerably. Modified amyloid-β and Hb-AGE induce the strongest aggregations whereas fibrin peptide 13 is relatively weak. In contrast, EC_{50} data show that Hb-AGE and BSA-AGE are the most effective proteins inducing aggregation at relatively low concentrations. Combinations of different amyloid proteins added in suboptimal concentrations showed that one protein could enhance the activation by the other. Saturating concentrations failed to show additive effects. Each of the modified proteins was positive in the Thioflavin T binding and Congo red assays but it is difficult to assess the role of amyloid in quantitative terms. Amyloid preparations are highly heterogenous in nature, varying from small, soluble oligomeric species and amorphous aggregates to large insoluble fibrils. Their structural similarities are limited to binding to Congo red, Thioflavin T and tPA but the precise nature of the binding epitopes for these compounds and for the platelet receptors that respond to amyloid remains to be elucidated.

A better insight in the activating properties of amyloid proteins is also crucial for our understanding of conformational diseases. In Alzheimer's disease, amyloid plaques correlate poorly in number, appearance and distribution with the clinical progression of brain injury and the small oligomeric species generated from a variety of proteins are better inducers of neuronal damage than the mature amyloid fibrils (38). Removal of insoluble fractions by high speed centrifugation preserved the property to activate platelets in most of the amyloid containing proteins. An exception was fibrin peptide 12 which lost most of its biological properties after centrifugation. Thus, both the soluble and insoluble form of amyloid proteins contain platelet activating epitopes.

CD36 is a multiligand receptor that binds modified proteins such as amyloid-β and glycated proteins, each known to contain amyloid properties. The recent demonstration of amyloid in oxidized low density lipoprotein (oxLDL) is in line with these observations(39) since oxLDL is a potent platelet activator (11). CD36 has a short cytosolic tail and no recognized signaling motif. It is therefore possible that CD36 is simply functioning as an adhesion receptor and thereby bringing the misfolded proteins to other, low affinity signaling receptors. Candidates for such a role are multiligand receptors such as low density lipoprotein receptor-related protein (LRP) and RAGE (40) which bind proteins with amyloid but their contribution to platelet activation is still uncertain. The results also raise the possibility that the absence of CD36 in 5 % of the Asian population offers protection against the platelet-activation component of amyloid-based diseases, a result that has important implications (41).

Recent publications described the presence of amyloid-β in platelet-derived microparticles in healthy subjects (42), in patients with atherosclerotic disease (43) and

diabetes mellitus (44). These particles are a source of tissue factor, which is the prime initiator of coagulation. This property, together with the capacity of amyloid to activate platelets shown in this study, makes these particles potent triggers for a combined activation of the coagulation cascade and formation of a platelet thrombus.

Protein misfolding and the generation of amyloid occurs in Alzheimer's disease, atherosclerosis, diabetes mellitus and systemic amyloidosis (45). Our observation that amyloid activates platelets suggests that protein misfolding should be considered as a risk factor for thrombotic disease.

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Chapter three

Analysis of misfolded protein-specific antibodies

Initial preliminary report

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Abstract

An increasing number of highly debilitating diseases, such as Alzheimer's disease, is associated with protein misfolding. No effective treatment is presently available for these diseases. Protein misfolding diseases share common characteristics including protein misfolding, aggregation, accumulation and tissue damage. Targeting harmful protein aggregation by antibody therapy for these disorders is considered as a promising approach based on animal studies, particularly for Alzheimer's disease. However, clinical trials, using an active immunization strategy had to be terminated due to severe inflammatory side-effects (meningoencephalitis) and even death. Novel insights and new strategies are clearly required in order to develop effective and safe antibody therapy. The use of antibodies that do not recognize native proteins is still considered a good approach. Intravenous immunoglobulins (IVIg) is a collection of antibodies purfied from a large number of human volunteers that is already succesfully used in various inflammatory syndromes. A recent study showed that IVIg is also beneficial in Alzheimer's disease. How IVIg exert its effect is yet unknown. Thus, we investigated the capacity of IVIg to bind misfolded proteins. In addition, we immunized mice with multiple unrelated misfolded proteins in order to test whether conformational antibodies can be obtained that do not recognize native proteins. Indeed, we found that IVIg contained antibodies specific for misfolded proteins. Upon immunizations and hybridoma selections, we obtained 6 monoclonal antibodies. Five of them (2E2, 4F4, 7H1, 7H9 and 8F2) showed polyspecificity for multiple misfolded protein ligands and one (7H2) bound only to misfolded immunoglobulins. Five of the clones were of IgM isotype. Further analysis with 7H2 revealed its specificity for different subsets of misfolded γ-globulins*. The binding of 7H2 to misfolded γ-globulins was inhibited by the dye Congo red but not by dye Thioflavin T or tissue-type plasminogen activator (tPA), three molecules that we previously shown to bind common epitopes on misfolded proteins. This suggests that similar binding sites for 7H2 and Congo red are present, but different for Thioflavin T or tPA. Both IVIg and hybridoma antibodies interfered with misfolded proteins induced platelet aggregation. In conclusion, our data suggest that antibodies against misfolded proteins can be obtained. Such antibodies may neutralize the potential harmful effects of misfolded proteins. The beneficial effects of administration of IVIg may similarly be explained by the presence of antibodies to misfolded proteins.

* γ-globulins corresponds to immunoglobulins purified from human blood (Sigma)

Abbreviations: Hb-AGE, glycated hemoglobin; BSA-AGE, glycated bovine serum albumin; tPA, tissue-type plasminogen activator; IVIg, intravenous immunoglobulin; FP13, fibrin peptide 13; γ-glubulins, human immunoglobulins.

Introduction

Protein misfolding diseases are a group of previously unrelated disorders including Alzheimer's disease, systemic amyloidosis and diabetes mellitus (1;2), which share common characteristics, such as misfolding and accumulation of proteins that result in inflammation and tissue damage (3;4). Misfolding is a generic propensity, which can occur in any protein (5); it is accelerated by certain mutations (6) and by exposure of proteins to specific environmental changes such as increased temperature (7), high or low pH (8), agitation (9), elevated glucose (10) or oxidative agents (11). Upon misfolding proteins lose their native conformation and seek aggregation. During the complex process of aggregation, which is extremely depending on conditions, different structural assemblies are generated from low molecular weight components through oligomeric intermediates to fibrils and mixtures thereof (12). The fibrils are termed amyloid and the disease they are linked to is amyloidosis. Approximately 25 disorders have been classified as amyloidosis, but it is anticipated that a number of other diseases, like atherosclerosis (13) and some with yet unexplained etiology are also associated with protein misfolding and aggregation, not necessarily composing amyloid fibrils according to the classical definition.

Amyloids have well-defined characteristics. They are rich in β-sheets, bind Congo Red and Thioflavin derivatives, have filamentous morphology and display cross-β structure motif in X-ray diffraction (14). The term cross-β refers to the stacking of β-sheets perpendicular to the fibril axis. We recently showed that tissue-type plasminogen activator (tPA) selectively binds to proteins with amyloid properties (2). Structural characterization and biochemical analysis of fibril assembly revealed that amyloid develops through a transition of soluble oligomers into intermediate elements that form the fibrils. Consequently, most amyloid protein aggregates are heterogeneous in nature, and the exact epitopes for Congo Red, Thioflavin T and tPA remain to be identified, but it seems that they bind not exclusively to amyloid fibrils as classically defined.

The exact mechanisms that underlie the pathology of protein misfolding diseases and the role of different structural constituents of protein aggregates during the course of the disorders are still targets of intense research. Besides the environmental stress that

accompanies the growth of plaques of protein aggregates and amyloid, direct cellular effects have been implicated. These include cell activation (15), inflammatory responses (16) and induction of cell toxiciy and cell death (17). We have recently described that misfolding of proteins induces platelet activation (18). It is thought that these effects are unrelated to the specific amino acid sequence of the proteins, but caused by their common structural elements (19). As a result of misfolding and aggregation, proteins adopt unique structural pattern(s). These patterns can be distinguished by platelet receptors CD36 and GPIbα (18), by receptors of other cell types such as receptor for advanced glycation end products, (RAGE) and others (20-22); tissue-type plasminogen activator (tPA) (2), or by amyloid-specific dyes such as Congo Red and Thioflavin T (23). The exact nature of the recognition of misfolded proteins by tPA, Congo red and Thioflavin T is not entirely understood, but it is likely that the increase in β-sheets is a crucial element in the recognition of misfolded proteins by these binding components. Taken togther, the observations thus far have indicated that misfolding of polypeptides leads to exposure of common structural patterns, i.e. conformational epitopes which initiate the aggregation of non-native peptides and pathological processes.

To target protein aggregation in protein misfolding diseases, researchers attempted to generate specific antibodies against the harmful compounds such as oxidized low density lipoprotein and amyloid-β (24;25). The identification of specific immunoreactive structures in oxidized LDL, which are responsible for inflammation, has made it possible to develop novel therapeutic approaches for treatment of atherosclerosis based on active (vaccines) and passive (antibodies) immunization. This is under development and may emerge as a novel immunomodulating atheroprotective strategy (26-28). Monoclonal antibodies have been raised by passive immunization against critical regions of the Alzheimer's peptide amyloid-β that participate in the aggregation process. While these antibodies succesfully reduced amyloid pathology in some studies (29) and references therein), others reported inflammatory side-effects, i.e. meningoencephalitis upon series of injections into transgenic mice that serve as a model for Alzheimer's disease (30). The effectivity of passive immunization in Alzheimer's patients is yet unknown, though the first clinical trials with passive immunizations are on the way. Active immunization strategies with amyloid-β were also promising in animal models (31), but unfortunately, after the early success of animal models, the first human trials had to be terminated because of unexpected severe meningoencephalitis in Alzheimer's patients (32).

Through mechanisms that are incompletely understood, vaccines can overcome tolerance, immunized transgenic animals showed reduced amyloid-β deposition (33), improved the cognitive function in the brain (34), and mucosal immunizations of mice against other

misfolded proteins showed delay of disease (35). Possible mechanisms have been suggested explaining the reduction of amyloid-β deposition in the brain. These include neutralization of amyloid-β oligomers (36) as well as dissolution of amyloid-β fibrils (37). It has also been postulated that amyloid-β specific antibodies initiate Fc receptor mediated phagocytosis by microglial cells (38) and that the administration of amyloid-β-specific antibodies results in an efflux of amyloid-β from the brain to the blood (39).

A few studies have addressed the possibility to generate antibodies which recognize structural epitopes *common* to misfolded proteins and that can distinguish between the harmless native and the damaging non-native conformation. O'Nuallain and coworkers have shown that mouse monoclonal antibodies that are generated against sonicated amyloid-β, recognize conformation-specific epitopes of the fibrillar form and show specificity for other non-native proteins (40). Additionally, Kayed and colleagues produced conformational antibody that recognizes soluble oligomers from many types of amyloid proteins, regardless of the sequence (41;42). These observations suggest that fibrillar and oligomeric misfolded proteins contain distinct conformational epitopes.

Normal human serum contains naturally occurring antibodies against amyloid-β (43) and the level of antibodies against amyloid-β is decreased in patients with Alzheimer's disease (44). Intravenous immunoglobulins preparations (IVIg) contain human immunoglobulins purified from numerous healthy donors and has been succesfully used to treat various immune deficiencies, autoimmune disorders and inflammatory syndromes (45;46). According to a recent pilot study, application of IVIg improved the cognitive functions of Alzheimer's disease patients, moreover, the treatment resulted in decreased total amyloid-β levels in the cerebrospiral fluid and increased levels the serum (47). The machinery behind the beneficial effect of IVIg in Alzheimer's disease is not clear, it might act through enhancing the clearance (from the cerebrospiral fluid to the circulation) or neutralizing the toxic effect of amyloid-β.

These findings lead us to address the following questions. Firstly, whether conformational antibodies are present in IVIg, which could explane the observed beneficial effects of IVIg in Alzheimer's disease. Secondly, whether it is possible to raise conformational antibodies by multiple injection of unrelated proteins that have misfolded state in common. Thirdly, whether conformational antibodies share similar recognition site(s) for structural patterns on proteins with platelet receptors, tPA, Congo red and Thioflavin T. To investigate these matters, we immunized mice with various misfolded proteins and selected hybridoma clones. The binding of hybridoma antibodies and intravenous immunoglobulins (IVIg) to misfolded proteins was analyzed and their effect on misfolded proteins induced platelet activation was tested. In this study we show that IVIg contains IgG antibodies that specifically

recognize misfolded but not native proteins; moreover, IgM antibodies are found in mice with similar properties.

Methods

Materials - We purchased the following items: Amyloid-β peptide 1-40 DAEFRHDSGYE VHHQKLVFFAEDVGSNKGAIIGLMVGGVV (amyloid-β), fibrin peptide FP13 KRLEVDIDIGIRS, fibrin peptide FP6 IDIKIR, IAPP KCNTATCATQRLANFLVHSSNNFGAILSSTNVGSNTY from the Peptide Synthesis Facility, Netherlands Cancer Institute, Amsterdam, The Netherlands. Hemoglobin (H7379-1G), human γ-globulins (G4386), 1,1,1,3,3,3-hexafluoro-2-propanol from Sigma, St.Louis, MO, USA. Trifluoro-acetic acid from Pierce Biotechnology, Rockford, IL, USA. D-glucose-6-phosphate disodium salt hydrate from ICN; Aurora, OH, USA. Recombinant Chicken Serum Amyloid was produced using the bacterial expression system and a glutathione S-transferase (GST) pGEX-2T vector (Amersham Pharmacia Biotech). The GST-rchSAA fusion protein was purified using affinity chromatography with glutathione Sepharose 4 B bead solution (Pharmacia). Intravenous immunoglobulin (IVIg) was from Baxter, Healthcare Corporation, Glandale, CA, USA.

Preparation of misfolded proteins

1. *Aged:* Lyophilized amyloid-β, FP13, FP6, γ-globulins, TTR11, IAPP and chicken serum albumin were dissolved at 10 mg/ml in 1,1,1,3,3,3-hexafluoro-2-propanol and trifluoro-acetic acid in a 1:1 volume ratio. The solvents were evaporated under an air stream and the polypeptides were dissolved in PBS at the following final concentration: Amyloid-β and FP6 at 10 mg/ml, FP13 at 2 mg/ml; γ-globulins and chicken serum albumin at 1 mg/ml. Then, amyloid-β and γ-globulins were incubated for 72 h at 37°C (aged), and stored at room temperature or at -20°C, respectively. TTR11, IAPP, FP6 and FP13 were kept at room temperature.
2. *Glycated:* Glycated bovine serum albumin (BSA-AGE) and hemoglobin (Hb-AGE) were prepared by incubating BSA (100 mg/ml) and hemoglobin (10 mg/ml) for 70 and 32 weeks (respectively) at 37°C in PBS containing 1 M of glucose-6-phosphate and 0.05% of NaN_3. After incubations, solutions were extensively dialyzed against PBS and subsequently stored at 4°C. Protein concentrations were determined with Advanced protein-assay reagent ADV01 (Cytoskeleton, Denver, CO, USA). Glycation and formation of advanced glycation end-products (AGE) was confirmed by measuring intrinsic fluorescent signals from advanced glycation end-products; excitation wavelength 380 nm, emission wavelength 435 nm.

3. *NaOH- and HCl-denatured:* First, lyophilized γ-globulins were dissolved at 10 mg/ml in a solution containing 10 mM HEPES, 4 mM KCl, 137 mM NaCl (pH7.3). Afterwards, the solution was incubated with 11.5 mM NaOH or HCl for 40 minutes on 37°C. To neutralize the preparation, 11.5 mM HCl or NaOH was added, respectively. For control, 11.5 mM NaOH and HCl was first premixed and then added to the γ-globulins. Finally, all solutions were diluted to 5 mg/ml.
4. *Heat-denatured*: For the preparation of heat-denatured γ-globulins solution, we used a previously described procedure (48). Briefly, lyophilized γ-globulins were dissolved to 1 mg/ml in 67 mM sodium phosphate buffer and 100 mM NaCl (pH 7.0). The solution was gradually heated from 30 to 85°C over a period of 12 minutes and afterwards cooled to 4°C for 5 minutes. This heating procedure was repeated 4 times.
5. *Oxidized*: Lyophilized γ-globulins were dissolved to 1 mg/ml in 10 mM sodium phosphate buffer (pH 7.2) and was incubated with 4 mM ascorbic acid and 40 μM $CuCl_2$ at room temperature overnight. The oxidative reaction was stopped by addition of 1 mM EDTA and dialyzed extensively against PBS.

Characterization of misfolded properties – To confirm the presence of misfolded proteins in our preparations before injection into the mice, tissue-type plasminogen activator (tPA) binding ELISA, Thioflavin T and Congo red fluorescence measurements, transmission electron microscopy (TEM) imaging and X-ray diffraction analysis were used (data not shown) (18;49).

Immunizations – Two Balb/c mice was immunized with 100 μl 1 mg/ml amyloid-β and 100 μl complete Freund's adjuvant. After three weeks, a first boost of 50 μg amyloid-β in H_2O-Specol (ID-DLO, Lelystad, The Netherlands) was given, followed by a second boost after 4 weeks. Five weeks after the second boost, the mice were given two additional boosts with 50 μg amyloid-β in PBS (intravenously). Fourty-nine weeks later, the mice were immunized with 50 μg chicken serum amyloid A in H_2O-Specol. Four weeks later, the mice were immunized with 50 μg Hb-AGE. Finally, 4 weeks later the mice was boosted twice intravenously with 50 μg fibrin peptide (FP6) in PBS. Three days after the final boost, the mice were sacrifized and the spleen was used to prepare hybridoma cells. The affinity of the preimmune sera as well as the sera from immunized mice were tested for binding of amyloid proteins, amyloid-β, FP13, an 11 amino-acid residues peptide from transthyretin (TTR11) and islet amyloid polypeptide (IAPP). The preimmune sera did not contain antibodies against these misfolded proteins, whereas after immunization, antibody titers against misfolded proteins in total serum were increased (data not shown).

Hybridoma screen – Hybridoma clones were screened for production of misfolded protein antibodies. First, 768 clones in 96-wells plates were screened for the presence of antibodies that bind to immobilized aged FP13 and γ-globulins. For this purpose, FP13 and γ-globulins were diluted together in H_2O to 5 μg/ml. Microlon high-binding ELISA plates (Greiner, Bio-One GmbH, Frickenhausen, Germany) were filled with 50 μl of this solution and air-dried overnight at 37°C. Plates were blocked with Blocking reagent (Roche Applied Science, Basel, Switzerland). One hundred μl of hybridoma cell culture supernatants containing 10% fetal calf serum was transferred to the plates and incubated for 1 h at room temperature (RT) while shaking. Plates were washed with Tris-buffered saline pH 7.3 (TBS) with 0.1% Tween-20 (wash buffer), and subsequently overlayed with peroxidase-coupled rabbit anti-mouse immunoglobulins (1:2000, RAMPO, DAKO, Denmark) in PBS/0.1% Tween-20, for 30 minutes at room temperature while shaking. After extensive washing, bound RAMPO was visualized with tetramethylbenzidine (TMB, Biosource, Nivelles, Belgium). The reaction was stopped after 5 minutes with 1% H_2SO_4 in H_2O. Plates were read at 450 nm. Clones were included in further screening trials when signals reached at least 1.5x background levels. The selected hybridoma clones were purified using thiophillic agarose (50).

Ligand specificity test for hybridoma antibodies and intravenous immunoglobulins (IVIg) – In order to test the specificity of the hybridoma clones, 50 μl of 5 μg/ml aged γ-globulins, FP13, amyloid-β and Hb-AGE were air-dried on 96 well ELISA plates (Greiner, Bio-One GmbH, Frickenhausen, Germany) overnight at 37°C. For controls, 50 μl of 5 μg/ml native γ-globulins, FP13, amyloid-β and hemoglobin were coated on immobilizer ELISA plates (Exiqon, Vedbæk, Denmark) for 1 hour at room temperature while shaking. Antibodies showed similar binding affinities to misfolded proteins on Greiner and Exiqon plates. For IVIg binding experiments 5 μg/ml BSA-AGE, Hb-AGE, aged amyloid-β and their native controls (BSA, Hb and freshly dissolved amyloid-β) was coated on immobilizer ELISA plates for 1 hour on room temperature. After that, the plates were blocked with Blocking reagent at room temperature for 1 hour, which was followed by incubation with 100 μl of 1:500 purified hybridoma antibodies or IVIg (1:200) in PBS/0.1% Tween-20 for 1 hour at room temperature. After washing the plates, horseradish peroxidase conjugated rabbit anti mouse (RAMPO, 1:2000) or rabbit anti human secondary antibody (RAHPO, 1:1000) in PBS/0.1% Tween-20 was added for 30 minutes at room temperature while shaking. Finally, after extensive washing, TMB was added to the wells and finally, the reaction was stopped with 1% H_2SO_4. Plates were read at 450 nm.

Thioflavin and Congo red measurements – It has been described that fluorescent dyes Thioflavin T and Congo red have capacity to bind misfolded proteins (23;51), thus we

used the previously reported protocol to confirm the presence of misfolded proteins in our preparations (48). Briefly, 5 mM Thioflavin T and Congo red solution was made in 50 mM glycine buffer (pH 9) and PBS, respectively. Then, the misfolded proteins and controls were added at a final concentration of 25 µg/ml. After 30 minutes of incubation at room temperature, the fluorescence intensity of the dyes were measured in a Hitachi F4500 fluorescence spectrophotometer. In Thioflavin T measurements 435 nm excitation and 485 nm emission; in Congo red measurements 544 nm emission and 585 nm emission fluorescence wavelengths were used.

Tissue-type plasminogen activation (tPA) mediated plasmin formation assay – tPA binds misfolded proteins (2), therefore we used this assay to investigate whether γ-globulins preparations contain misfolded proteins. Aliquots of 25 µg/ml misfolded γ-globulins and controls were mixed with 400 pM tPA, 20 µg/mL plasminogen and 0.42 mM plasmin-specific chromogenic substrate S-2251 in Hepes-buffered saline (20 mM HEPES, 4 mM KCl, 3 mM $CaCl_2$, pH 7.4). The conversion of plasminogen to plasmin was inferred from the liberation of p-nitroaniline at 405 nm at 1 minute intervals for 3 hours at 37°C in 96-well plates (Corning Incorporation, NY) (2).

Competition ELISA for 7H2 antibody binding – To study the nature of the epitope of aged γ-globulins for hybridoma 7H2 and that whether this binding site for 7H2 are the same as for Congo red, Thioflavin T or tissue-type plasminogen activator, we used an ELISA setup. Five µg/ml aged γ-globulins were air-dried on high binding ELISA plates (Greiner) for overnight, at 37°C. Then the plates were blocked, washed and the wells were filled with a mixture of dilution serie of Congo red (0.005, 0.05, 0.5, 5, 50, 500 µM), Thioflavin T (0.005, 0.05, 0.5, 5, 50, 500 µM), tPA (0.001, 0.01, 0.1, 1, 10, 100 nM) and 7H2 antibodies (1:400) for 1 hour at room temperature while shaking. After washing with PBS/0.1% Tween-20, the wells were incubated with either RAMPO (1:2000, for Congo Red and Thioflavin T wells) or rabbit polyclonal anti-tPA antibody (385 R, 1:500 from American Diagnostica Inc., CT, USA; for tPA wells) for 1 hour at room temperature, which was followed by washing. In the tPA wells, peroxidase-coupled swine-anti-rabbit polyclonal immunoglobulins were added (1:2000, DAKO, Denmark) and incubated for 1 hour at room temperature. Then after extensive washing, TMB was added to the wells and the colour formation was stopped by addition of 1% H_2SO_4. The plates were read at 450 nm.

Platelet isolation and aggregation - Platelets were isolated and aggregation test was performed as described earlier (18). After centrifugation (150 g, 15 minutes, 20°C), the platelet rich plasma (PRP) was removed, its pH was lowered to 6.5 with 10% ACD buffer (2.5% trisodium citrate, 2% D-glucose and 1.5% citric acid) to avoid platelet activation. Then,

the PRP was centrifuged (300 g, 15 minutes, 20°C). The pellet was resuspended in pH 6.5 HEPES-Tyrode buffer (5 mM KCl, 145 mM NaCl, 0.5 mM Na_2HPO_4, 1 mM $MgSO_4$, 10 mM HEPES and 5 mM glucose) and was immediately centrifuged (330 g, 15 minutes, 20°C) after adding 10 ng/ml PGI_2 to the solution. The platelet pellet was resuspended again in pH 7.2 HEPES-Tyrode buffer and the final platelet number was adjusted to $2x10^5$ THR/µl. Prior to the experiment, the platelets were kept at 37°C for at least 30 minutes to ensure their resting state. The aggregation of the platelets was monitored for 15 minutes at 37°C with a stirring speed of 900 rpm in an aggregometer (Chrono-Log Corporation, Havertown, PA, USA). For this, we used $2x10^5$ platelet/µl. First, 270 µl of platelet solution was pipetted in the aggregometer and after approxmiately 60 seconds of stirring, 30 µl of protein solution or control was added. As blank, we used unstirred Hepes Tyrode buffer. In blocking experiment with monoclonal antibodies, equivalent amounts of the 5 purified monoclonal antibody solutions were mixed (mHAb). Then, mHAb or intravenous immnoglobulins (IVIg) were preincubated with misfolded proteins for 15 minutes at room temperature. The used protein and antibody concentrations are expressed as final ones in the platelet suspension. The aggregation is expressed as the percentage of the light transmission (0-100%). As control, thrombin receptor activating peptide (TRAP) was added with 4 µM to induce platelet aggregation. The platelets were used for not more than 3 hours after isolation.

Results

Intravenous immunoglobulins (IVIg) contain antibodies for misfolding proteins – In this study, we used the following proteins: amyloid-β, bovine serum albumin (BSA), hemoglobin (Hb), a synthetic peptide derived from the sequnce of fibrin peptide FP13 and human immunoglobulins (γ-globulins). To obtain misfolded preparations, we used the following previously described protocols. We misfolded proteins and peptides by glycation (BSA-AGE and Hb-AGE) (49) and ageing at elevated temperature (aged amyloid-β, FP13 and γ-globulins). Amyloid-like characteristics were confirmed by the binding of the small dyes Thioflavin T and Congo red and by analysis of the activation of tissue-type plasminogen activator (tPA) mediated plasmin formation (data not shown). We previously found that tPA is activated by misfolded proteins with amyloid-like properties.

To investigate whether IVIg also contains antibodies that bind misfolded proteins, BSA-AGE, Hb-AGE, aged amyloid-β and their native controls were coated on ELISA plates and the binding of IVIg was determined. IVIg demonstrated strong binding (OD 1.18, 1.05

and 0.57 respectively) to the misfolded forms of the proteins (BSA-AGE, Hb-AGE and aged amyloid-β). However, the binding of IVIg to native controls BSA, Hb and freshly dissolved amyloid-β was weak (OD 0.27, 0.28 and 0.07)(Figure 1). The isotype of the bound IVIg antibodies was determined by ELISA (data not shown) and found to be exclusively IgG. Taken together, IVIg contains antibodies that can distinguish misfolded from native conformation of multiple proteins.

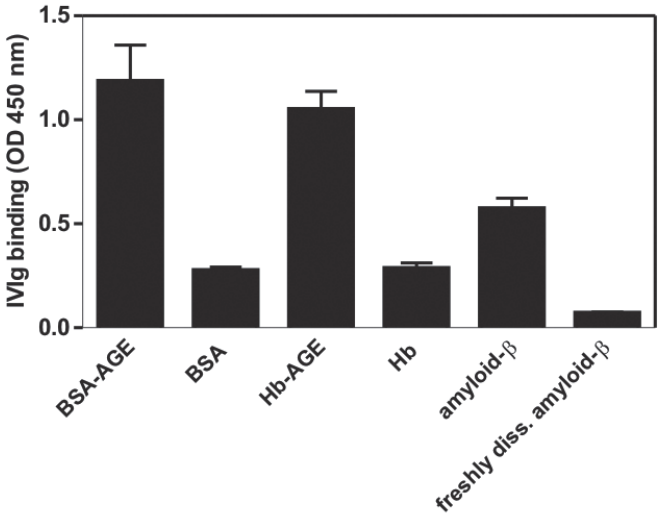

Figure 1. Intravenous immunoglobulins (IVIg) bind misfolded proteins - Misfolded proteins (BSA-AGE, Hb-AGE, aged amyloid-β) and native controls (BSA, Hb, freshly dissolved amyloid-β), all at 5 μg/ml were coated on ELISA plate and IVIg (1:200) was added. Misfolding of proteins triggers increased binding of IVIg (BSA-AGE/BSA: 4.3 fold, Hb-AGE/Hb: 3.75 fold, aged amyloid-β/freshly dissolved: 9.5 fold increase).

IVIg diminishes misfolded proteins-induced platelet aggregation – We have recently reported that misfolded proteins activate platelets (18). In order to test the neutralizing effect of IVIg on misfolded proteins, we tested the influence of IVIg on platelet aggregation induced by misfolded proteins. Preincubation of aged amyloid-β with 2.6, 1.3 and 0.65 mg/ml IVIg resulted in concentration dependent reduction of platelet aggregation (Figure 2A). In contrast, the addition of IVIg did not influence the platelet stimulatory effect of 4 μM thrombin receptor activating peptide TRAP (Figure 2B). IVIg did not completely inhibit but rather delayed the effect of amyloid-β, since the early platelet activation feature i.e. shape change was still observed, although the degree of aged amyloid-β induced aggregation (77%) was strongly (2.6 mg/ml: 21%, 1.3 mg/ml: 27%, 0.65 mg/ml: 41%) reduced in the presence of IVIg. Similar inhibition was observed with 2,6 mg/ml IVIg and Hb-AGE (89%±12%;

+IVIg: 20%±0.6%) and BSA-AGE (71%±19%; +IVIg: 11%±7%) induced platelet aggregation (Figure 2C). The inhibitory effect of IVIg was somewhat stronger on glycated proteins (Hb-AGE: 22%±15%; BSA-AGE: 16%±7%) than on aged amyloid-β (29%±15%) induced platelet aggregation (Figure 2D). Taken togeter, IVIg can neutralize the stimulatory effect of misfolded proteins but not that of TRAP.

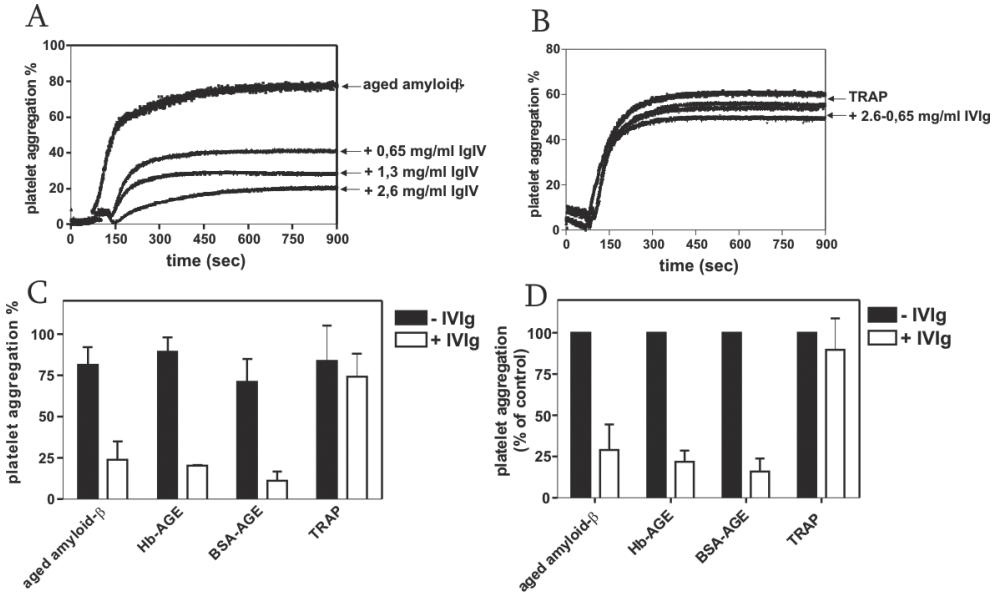

Figure 2. Misfolded proteins-induced platelet aggregation is blocked by IVIg - Platelet aggregation induced by aged amyloid-β is dose-dependently inhibited by IVIg (A), but TRAP-induced aggregation is not influenced (B). IVIg (2.6 mg/ml) blocks amyloid-β, Hb-AGE and BSA-AGE induced platelet aggregation; but has no effect on TRAP-induced aggregation (C, D).

Selection of hybridoma clones – In order to investigate whether it is possible to induce the formation of conformational antibodies against various misfolded proteins, we immunized mice with aged amyloid-β, chicken serum albumin, glycated hemoglobin (Hb-AGE) and FP6. After sacrifizing the animals and taking out their spleen, hybridoma cells were prepared and supernatants were tested for binding to aged FP13 and γ-globulins, two proteins that were composed of aggregated misfolded proteins but were not used during the immunizations. Through this selection step, we aimed to pick clones that produce antibodies with specificity for the misfolded, non-native protein structure rather than towards their specific amino acid composition. Six antibody clones, 2E2, 4F4, 7H1, 7H2, 7H9 and 8F2 were chosen, from which 4F4, 7H1, 7H2 and 8F2 were of IgM isotype; 2E2 had IgG1 isotype, and 7H9 appeared to be a dual clone with IgM and IgG2b isotype specificity (Table 1.).

Table 1. Isotype of the selected monoclonal hybridoma antibodies

Hybridoma clones	Isotypes
2E2	IgG1
4F4	IgM
7H1	IgM
7H2	IgM
7H9	IgM / IgG2b
8F2	IgM

Hybridoma clones produce polyreactive antibodies for various misfolded proteins – The 6 selected antibodies were tested for their ability to bind misfolded proteins. For this; aged γ-globulins, FP13, amyloid-β and Hb-AGE and native controls were coated on ELISA plates and the binding of the hybridoma antibodies was determined. Figure 3 demonstrates that 2E2, 4F4, 7H1, 7H9 and 8F2 showed ability to bind more than one of the coated aged γ-globulins, FP13, amyloid-β and Hb-AGE; although the ligand specificity of the different clones with their binding affinities were different. Higher than 2 fold relative binding was showed with aged FP13 and amyloid-β with 2E2; aged FP13 with 4F4:, age amyloid-β and Hb-AGE with 7H1, aged γ-globulins with 7H2, aged γ-globulins, amyloid-β and FP13 with 7H9 and 8F2 (Figure 3). The monoclonal antibody that recognized one ligand, aged γ-globulins was 7H2. None of the antibodies bound to buffer or native controls. Taken together, the selection resulted in 5 antibodies (2E2, 4F4, 7H1, 7H9, 8F2) that revealed polyspecificity for various misfolded proteins and one (7H2) that bound exclusively to aged γ-globulins.

Antibody 7H2 exclusively binds to particular subsets of misfolded γ-globulins – Antibody 7H2 appeared to be highly specific for aged γ-globulins and did not bind the native form or other misfolded proteins. Moreover, its isotype was IgM, which is comparable to the 4 other clones. Therefore clone 7H2 was chosen to be the subject for further investigations of the ligand specificity and the epitope for the interaction between 7H2 and misfolded γ-globulins. First, different techiques such as NaOH-, HCl-, heat-denaturation and oxidation were used to modify γ-globulins. Then, the diverse γ-globulins preparations were coated at the same concentration (5 μg/ml) on ELISA plates and the binding of 7H2 was measured. As it is seen on Figure 4A, 7H2 recognized aged and NaOH-denatured γ-globulins but not HCl-, heat-denatured, oxidized or native forms. To investigate the impact of misfolding on the ligand specificity of 7H2, the presence and quantity of misfolded proteins in the γ-globulins preparations were measured by Thioflavin T, Congo Red (Figure 4B) and tPA-mediated plasmin formation assays (Figure 4C).

Thioflavin T and Congo red have the capacity to distinguish misfolded proteins and this interaction triggers the increase of fluorescence of the dyes (23).

The aged, NaOH and HCl-denatured γ-globulins bound both Congo red and Thioflavin T, although NaOH-denatured preparation triggered reduced fluorescent intensity of both dyes compared to aged. Native, heat-denatured and oxidized γ-globulins did not bind Congo red or Thioflavin T. Due to its capacity to interact with misfolded proteins and become activated upon binding (2), tPA-mediated plasmin formation assay was also used to test the different γ-globulins. Aged, NaOH, HCl- and heat-denatured preparations provoked tPA activation, wherease oxidized and native γ-globulins did not have any effect (Figure 4C).

Because Congo red, Thioflavin T and tPA bound misfolded γ-globulins, we used these molecules to examine the binding site of aged γ-globulins for antibody 7H2. In the range of 5 nM to 0.5 μM, Congo red did not influence the reaction, however, above 0.5 μM of added Congo red, antibody 7H2 lost the capacity to bind to aged γ-globulins (Figure 4D); whereas Thioflavin T (Figure 4E) and tPA (Figure 4F) did not interfere with the reaction at any concentration. Taken together, antibody 7H2 bound a particular subset of misfolded γ-globulins that are present in aged and HCl-treated samples. Additionally, these samples triggered binding of Congo red, Thioflavin T and tPA. These data suggest that the epitope for the interaction of aged γ-globulins and 7H2 is the same as for Congo red but different from that for Thioflavin T and tPA.

Misfolded protein-induced platelet aggregation is inhibited by hybridoma antibodies –To study the capacity of hybridoma antibodies to opsonize misfolded proteins and thus interfere with the induced platelet aggregation, platelet aggregation tests were performed in the presence or absence of hybridoma antibodies, which were mixed with equal amounts beforehand. Amyloid-β provoked maximal platelet aggregation at a concentration of 50 μg/ml. Preincubation of 100 μg/ml antibodies with amyloid-β resulted in complete inhibition of platelet aggregation (Figure 5A), whereas 50 or 25 μg/ml antibodies reduced the aggregation to 10% and 20%, respectively. Presence of similar doses of antibodies did not affect thrombin receptor activating peptide (TRAP, 4 μM) induced platelet aggregation (Figure 5B). Taken together, hybridoma antibodies disturb the interaction of platelets and amyloid-β through binding to the epitope for platelet interaction; which is apparently absent in TRAP.

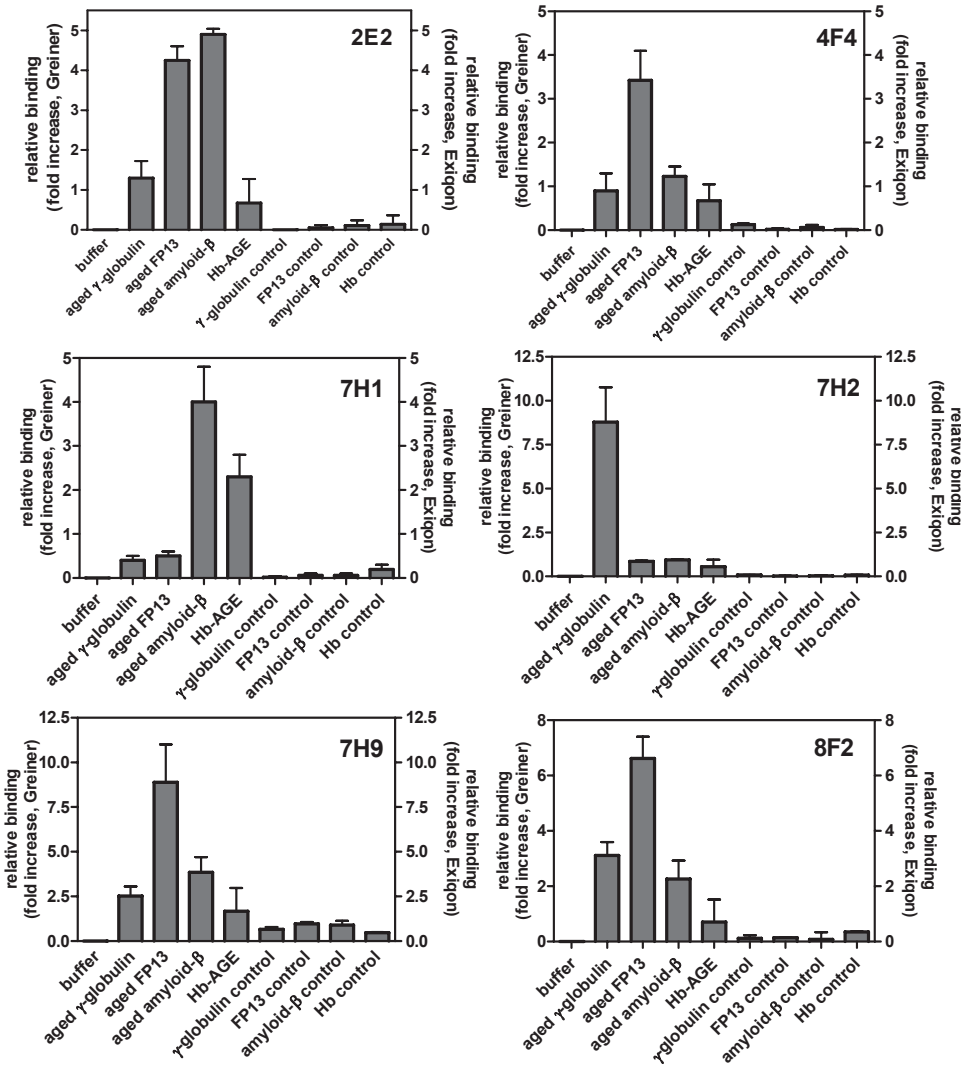

Figure 3. Monoclonal antibodies have polyspecificity for multiple misfolded proteins - Antibodies 2E2, 4F4, 7H1, 7H9 and 8F2 bind various proteins ligands, whereas 7H2 interacts with only misfolded γ-globulins. None of the antibodies recognized the native forms of the polypeptides.

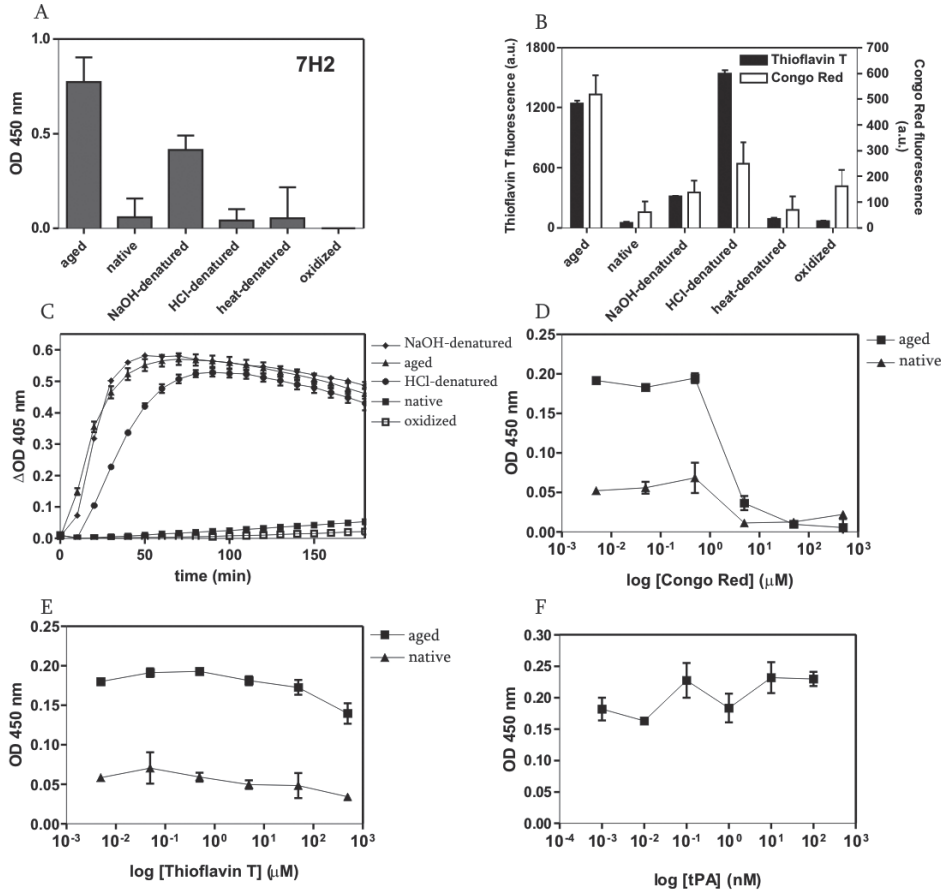

Figure 4. Monoclonal antibody 7H2 recognizes a specific subset of misfolded γ-globulins. Antibody 7H2 binds to aged and NaOH-denatured γ-globulins (A). Various γ-globulins (5 μg/ml) were coated on ELISA plate and the binding of (1:500) 7H2 was measured after 1 hour of incubation. Congo red and Thioflavin T (both 5 μM) bind aged, HCl- and NaOH-denatured γ-globulins (B). Tissue-type plasminogen activator (tPA, 400 pM) is activated by NaOH-denatured, aged, HCl-denatured and heat-denatured γ-globulins (all 25 μg/ml) (C). None of 7H2, Congo red, Thioflavin T and tPA binds to oxidized and native γ-globulins. Congo red, from 5 nM to 0.5 μM does not interfere with the binding of 7H2 to aged γ-globulins, however from 5 μM on, the dye entirely hampers the interaction (D). The same range (5 nM-500 μM) of Thioflavin T (E) or 1pM-100 nM tPA (F) does not have effect on the binding of 7H2 (1:400) to 5 μg/ml aged γ-globulins.

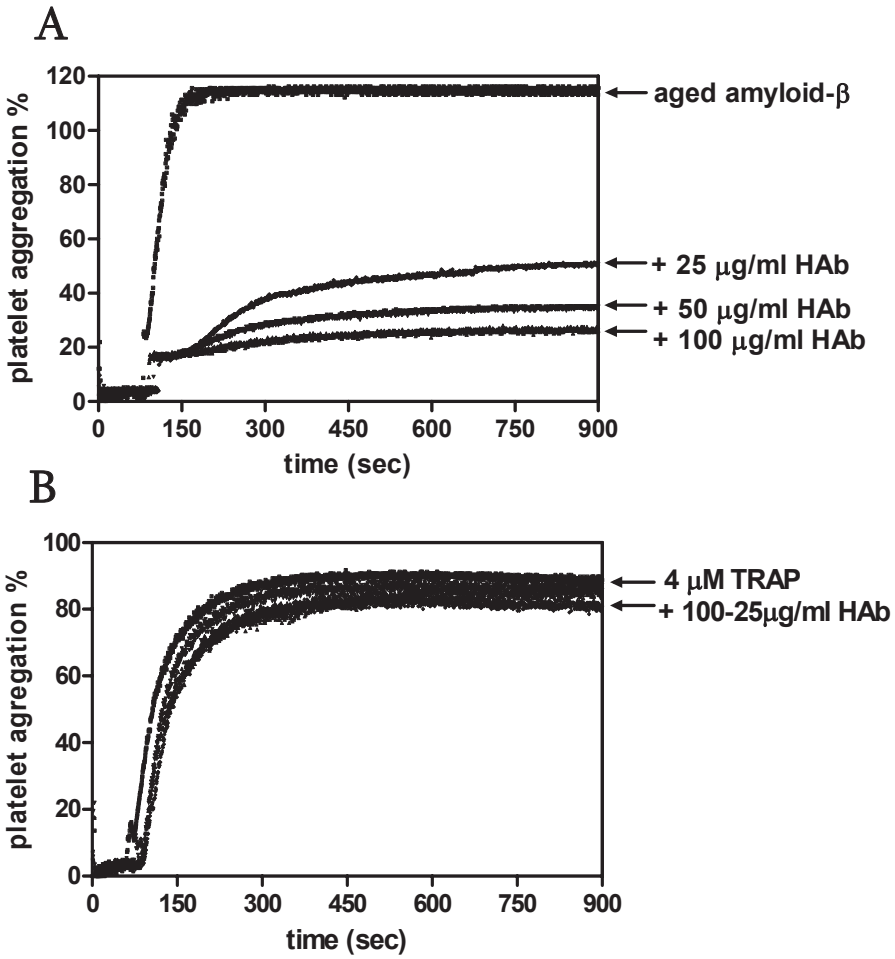

Figure 5. Monoclonal antibodies abolish amyloid-β induced platelet aggregation - Platelet aggregation provoked by 50 μg/ml amyloid-β is precluded by the addition of 100 μg/ml hybridoma antibody suspension (HAb), whereas 50 and 25 μg/ml antibody solutions have incomplete blockage (A). The thrombin receptor activating peptide (TRAP, 4 μM) induced aggregation is not effected by the addition of 25, 50 and 100 μg/ml HAb (B).

Discussion

The present work demonstrates that IgG antibodies with polyreactivity towards misfolded proteins are found in intravenous immunoglobulin preparations. In mice, conformation-specific IgM antibodies can be raised. One of the hybridoma clones, 7H2 antibody react only with specific subsets of misfolded γ-globulins through an epitope that is similar to that of the Congo red but not of Thioflavin T or tPA. Misfolded protein-specific mouse monoclonal or human polyclonal antibodies inhibit the stimulatory effect of misfolded proteins on platelets.

Antibodies with IgG isotype were found in IVIg, which recognized various unrelated misfolded proteins without binding to their native form. The fact that IgG antibodies with specificity for modified and misfolded self antigens are present in the blood of healthy subjects without triggering pathologic processes suggests a natural role for these antibodies in the maintenance of immune system homeostasis, that requires further investigation.

Recent observations suggest that misfolded proteins contain conformational epitopes and that antibodies that are generated against misfolded proteins in animals can distinguish between different structural appearances of proteins (40;41). Our results are in aggreement with these findings. Monoclonal antibodies that were generated against multiple injected misfolded proteins recognize the misfolded form of unrelated proteins. The isotype of the antibodies was IgM and IgG with the prevalence of IgM. Polyreactive antibodies that are described in the study of O'Nuallian are also of the IgM subtype (40). Interestingly, the mentioned multiple ligand-specificity and the IgM isotype characteristics of our clones resemble natural polyreactive IgM antibodies (52;53) that mediate the clearance of self-antigens such as altered proteins, lipids or apoptotic cells and participate in the primary immune reponses against bacteria (54;55). Apoptotic cells expose misfolded proteins on their surface and microbes such as bacteria and fungi express amyloid coat which improve their invading capacity (56). From these observations it can be hypothesized that the interaction of natural antibodies with their ligands may be in fact mediated by misfolded proteins. Though the characteristics of human antibodies found in IVIg are similar to those found in mice, the former is exclusively composed of IgG, while the latter is IgM. The fact that misfolding of proteins triggers class switch in B cells in man but not in mice might reflect the differences in immune responses between mice and man (57).

The binding of 7H2 antibody to misfolded γ-globulins was inhibited by the addition of Congo Red but was not affected by Thioflavin T and tPA. These results suggest that 7H2 and Congo red interact with a similar epitope on aged γ-globulins. Congo red has been used

as a gold standard to identify amyloid fibrils and it has recenty been observed to interfere with amyloid fibril formation and to protect against amyloid toxicity (58;59); although, the exact binding site for Congo red on its ligands remains unknown. Although Thioflavin T and tPA also recognize misfolded proteins, they could not interfere with the 7H2 binding to aged γ-globulins suggesting dissimilar binding sites for these molecules. Since aged γ-globulins interact with Congo red, Thioflavin T and tPA, there might be different conformational epitopes that become exposed on the protein as a consequence of its aging.

Platelet aggregation provoked by amyloid-β was prevented by the addition of hybridoma antibodies and IVIg, whereas they did not have any effect on TRAP induced aggregation. We have recently described that activation of platelets by misfolded proteins is mediated by two receptors, CD36 and GPIbα and that this activation is abolished by tPA and soluble receptor for advanced glycation end product (sRAGE) (18). Our data indicate that the addition of hybridoma antibodies and IVIg preparations (similarly to previous results with tPA and sRAGE) neutralized those binding sites for CD36 and GPIbα platelet receptors, which resulted in reduced of aggregation. However, inhibition of platelet aggregation with IVIg resulted in a remaining platelet aggregation and a significant shape change, whereas with the hybridoma antibodies it was complete. This might be explained by the multivalent nature of the pentameric IgM when compared to IgG; the epitopes for CD36 as well as for GPIbα might be entirely masked by the extensive sized IgM but only partly by IgG.

Our findings reveal new insight into immune responses against misfolded proteins and the possible advantageous role of conformational antibodies in misfolded proteins diseases-related thrombotic events. The presence of antibodies agaist modified proteins in healthy subjects suggest that these antibodies play a role in homeostasis preventing accumulation of misfolded proteins by capturing them directly in the circulation. Moreover, the lack of these antibodies might be crucial in the progress of protein misfolding diseases. While this study was in progress, O'Nuallain et al. (60) published that IVIg contained polyreactive antibodies with specificity for fibrillar forms of amyloid proteins. They were able to isolate these antibody fractions on specific fibril-coated beads. Furthermore, enriched IVIg obtained in this way could be used to stain amyloid in a mouse model of systemic amyloidosis. Our and the above mentioned work contributes to finding new perspectives regarding the treatment of the evolving group of protein misfolding diseases.

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Chapter four

Structural and functional characterization of amyloid-β preparations

Initial preliminary report

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Abstract

Alzheimer's disease is a protein misfolding disorder that is characterized by aggregation and accumulation of amyloid-β (Aβ) peptide in brain parenchyma and cerebrovasculature. The aggregation aspects of amyloid-β are crucial for the pathology caused by the peptide. The most prevalent amyloid-β variants, Aβ1-42 and Aβ1-40, which are both associated with the pathology of Alzheimer's disease, have similar aggregation steps but also main structural dissimilarities. The 25-35 and 17-20 segments of Aβ are known to influence their aggregation kinetics and contribute to pathology.

To study whether there is association between the structural arrangement and functional characteristics of Aβ regarding their platelet stimulating capacity, we investigated the aggregation and platelet activating characteristics of various Aβ peptides.

We have found that Aβ1-42 treated with acid (HCl) develops fibrillar species, while under similar conditions; Aβ1-40 lacks these structural components. However, they both developed non-fibrillar aggregates upon incubation with buffer only. With the exception of Aβ17-20, all small Aβ fragments, Aβ17-20, Aβ16-22 and Aβ25-35 do not form visible aggregates or fibrils under the applied conditions. Platelet aggregation was observed with the freshly dissolved and buffer-treated Aβ1-42. However, fibrillar Aβ1-42 lacked this capacity. Aβ1-40 and the smaller Aβ peptides Aβ17-20, Aβ16-22 and Aβ25-35 did not provoke platelet aggregation, with the exception of Aβ17-20. Our data suggest that platelet stimulatory capacity of Aβ resides in the non-fibrillar fractions. Since these constituents of Aβ are found in the blood of healthy subjects and their levels are increased in Alzheimer's patients, these results implicate a novel platelet stimulatory role for Aβ.

Introduction

Alzheimer's disease (AD) is associated with the presence of extracellular amyloid plaques, cerebrovascular amyloid, and intracellular neurofibrillary tangles located in the brain (1). The amyloid-β (Aβ) peptide, which results from proteolytically cleaved amyloid precursor protein (APP) has the capacity to aggregate into large amyloid fibrils, and forms the major component of the amyloid deposits that are the main hallmark of Alzheimer's disease (2;3).

According to the amyloid cascade hypothesis, the aggregation process of Aβ includes subsequent stages of polymerization from monomers through soluble oligomers to ultimate insoluble fibrils (4;5). During the different phases of aggregation, Aβ forms a mixture of different aggregate morphologies including dimers, small soluble oligomers, protofibrils, diffuse plaques and fibrillar deposits that are all rich in β-sheets (6;7). Each of the different amyloid morphologies exposes unique structural pattern and most likely distinct structural and functional epitopes. According to the amyloid cascade hypothesis, the fibrillar Aβ is the final product of the aggregation cascade, which accumulates in plaques and causes damage in the brain (8). However, there are concerns about amyloid cascade hypothesis since recent studies have demonstrated that the soluble Aβ, which can integrate into the cell membrane and form channels (9), is more toxic than the fibrillar form of the amyloid peptides (10). Moreover, the amount of cortical fibrillar plaques in Alzheimer's patients is not always associated with symptoms of cognition disorders (11;12). Thus, soluble Aβ, rather than fibrillar species are now supposed to participate in the progression of Alzheimer's disease; albeit, the principal mechanisms are not completely understood.

The aggregation aspects of Aβ are crucial for the pathology of the peptide. The peptides found in vivo are either 40 or 42 amino acids in length (Aβ1-40 and Aβ1-42 respectively). In vitro the kinetics of aggregation of these peptides is different and there are also important segments of Aβ, which influence the aggregation kinetics and outcome. This is considered to attribute to the neurotoxicity, such as for example the 25-35 fragment that has neurotoxic activity (13). Previous analysis of the carboxyl terminal region of Aβ1-40 revealed that besides 25-35, the 17-20 core contribute to neurotoxicity and aggregation capacity (14;15).

Though the two most prevalent Aβ variants, Aβ1-42 and Aβ1-40 share similar aggregation steps, they have important structural differences (16). While Aβ1-42 forms pentamers and larger multimers, Aβ1-40 develops shorter assemblies such as dimers, trimers and tetramers (17). Although Aβ1-42 aggregates faster than Aβ1-40, they are both associated with amyloid pathology. Cerebral amyloid angiopathy deposits contain a mixture of Aβ1-42

and Aβ1-40 (18), the early senile plaques contain mainly Aβ1–42, whereas plaque maturation is associated with the progressive appearance of the shorter Aβ1–40 (19). The concentration of Aβ1-42 in the plasma of AD patients is six times higher than in healthy subjects, whereas the level of Aβ1-40 remains the same (20). In healthy individuals, the soluble, non-aggregated Aβ peptide is continuously released in the blood under normal conditions; approximately 90% of secreted Aβ consists of Aβ1-40 and only 10% of the Aβ1-42 form. Aβ levels are tightly regulated by amyloid degrading-enzymes and blood-born inhibitory factors (21). These observations suggest that in the blood, Aβ1-40 and Aβ1-42 do not necessarily trigger pathologic processes given that it is constantly present, while their levels are changed in the brain of AD.

Though the majority of the plasma-derived Aβ peptide is produced by platelets (22), little is known about platelet-released Aβ and its physiological role in haemostasis. Aβ, as well as its precursor protein, APP, is released upon platelet stimulation with agonists such as thrombin and collagen (23). It has been reported that the main species of Aβ released by activated platelets is Aβ1-40. We have shown that the aged Aβ1-40, which exposed amyloid characteristics such as Thioflavin T, Congo red binding, capacity to activate tissue-type plasminogen activator (tPA) and comprised fibrillar structure in electron micrographs; induced platelet activation, whereas the freshly prepared, non-filamentous Aβ1-40 failed to stimulate the platelets (24).

In the past decade, researchers mainly focused on revealing structural species of Aβ concerning its neurotoxic properties, although, the peptide derives from platelets and therefore it is constantly present in the blood. In spite of the fact that the aggregated Aβ harms primarily the brain of Alzheimer's disease patients, our previous work suggests that Aβ acts as a stimulator for platelets, which might be dependent on its conformation. Therefore, the present study aims to enlighten the association between the structural arrangement and functional characteristics of Aβ peptides regarding their platelet stimulating capacity by investigating the aggregation and platelet activating characteristics of various Aβ peptides. In this work, Aβ1-42, Aβ1-40, Aβ17-20, Aβ16-22 and Aβ25-35 peptides were dissolved in buffer or HCl to get freshly dissolved (t=0), buffer-(HBS) or acid-treated (HCl) preparations, and then they were used to analyse their stimulatory activities on human platelets.

Methods

Substances - Aβ17-20 (LVFF), Aβ16-22 (KLVFFAE), Aβ25-35 (GSNKGAIIGLM), Aβ1-40 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV) and Aβ1-42 (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA) were synthesized at Netherlands Cancer Institute, Amsterdam, The Netherlands. 1,1,1,3,3,3-hexafluor-2-propanol (HFIP), dimethyl sulfoxide (DMSO), Thioflavin-T, 8-anilino-1-naphthalenesulfonic acid (ANS) were purchased from Sigma; St.Louis, MO, USA. Congo red was obtained from Aldrich Chemical Company. Pefachrome-PL was from Kordia (Leiden, The Netherlands).

Preparation of peptides - Amyloid-β (Aβ) peptides were first dissolved to 1 mM in HFIP, which was afterwards evaporated under nitrogen gas. Then, the peptide layer was resuspended in 100 µl DMSO and then diluted in PBS (t=0), HBS or in 10 mM HCl to the concentration of 400 µg/ml. The samples prepared in HBS and HCl were incubated for at 24 hours at 4°C and 37°C, respectively (Table 1.).

Thioflavin T, Thioflavin S, Congo red and ANS binding - Thioflavin T (ThT) and Thioflavin S (ThS) were dissolved in 50 mM glycine buffer at pH 9 to a concentration of 25 µM. Twenty-five µM Congo red was prepared by incubation of the dye with PBS for 30 minutes at 37°C. 8-anilino-1-naphthalenesulfonic acid (ANS) was diluted in PBS to 50 µM. Ten µl of 400 µg/ml Aβ peptide solution was diluted in 90 µl staining in the wells of black ELISA plate (Greiner Bio-one, Frickenhausen, Germany). For the fluorescence measurements, the samples were incubated for 10 minutes at room temperature and measured in microplate fluorometer (Fluoroskan Ascent, Thermo Electron Corporation, Waltham, MA, USA). The used excitation and emission wavelengths were λex: 544 nm and λem: 590 nm (CR); λex: 435 nm and λem: 485 nm (Tht); λex: (ThS) and λex: 380 nm and λem: 460 nm (ANS).

Transmission Electron Microscopy - For the analysis by transmission electron microscopy, 5 µl of Aβ was applied on copper-coated grids. After subsequent washing steps with PBS and water, the grids were incubated with 1% methyl-cellulose uranyl pH 4 for 5 minutes at 22°C. The grids were analyzed with a JEM-1200EX electron microscope (JEOL, Tokyo, Japan) at 10.000 x magnification.

tPA-mediated plasminogen activation - Aliquots of 40 µg/mL Aβ or controls were mixed with 400 pM tPA, 20 µg/mL plasminogen and 0.42 mM plasmin-specific chromogenic substrate Pefachrome in Hepes-buffered saline (20 mM HEPES, 4 mM KCl, 3 mM CaCl2, pH 7.4). The conversion of plasminogen to plasmin was inferred from the liberation of p-nitroaniline at 405 nm at 1 minute intervals for 3 hours at 37°C in 96-well plates (Corning Incorporation, NY)(25). As positive control, heat-denatured ovalbumin was used, prepared

as follows. A solution of 1 mg/ml ovalbumin (Sigma, MO, USA) in 67 mM sodium phosphate buffer with100 mM NaCl (pH 7.0) was gradually heated from 30°C to 85°C over a period of 12 minutes The slope of the curves were calculated with GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Platelet isolation - Freshly drawn venous blood from healthy volunteers with informed consent was collected into 0.1 volume 130 mmol/L trisodium citrate. The donors claimed not to have taken any medication during 2 weeks prior to blood collection. After centrifugation (15 minutes, 150g, 20°C), the platelet rich plasma (PRP) was 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 minutes, 300g, 20°C), the 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 PGI_2 (final concentration) was added and the wash step was repeated. The platelet pellet was then resuspended in Hepes-Tyrode buffer pH 7.2 to a final platelet count of $2x10^{11}$ THR/L. Before the start of the experiments, the platelets were kept at 37°C for at least 30 minutes to achieve a resting state.

Platelet aggregation - Platelet aggregation was followed in an aggregometer (Chrono-Log Corporation, Havertown, PA, USA) for 15 minutes at 37°C at 900 rpm. A volume of 270 µl platelet suspension was incubated with 30 µl solution containing samples for analysis at indicated concentrations.

Table 1. Names, sequences and treatments of Aβ peptides

Peptides	Sequences	Treatments
Aβ17-20 t=0	LVFF	freshly dissolving in PBS
Aβ17-20 HBS		dissolving in HBS, incubation for 24 hours at 4°C
Aβ17-20 HCl		dissolving in HBS, incubation for 24 hours at 37°C
Aβ16-22 t=0	KLVFFAE	freshly dissolving in PBS
Aβ16-22 HBS		dissolving in HBS, incubation for 24 hours at 4°C
Aβ16-22 HCl		dissolving in HBS, incubation for 24 hours at 37°C
Aβ25-35 t=0	GSNKGAIIGLM	freshly dissolving in PBS
Aβ25-35 HBS		dissolving in HBS, incubation for 24 hours at 4°C
Aβ25-35 HCl		dissolving in HBS, incubation for 24 hours at 37°C
Aβ1-40 t=0	DAEFRHDSGYEVHHQKLVFFAEDVG	freshly dissolving in PBS
Aβ1-40 HBS	SNKGAIIGLMVGGVV	dissolving in HBS, incubation for 24 hours at 4°C
Aβ1-40 HCl		dissolving in HBS, incubation for 24 hours at 37°C
Aβ1-42 t=0	DAEFRHDSGYEVHHQKLVFFAEDVG	freshly dissolving in PBS
Aβ1-42 HBS	SNKGAIIGLMVGGVVIA	dissolving in HBS, incubation for 24 hours at 4°C
Aβ1-42 HCl		dissolving in HBS, incubation for 24 hours at 37°C

Results and discussion

The intent of this work was to obtain different structural species of Aβ peptides and investigate their properties on human platelets. To achieve this, we applied a previously reported approach (26) to generate Aβ1-42 samples that are rich in oligomeric and fibrillar species. We also included the freshly dissolved form of the peptide. First, we analyzed their structural appearances by using fluorescent dyes and transmission electron microscopy. Then, we tested their potential to mediate plasmin formation by activating tissue-type plasminogen activator (tPA). Finally, we studied whether they induce platelet aggregation. In order to measure amyloidogenic properties, we used the same conditions to prepare and analyse other Aβ fractions (Aβ17-20, 16-22, 25-35 and 1-40), which were subsequently tested in platelet aggregation assay. A list of the peptides with their names, sequences and the applied treatments are provided in Table 1.

Characterization of Aβ1-42 Preparations - Freshly dissolved Aβ1-42 (Aβ1-42 t=0,) buffer-treated Aβ1-42 (Aβ1-42 HBS) and HCl-treated Aβ1-42 (Aβ1-42 HCl) samples were added to fluorescent dyes (Thioflavin T (27), Thioflavin S (28) and Congo red (29)) to determine their amyloid content and the conformational differences (1-anilino-8-naphtalene sulfonate, ANS (30;31)) of the samples (Figure 1A). Aβ1-42 t=0 and Aβ1-42 HBS showed equally low binding to Thioflavin T, Congo red and ANS contrary to Aβ1-42 HCl, which had high capacity to bind these dyes. However, there was no significant difference between the three in binding to Thioflavin S. This may reflect earlier findings that showed Congo red negative and Thioflavin S positive staining in diffuse plaques (32), which are rich in amorphous assemblies and which has been suggested to be the initial stage of senile plaques found in Alzheimer's brain (33). Aβ1-42 t=0 and Aβ1-42 HBS contained similar aggregates and were free of fibrils illustrated on transmission electron micrographs (Figure 1B), though the amount of aggregates was higher in the Aβ1-42 HBS than in the Aβ1-42 t=0. Fibrils were seen in the Aβ1-42 HCl sample. Furthermore, while Aβ1-42 t=0 and Aβ1-42 HBS were potent stimulators of tPA (V_{max} 0.583 ± 0.013 and 0.587 ± 0.011, Slopes 0.00605 ± 0.00064 and 0.00573 ± 0.00047), Aβ1-42 HCl failed to induce tPA-mediated plasmin formation (V_{max} 0.169 ± 0.008, slope 0.00141 ± 0.00031; Figure 1C), which is similar to previous studies (34). Taken together, Aβ1-42 t=0, Aβ1-42 HBS and Aβ1-42 HCl expose different properties by means of their amyloid contents and structural arrangement.

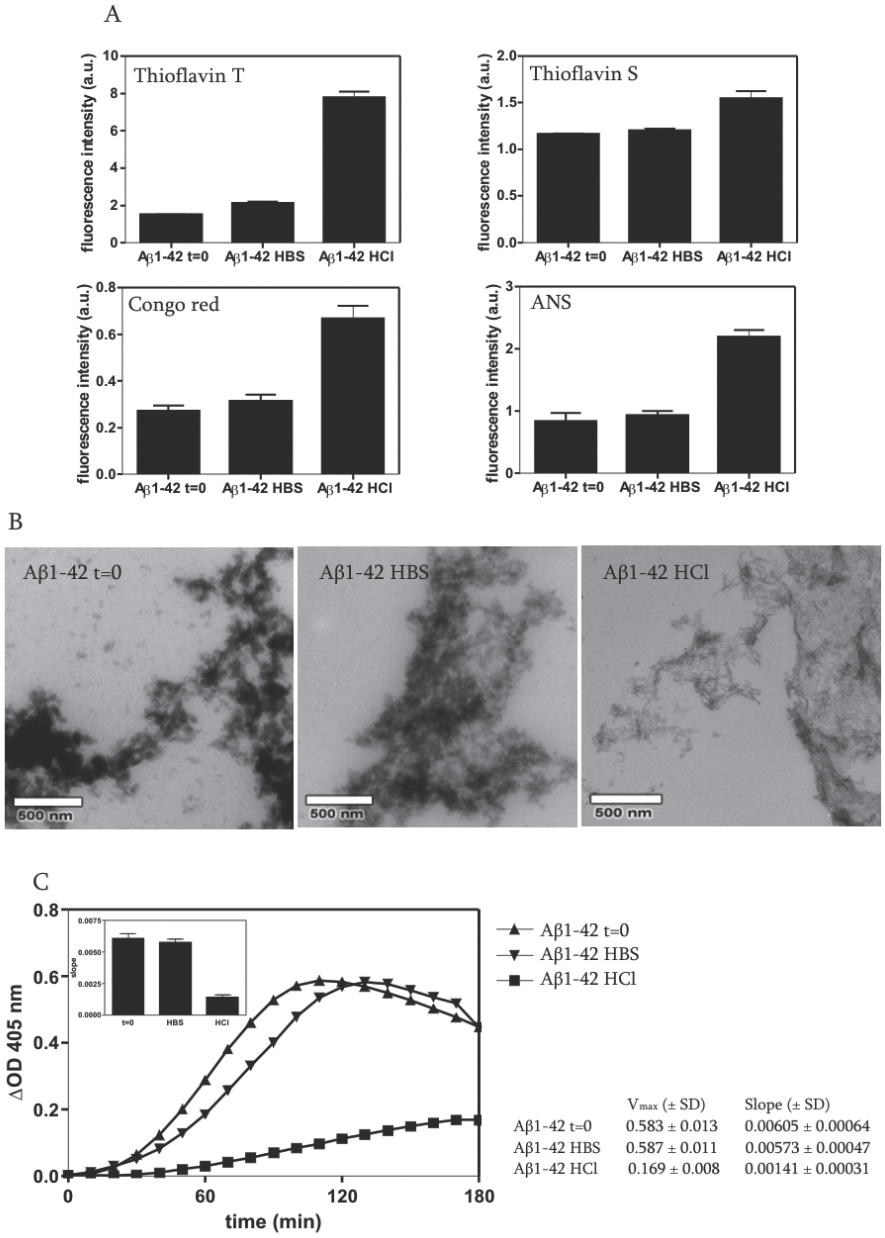


Figure 1. Different treatments induce structural diversity of Aβ1-42 samples - Structural characteristics of freshly dissolved (t=0), buffer- (HBS) and HCl-treated Aβ1-42 were analysed by Thioflavin T, Thioflavin S, Congo red and ANS binding, TEM and tPA-mediated plasmin formation test. A: Fluorescence of Thioflavin T, Congo red, Thioflavin S and ANS was measured in solution after addition of 40 μg/ml Aβ1-42 t=0, HBS and HCl samples to the dyes. The HCl-treated Aβ1-42 bound the dyes and increased their fluorescent intensity. B: Transmission electron micrographs showed different structural properties of the Aβ1-42 preparations; t=0 and HBS samples contain non-fibrillar aggregates, the HCl-treated one consists of fibrils. The scale bars correspond to 500nm. C: Aβ1-42 t=0 and HBS preparations induced fast tPA-mediated plasmin formation, the HCl-treated sample did not have effect.

Platelet aggregation is induced by non-fibrillar Aβ1-42 – Previously, we demonstrated that aged and fibrillar Aβ1-40 but not fresh Aβ1-40 induced platelet aggregation (24). When the three different Aβ1-42 forms were used (40 μg/ml), Aβ1-42 t=0 and HBS induced platelet aggregation (Figure 2). Aggregation induced by Aβ1-42 t=0 was rapid and resulted in approximately 70% aggregation; whereas the response stimulated by Aβ1-42 HBS was weaker (50%) and more prolonged. Aβ1-42 HCl failed to induce aggregation; however, it did provoke platelet shape change. Thus, there is a clear separation between Aβ1-42 t=0, HBS and HCl regarding their capacity to induce platelet aggregation, with preference for the non-fibrillar Aβ1-42 assemblies (Aβ1-42 t=0 and Aβ1-42 HBS), which are strong stimulators of platelets. The absence of platelet aggregation with the fibrillar Aβ1-42 HCl differs from what we have previously found with aged and fibrillar Aβ1-40, which might be explained with the lack of structural assemblies most likely generated during longer period of incubation.

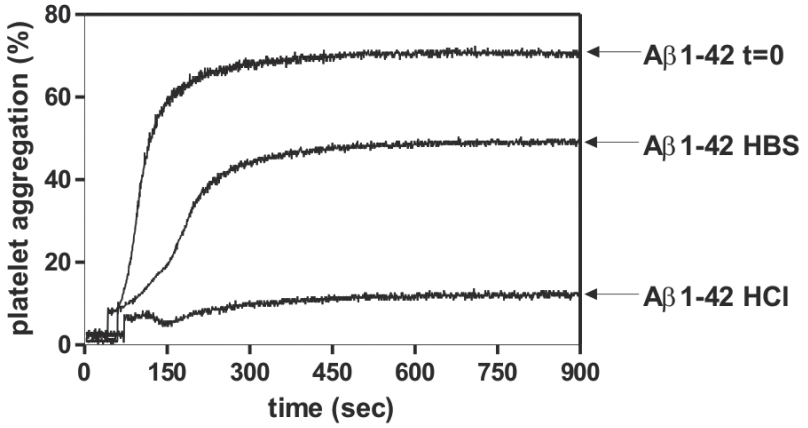
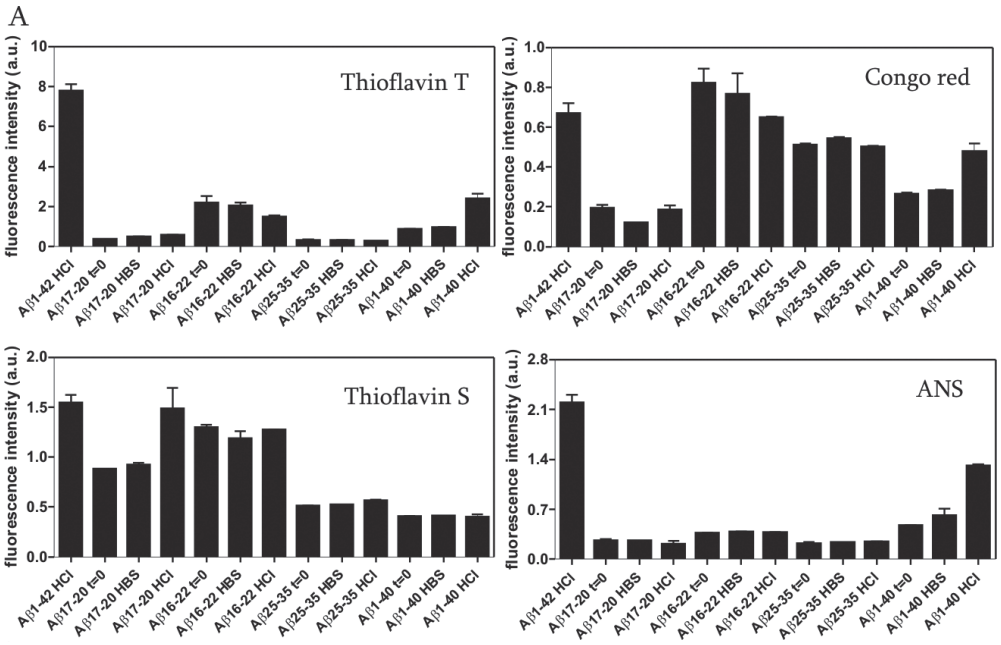


Figure 2. Non-fibrillar Aβ1-42 induces platelet aggregation. -Platelet aggregation was measured during stimulation of the washed platelets with Aβ1-42 t=0, Aβ1-42 HBS and Aβ1-42 HCl with the final concentration of 40 μg/ml. Platelet aggregation is induced by 40 μg/ml of Aβ1-42 t=0 and Aβ1-42 HBS but not by Aβ1-42 HCl sample.

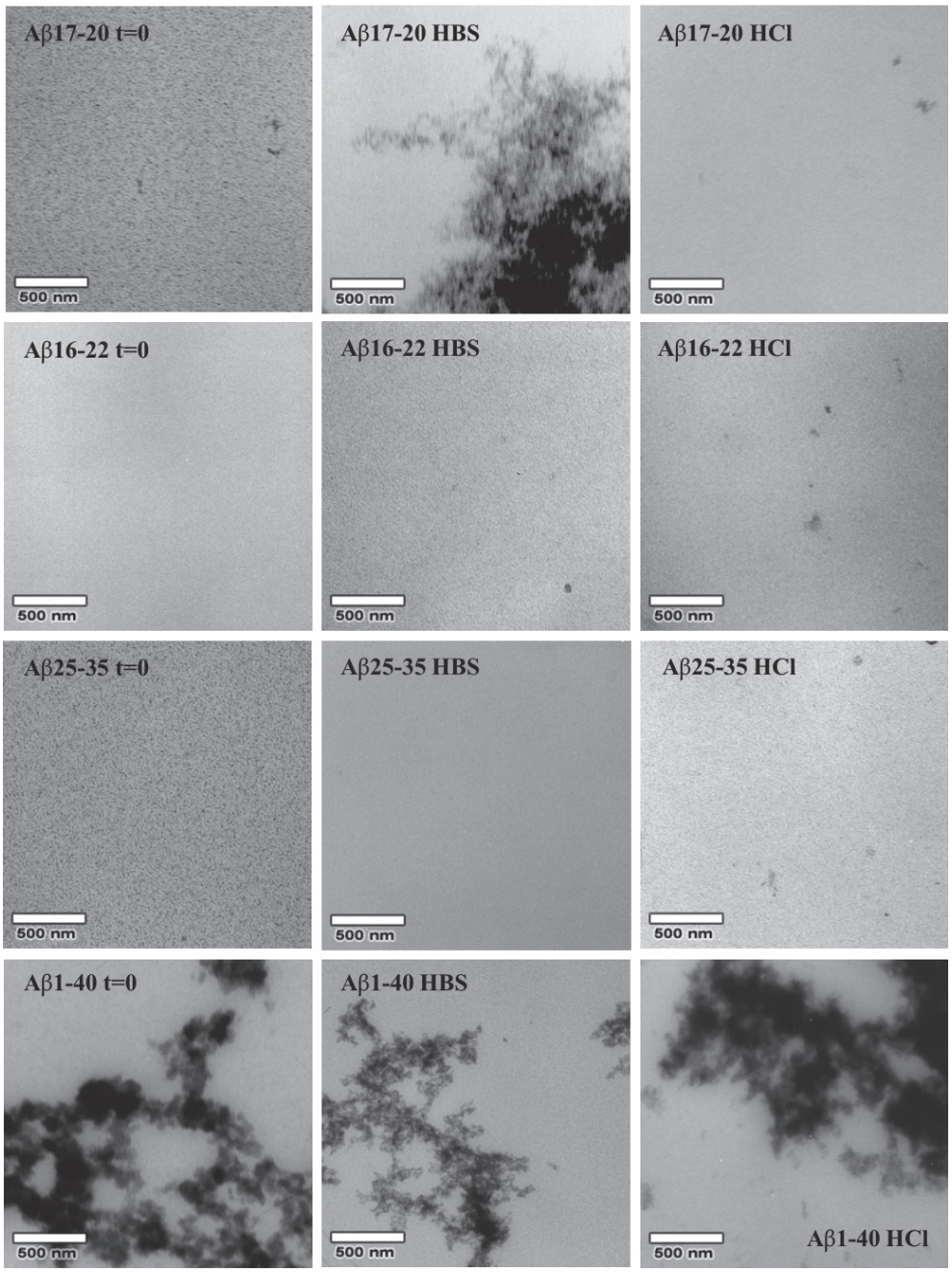
Characterization of Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40 Preparations – The observed differences in platelet response with variations of Aβ1-42 prompted us to analyze the structural influence on the aggregation and platelet stimulatory properties of other Aβ peptides, such as small segments of Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40. The selected 17-20 and 25-35 segments play crucial role in the aggregation rate of the peptide (35); while Aβ16-22 is reported to form amyloid fibrils under certain circumstances (36). In order to compare structural assemblies of Aβ17-20, Aβ16-22, Aβ25-35, and Aβ1-40, we first applied the same protocol as we used for Aβ1-42. Then, we tested their capacity to bind fluorescent probes Thioflavin T, Thioflavin S, Congo red and ANS (Figure 3). Aβ17-20 t=0, HBS and HCl all

failed to bind to Thioflavin T (fluorescent intensities: 0.3, 0.4, 0.5), Congo red (f.i.: 0.19, 0.11, 0.18) and ANS (f.i.: 0.25, 0.25, 0.20), but showed increased binding to Thioflavin S, particularly the HCl form, which demonstrated enhanced binding (f.i.: 1.4) to the dye compared to the t=0 (f.i.: 0.8) and HBS (f.i.: 0.9) forms. Aβ16-22 t=0, HBS and HCl illustrated significant binding to Thioflavin T (f.i.: 2.1, 2.0, 1.4) Thioflavin S (f.i.: 1.2, 1.1, 1.2) and Congo red (f.i.: 0.8, 0.8, 0.7) (Figure 3A), but it did not bind to ANS (f.i.: 0.36, 0.36, 0.38, respectively). Aβ25-35 t=0, HBS and HCl did not bind to Thioflavin T (f.i.: 0.35, 0.34, 0.27, respectively), Thioflavin S and ANS, but did to Congo red (f.i.: 0.53, 0.51, 0.49, respectively). The most diversity between the differently treated samples, regarding their affinity to Thioflavin T, Congo red and ANS, was seen with Aβ1-40. The Aβ1-40 t=0 and HBS forms did not show affinity to these fluorescent probes, while HCl bound them strongly (f.i.: Thioflavin T 2.3, Congo red 0.47, ANS 1.3).

For ultrastructural analysis, 400 µg/ml Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40 were applied to TEM. Aβ17-20, Aβ16-22, Aβ25-35 did not display any visible elements, except the Aβ17-20 HBS preparation, which revealed fibrillar composition (Figure 3B). All three Aβ1-40 preparations, t=0, HBS and HCl showed similar non-fibrillar aggregates, though their composition differed in quantity. None of the Aβ17-20, Aβ16-22 and Aβ25-35 preparations induced tPA-mediated plasmin formation (Figure 3C and D); however, Aβ1-40 t=0, HBS and HCl induced equally plasmin formation with similar kinetics (Figure 3D).



B


Aβ17-20 t=0
Aβ17-20 HBS
Aβ17-20 HCl
Aβ16-22 t=0
Aβ16-22 HBS
Aβ16-22 HCl
Aβ25-35 t=0
Aβ25-35 HBS
Aβ25-35 HCl
Aβ1-40 t=0
Aβ1-40 HBS
Aβ1-40 HCl
500 nm

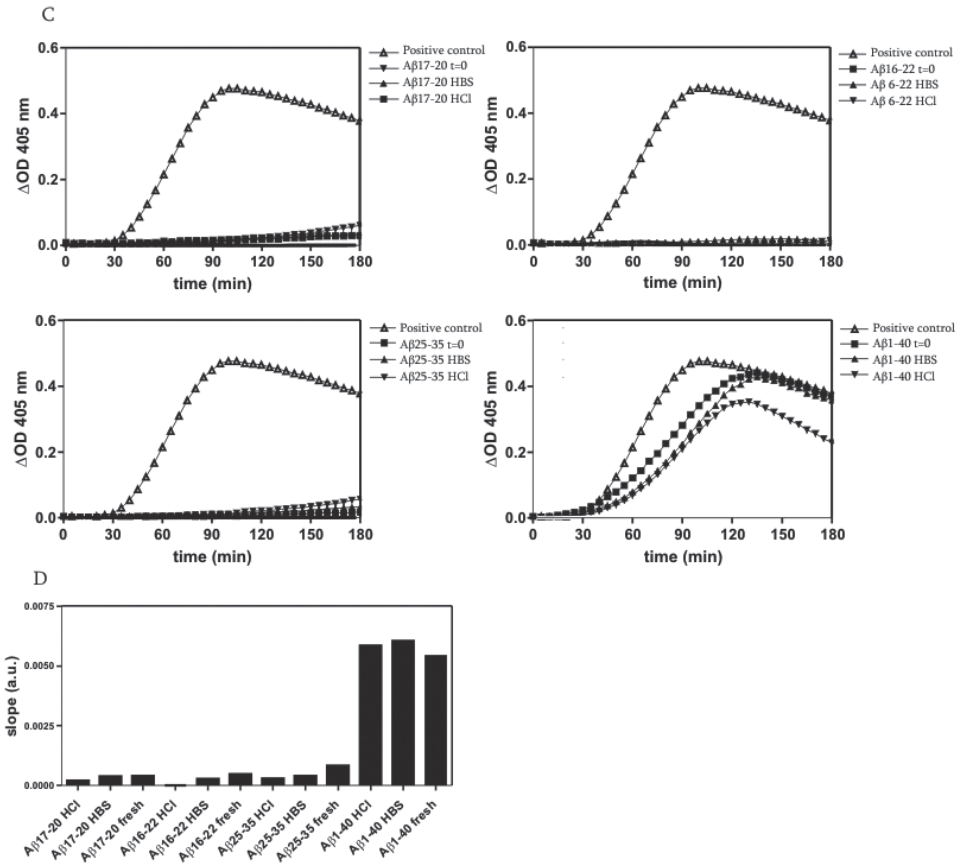


Figure 3. Differently treated Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40 present structural variations - A: Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40 show dissimilar binding to amyloid-specific dyes. Thioflavin T (25 μM), Thioflavin S (25 μM) Congo red (25 μM) and ANS (50 μM) dyes were added to 40 μg/ml Aβ preparations and fluorescent intensities were measured. B: TEM images with Aβ preparations. C: Aβ1-40 induces tPA-mediated plasminogen activation. Aβ preparations (40 μg/ml) were added to a premix of tPA, plasminogen and plasmin-specific chromogenic substrate. Plasmin generation was detected by measuring color formation (405 nm). As positive control, heat-denatured ovalbumin was used. D: The slopes of tPA-mediated plasminogen activation curves were calculated with GraphPad Prism.

In conclusion, our results suggest that under the applied conditions, Aβ17-20, Aβ16-22 and Aβ25-35 did not compose ultrastructural assemblies since they did not show visible particles on electron micrographs, except Aβ17-20 HBS, which showed non-fibrillar aggregates. However, these small segments clearly underwent conformational changes as they show different binding affinity to fluorescent probes Thioflavin T, Thioflavin S, Congo red and ANS. Previous reports described fibrillar Aβ17-20, Aβ16-22 and Aβ25-35 preparations after more than one week of incubation (37). According to these reports, amyloid fibrils exhibit a

variety of β-sheet organizations, depending on the peptide sequence. A common feature of the Aβ1-40 and Aβ1-42 sequences is the presence of hydrophobic segments (residues 17-22 and 25-35). Our data propose that small Aβ segments have different structural properties; however, under the applied conditions they neither formed large assemblies, nor activated tPA.

Aβ17-20 and Aβ1-40 are weak stimulators of platelet aggregation –To investigate the platelet stimulatory properties of the small Aβ peptides, 40 μg/ml Aβ17-20, Aβ16-22, Aβ25-35 and Aβ1-40 was added to platelets. Aβ17-20 t=0, HBS and HCl as well as Aβ1-40 HBS and HCl induced weak platelet aggregation with values between 5-15% (Figure 4). Neither Aβ16-22 and Aβ25-35 preparations, nor Aβ1-40 t=0 had any effect on platelets. Taken together, small Aβ segments and Aβ1-40 failed to induce significant platelet aggregation. When compared to the Aβ1-42 t=0 and HBS samples, which induced strong platelet aggregation, Aβ1-40 that was prepared in similar manner, did not result in comparable platelet activation capacity, though 1 week-old fibrillar Aβ1-40 was strong stimulator of platelet aggregation. These differences might be raised by structural variations, which initiate from the absence of amino-acids at the C-terminus of Aβ1-40 versus Aβ1-42 that has strong influence on the aggregation kinetics of the peptides.

Concluding remarks

In this preliminary study we aimed to investigate the amyloidogenic characteristics, as well as the platelet stimulatory capacity of Aβ peptides. Comparing the two most commonly expressed Aβ peptides, Aβ1-40 and Aβ1-42, we have found that Aβ1-42 develops fibrillar species after 24 hours of incubation with 10 mM HCl, while under similar conditions, Aβ1-40 lacks these structural components as it is indicated by less binding to Thioflavin T, Thioflavin S and ANS and images of electron micrographs. However, they both developed similar non-fibrillar aggregates upon incubation with buffer. Small Aβ fragments, Aβ17-20, Aβ16-22 and Aβ25-35 do not aggregate under the applied conditions, except Aβ17-20 HBS.

Interestingly, fibrillar Aβ1-42 loses its capacity to stimulate platelet aggregation, which was observed with the freshly dissolved and buffer-treated samples; nevertheless, Aβ1-40 did not induce significant platelet aggregation. Our data suggest that platelet stimulatory capacity of Aβ1-42 resides in the non-fibrillar fractions, which are however different from those of Aβ1-40. Small fragments of Aβ, however, do not stimulate platelet aggregation. The only exception is Aβ17-20 HBS, which is the single preparation that shows visible, non-

fibrillar aggregates.

To find the exact structural element of Aβ1-42 that induces platelet aggregation requires further studies which could examine low molecular weight assemblies of Aβ including oligomeric as well as monomeric preparations.

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Chapter five

Proinflammatory changes in HUVECs can be induced neither by native nor by modified CRP

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Abstract

The role of C-reactive protein (CRP) in atherosclerosis is controversial. It is not clear, either, if the presumed endothelium-activating effect of CRP resides in native CRP or in a conformational isoform of CRP known as modified CRP (mCRP). In the present study we evaluated and compared the effect of native CRP (nCRP), recombinant modified (r_mCRP) and urea-modified (u_mCRP) CRP on human umbilical vein endothelial cells (HUVECs).

CRP preparations were carefully analyzed by biochemical, immunological and cell-biological methods in order to avoid endotoxin or sodium-azide contamination as well as inappropriate conformational changes, which together had possibly been the main reason for the previously published controversial results. Neither nCRP nor mCRP showed significant cytotoxicity up to 100 μg/ml at 24 hours but high concentrations of CRPs induced cell death at 48 hours. r_mCRP but not nCRP nor u_m-CRP showed membrane binding to HUVEC by confocal microscopy. However, none of the CRP forms induced ICAM-1, VCAM-1, E-selectin expression or IL-8 production. MCP-1 production was weakly inhibited by high concentration of both native and recombinant modified CRP, analyzed by sandwich ELISA.

Neither native nor modified CRP could induce pro-inflammatory changes in the phenotype of HUVECs. Therefore our present findings do not support the notion that different isoforms of CRP alone have significant effects on inflammation of the vessel wall via an interaction with endothelial cells, although one cannot exclude the possibility that there may be significant differences amongst various types of endothelial cells in the response to CRP.

Introduction

C-reactive protein (CRP) is a sensitive marker of acute inflammation [1-3] and its elevated serum levels are proposed to be a sensitive cardiovascular risk factor [4-8]. However, the importance of CRP as a pro-inflammatory molecule and its predictive value in coronary heart diseases (CHD) has been controversially discussed. Danesh *et al.* reported two things on this issue, including data from a very large recent prospective study, and an updated meta-analysis of all other studies published since 2000 [9]. Values for CRP- among other inflammatory markers- have been found consistent decade to decade, suggesting that these inflammatory markers are sufficiently stable for potential use in the long-term prediction of CHD. However, it was concluded from the updated meta-analysis that baseline CRP values in the upper third of the general population distribution were associated with a more modest risk of a future coronary event than had previously claimed on the basis of the earlier, smaller studies. Wilson *et al.* [10] concluded the same: Elevated CRP provide no further prognostic information beyond traditional risk factor assessment to predict future major CVD/CHD in the study population of more than 4000 patients.

CRP has been claimed to contribute directly to endothelial dysfunction via upregulation of intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and E-selectin as well as release of IL-8, MCP-1 and IL-18 by endothelial cells and therefore proposed to play a pro-atherogenic role in atherosclerosis [11-13]. However, in recently published studies, it has been suggested that activation of endothelial cells and smooth muscle cells attributed to CRP was caused by biologically active contaminants, such as sodium-azide or LPS [14-20]. On the other hand, in a recent paper of Khreiss *et al.*, it is shown that conformational rearrangement of native CRP (nCRP), resulting into monomeric or so-called modified CRP (mCRP), is required to evoke cell activation by human coronary artery endothelial cells (HCAECs) [21]. These results are in disagreement with results from other laboratories showing that native CRP indeed upregulated adhesion molecule expression and adhesion in endothelial cells [22]. Devaraj *et al.* reported that native pentameric CRP is more potent with regard to IL-8, PAI-1 and PGF1-alpha release compared to the urea-modified CRP (u_mCRP) in human aortic endothelial cells [22].

A growing body of evidence indicates that inflammatory processes are involved in the pathogenesis of atherosclerosis from the initial step to the rupture of the vulnerable plaque [23-26]. Chronic inflammation such as during atherosclerosis is also accompanied by neovascularization. Postcapillary venules, which are part of the newly generated vessel-network in the atherosclerotic plaque, have an important role in the influx of inflammatory

cells and plasma proteins into the inflamed tissue, leading to plaque destabilization [27, 28]. Therefore in the present study, venous endothelial cells isolated from human umbilical cord (HUVECs) were used as a model for postcapillary venules.

There is a paucity of data comparing the biological activities exerted by native, urea-modified and recombinant modified CRP on venous endothelial cells. The purpose of the current study was to evaluate specific biological effects of commercially available CRP and modified CRP on human venous endothelial cells using well-characterized preparations of native and modified CRP.

Methods

Reagents - Purified native CRP from human serum was purchased from Sigma. To remove NaN_3 from the commercial CRP preparation (0.1% NaN_3), CRP was dialyzed twice against Tris-buffered saline with 2 mM calcium at 4°C (nCRP) for 24 hours. In some experiments the original azide-containing Sigma CRP was also used (a-nCRP). Urea-modified C-reactive protein (u_mCRP; azide-free) was prepared using the method of Potempa *et al.* [29]. Briefly, native CRP was incubated in the presence of 8 M urea and 10 mM EDTA solution at 37°C for 1 hour and then dialyzed overnight at 4°C into 25 mM Tris-HCl buffer at pH 8.3 to remove the urea. Recombinant modified CRP [30] (r_mCRP; azide-free) was kindly provided by L.A. Potempa (Immtech International, Inc, Vernon Hills, Ill.). The purity of CRP samples was confirmed by 12% SDS-PAGE under reducing conditions. Five μg protein was loaded on the gel and stained with Coomassie Brilliant Blue-R (the sensitivity for the detection of protein is <500 ng/ml) yielding a single protein band of 23 kDa. Endotoxin levels of all proteins were determined by LAL-assay and were below the detection limit (less than 6 pg LPS/mg CRP). Mouse monoclonal antibodies (mAb) 8D8 and 9C9 (kindly provided by L.A. Potempa) were used to detect epitopes of native CRP and modified CRP, respectively [31].

HUVEC isolation - HUVECs were digested from human umbilical cord vein by collagenase (Sigma Chemical Co., St. Louis, USA). Cells were seeded onto 0.5% gelatin (Sigma)-coated flasks and cultured in M199 (Gibco/Life Technologies Inc., Breda, The Netherlands) medium supplemented with 10% fetal calf serum (FCS) (Gibco), 100 IU/ml penicillin (Sigma), 100 ng/ml streptomycin (Sigma), 7.5 IU/ml heparin (Sigma), 2 ng/ml epidermal growth factor (EGF) (R&D, Abington, UK) and 250 pg/ml β-endothelial cell growth factor (β-ECGF) (BioSource, Camarillo, USA). The cells were used at passages 2 to 4.

Immunofluorescent microscopy - Binding of CRP to intracellular compartments: Endothelial

cells were seeded onto 0.5% gelatin coated slides. Following fixation with a 50% / 50% mixture (v/v %) of acetone-methanol, cells were incubated for 20 minutes at room temperature with 10 μg/ml nCRP, r_mCRP or u_mCRP in Tris-HCl-buffered physiological salt solution (pH 7.4) completed with 1% FCS and 2 mM $CaCl_2$. To screen binding of native and modified CRP, mAb 8D8 and 9C9 were used, respectively. Finally, the cell-bound antibodies were detected with goat anti-mouse Ig (GAM) antibody conjugated to the fluorescent dye Alexa 568 (Molecular Probes, Leiden, The Netherlands), and Hoechst 33342 (Molecular Probes) was used for nuclear counterstaining. Between incubations, cells were washed with Tris-HCl-buffered physiological salt solution (pH 7.4) completed with 2 mM $CaCl_2$. Analysis was performed by fluorescence confocal laser scanning microscopy (Olympus IX 81 + FV500 Scanning-unit). *Membrane binding of CRP:* - Endothelial cells were grown onto 0.5% gelatin-coated slides and subsequently incubated for 20 minutes at 4°C with 10 and 100 μg/ml nCRP, u_mCRP and r_mCRP in Tris-HCl- buffered physiological salt solution (see above). Following the washing step, cells were fixed with acetone- methanol. The extracellularly bound CRP was detected by mAb 8D8 and 9C9 as described above.

Cytotoxicity of CRP isoforms - The cytotoxic effects of CRP isoforms were measured on the basis of DNA-staining of cells remaining attached to the plates. Cells were seeded at a density of 10,000 cells per well onto 0.5% gelatin-coated 96 well plates and cultured for 24 or 48 hours in M199 medium completed with 2% FCS and growth factors in the presence of a-nCRP, nCRP, r_mCRP and u_mCRP. Then, cells were fixed, permeabilized and labeled for 30 minutes with SYBR-Green (1/10000) fluorescent nuclear staining diluted in physiological salt solution supplemented with 1% FCS, 2 mM $CaCl_2$ and 20 mM Tris-HCl (pH 7.4). The plate was read by Fluoroscan Ascent FL (Thermo Electron Co., Waltham, MA) with filters for excitation (485) / emission (538) at 500 ms integration time. Cell numbers were calculated on basis of a calibration curve. To validate plate-reader based measurements, cytotoxicity was also assessed by fluorescence microscopy (Olympus IX81) using automated cell counting (AnalySIS, Soft Imaging System GmbH, Münster, Germany) on the basis of nuclear staining. The two assays highly correlate (Pearson $r=0.993$, $p<0.0001$).

Proliferation assay with Alamar Blue - Cells were seeded at a density of 2,000 cells per well onto 0.5% gelatin-coated 96 well plates and were rendered quiescent for 24 hours in M199 medium completed with 2% FCS. Next, the cells were washed and incubated in medium supplemented with nCRP or mCRP. Thereafter, the cells were cultured for 24 hours, after which 10% v/v Alamar Blue was added (BioSource Europe, S.A., Nivelles, Belgium) to quantify the number of cells per well. The plate was read at 600 nm and 550 nm. For optimal results, optical density at 600 nm was subtracted from optical density at 550 nm.

Detection of adhesion molecules by cellular ELISA - Confluent cells on 0.5% gelatin-coated 96 well plates were treated with nCRP, r_mCRP and u_mCRP. Expression of ICAM-1 (CD54), PECAM-1 (CD31) and VCAM-1 (CD106) was assessed after 24 hours stimulation. Expression of E-selectin (CD62E) was measured after 4-hour stimulation. Fixed cells were labeled with mouse monoclonal antibodies against CD54 (Dako, Glostrup, Denmark), CD31 (MedSystem Diagnostics GmbH, Vienna, Austria), CD106 (BD, Pharmingen, Inc. San Diego, USA) and CD62E (Pharmingen), followed by a detection step using goat anti- mouse antibody conjugated to HRP (Dako). Peroxidase activity was detected using *o*-phenylenediamine dihydrochloride (OPD) substrate and measured at 492 nm.

MCP-1 and IL-8 sandwich ELISA - Confluent layers of HUVECs were cultured overnight in M199 medium supplemented with 2% FCS and growth factors. Next, the cells were cultured for 48 hours in the presence of nCRP, r_mCRP and u_mCRP. IL-8 and MCP-1 production were determined from the supernatants by sandwich ELISA as described previously [32].

Statistics - All experiments were performed three times and in triplicate, unless otherwise indicated. Data are presented as mean ± SD. Unpaired t-test or one-way ANOVA were used for statistical analysis. Correlation was assessed by Pearson correlation test. The level of significance was set at $p < 0.05$.

Results

Analysis of CRP isoforms - Taylor *et al.* [14] and Liu *et al.* [20] showed that activation of endothelial cell by CRP is an artefact caused by azide and/or LPS. Based on these studies all CRP preparations were carefully characterized structurally and functionally on the basis of their antigenicity and binding capacity to their biological ligands using ELISA and fluorescence microscopy techniques. Endotoxin content of each CRP preparation was below detection limit and CRP preparations were azide-free as described previously in the Methods section. Antigenic and ligand binding properties of nCRP, u_mCRP and r_mCRP fit in the data of literature [29, 31, 33, 34] (data not shown). nCRP recognized nucleus (Figure 1A) whereas u_mCRP and r_mCRP bound to cytoplasm of HUVECs (Figure 1C and E, respectively), which is in concordance with other publications [35-37].

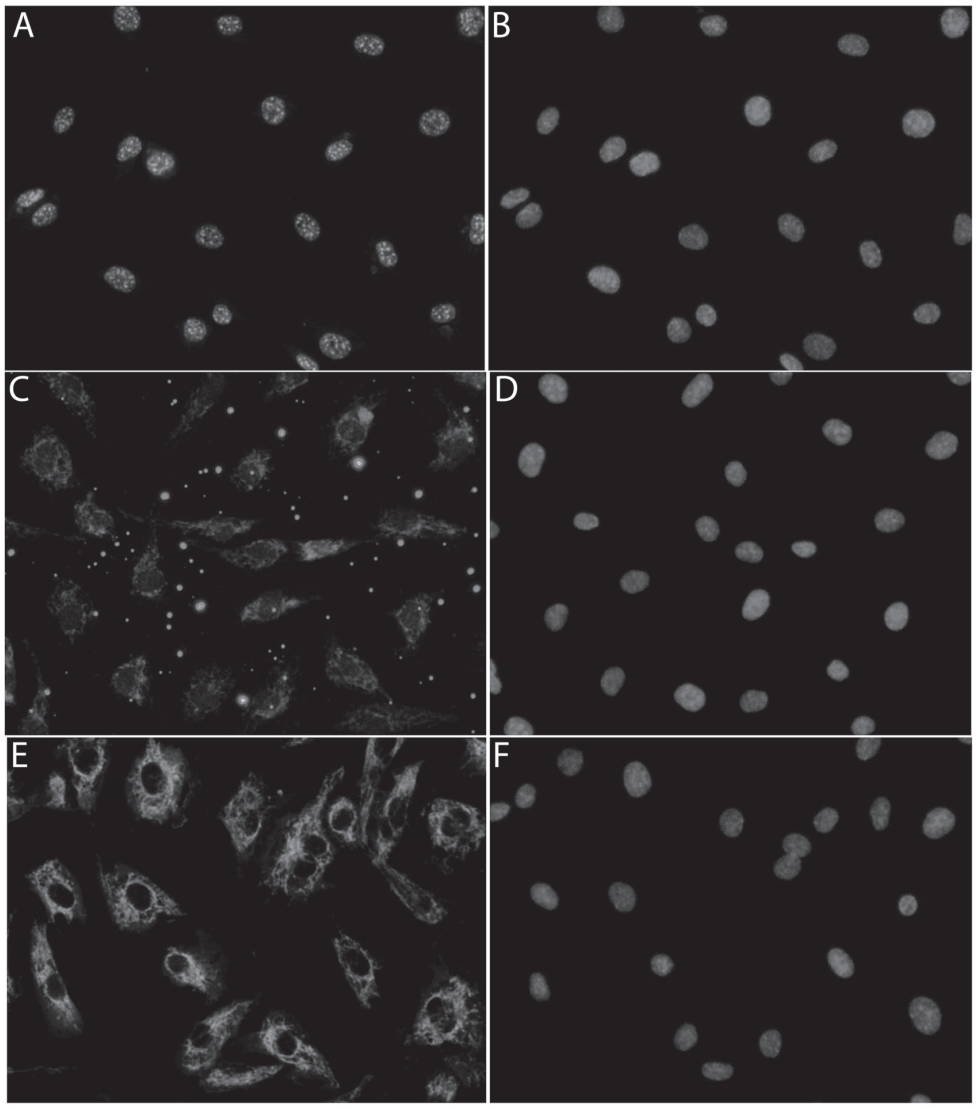

Figure 1. Substrate specificity of the various CRP isoforms. HUVECs were fixed, permeabilized and subsequently incubated with nCRP (A, B), umCRP (C, D) or rmCRP (E, F) at 10 mg/ml then monoclonal antibodies 8D8, specific for native CRP, and mAb 9C9, specific for modified CRP, were added. Finally, CRPs were detected by Alexa568 labeled goat-anti-mouse antibody (A, C, E), while the nuclei were visualized by Hoechst 33342 (B, D, F) on the same sample. A representative of five independent experiments is shown.

Viability of HUVECs following nCRP and r_mCRP treatment - Several studies have reported that CRP reduces cell viability [38, 39]. Apart from theoretical significance, viability could strongly influence the measurement of other markers. Thus, we assessed the cytotoxic effect of CRP preparations on HUVECs at 24 and 48 hours. Serial dilutions of azide-free nCRP,

azide-containing a-nCRP, r_mCRP and u_mCRP induced no cytotoxicity at 24 hours (data not shown). At 48 hours, nCRP, a-nCRP and u_mCRP reduced the viability at 50 to 100 μg/ml (Figure 2), despite only a-nCRP contained azide. Importantly, no dose-dependent cytotoxicity of sodium-azide was observed in the range that was equal to the azide content of the appropriately diluted commercial CRP preparation (data not shown).

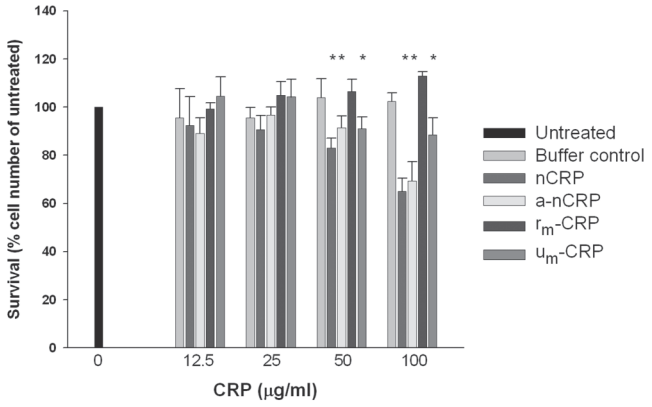

Figure 2. Cytotoxicity of CRP - Cells were cultured for 48 hours in the presence of 0 to 100 ng/ml nCRP, a-nCRP, umCRP, rmCRP and dialysis buffer control of nCRP. Afterwards, the cells were fixed, permeabilized and incubated with SYBR-Green fluorescent dye. Results are presented as percentage of attached cells following treatment compared to untreated cells. Asterisk shows significant differences at $p<0.05$ level. A representative of three independent experiments is shown.

Binding properties of r_mCRP, nCRP and u_mCRP to the cell membrane of HUVECs - In order to determine the extracellular binding ability of native and modified CRP, intact HUVECs were incubated with nCRP, r_mCRP or u_mCRP at 10 or 100 μg/ml concentration followed by fixation to preserve the membrane-bound molecules. Only r_mCRP exhibited clear membrane binding to HUVECs at 10 μg/ml concentration, whereas no detectable binding of nCRP and u_mCRP was observed (Figure 3). At higher concentration, however, native and modified CRPs showed strong background staining of the intercellular spaces, which masked any possible cellular staining (data not shown).

Effect of native and modified CRP on activation of HUVECs - To determine the effects of CRP on cell proliferation, metabolic activity of HUVECs was measured by Alamar Blue colorimetric assay at 24 hours. Neither nCRP nor r_mCRP influenced the proliferation of cells (data not shown).

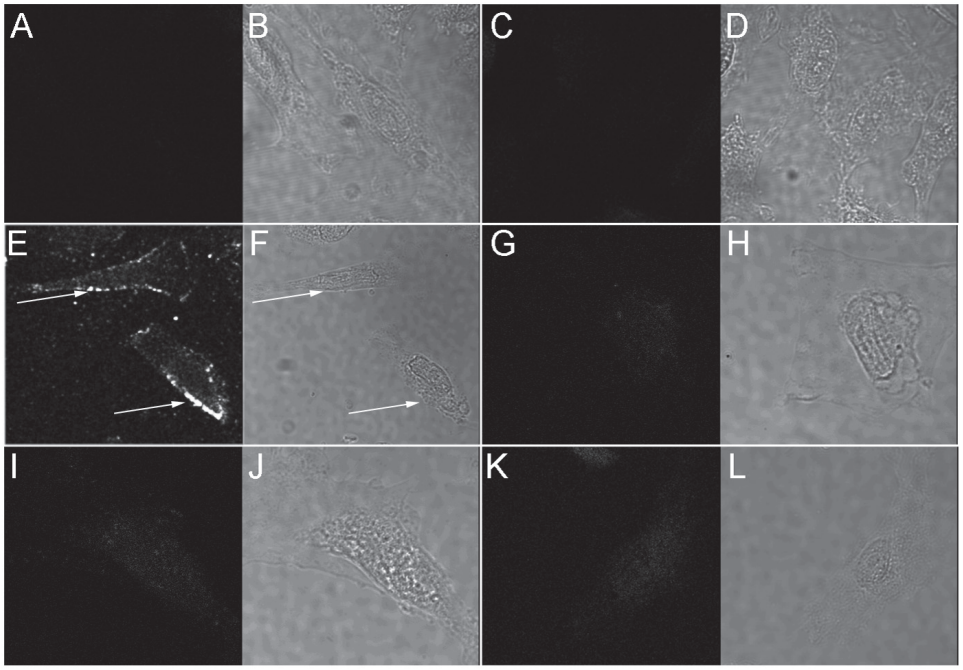

Figure 3. Plasma membrane binding of CRP to HUVECs - HUVECs were incubated with nCRP (A, B, C, D), rmCRP (E, F, G, H) or umCRP (I, J, K, L) at 10 ng/ml for 20 minutes at 4°C. Then cells were washed, fixed and incubated with mAb 9C9, directed against modified CRP (A, B, E, F, I, J) or mAb 8D8, directed against native CRP (C, D, G, H, K, L). Confocal fluorescence (A, C, E, G, I, K) and phase contrast (B, D, F, H, J, L) photos were taken. White arrows show the membrane-bound rmCRP. A representative of three independent experiments is shown.

Effect on expression of adhesion molecules - Enhanced expression of ICAM-1, VCAM-1 and E-selectin is a well described marker of pro-inflammatory changes on endothelial cells. Untreated HUVECs expressed low / undetectable levels of ICAM-1, VCAM-1 and E-selectin (Figure 4), whereas platelet endothelial cell adhesion molecule (PECAM-1) was highly expressed. LPS treatment of HUVECs resulted in a strong increase in expression of ICAM-1, VCAM-1 and E-selectin compared to the baseline expression level (Figure 4). However, incubation of HUVEC with nCRP, r_mCRP and u_mCRP in the concentration range of 25 to 100 μg/ml induced no significant change in the expression (Figure 4). Neither LSP nor CRP could change the level of PECAM-1 (data not shown). Similar results were found in the presence of human serum (data not shown).

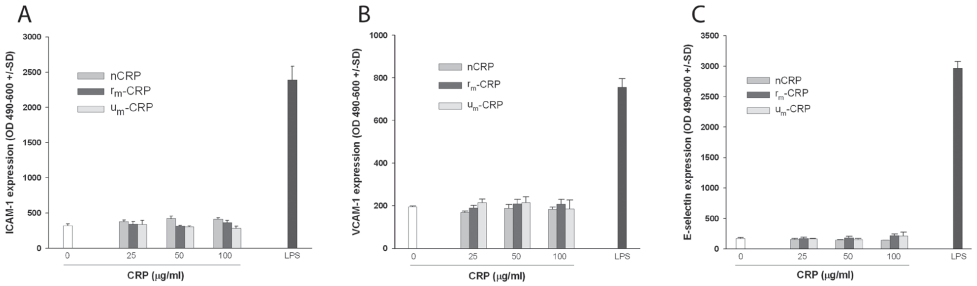

Figure 4. Effect of CRP on the expression of ICAM-1, VCAM-1 and E-selectin. Expression of adhesion molecules was determined by cellular ELISA on confluent layers of HUVECs. ICAM-1 (A) and VCAM-1 (B) expression were measured after 24 hours and E-selectin (C) expression was measured after 4 hours exposure to serially diluted nCRP, rmCRP and umCRP, respectively. Addition of LPS (1 ng/ml) was used as a positive control. Results are shown as mean ± SD. None of the values, except LPS, differed significantly from untreated control. A representative of four independent experiments is shown.

Effect on chemokine production - Production of IL-8 and MCP-1 is also a sensitive and accepted pro-inflammatory marker of endothelial cells. CRP has been reported to increase secretion of IL-8 and MCP-1 by human coronary arterial cells (HCAECs) [21]. To determine the ability of different CRP isoforms to evoke production of IL-8 and MCP-1 by HUVECs, cells were cultured for 48 hours in the presence or absence of nCRP and r_mCRP. Analysis of the supernatants for secretion of IL-8 did not show any measurable changes, compared to the baseline production of IL-8 (Figure 5A). Interestingly, CRP slightly decreased the production of MCP-1 in a dose-dependent manner (Figure 5B). As a positive control, LPS (1 μg/ml) induced an approximately 15-fold increase in IL-8 and 5-fold increase in MCP-1 production.

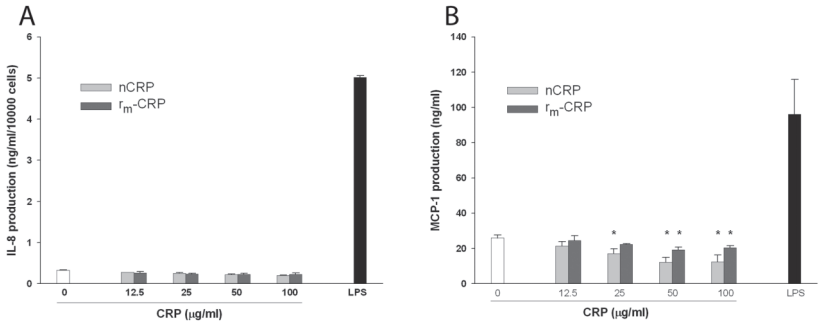

Figure 5. Effects of CRP on IL-8 and MCP-1 release by HUVECs. Cells were cultured for 48 hours in the presence or absence of nCRP, rmCRP and LPS (1 ng/ml), respectively, in concentrations as indicated. Supernatants were harvested and analyzed for IL-8 (A) and MCP-1 (B) by sandwich ELISA. Results are expressed as mean ± SD. ANOVA with Bonferroni's post test were used for statistical analysis. Samples with significant ($p < 0.05$) difference from untreated cells are marked with asterisk in the graphs. A representative of three individual experiments is shown.

Discussion

In the present study we showed that neither native nor modified CRP can induce pro-inflammatory signals in HUVECs. Viability, proliferation and expression of ICAM-1, VCAM-1 and E-selectin were not changed in response to well characterized CRPs within 24 hours, although membrane binding capacity of r_m-CRP was detected. At 48 hours we observed cytotoxicity and slightly reduced MCP-1 production at very high CRP concentrations (50-100 μg/ml), while there were no changes in the IL-8 secretion.

Native, pentameric CRP has been identified as a potent pro-inflammatory molecule, which can promote atherogenesis [11, 12]. In these studies expression of adhesion molecules (ICAM-1, VCAM-1, E-selectin) as well as pro-inflammatory cytokines (MCP-1 and IL-8) were determined. Recently, other proteins with enhanced expression induced by CRP have also been described [13, 40, 41]. Liang *et al.* elegantly presented data on the signalling pathway of CRP in endothelial cells [42]. Meanwhile, increasing numbers of criticisms have been raised on the use of commercial preparations of CRP concerning to the incompletely defined and biologically active contaminants, such as sodium azide and LPS. Contamination with LPS was excluded in most of the studies [12, 30, 42, 43], however, bacterial lipoproteins and other microbial compounds are very hard to detect. Several examples in the literature demonstrated convincingly that minute contamination can lead to alternative conclusions [14-19, 44, 45]. Taylor *et al.* and Liu *et al.* claim that sodium azide, LPS or other bacterial products of the CRP preparations are responsible for the reported effects of CRP, including antiproliferative, antimigratory, proapoptotic, and antiangiogenic effects, as well as changes in the expression of endothelial NO synthase, ICAM-1, MCP-1, IL-8 and von Willebrand factor [14, 20]. Most of the above mentioned studies concentrated on native CRP. Khreiss *et al.* showed that native CRP failed to activate endothelial cells but modified CRP, the conformational variant of pentameric CRP significantly upregulated the expression of adhesion molecules [21], whereas Devaraj *et al.* [22] found the opposite. While Khreiss *et al.* used citraconylated recombinant modified CRP, Devaraj *et al.* applied urea-modified CRP. Using both forms of modified CRP we now show that these are different in the membrane binding capacity and in the late cytotoxicity, raising the question: which modified CRP resembles most the physico-chemical characteristics of the modified CRP identified in inflamed tissues as well as in the wall of human normal blood vessels [46, 47].

Khreiss *et al.* and Devaraj *et al.* used endothelial cells of arterial origin to compare the effects of native and modified CRPs [21, 22], thus we asked which form of CRP is able to stimulate HUVECs. Based on the studies of Liu *et al.*, Taylor *et al.* and Khreiss *et al.* [14, 20,

21], we carefully characterized our CRP preparations to exclude any contamination as well as inappropriate conformational variants (e.g. native CRP in modified CRP preparations and *vice versa*). We could confirm the findings Liu *et al*. and Taylor *et al.* [14, 20], which showed that native CRP cannot evoke pro-inflammatory changes in HUVECs, and we extended these results to modified CRP, too. They find that cytotoxicity of CRP is due to azide contamination. In contrast, we showed that extensively dialyzed CRP preparations can also have some toxic properties at high (50-100 μg/ml) CRP concentrations. We observed strong extracellular matrix staining of CRPs at these concentrations (data not shown), which raises the possibility that CRP may compete matrix binding receptors of the cells, thus inhibiting proper attachment. This effect may also explain the slightly decreased MCP-1 production.

In contrast to Khreiss *et al.* [21], we did not find any pro-inflammatory changes induced by CRP. The reason for the discrepancies between the studies, where pro-inflammatory properties of CRP have or have not been demonstrated, is largely unknown. Here we list some of the major possibilities. Endothelial cells (ECs) of different origin are quite different. The above mentioned studies used HUVECs, aortic ECs (HAECs), coronary artery ECs (HCAECs), and even bovine aortic ECs (BAECs). Phenotype of ECs depends on the number of passages, too. Most studies use ECs between passage 2 to 4, but some until passage 3 [43, 48]. There are even more substantial differences in the cell culture medium. M199, M200, RPMI and EGM-MV was used as basal medium, completed with 1 to 20% of fetal bovine serum (FBS). This later has critical importance, because FBS may contain substantial amount of endotoxin with very high batch-to-batch variance [49]. Moreover, Wadham *et al.* showed that High-Density Lipoproteins (HDL) can neutralize the effect of CRP [43]. None of the CRP studies refers to the HDL concentration of the FBS. Recombinant growth factors as well as bovine brain extract (Endothelial Cell Growth Supplement) were typically used as growth stimulus. To assess the effects of CRP, in different studies a wide variety of evaluation methods was applied (FACS, cellular ELISA, Western-blot, RT-PCR, mRNA microarray, sandwich ELISA, etc.), which are not equivalent in the sensitivity nor in the time-kinetics. Finally, CRP preparation itself serves as a source of divergence. We must stress that endothelial cells are highly sensitive for endotoxin. According to Wadham *et al.*, the LPS at concentration up to 1 ng/ml was insufficient to induce adhesion molecule expression [43]. In our experiments, however, the sensitivity was between 50-500 pg/ml, showing quite wide variance amongst individual HUVEC cultures. Moreover, other bacterial contaminants (e.g. C polysaccharide from pneumococcus, a substrate of CRP after which it was named) are very rarely assayed, although a major known function of CRP is the opsonization of bacterial compounds.

Taken together our results show that neither native nor modified CRP have pro-inflammatory capacity on HUVECs, which does not completely rule out that CRP cannot evoke any phenotypical changes on different endothelial cells, assayed in different conditions.

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Chapter six

Protein kinase inhibitor induced endothelial cell cytotoxicity and its prediction based on calculated molecular descriptors

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Abstract

Protein kinase inhibitors as potent signal transduction therapeutic compounds make up a very rapidly expanding group of anti-cancer drugs. These agents may be toxic for endothelial cells, one of the most exposed cell types; however, very few experimental data exist on the cytotoxicity of protein kinase inhibitors. Thus, the aim of this study was to set up an appropriate test system for endothelial cells and to assess the structure-related cytotoxic effects of a selected library of protein kinase inhibitors.

The inhibitor library contains several lead molecules with different basic structure and a set of modified derivatives of the lead compounds. Microscopy-based morphology test and quantitative analysis based on fluorescence nucleic-acid determination were used to determine cytotoxicity. Using this new method, the analysis of 71 protein kinase inhibitors and two control compounds showed that a significant proportion (46%) of the inhibitor library has no toxic effect on endothelial cells even at 20 micromole/L concentration. The toxicity of protein kinase inhibitors did not correlate directly with the structural features of the molecules. However, we successfully built up a model based on 15 calculated molecular descriptors (selected from 25000 descriptors), which is capable of predicting cytotoxicity with acceptable probability.

Although endothelial cell cytotoxicity can be useful for the abolishment of tumor neovascularization, endothelial dysfunction/injury may also cause serious side effects such as edema, thrombus formation, shock and atherosclerosis. Our results show that the cytotoxic effects of protein kinase inhibitors should take into account for optimal drug development to overcome these side effects.

Abbreviations: HUGO, Human Genome Organization; VRGF-R, vascular endothelial growth factor receptor; EGF-R ,epitdermal growth factor receptor; bFGF-R, basic fibroblast growth factor receptor; PDGF-R, platelet derived growth factor receptor; TGF-β, transforming growth factor beta; IL, interleukin; PKI, protein kinase inhibitor; NCL™, Nested Chemical Library; DMSO, dimethyl-sulphoxide; IC_{50}, half-maximal inhibitory concentration; PBS, phosphate buffered saline; FCS, fetal calf serum; β-ECGF, beta endothelial cell growth factor; MSA, microscopy scoring assay; HBSS, Hank's Balanced Salt Solution; LD_{50}, Half-lethal dose; HUVECs, Human umbilical cord vein endothelial cells; PDE IV, phosphodiesterase IV.

Introduction

The HUGO (Human Genome Organization) project revealed that the human genome contains about 32.000 genes, which control the expression of 250.000-300.000 proteins (1). About 20-25 percent of the expressed proteins are involved in signal transduction, providing an attractive target group for signal transduction therapy. The relevance of this target group is further supported by the findings that ~80 percent of diseases (such as cancer, infectious diseases, atherosclerosis, arthritis, neurodegenerative disorders, etc.) can be related to communication abnormalities and impaired signal transduction (2, 3). As a result of development of molecular biology and protein chemistry in recent years, signal transduction therapy has emerged. The aim of this new approach is to repair signaling defects involved in cell communication. A reasonable therapeutic target may be the group of receptor tyrosine kinases and cytosolic protein kinases.

The first agent for signal transduction therapy - a BCR-Abl kinase inhibitor - was launched in May 2002 (4, 5). The drug inhibits tumor cell proliferation with 90 percent efficacy. Almost 200 other compounds with kinase inhibitory activity are being developed for signal transduction therapy, which definitely indicates the influence of this treatment modality on drug design (6, 7).

Amongst receptor tyrosine kinases, vascular endothelial growth factor receptor (VEGF-R), epidermal growth factor receptor (EGF-R), basic fibroblast growth factor receptor (bFGF-R) and platelet derived growth factor receptor (PDGF-R) have high importance because these receptors are involved in the signaling pathways of the proliferation and angiogenesis (8-11).

Beside the well known barrier function between blood and tissues, endothelial cells play a crucial role in the regulation of blood pressure, thrombosis, inflammation and vessel organization. Considering this multi-functionality of the endothelial cells, their role in tumor therapy is essential. On the one hand, sufficient blood supply is necessary for tumor growth, thus, neovasculogenesis inside tumors is an absolute requirement (12, 13). Tumor cells produce several growth factors such as VEGF, transforming growth factor (TGF)-β and angiopoietin to accelerate endothelial cell proliferation and angiogenesis (14-16). Suppressing VEGF production with interleukin-(IL)-10 or angioarrestin reduces capillarization of solid tumors, which may lead to total destruction of the tumor tissue (17, 18). In this point of view, inhibiting or killing endothelial cells in a strictly controlled manner can be beneficial, which concept was initially proposed by Judah Folkman and his colleagues more than 30 years ago (19).

Nevertheless, endothelial cells are one of the most vulnerable cell types. Since endothelial cells line all the blood vessels, the injury of endothelium may cause serious acute effects such as shock (20), edema (21) or thrombosis (22). Dysfunction or injury of the endothelial cells also play a pivotal role in chronic pathogenic processes such as atherosclerosis (23), vasculitis (24) and chronic venous insufficiency (25). All these malfunctions of endothelial cells can be triggered by the exposure to adverse drug effects, especially to cytotoxic drugs used in cancer therapy (26).

Thus, the purpose of this study was to perform a structure-and-function analysis for testing well-known protein kinase inhibitor (PKI) drugs and their chemically modified derivatives on human endothelial cells. The analysis may be important in developing drugs with minimal endothelial cytotoxicity and give some explanation for the distinct side effects of different PKI compounds.

Methods

Protein kinase inhibitor (PKI) library - PKI library for this study was obtained from Nested Chemical Library (NCL™) of Vichem Ltd. The core selection was based on the PDGF-R, EGF-R, VEGF-R inhibiting properties of previously described prominent lead compounds and their derivatives. Thus, 2,3-dihydro-1H-imidazo[5,1-b]quinazolin-9-ones (27-29), 4-phenylamino-quinazolines (30), 2-oxindoles (31), and a series of 2-phenylamino-pyrimidine variants (32-34) were synthesized for the study (see Table 2) and dissolved in DMSO at 2 mM concentration. Half-maximal inhibitory concentration (IC_{50}) values of PDGF-R and EGF-R inhibitors were obtained from the literature, calculated by in vitro kinase assays.

Endothelial cell culture - Human umbilical cord vein endothelial cells (HUVECs) were prepared from fresh umbilical cord as described earlier (35). Briefly, catheterized vein was washed by PBS, digested by 1 mg/ml type II collagenase (Sigma-Aldrich, St. Louis, MI) for 25 min at 37°C. Cells were washed out by M199 containing 10% FCS, centrifuged and plated into a 25 cm^2 tissue culture flask (Corning Inc., New York, NY) precoated with 0.5% gelatin. Cells were cultured in M199 (Gibco Invitrogen Inc., Carlsbad, CA) containing 10% FCS (Gibco), 2 ng/ml EGF (BioSource Europe, Nivelles, Belgium), 250 pg/ml β-endothelial cell growth factor (β-ECGF) (R&D System, inc., Minneapolis, MN), 7.5 U/ml heparin (Sigma) and penicillin/streptomycin (Sigma). Purity of the cell cultures was routinely checked by fluorescence microscopy using von Willebrand-factor (Sigma) immunostaining. Cells were used at passages 2 to 4.

Cytotoxicity assays - Cells were plated onto 96-well cell culture plates at 10 000 cells/well density. After 24 h of incubation, PKI-s were added in three parallels of 6 dilution steps (20 000 to 82 ng/ml, 1:3 serial dilution). Untreated cells (from 10 000 to 1000 cells/well) were applied to obtain a standard curve. DMSO dilutions were used to rule out the effects of the solvent. After another 24 h, cytotoxicity (referring to both necrosis and apoptosis, independent of the pathogenetic pathway) was measured by two different methods. First, the plate was scored (0 to 4) well-by-well using phase contrast microscopy (microscopy scoring assay, MSA). This method reflects to both cell number and morphology. After scoring, cells were washed by Hank's Balanced Salt Solution (HBSS), fixed in methanol/acetone (1:1) for 10 min, stained with SYBR-Green (Molecular Probes Invitrogen Inc., Carlsbad, CA) nucleic acid dye for 15 min and 485/535 nm fluorescence was read by Fluoroskan Ascent FL (Thermo Electron Corp., Vantaa, Finland) at 500 ms integration time. Half-lethal dose (LD_{50}) was defined as the concentration of PKI at which fluorescence of 5000 (half of the maximal 10000) cells is reached (SYBR-Green Assay) or the concentration at which value 2 is reached (MSA). Every PKI was tested in two independent experiments; the combined LD_{50} values were calculated as the mean of LD_{50} values from the SYBR-Green and the MSA tests. For the analysis of the cytopathic effects of 29 PKI molecules, HUVECs were plated onto 96-well plate at the density of 10000 cells/well and stimulated with PKI-s as described before. After 24 hours of incubation, the cells were fixed and stained with Hoechst 33342 and SYPRO-Red (both from Molecular Probes). The vacuolization and/or apoptosis were determined by Olympus IX81 inverted fluorescence microscope.

Building and validation of a molecular model - The 73 compounds were categorized into four groups according to the LD_{50} values (Table 1). The dataset was divided into two main parts: a work set (49 molecules), and an external validation set (24 molecules). The former one was used to build the model, the latter one is to validate the obtained model (these molecules were not used in the model building). More than 25 000 molecular descriptors were calculated by Dragon software (36) and our in-house developed software (this program calculates different simple topological descriptors, which are pair and auto correlation functions of any atom type in the 2D or 3D space - we call these histogram descriptors) (36). Constant descriptors and one descriptor from each pair of highly correlated descriptors were removed from the dataset. Descriptors that showed poor correlation with biological data were also deleted and the number of descriptors was reduced to 71. This dataset was analyzed by Statistica 6.0 (StatSoft Inc. 2001), where *General Discriminant Analysis* model was built by backward stepwise descriptor selection method (maximum number of cycles was set to 1000). The final model contained 15 descriptors (Table 2.) The predictive capability of

the obtained model was tested by predicting the biological data of the external validation set of molecules.

Statistical analysis - LD_{50} values were calculated by GraphPad Prism 4.02 (GraphPad Software Inc.) using sigmoidal dose-response regression. Correlation between variables was assessed by Spearman's or Pearson's correlation tests depending on the distribution of the parameters. Comparison of predicted and measured values in the external validation data set was performed by ^{2} analysis.

Results

New quantitative method for endothelial cell toxicity - Endothelial cells have well-known cobblestone morphology when they reach confluence. In cell culture, both apoptosis and necrosis of endothelial cells are preceded by decreased homotypic adhesion and decreased attachment to the extracellular matrix. MSA is sensitive for the altered cell-morphology and for impaired adherence to the plastic surface. HUVEC cells were plated at 10000/well concentration in 96-well plates, cultured in the presence of PKI-s as described above and cell cultures were scored by values 0 to 4 (0: No adherent cells in the well, 4: confluence with cobblestone morphology, 1,2,3: intermediate confluence and morphology values). Supporting MSA, a more objective and quantitative but less sensitive test was introduced. After MSA, cells were stained with SYBR-Green nucleic acid dye and the fluorescence values were read by plate format fluorescence reader. Fluorescence values reflect to the adherent cell number in the well. Cytotoxicity was described by LD_{50} values calculated from the dose-response curves of the compounds. Representatives of such dose-response curves are shown in Figure 1. LD_{50} values calculated from the morphology scoring analysis (MSA) and from the SYBR-Green fluorescence test have been proved to be in strong correlation (Pearson $r=0.8741$, $p<0.0001$, data not shown).

Screening Protein Kinase Inhibitor library for endothelial cell toxicity - MSA and fluorescence cytotoxicity assays were used to screen PK-inhibitors. 73 compounds were tested and LD_{50} values were calculated (Table 1). In case of 4 out of 73 compounds, we found dissimilarities between SYBR-Green fluorescence and MSA values. To specify these results and the cause of discrepancy, treated cells were stained with Hoechst 33342 and SYPRO-Red to follow changes in morphology by visualizing the nucleus and the cytoplasm in details. Altered cell morphology with increased cytoplasmic vacuolization was detected (Figure 2B.). Morphology

of these cells can be related to non-apoptotic programmed cell death (37, 38). Because of the proved cytotoxic effect without early detachment of the cells, we calculated LD_{50} values based solely on MSA in the case of these 4 inhibitors. Another 25 PKI-s were also subjected to fluorescence morphological tests and found correlation between vacuolization and the combined LD_{50} values (data not shown).

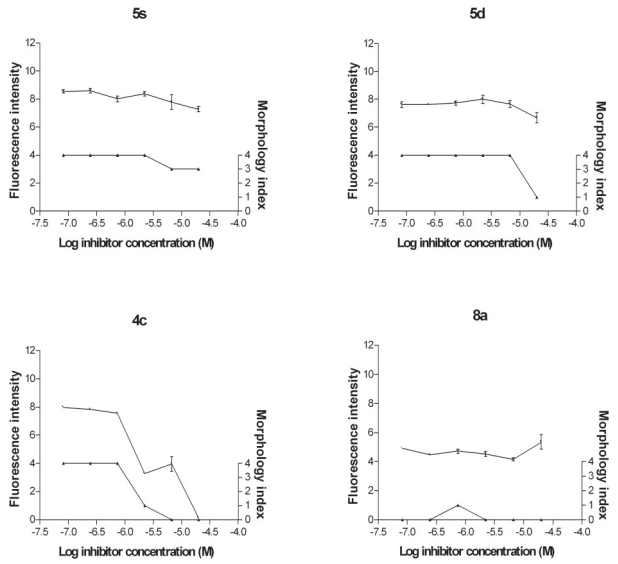


Figure 1. Typical dose-response curves of PKI-s - Cells were seeded onto 96-well plates at 10 000 cells/well density. After 1 day cells were treated with serially diluted PKI-s for 24 hours. Each well was scored by the same investigator then stained with SYBR-Green nucleic acid dye. Fluorescence values and microscopy scores were plotted against the logarithm of concentration. One representative plot of each cytotoxicity group (group 0 (Supplementar Data 5s), group 1 (Supplementary Data 5d) group 2 (Supplemntary Data 4c) and group 3 (Supplementary Data 8a)) is shown. Rectangles correspond to fluorescence intensity, triangles correspond to morphology index of the PKI treated samples.

Table 1. Short-period (24h) cytotoxicity of protein kinase inhibitors. Categories were defined in 2000-20000 nM concentration range

Group*	Effect	LD_{50} (nM)	Number of compounds
0	no	> 20 000	35
1	minor	10 000 – 20 000	23
2	moderate	2 000 – 10 000	12
3	strong	< 2 000	3

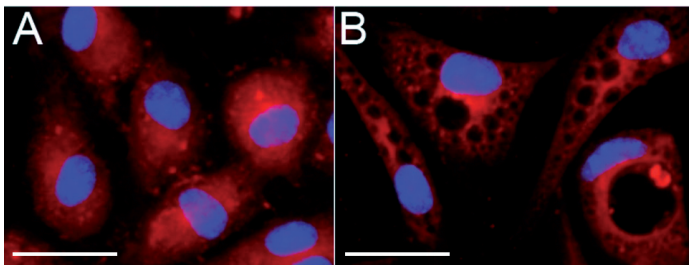


Figure 2. Vacuolization of endothelial cells - 4 PKI-s out of the 73 tested compounds induced cytoplasmic vacuolization as a marker of non-apoptotic programmed cell death. One representative of the non-toxic compounds, as a negative control (A) and a representative of vacuola inducing compound (B) are shown. Cellular proteins were stained with SYPRO-Red, while the nuclei were labeled with Hoechst 33342. (Scale bar represents 50 μm.)

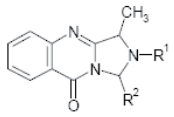
Structure/activity analysis - Four structural families of known VEGF-R, EGFR or PDGF-R inhibitors were selected for the experiments, based on core structures such as 2,3-dihydro-1H-imidazo[5,1-b]quinazolin-9-ones, 4-phenylamino-quinazolines, 2-oxindoles and 2-phenylamino-pyrimidines. 2,3-Dihydro-1H-imidazo[5,1-b]quinazolin-9-ones were originally developed as potential phosphodiesterase (PDE) IV inhibitory (cAMP inhibitory) drugs showing antithrombotic properties in *in vivo* animal tests. Their antithrombotic activity was weaker than that of the imidazo[2,1-b]quinazoline drug Anagrelide, but significantly more active than sulfinpyrazone or acetylsalicylic acid, in ADP and collagene induced platelet aggregation models (29).

Since antithrombotic and other biological effects of quinazolines can be attributed to biosterism with adenine, kinase inhibitory activity of 2,3-dihydro-1H-imidazo[5,1-b]quinazolin-9-ones had also been tested. Several active compounds were found showing low micromolar LD_{50} values, e.g. SU1835 in VEGF-R (Table 2,1e) and SU1822 (Table 2,1a) in PDGF-R assays (27, 28). There are two well-known compounds in the aminoquinazoline group: Gefitinib (Table 2,2e) (ZD1839) and Erlotinib (2d) (OSI774).

Both Erlotinib and Gefitinib showed significant endothelial cell toxicity. Increased toxicity can be observed in case of compounds that were selected from early stage developed quinazolines (Table 2,2a-b). 2-Oxindole based compounds are potent VEGF-R inhibitors and promising novel drug candidates (39). Only minor toxicity was found in this group, the average LD_{50} values are 10 μM. The fourth group of inhibitors was split into five parts depending on the substituents. Imatinib (Table 2,5n) (also known as STI-571 or CGP57148), the lead compound of its group, showed no cytotoxicity. Most of the compounds from this group have no/low endothelial toxicity (LD_{50} > 15 μM), but there are some molecules containing special substituents out of this range. For example, introduction of chloroacetyl-

or chloromethyl-benzoyl moiety resulted in significant toxicity, which can be attributed to alkylating properties (Table 2,4a-b,5a-b). Compounds with nitro-function(s) were generally highly toxic for endothelial cells. Surprisingly, the isoquinolin-5-yl-sulphonyl group was very toxic, which can not be explained on a simple structural basis. CGP60474 (Table 2,8a), a known cytotoxic PKI compound, was used as a positive (i.e. toxic) control (Figure 1D). To assess drugs that are registered as human therapeutic agents, the previously mentioned antithrombotic Anagrelide and a neovascularization inhibitor Thalidomide (40) (Table 2,9a-b) were also tested. Neither drugs showed any short-period toxicity on endothelial cells.

1



Compound	R^1	R^2	Average LD_{50} (nM)	SD	VEGFR2 * IC_{50} (nM)	EGFR * IC_{50} (nM)	PDGFR * IC_{50} (nM)
1a	benzyl	3.4-dihydroxyphenyl	10999	1932	-	4000	77470
1b	3,4-methylenedioxybenzyl	3-hydroxyphenyl	11552	383	-	-	-
1c	3,4-methylenedioxybenzyl	4-hydroxyphenyl	15828	4029	-	-	-
1d	dimethyl (quaterner)	hydrogen	>20000	0	-	-	-
1e	benzyl	4-hydroxyphenyl	>20000	0	3400	100000	50000
1f	ethyl	3.4-dihydroxyphenyl	>20000	0	-	-	-

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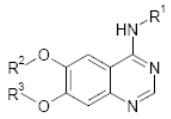
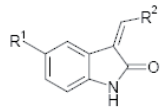


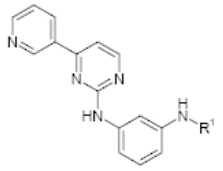
Compound	R^1	R^2	R^3	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
2a	3-bromophenyl	methyl	methyl	5073	2483	-	0.025	-
2b	3-chlorophenyl	methyl	methyl	7086	498	-	0.31	-
2c	2-phenylethyl	methyl	methyl	10384	681	-	-	-
2d	3-ethinylphenyl	2-methoxyehyl	2-methoxyehyl	10981	6560	-	20	-
2e	3-chloro-4-fluorophenyl	3-(4-morpholinyl)-propyl	methyl	11880	5717	-	23	-

3



Compound	R^1	R^2	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
3a	3-(1-piperidinyl)-1-oxo-propyl-amino	3-(5-methoxy)-indolyl	9539	2222	-	-	-
3b	chloro	3,5-bis-(2-propyl)-4-hydroxyphenyl	9742	1936	4300	9500	60400
3c	hydrogen	4-dimethylaminophenyl	9791	1867	800	-	19400
3d	chloro	2-pirrolyl	11239	1593	3000	-	85400
3e	hydrogen	3,5-bis-(2-propyl)-4-hydroxyphenyl	11641	649	8500	15300	17500
3f	hydrogen	2-(3,5-dimethyl)-4-(3-carboxypropyl)-pirrolyl	15081	4520	1040	100000	20260
3g	hydrogen	2-(3,5-dimethyl)-pirrolyl	17460	4399	2430	100000	60
3h	hydrogen	4-(2-propyl)-phenyl	>20000	0	5200	18500	24200

4



Compound	R^1	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
4a	2-chloroacetyl	321	46	-	-	-
4b	4-chloromethylbenzoyl	1995	612	-	-	-
4c	(nitro)	2156	94	-	100000	100000
4d	3,4,5-trimethoxybenzoyl	11287	865	-	-	-
4e	hydrogene	11289	38	-	50000	-
4f	4-hydroxy-5-(1-imino-ethyl)-pyrimidin-6-yl	13090	9772	-	-	-
4g	4-methylbenzenesulphonyl	12321	2751	-	-	-
4h	3-Methyl-isoxazolo[5,4-d]pyrimidin-4-yl	13334	9428	-	-	-
4i	2-(4-Methyl-piperazin-1-yl)-acetyl	15259	4474	-	-	-
4j	4-methylbenzolyl	17375	3165	-	100000	800
4k	nicotinoyl	18687	1857	-	100000	50000
4l	1,8-naphtiridine-2-yl-carbonyl	>20 000	0	-	-	-
4m	1,8-naphtiridine-2-yl-thiocarbonyl	>20 000	0	-	-	-
4n	1,6-naphtiridine-2-yl-carbonyl	>20 000	0	-	-	-
4o	1,6-naphtiridine-2-yl-thiocarbonyl	>20 000	0	-	-	-
4p	4-(4-Methyl-piperazin-1-ylmethyl)-benzoyl	>20 000	0	-	100000	200
4q	4-cyanophenyl	>20 000	0	-	100000	1500
4r	cyclohexylcarbonyl	>20 000	0	-	100000	800
4s	(isoquinolin-5-yl)-sulphonyl	>20 000	0	-	-	-
4t	2-methoxybenzoyl	>20 000	0	-	100000	1000

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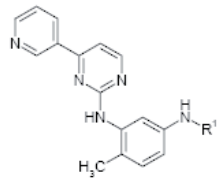
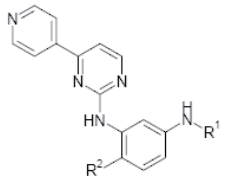


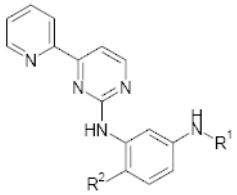
Compound	R^1	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
5a	2-chloroacetyl	2750	151	-	-	-
5b	4-chloromethylbenzoyl	9888	385	-	-	-
5c	3,4,5-trimethoxybenzoyl	11546	1231	-	-	-
5d	4-methylbenzolyl	16840	2740	-	100000	10
5e	3-Methyl-isoxazolo[5,4-d]pyrimidin-4-yl	19125	1237	-	-	-
5f	(thiophene-3-yl)-cardonyl	>20 000	0	-	-	-
5g	cyclohexylcarbonyl	>20 000	0	-	-	-
5h	(isoquinolin-5-yl)-sulphonyl	>20 000	0	-	-	-
5i	2-naphtoyl	>20 000	0	-	100000	50
5j	2-methoxybenzoyl	>20 000	0	-	100000	300
5k	benzoyl	>20 000	0	-	65000	100
5l	3,5-diacetylphenyl-aminocarbonyl	>20 000	0	-	-	-
5m	3,5-diacetylphenyl-aminocarbonyl-bis-guanylhydrazon	>20 000	0	-	-	-
5n	nicotinoyl	>20 000	0	-	-	-
5o	4-hydroxy-5-(1-imino-ethyl)-pyrimidin-6-yl	>20 000	0	-	-	-
5p	1,8-naphtiridine-2-yl-carbonyl	>20 000	0	-	-	-
5q	2-(4-Methyl-piperazin-1-yl)-acetyl	>20 000	0	-	-	-
5r	4-methylbenzenesulphonyl	>20 000	0	-	-	-
5s	4-(4-Methyl-piperazin-1-ylmethyl)-benzoyl	>20 000	0	-	100000	50
5t	4-chlorobenzoyl	>20 000	0	-	100000	100

6



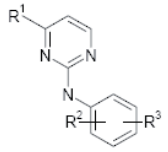
Compound	R^1	R^2	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
6a	(isoquinolin-5-yl)-sulphonyl	hydrogen	3761	465	-	-	-
6b	4-(4-Methyl-piperazin-1-ylmethyl)-benzoyl	methyl	8348	2377	-	-	-
6c	3,4,5-trimethoxybenzoyl	hydrogen	15260	6703	-	-	-
6d	3,4,5-trimethoxybenzoyl	methyl	>20 000	0	-	-	-
6e	(thiophene-3-yl)-cardonyl	methyl	>20 000	0	-	-	-

7



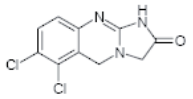
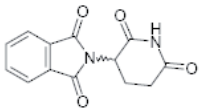
Compound	R^1	R^2	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
7a	4-(4-Methyl-piperazin-1-ylmethyl)-benzoyl	hydrogen	11405	150	-	-	-
7b	3,4,5-trimethoxybenzoyl	methyl	>20 000	0	-	-	-
7c	4-(4-Methyl-piperazin-1-ylmethyl)-benzoyl	methyl	>20 000	0	-	-	-

8



Compound	R^1	R^2	R^3	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
8a	2-(3-hydroxypropylamino)-pyridin-4-yl	hydrogen	3-chloro	<82	0	-	100000	100000
8b	3,4-dimethoxyphenyl	methyl	3-(4-(4-Methyl-piperazin-1-ylmethyl))-benzamido	4503	1691	-	-	-
8c	4-(imidazol-1-yl)-phenyl	methyl	3-(4-(4-Methyl-piperazin-1-ylmethyl))-benzamido	9344	4642	-	-	-
8d	3-pyridyl	hydrogen	4-(4-(4-Methyl-piperazin-1-ylmethyl))-benzamido	>20 000	0	-	-	-

9

Compound	Name	Structure	Average LD_{50} (nM)	SD	VEGFR2 IC_{50} (nM)	EGFR IC_{50} (nM)	PDGFR IC_{50} (nM)
9a	Anagrelide		>20000	0	-	-	-
9b	Thalidomide		>20000	0	-	-	-

Prediction of cytotoxicity by an in silico *model* - Comparing the chemical structures of PKI-s no simple structural features (core structure, lipophilicity, acidity, special functional groups, etc.) can be found which could be attributed to endothelial cytotoxicity (see Table 2). However, by a selected set of calculated molecular descriptors (Table 3), we succeeded to build up a model by which cytotoxicity can be reliably predicted (Table 4). When the predicted and the measured values were compared, the χ^2 was 16.23 and the critical value of χ^2 was 14.68 (degree of freedom = 9, p=0.0622).

Table 3. List of molecular descriptors used for endothelial cell cytotoxicity prediction model. Explanation to the histogram descriptors (N_AT1_AT2_X): N is the dimension of the descriptor, AT1 and AT2 are the atom types and X is the 2D or 3D distance between AT1 and AT2. Any: Any atom type, C.2: sp2 C, N.ar: aromatic N, Het: Hetero atom, H.c: Hydrogen attached to C, Hal: Halogen atom, Hev: Heavy atom.

Descriptor code*	Descriptor
2_Any_Cl_8	
2_C.2_N.ar_12	
2_C_N.ar_12	
2_C_N.ar_8	
2_Het_H.c_6	
2_Het_Hal_13	Histogram descriptors
2_Hev_Hal_7	
3_C_Cl_9	
3_Hal_H_1	
3_Hal_H_12	
3_Het_Hal_11	
GATS7p	Geary autocorrelation - lag 7 / weighted by atomic polarizabilities
Lop	Lopping centric index
R2m+	R maximal autocorrelation of lag 2 / weighted by atomic masses
Solub	Calculated water solubility (49)

Correlation between cytotoxicity and protein kinase inhibition - When cytotoxic effects were compared with the literature data of EGF-R and PDGF-R inhibitory capacity of 22 or 21 compounds, respectively, significant correlation has been found. Interestingly, cytotoxicity showed positive correlation with EGF-R inhibition (Spearman r=0.6236, p=0.0019) (Figure 3A) but negative correlation with PDGF-R inhibition (Spearman r=-0.6201, p=0.0027) (Figure 3B).

Table 4. Prediction of the cytotoxicity by the model based on 15 molecular descriptors. Prediction by our model corresponds to the measured cytotoxicity in the validation set. The predicted and the measured values were compared, the χ^2 was 16.23 and the critical value of χ^2 was 14.68 (degree of freedom = 9, p=0.0622)

		Predicted by our model			
		Group 0	**Group 1**	**Group 2**	**Group 3**
Measured	**Group 0**	7	2	1	0
	Group 1	2	3	1	0
	Group 2	3	1	1	0
	Group 3	0	0	1	1

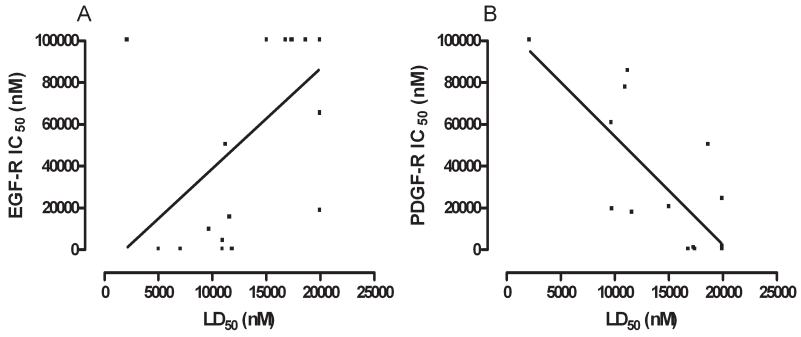


Figure 3. Correlation of cytotoxicity with receptor tyrosine kinase inhibition - LD50 values measured by our cytotoxicity tests and IC50 values of EGF-R and PDGF-R inhibitors obtained from the literature were used for the calculation of correlation. Strong positive correlation was found between cytotoxicity and EGF-R inhibition (A). PDGF-R inhibition showed similarly strong but negative correlation with cytotoxicity (B).

Discussion

Injury of the endothelium by protein kinase inhibitors, as well as by other drugs used in cancer therapy, can be a substantial pathogenetic factor in several harmful side-effects of these agents (20-22, 41). Here we set up a combined test, by which cytotoxic

effects can be measured on endothelial cells. This method is based on microscopic scoring and fluorescent nucleus counting tests. Combination of these tests generates a sensitive, repeatable, simple measurement system. Since endothelial cells can easily be detached by the least noxious stimulus, the number of the attached cells reflects to the viability (35). However, endothelial cell phenotype can be altered, leading to increased permeability or production of pro-inflammatory and/or pro-thrombotic factors without major morphological changes or detachment. Thus, by using this method, we may even underestimate the side-effects of the tested compounds. Our cytotoxicity tests are mostly based on precise cell number determination, while the exact process of the cell death (e.g. necrosis, apoptosis) cannot be directly studied. However, necrosis usually involves quick energy depletion of the cells leading to swollen morphology and cell death within few hours of exposure. We could not observe any sign of cytotoxicity (data not shown) within 4 hours after treatment, which supports the idea that programmed cell death may be responsible for the decreased cell number. Moreover, some of the compounds (4 out of 73) induced extensive cytoplasmic vacuolization but not detachment within 24 hours. The characteristics of these cells are similar to non-apoptotic programmed cell death described by Clarke (38) and related to cytotoxic effects of different agents (37). Our cytotoxicity test was further validated by a known cytotoxic PKI, CGP60474, which was developed by Novartis as a potent cyclin dependent kinase inhibitor (42). The development was stopped in an early phase because of its high cellular toxicity, which coincide well with the finding that CGP60474 was the most toxic compound in our assay.

Using this method we determined the LD_{50} of a library of 71 protein kinase inhibitors and another two structuraly related drugs. 54% of the PKI molecules have cytotoxic effects. Five compounds of the library are registered drugs. Two aminoquinazolines – Erlotinib and Gefitinib – are potent EGF-R inhibitors, which are used in the treatment of non-small-cell lung cancer (43, 44). The endothelial cell cytotoxicity of the later developed Gefitinib may well explain the serious side effects observed during treatment (45). Imatinib is the foremost launched signal transduction drug in the treatment of human myelogenous leukemia (46) having significant PDGF inhibitory activity (IC_{50}=380 nM) showed no cytotoxicity. Anagrelide and Thalidomide, although having no significant PKI activity, were selected to expand the group of registered drugs. Anagrelide is structurally related to 2,3-Dihydro-1H-imidazo[5,1-b]quinazolin-9-ones with anti-thrombotic properties, while Thalidomide is a known inhibitor of neovascularization, neither of them were cytotoxic. The latter is a good example that a drug with anti-neovascularization activity is not necessarily directly toxic to endothelial cells in general, which observation may well be converted into the anti-cancer drug development.

The differences in the cytotoxicity amongst PKI-s raise the question which molecular properties are the most important determinants. Since there is not a single definite determinant but rather there are several molecular descriptors which together specify an active structure it implicates that very strict molecular interactions between PKI and cellular molecules (hypothetically protein kinases) may occur. This observation is further supported by the findings that there is strong positive correlation between EGF-R inhibition and cytotoxicity and similarly strong negative correlation between PDGF-R inhibition and cytotoxicity (Figure 3).

Acceptable estimations can be obtained about endothelial cytotoxicity of kinase inhibitors in the therapeutic concentration range (47, 48) with the application of calculated molecular descriptors. The external validation set of compounds could be classified with high reliability into the four arbitrary toxicity groups.

Our results highlight the importance of the endothelial cell cytotoxicity measurements of drug-candidate compounds. The cytotoxicity data in the literature are insufficient or not available, although the high proportion of the toxic compounds in our protein kinase inhibitor library implicates that such tests should be included in the process of drug development. This could be theoretically achieved by two non-exclusive ways: 1. Measuring every drug candidates in a high-throughput endothelial cytotoxicity screening. 2. By the extension of our database with the cytotoxicity measurement of several-hundred compounds, improving the molecular model to an *in silico* cytotoxicity prediction.

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Chapter seven

Summary and remarks

This thesis comprises the results of two separate projects. The first topic is the molecular and cellular aspects of protein misfolding. The studies were performed at the University Medical Center Utrecht, The Netherlands. The studies on the role of endothelial cells in inflammation of the second part were performed at the Semmelweis University of Budapest, Hungary. Here, I will provide a summary of the work in both laboratories. At the end, a synopsis will be given to illustrate the current state on the knowledge of disease progression of Alzheimer's disease and atherosclerosis with the aim to highlight and link similar features of cellular mechanisms and protein aggregation.

Summary of the thesis

Protein misfolding is a generic propensity of proteins. Protein misfolding diseases are linked by common principles of protein misfolding and aggregation that are associated with the development of plaques and tissue damage (1). The expanding group of protein misfolding diseases comprises various, and at first site quite diverse disorders such as Alzheimer's disease, systemic amyloidosis and diabetes mellitus (2). There is also an increasing number of previously unrelated disorders such as atherosclerosis (3) or sickle cell anemia (4) that are now identified or being recognized as protein misfolding diseases based on common characteristics. However, at present there is no adequate therapy for these, mostly highly, debilitating diseases. The aim of this thesis was to increase our understanding of protein misfolding diseases, with emphasis on the commonalities and differences. Ultimately, this should lead to the development of safe therapeutics.

In most of the protein misfolding disorders, increased levels of platelet reactivity have been observed (5-7), but the mechanisms of the platelet activation are not entirely understood. In chapter II, we addressed the question whether misfolded proteins have the capacity to activate platelets. We illustrate that unrelated misfolded proteins induce platelet aggregation and that this is mediated by specific surface receptors, i.e. CD36 and GPIbα (8). Our results revealed a novel stimulatory mechanism for platelets by misfolded proteins. Since misfolded proteins are detected in the blood of Alzheimer's patients in the pico- and nanogram range (9;10), one can argue with the relevance of our results showing platelet activation upon addition of micrograms of misfolded proteins. Nevertheless, misfolded proteins aggregate and accumulate into plaques, which imply a higher local protein concentration; therefore the measured blood levels are not reflecting the occurring protein misfolding. The evaluation of the amount of misfolded proteins in these plaques remains

a demanding task. Why platelets are activated remains to be established as well as their potential beneficial and/or pathological role in misfolding diseases remains to be established. Since misfolding of cellular proteins occurs during apoptosis (11) and cellular infection (12), one intriguing option is that platelets aid in the clearance of misfolded proteins and maintenance of homeostasis.

Besides platelets, there are other blood-born substances that are capable of the recognition of misfolded proteins. Chapter III demonstrates that pooled human intravenous immunoglobulins (IVIg), which are purified from numerous healthy donors and are successfully applied in the therapy of autoimmune, inflammatory diseases (13;14) as well as Alzheimer's disease (15), have the capacity to bind unrelated misfolded proteins. We found that IVIg inhibited platelet aggregation induced by misfolded proteins. These_findings raised the question whether unrelated misfolded proteins share common epitopes that can be recognized by conformational antibodies directed against these structural patterns. To investigate the possibility to generate conformational antibodies, we injected various misfolded proteins into mice. Our data show that antibodies are generated, which can recognize multiple misfolded proteins, although in contrast to human IgG, these monoclonal antibodies were of IgM isotype. Therefore, it is possible that IgG antibodies are present in healthy subjects ready to opsonize misfolded proteins, which implies a role of these human IgG antibodies in clearance. This is supported by the findings of Istrin et al, who showed that IVIg dissolves amyloid-β fibrils, enhances microglial migration towards deposits, and mediates phagocytosis of amyloid-β (16). In mice however, misfolded proteins did not trigger class switch of B cells since their predominant isotypes were IgM. These findings suggest significant differences in immune responses between mouse and man. (17). Nevertheless, there is disagreement about whether conformational antibodies exist mostly because of the complex structural contents of misfolded proteins and the opposing results that are available on the topic. Kayed and Glabe generated oligomeric structure specific polyclonal rabbit antibodies against amyloid-β that crossreact with α-synuclein, islet amyloid polypeptide, polyglutamine, lysozyme, insulin and prion peptide (18). O'Nuallain and coworkers described monoclonal IgM antibodies that appeared to recognize a common conformational epitope in fibrillar amyloid protein including amyloid-β, β_2-microglobulin, islet amyloid polypeptide, transthyretin polyglutamine and lysozyme (17). One interesting speculation about our data is that in mice tolerance against these misfolded proteins was not broken (see IgM isotype of the misfolded protein-specific monoclonal antibodies); whereas in men, similar antibodies comprise the circulating IgG fraction of the blood, perhaps because IVIg is purified from human subjects that are in early stage to develop protein misfolding diseases yet without any detectable indications.

Clearly, further studies are required in order to understand the nature and role of such conformational antibodies, like IVIg.

The aggregation aspects of amyloid-β are crucial for the pathology of the peptide. Soluble Aβ, rather than fibrillar species are supposed to participate in the progression of pathology since the amount of fibrillar plaques in Alzheimer's patients is not correlating with the severity of cognition disorders (19); although, the principal mechanisms are not completely understood. In the study described in chapter IV, we investigated the amyloidogenic characteristics, as well as the platelet stimulatory capacity of amyloid-β (Aβ). In order to study the relationship between the aggregation kinetics and platelet stimulatory capacity, we used Aβ1-42, Aβ1-40, Aβ17-20, Aβ16-22 and Aβ25-35 fragments. We found that freshly dissolved and buffer-treated Aβ1-42 comprised non-fibrillar aggregates and induced strong platelet aggregation, which was absent in the fibrillar preparation. Aβ1-40, which had non-fibrillar aggregates, as well as Aβ17-20, Aβ16-22 and Aβ25-35 that lacked visible structural components did not have platelet stimulatory capacity. These data suggest that platelet stimulatory capacity of Aβ resides in the freshly dissolved and non-fibrillar fractions of Aβ1-42. This is somewhat different from previous data of chapter 2, where aged but not freshly dissolved preparation provoked maximal platelet aggregation, which can be possibly explained by main differences in fibrillar and non-fibrillar structural assemblies of the peptides. It is also interesting to note that Aβ1-42 and Aβ1-40 develop dissimilar structural species under certain conditions such as low pH. The observed results together with data of others showing that expression of Aβ1-42 but not Aβ1-40 is essential for amyloid deposition in transgenic mice models (20) raise the possibility for distant protein aggregation mechanisms as well as biological activity for Aβ1-42 and Aβ1-40.

Proteins can acquire dissimilar properties when they undergo conformational changes or lose their native appearances (21). It has been reported that C-reactive protein (CRP), which is pentameric in its native conformation, gains function when loses its native conformation (22). CRP is an acut phase molecule; its level is raised in the blood during inflammation including atherosclerosis or infection occurring in the body (23;24). Khreiss et al. showed that the native nCRP failed to activate endothelial cells but modified mCRP, the conformational variant of native pentameric CRP significantly up-regulated the expression of adhesion molecules (25), whereas Devaraj et al. found the opposite (26). Chapter V illustrates that neither nCRP nor mCRP evoked pro-inflammatory changes in human umbilical vein endothelial cells (HUVEC), and shows that CRP preparations have toxic properties at high (50–100 μg/ml) CRP doses. We observed that recombinant modified CRP exhibited clear membrane binding to HUVECs, whereas no detectable binding of nCRP and urea-

modified CRP was observed. These results show differences between native and modified CRP in their binding capacity to diverse endothelial compartments, although the previously found differences in proinflammatory cytokine release or adhesion molecule expression by HUVECs induced by nCRP and mCRP are not confirmed by our data.

Early endothelial injury and disfunction are early events in the development of diseases such as Alzheimer's disease (27), atherosclerosis (28), type 2 diabetes (29) and even in cancer (30). In these diseases, levels of endothelial growth factors (vascular endothelial growth factor ,VEGF; transforming growth factor, TGF-β and angiopoietin) are increased (31), which accelerate endothelial cell proliferation and angiogenesis. Therefore, suppression of solid tumor capillarization in a strictly controlled manner that leads to destruction of the tumor tissue can be beneficial for tumor therapy. Receptor tyrosine kinase inhibitors are potent signal transduction therapeutic compounds used in tumor therapy; however, their cytotoxic effect has not been studied yet. In chapter VI we describe a system that is developed to screen these compounds to measure the structure-related cytotoxic effects of a selected library of protein kinase inhibitors. Using microscopic and fluorescent cytotoxicity measurements, we determined the half-lethal dose (LD_{50}) of a library of 71 protein kinase inhibitors and another two structuraly related drugs. Fifty-four percent of the protein kinase inhibitors have cytotoxic effects and five compounds of the library are registered drugs, Erlotinib, Gefitinib, Imatinib, Anagrelide and Thalidomide. We found a strong positive correlation between endothelial growth factor receptor (EGF-R) inhibition and cytotoxicity and equally strong negative correlation between platelet-derived growth factor (PDGF-R) inhibition and cytotoxicity. These data provide possible explanations for side-effects of Gefitinib (32) and CGP60474 (33) and highlight the importance of the endothelial cell cytotoxicity measurements of drug-candidate compounds.

Concluding remarks

Protein misfolding diseases share common characteristics such as protein aggregation, accumulation and tissue damage. The role of protein misfolding is increasingly recognizd and the exact etiology and machinery regarding development of these diseases is presently under extensive investigation. This thesis contributes to our understanding of the role of misfolded proteins. In particular, together with other studies, they link well established protein misfolding diseases and as yet less established misfolding diseases, like atherosclerosis. Here, we highlight the similarities of these seemingly two unrelated diseases,

Alzheimer's disease and atherosclerosis, with protein misfolding in common.

One of the most thoroughly studied neurodegenerative disorders is Alzheimer's disease. Although the molecular basis of the syndrome is not clearly understood, a neuroinflammatory process, triggered by amyloid-β, plays a central role in the neurodegenerative process (34). In the brain interstitial fluid, the level of amyloid-β, which is continuously produced by neurons, is increased upon stress (35). In Alzheimer's disease, amyloid-β aggregates into fibrils and ultimately forms amyloid plaques in the brain parenchyma and in the cerebrovasculature (36;37). Amyloid-β activates astrocytes via LDL-receptor related protein (LRP) to degrade amyloid-β (38). Under healthy conditions, microglia cells perform general maintenance and clean cellular debris (39); however in Alzheimer's brain they become activated cells that phagocyte and degrade aggregated amyloid-β (40) via receptor complex of CD36, scavenger receptor A (SR-A), CD47 and integrins (41). Activated microglia cells release proinflammatory mediators such as IL-1β, TNFα, nitric oxide (NO) and reactive oxygen species (ROS), which result in neuroinflammation (42). Amyloid-β levels are in equilibrium between the plasma and the brain, which is mediated at the blood-brain barrier by two receptors, LDL-receptor related protein (LRP) and receptor for advanced glycation end product (RAGE) (43). During plaque development, the balance mechanism shifts towards the brain (44). Plaque development is associated with elevated levels of amyloid-β in the blood (45). Activated platelets release amyloid-β (46), and in fact platelets are the main source of amyloid-β in the blood (47). Individuals who have had multiple small strokes have a higher prevalence of Alzheimer's disease compared to individuals who have taken anti-platelet drugs (48). These findings suggest a role for platelet-derived amyloid-β in the development of Alzheimer's disease. We have reported that blood platelets are activated by misfolded proteins including amyloid-β as well as glycated proteins and fibrin fragments whereas native controls did not induce platelet activation.

Alzheimer's disease and atherosclerosis are linked by several shared risk factors such as hypertension, elderly age and polymorphisms such as CD36 and lipoprotein gene mutations (49-52). Furthermore, it has been shown that atherosclerosis was associated with an increased risk for dementia during a follow-up study (53). Both diseases are characterized by the development of protein-rich deposits that are termed plaques. Although the site is different, protein aggregation and deposition are common hallmarks of Alzheimer's and atherosclerotic plaques (54-58). Atherosclerotic plaques develop in the intima layer of arteries and capillaries (59), whereas senile plaques are found in the brain parenchyma and cerebrovasculature (60). The first set of atherosclerotic events is the injury or dysfunction of the endothelium, which is also an important aspect in the development of Alzheier's disease

(61). In atherosclerosis, it is followed by monocyte transmigration through endothelial adhesion molecules (E-selectin, intercellular adhesion molecule, ICAM; vascular cell adhesion molecule, VCAM) (59). In the subendothelium, monocytes differentiate into macrophages, which, similarly to microglia cells in the brain, are activated by misfolded proteins, such as oxLDL through CD36 and SR-A, release IL-1β, TNFα and ROS, and phagocyte oxLDL (62-64). These processes lead to ultimate inflammation of the vessel wall. Importantly, *in vitro* studies using coincubation of platelets and macrophages establish a link between platelet phagocytosis and macrophage activation via generation of amyloid-β (65). A recent publication suggests that platelets that enter the atherosclerotic plaque from neovessels release amyloid-β and are phagocytosed by macrophages through CD36 (66), which supports the importance of misfolded proteins in the development of atherosclerosis.

In conclusion, despite of major differences concerning the site of injury as well as cellular repertoire between Alzheimer's and atherosclerotic plaques, there are numerous similar patterns regarding protein aggregation and scavenger receptors mediated cellular mechanisms throughout the progress of Alzheimer's disease and atherosclerosis (see Figure 1.). Moreover, there are several indications that protein aggregation is associated with comparable events in other protein misfolding disorders and vice versa, a number of diseases with as yet unknown etiology may involve protein misfolding as the underlying cause.

Taken together, the findings of this thesis reveal important details in generic molecular and cellular aspects of protein misfolding diseases. The described cellular mechanisms induced by misfolded proteins propose common pathways of cell activation. Our data indicate that generic structural properties can be targeted by antibodies and soluble molecules, which might provide possibilities for effective therapeutic approaches of this expanding group of disorders.

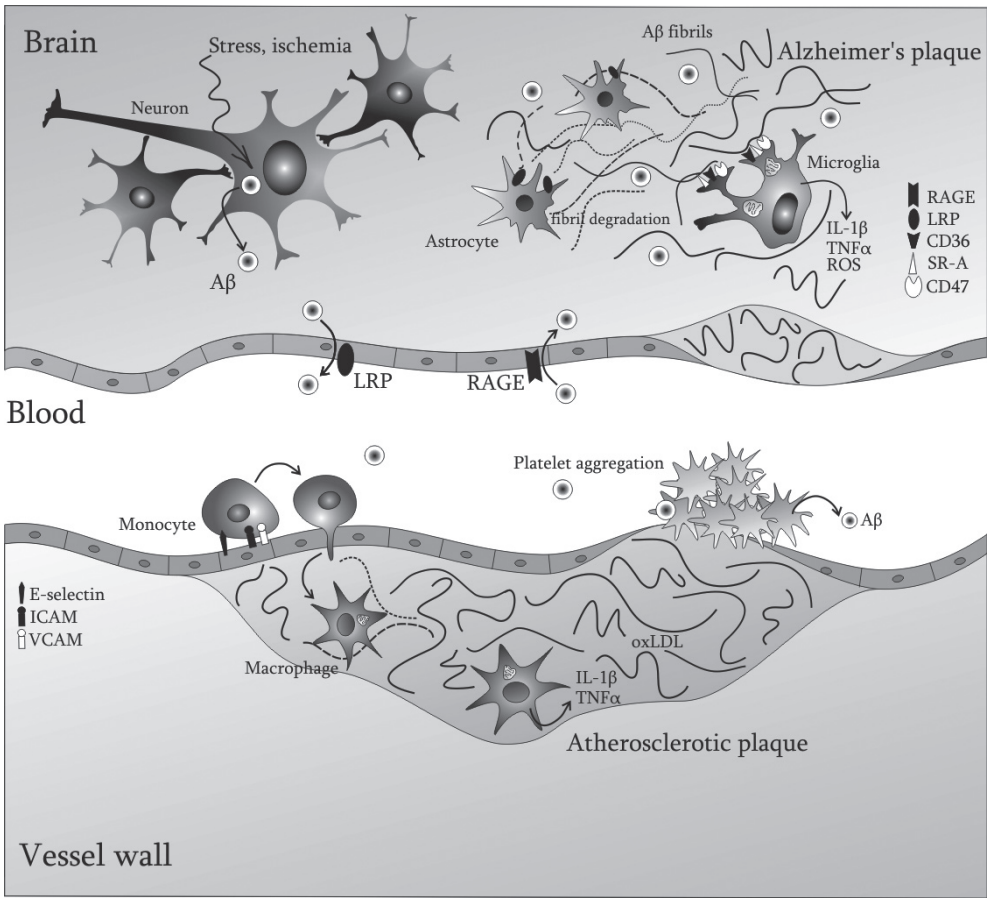

Figure 1. Similarities in cellular mechanisms and protein aggregation processes of Alzheimer's disease and atherosclerosis. Both diseases are associated with enhanced protein aggregation and deposition into plaques. Common inflammatory processes such as cytokine production are initiated by microglia in the brain and macrophages in the vessel wall. These cells have capacity to phagocytose misfolded proteins. Misfolded proteins stimulate platelet aggregation,and in addition, platelets are main sources of amyloid in the blood.

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Nederlandse Samenvatting

Dit proefschrift is het resultaat van twee verschillende projecten. Het eerste onderwerp behandelt de moleculaire en cellulaire aspecten van eiwitmisvouwing. Dit onderzoek werd gedaan aan het Universitair Medisch Centrum Utrecht, in Nederland. Het onderzoek naar de rol van endotheelcellen bij ontsteking, beschreven in het tweede deel van dit proefschrift, werd gedaan aan de Semmelweis Universiteit van Budapest, in Hongarije. Hier geef ik een samenvatting van het werk gedaan in beide laboratoria. Aan het einde van de samenvatting volgt een korte beschrijving van de huidige inzichten met betrekking tot de biologie van de ziekte van Alzheimer en atherosclerose, met als doel licht te werpen op overeenkomstige cellulaire processen en met name de rol van aggregatie van eiwitten.

Deel één

Alle eiwitten hebben van nature de neiging tot misvorming: in de biologie misvouwing genoemd. Eiwitmisvormingsziekten (eiwitmisvouwingsziekten) hebben gemeenschappelijke kenmerken zoals eiwitmisvouwing en de vorming van eiwit aggregaten, die zich ophopen in het lichaam. Dergelijke ophopingen worden ook wel plaques genoemd. Het ontstaan van plaques wordt inmiddels verondersteld een belangrijke en essentiële bijdrage te vormen van het ziekteproces. Het verhinderen van plaquevorming wodt daarom gezien als de aanpak om het ziekteproces te vertragen. De steeds groter wordende groep van eiwit misvouwingsziekten bestaat uit uiteenlopende aandoeningen zoals de ziekte van Alzheimer en suikerziekte. Er is ook een steeds groter wordende groep van aandoeningen die in eerste instantie niet in verband werd gebracht met eiwitmisvouwing, zoals aderverkalking (atherosclerose). Hiervan wordt meer en meer onderkend dat ook hier eiwitmisvouwings onderdeel is van het ziektebeloop. Momenteel is er echter nog geen effectieve behandelingsmethode voor deze over het algemeen slopende aandoeningen. Het is het doel van dit proefschrift om onze kennis over deze eiwit misvouwingsziekten te vergroten. Dit zal uiteindelijk moeten leiden tot de ontwikkeling van veilige behandelingsmethoden.

Bij de meeste aandoeningen waarbij eiwitmisvouwing een rol speelt, is er een verhoogde reactiviteit van bloedplaatjes te zien. Aangenomen wordt dat deze bloedplaatjes een rol kunnen spelen in de ziekte. De exacte oorzaak van de verhoogde activatie wordt echter nog niet volledig begrepen. In hoofdstuk twee behandelden we de vraag of misvouwen eiwitten bloedplaatjes kunnen activeren. We konden laten zien dat willekeurige misvouwen eiwitten plaatjesaggregatie kunnen induceren. Verder vonden we aanwijzingen hoe dit proces plaatsbindt. Op de wand van bloedplaatjes zitten specifieke receptoren die van belang zijn

en blijkbaar direct of indirect misvouwen eiwitten binden. Onze resultaten hebben een nieuw mechanisme van bloedplaatjesstimulatie onthult. Waarom de bloedplaatjes worden geactiveerd blijft nog onduidelijk. Ook moet de mogelijk gunstige of juist pathologische rol van dit proces in deze eiwit misvouwingsziekten nog nader onderzocht worden. Omdat de misvouwing van eiwitten plaatsvindt tijdens celdood of bij een infectie, is één mogelijkheid dat bloedplaatjes assisteren bij het opruimen van misvouwen eiwitten en zo bijdragen bij het in stand houden van een stabiele eiwithuishouding in het lichaam.

Naast bloedplaatjes zijn er ook andere cellen en eiwitten in ons lichaam die misvouwen eiwitten herkennen. In hoofdstuk drie wordt beschreven dat antistoffen, welke reeds als geneesmiddel worden ingezet bij autoimmuun- en inflammatoire aandoeningen, waaronder bij de ziekte van Alzheimer, in staat zijn om misvouwen eiwitten te binden. We ontdekten dat een bepaalde fractie van deze antistoffen verantwoordelijk is voor de binding en dat deze fractie in staat bleek te zijn om de door misvouwen eiwitten veroorzaakte aggregatie van bloedplaatjes te remmen. Deze bevindingen riepen de vraag op of we ook de aanmaak van dergelijke antistoffen konden stimuleren. Daartoe hebben we muizen ingespoten met verschillende misvouwen eiwitten. De resultaten lieten zien dat er verscheidene antistoffen gegenereerd konden worden die meerdere misvouwen eiwitten kunnen herkennen. Een veronderstelling is op basis van deze resultaten dat er in gezonde mensen al antistoffen aanwezig zijn die in staat zijn om te assisteren om misvouwen eiwitten op te ruimen. Deze veronderstelling wordt ondersteund door de bevindingen van de onderszoeksgroep van Istrin. Deze groepheeft laten zien dat antistoffen misvouwen eiwitten die een rol spelen bij de ziket van Alzheimer kan oplossen en de werking van hersencellen kan versterken. Ook de onderzoeksgroep van O'Nuallain en anderen hebben antistoffen beschreven die misvouwen eiwitten specifiek leken te herkennen. Al deze bevindingen suggereren dat antistoffen tegen misvouwen eiwitten kunnen bestaan. Er zijn meer studies nodig om de werking en rol van dergelijke antistoffen volledig te kunnen begrijpen.

De ziekte van Alzheimer wordt in belangrijke mate veroorzaakt door de aggregerende eigenschappen van het eiwit amyloid-ß (A ß). Onderzoek van de afgelopen jaren suggereert dat het juist de kleine aggregaten zijn, die in tegenstelling tot de aggregaten die lange fibrillen vormen, nog oplosbaar zijn bij dragen aan het voortschrijden van de ernst van de ziekte. In patienten komt de hoeveelheid plaques namelijk niet overeenkomt met de ernst van het geheugenverlies en andere verschijnselen. De mechanismen hierachter worden echter nog niet volledig begrepen. In de studie zoals beschreven in hoofdstuk 4, hebben we de verschillen vormen van het Aß onderzocht. We ontdekten dat vers opgelost en met buffer behandeld Aß bestond uit oplosbare aggregaten en dat deze sterk de klontering van bloedplaatjes

stimuleerden, wat niet het geval was bij fibril-vormende aggregaten. De resultaten uit dit hoofdstuk suggereren dat de stimulerende werking van Aß op bloedplaatjes veroorzaakt wordt door de vers opgeloste niet fibrilvormige Aß. Dit verschilt enigszins van vorige data, waarbij een oud niet vers opgelost preparaat, maximale aggregatie van bloedplaatjes veroorzaakte. Dit kan mogelijk worden verklaard door belangrijke verschillen in het de wel of niet hebben van een fibrilvorm van het Aß. Een andere interessante observatie is dat onder bepaalde condities, zoals een zure omgeving Aß andere vormen aanneemt. Deze resultaten in combinatie met die van anderen, laten zien dat vorm van Aß essentieel is voor de ophoping van amyloid. Dit schetst de mogelijkheid van twee verschillende mechanismen van eiwitaggregatie als wel verschillende biologische activiteit tussen verschillen (mis)vormen van Aß.

Deel twee

Eiwitten kunnen andere eigenschappen krijgen wanneer ze vormveranderingen ondergaan of hun oorspronkelijke vorm verliezen. Een voorbeeld is 'C-reactive protein' (CRP). Dit eiwit vormt een vijfhoek. Wanneer het deze natuurlijke vorm verliest krijgt het een extra functie. CRP is normaal een zogenaamd "acute fase" eiwit. Dit betekent dat het in bloed in verhoogde hoeveelheden voorkomt tijdens ontstekingen. Bijvoorbeeld bij aderverkalking. De onderzoeksgroep van Khreiss heeft laten zien dat de normale vorm van CRP, (natief CRP of nCRP) niet in staat was endotheelcellen (de binnenbekleding van bloedvaten) te activeren, maar gemodificeerd CRP (mCRP), de variant van het vijfhoekige CRP endotheelcellen juist wel stimuleert. De onderzoeksgroep van Devaraj vond echter het tegenovergestelde. In hoofdstuk vijf laten wij zien dat noch nCRP noch mCRP een ontstekingsreactie kunnen opwekken in endotheelcellen. Ook laten we zien dat CRP in hoge dosis schadelijk is. We zagen duidelijke binding van mCRP aan cellen, in tegenstelling tot nCRP. Deze resultaten laten duidelijke verschillen zien tussen de verschillende vormen van CRP, met betrekking tot binding aan endotheel. De eerder gevonden verschillen met betrekking tot de afgifte van ontstekings worden niet ondersteund door onze resultaten.

Beschadiging en het slecht functioneren van endotheelcellen die de bloedvatwand bekleden, zijn de eerste stappen bij het ontstaan van aandoeningen zoals de ziekte van Alzheimer, aderverkalking, suikerziekte en zelfs kanker. Bij deze aandoeningen zijn de waarden van endotheelspecifieke groeifactoren, bijvoorbeeld 'vascular endothelial growth' factor (VEGF), 'transforming growth factor' (TGF-ß) en angiopoietine) verhoogd. Dit versnelt

de vermenigvuldiging van het endotheel en daardoor de groei van bloedvaten. Daarom veronderstelt men dat een gereguleerde remming van endotheelcellen gunstig kan zijn bij de behandeling van kanker. Groeifactoren werken via binding aan endotheel en activatie van receptoren (“receptor kinases”), binders op de buitenkant van de endotheelcellen. Er zijn krachtige stoffen die de signering van groeifactoren via deze receptoren remmen. De schadelijke effecten van deze stoffen waren echter nog niet goed bestudeerd. In hoofdstuk zes beschrijven we een methode die is ontwikkelt om de schadelijke effecten van een verzameling van dergelijke remmers te testen. Met gebruik van microscopie en fluorescentie hebben we de half maximale dosis van een verzameling van 71 remmers en 2 sterk gelijkende medicijnen bepaald. Vierenvijftig procent van de remmers had schadelijke effecten. Vijf van deze stoffen uit de verzameling zijn al geregistreerde medicijnen: Erlotinib, Gefitinib, Imatinib, Anagrelide en Thalidomide. We vonden een positief verband tussen de remming van de receptor voor EGF en de schadelijke activiteit. Verder vonden we een negatief verband tussen de remming van de receptor voor een andere groeifactor, ‘platelet derived growth factor receptor’ (PDGF) en de schadelijkheid. Deze resultaten geven een mogelijke verklaring voor de bijwerkingen van veel gebruikte medicijnen als Gefitinib en CGP60474 en tonen het belang aan van metingen van de schadelijkheid op endotheel van nieuwe kandidaatmedicijnen.

Összefoglaló

A jelen disszertáció két témakörben folyatott kutatás eredményeit összegzi. Az első téma a a fehérjék konformációs változásait kísérő molekuláris jelenségeket és azok sejttani hatásait vizsgálja. A tanulmány az utrechti University Medical Centerben készült Hollandiában. A második rész az endotél sejtek szerepét írja le gyulladásos betegségekben. A kísérletek a budapesti Semmelweis Egyetemen folytak Magyarországon. Az összefoglaló célja, hogy bemutassa a két laboratóriumban kapott eredményeket.

Első rész

A fehérjék konformációja, azaz szerkezete alapvetően meghatározza funkciójukat. A natív szerkezet a fehérjék azon állapota, amelyben a fehérjék működőképesek. Natív konformációjukat elvesztve a fehérjék kitekerednek, amit egy megváltozott szerkezet kialakulása követhet. Ez a jelenség minden fehérjére jellemző folyamat. A konformációs betegségek kifejezés egy olyan betegségcsoportot jelöl, melynek közös jellemzői a fehérjék szerkezetváltozása, az aggregáció, az ezt követő fehérjelerakódás és a szövetek károsodása. Az ebbe a csoportba tartozó betegségek nagyon eltérőek és számuk az elváltozások részletesebb megismerésével egyre nő. A legismertebb konformációs betegségek közé tartozik az Alzheimer kór, az amiloidózis és a cukorbetegség, de a legújabb tanulmányok az érelmeszesedést és a sarlósejtes vérszegénységet is ide sorolják az újabban megfigyelt kóros fehérjeaggregáció és -lerakódás miatt. Bár az ide tartozó betegségek tünetei súlyosan érintik a betegek életminőségét, kezelésükre még nem találtak hatékony megoldást. A tanulmány célja a konformációs betegségekhez kapcsolódó folyamatok megértése, azok hasonlóságainak és különbségeinek leírása, melyek segíthetséget nyújthatnak egy megfelelő terápia kidolgozásához.

Több konformációs betegségben is megfigyelhető a vérlemezkék túlzott mértékű aktivitása, ennek okai azonban ismeretlenek. A disszertáció második fejezete azt tárgyalja, hogy van-e a megváltozott konformációjú fehérjéknek vérlemezke aktiváló hatásuk. A bemutatott eredmények azt mutatják, hogy olyan, aminósavsorrendjükben teljesen különböző fehérjék, amelyek megváltozott konformációval rendelkeztek, vérlemezke aktivációt váltottak ki. Ugyanezen fehérjék natív konformációban nem rendelkeztek aktiváló hatással. A kutatás során két olyan receptort is találtunk a vérlemezkék sejtfelszínén, amelyek részt vesznek a folyamat kiváltásában, ezek egyike a több liganddal is rendelkező CD36 és az agglutinációs reakciókban megismert GPIbα. Megváltozott konformációjú fehérjék kimutathatóak Alzheimer kórban szenvedő betegek vérében piko- vagy nanogramnyi mennyiségben, a mi

eredményeink viszont mikrogramos nagyságrendű fehérje hatására történő vérlemezke aktivációról számolnak be, megkérdőjelezi a tanulmány fiziológiás relevanciáját. Azonban a megváltozott konformációjú fehérjék egyik jellemzője az aggregáció és az ezzel összefüggő lerakódások képződése, ahol a fehérjék magas koncentrációban vannak jelen. Emiatt a vérben keringő megváltozott konformációjú fehérjék koncentrációja ad teljes képet a szervezetben máshol is előforduló folyamatokról. A lerakódások koncentrációjának méréséhez egyenlőre nem áll rendelkezésünkre megfelelő módszer. A dolgozatban tárgyalt mehanizmus azonban egy új vérlemezke aktivációs útvonalat ír le, amelyből izgalmas következtetések vonhatóak le a vérlemezkék potenciálisan jótékony vagy éppen a konformációs beteségekben játszott patológiás szerepét illetően. A fehérjék konformációs változásai ugyanis nem csak betegségekben, de a természetes sejthalál (apoptózis) illetve sejtes fertőzések (például malária) során is bekövetkeznek, így a tanulmányban leírt aktivációs folyamat egyik lehetséges jelentősége a vérlemezkék eltakarító illetve a fertőzés során játszott védelmi szerepe lehet,

A vérlemezkék mellett más, vér eredetű anyagok is rendelkeznek megváltozott konformációjú fehérje felismerő képességgel. A harmadik fejezet arról számol be, hogy a számos egészséges donorból tisztított intravénás immunglobulin készítmény (IVIg), amelyet sikeresen alkalmaznak autoimmun és gyulladásos megbetegedések illetve az Alzheimer kór kezelésére, is rendelkezik ilyen képességgel. Kísérleteinkben IVIg-et alkalmazva gátoltuk a megváltozott konformációjú fehérjék vérlemezke aktivációs hatását. Eredményeink egyik lehetséges magyarázata az, hogy a megváltozott konformációjú fehérjék olyan ismétlődő struktúrális mintázatokból felépülő epitóppal rendelkeznek, amelyet úgynevezett konformációs antitestek tudnak felismerni. Hogy igazoljuk a konformációs antitestek létezését, különböző megváltozott konformációjú fehérjéket oltottunk egerekbe. Azt találtuk, hogy az egerekben termelt antitestek az emberi antitestekhez hasonlóan felismertek megváltozott konformációjú fehérjéket, azonban ezek nem az IVIg-ben megtalálható IgG, hanem IgM izotípussal rendelkeztek, ami a többnyire a saját fehérjék ellen termelődő természetes antitestekre jellemző. Lehetséges, hogy az általunk talált, egészséges emberekből származó IgG antitestek szerepet játszanak a megváltozott konformációjú fehérjék opszonizációjában, illetve eltakarításában. A feltevésünket az Istrin és munkatársai által leírt kutatási eredmények is megerősítik. Ezek szerint az IVIg hatására a már aggregálódott amiloid-béta fehérje, amely az Alzheimer kór fő okozója, feloldódik, a fehérje fagocitózisa is fokozódik. Az egérben végzett kísérletekben azonban a megváltozott konformációjú fehérjék nem váltottak ki teljes ellenanyagválaszt, más szóval nem törték át az immunológiai toleranciát, ugyanis nem eredményeztek IgM-IgG izotípusváltást. Lehetséges, hogy az egészséges emberekből származó IVIg preparátumban lévő konformációt felismerő IgG antitestek olyan donoroktól

származnak, akik a konformációs betegségek egy korai, még nem kimutatható fázisában vannak. Az egerekben kapott IgM és a humán IgG ellenanyagok izotípus eltérése elemi különbségekre utal az egyes fajokban zajló konformáció-ellenes immunválaszt illetően. A szóban forgó konformációs antitestek létezésére vonatkozóan azonban közel sincs egyetértés a kutatók körében. Ennek okai főleg a megváltozott konformációjú fehérjék több szerkezeti komponensből álló, bonyolult összetétele és a témában közölt, egymásnak sokszor ellentmondó publikációk megjelenésében keresendők. Kayed és Glabe kísérleteik során olyan, nyúlban termeltetett amiloid-béta elleni antitestet találtak, amelyek felismertek *oligomer* szerkezetű, egymástól független aminósavszekvenciával rendelkező fehérjéket is. O'Nuallain és csoportja hasonló, de IgM izotípussal rendelkező ellenanyagokról számol be, melyek inkább a különböző *fibrilláris* szerkezetű fehérjék konformációs epitópjainak megkötésére képesek. Ahhoz azonban, hogy megértsük a konformációs antitestek jellegét és szerepét, további kísérletek szükségesek.

Az Alzheimer kórt okozó amiloid-béta fehérje aggregációs képessége alapvetően meghatározza a peptid patológiás szerepét. A legújabb eredmények szerint az oldható (szolúbilis) és nem az oldhatatlan (fibrilláris) amiloid-béta frakció játszik szerepet az Alzheimer kór kialakulásában, ugyanis az agyban talált fibrilláris plakkok száma és a betegség súlyossága között nem találtak összefüggést. A negyedik fejezetben az amiloid-béta (Aβ) fehérje szerkezeti jellemzői és a vérlemezke aktiváló hatása közötti összefüggést vizsgáltuk. A tanulmányban a következő Aβ fragmentumokat használtunk: Aβ1-42, Aβ1-40, Aβ17-20, Aβ16-22, Aβ25-35. Azt találtuk, hogy a frissen feloldott illetve a pufferben inkubált Aβ1-42, amely nem tartalmazott fibrillumokat, erős vérlemezke aktiváló hatással rendelkezett. A fibrilláris Aβ1-42, és az Aβ1-40, illetve a rövid Aβ17-20, Aβ16-22 és Aβ25-35 fragmentumok nem aktiválták a vérlemezkéket. Bár az eredményeink bizonyításához további vizsgálatok szükségesek, az adatok azt sugallják, hogy a vérlemezke aktiváló képesség, a 42 aminósav hosszúságú amiloid-béta (Aβ1-42) fehérje esetében legalábbis a nem fibrilláris frakciókban rejlik. Ez többé-kevésbé ellentmond annak a második fejezetben tárgyalt adatnak, miszerint az egy hétig inkubált Aβ1-40 erős vérlemezke stimulaló hatással rendelkezett, a frissen feloldott peptidnek azonban nem volt hasonló hatása. Ez azzal magyarázható, hogy a két amiloid-béta peptid, az Aβ1-42 és az Aβ1-40 hasonló körülmények között különböző szerkezeti összetételt mutattak, így feltehetően másként is viselkednek. Fontos megjegyezni, hogy egyes állatkísérletek szerint csak az Aβ1-42 expressziója elengedhetetlen feltétele az amiloid lerakódásnak, ami az Aβ1-40 esetében nem volt igaz. Ennélfogva lehetséges, hogy a két, különböző aggregációs képességgel rendelkező amiloid-béta peptidnek (Aβ1-42 és Aβ1-40) eltérő biológiai aktivitása és szerepe van.

Második rész

A konformációváltozás és a natív struktúra elvesztése hatással lehet a fehérjék természetére. A C-reaktív protein (CRP) egy olyan, gyulladás esetén felszabaduló, azaz akut-fázis fehérje, melynek koncentrációja a vérben ateroszklerózis illetve fertőzés esetén drasztikusan megemelkedik. A CRP natív formában pentamer, vagyis öt alapegységből álló szerkezettel rendelkezik. Khreiss és munkatársai csoportjában olyan eredmények születtek, melyek szerint a szerkezetileg módosult CRP, amelyben az alegységek nem alkotnak pentamert, tehát monomer formában vannak jelen, stimulálta az endotél sejteket, a kezelés következtében azok gyulladásos adhéziós molekulákat fejeztek ki a sejtfelszínen. A natív CRP fehérje ugyanakkor semmilyen hatást nem váltott ki. Devaraj viszont ennek épp ellenkezőjét találta laboratóriumában. A mi kísérleteinkben, amelyekben különös figyelmet fordítottunk a preparátumok szennyezésének mérésére és amiről az ötödik fejezetben olvashatnak, sem a natív, sem a módosított forma nem váltott ki az előzetes eredményekhez hasonló gyulladásos hatást endotél sejteken. A CRP minták magas koncentráció esetén toxikusnak bizonyultak. Mikroszkópos vizsgálataink során a rekombinánsan előallított, módosított CRP mindig a sejtek membránjához kötődött, míg a natív illetve egy másik technikával (urea) módosított CRP preparátum esetén nem találtunk kimutatható kötődést. Eredményeink a natív és módosított CRP közötti különbséget ugyan igazolják, ami az endotélsejtekhez való kötődést illeti; azonban nem támasztják alá a fent leírt Khreiss-féle adatokat, ugyanis a mi esetünkben az endotél sejtek nem termeltek gyulladásos citokineket és adhéziós molekulákat sem fejeztek ki a sejtfelszínen.

Az erek legkülső, a vérrel állandóan kapcsolatban lévő rétege, az endotélium, melynek sérülése és működési zavara számos betegség, például az Alzheimer kór, az ateroszklerózis, a cukorbetegség és a rák egyik legkorábbi eseményei közé tartozik. Ezekben a betegségekben az endotéliális sejtekre ható növekedési faktorok (VEGF, TGF-β és angiopoietin) emelkedett szintjét mutatták ki, amelyek a sejtek osztódását és új vérerek képződését simulálják. A rákos megbetegedések során előforduló tumor kapillarizáció szigorúan kontrollált módon való megfékezésének és a rákos szövet elpusztításának hatékony szerep juthat a rákterápiában. A környezetükben fellépő változások hatására a sejtekben bonyolult, kaszkádszerű jelátviteli folyamatok indulnak el, amelyek többek között a sejtek osztódásáért is felelősek. A receptor tirozin kináz gátlók a rákterápiában már használatos, jelátvitelt gátló szerek. Ugyanakkor, meglepő módon, ezek endotélsejtekre kifejtett toxikus hatása nem ismert. A negyedik fejezetben egy olyan általunk kifejlesztett módszert ismertetünk, amely képes a kiválasztott gátlószerek szerkezet-függő toxikus hatásának tesztelésére. Mikroszkópos illetve fluoreszcens

mérések segítségével megállapítottuk egy 71 vegyületből álló protein kináz gátlószer könyvtár és két, szerkezetükben hasonló, már használatban lévő tumorgátlószer halálos dózisait (LD_{50}). A gátlószerek ötvennégy százaléka rendelkezett mérgező hatással, ebből öt vegyület már regsztrált gyógyszer volt (Erlotinib, Gefitinib, Imatinib, Anagrelide és Thalidomide). Eredményeink analízise során erős pozitív összefüggést találtunk az endotéliális növekedési faktor receptor (EGF-R) gátló- és citotoxikus hatása között; ellenben a vérlemezke-eredetű növekedési faktor receptor (PDGF-R) gátló- és citotoxikus hatása között negatív korrelációt fedeztünk fel. Ezek az eredmények lehetséges magyarázattal szolgálnak a már használt rák ellenes szerek, például a Gefitinib és a CGP60474 esetében leírt és mindeddig magyarázat nélküli mellékhatásokra és felhívják a figyelmet a potenciális gyógyszerek endotélsejteken végzett toxicitási tesztelésének fontosságára.

Acknowledgement

Studying abroad is the best thing that can happen in your life. This statement is true, even if it is not always fun. But it's worth it for several reasons. First of all, you can learn a new language and culture. Second, you extend your knowledge in your scientific field. Third, you make new friends. Fourth, you get know yourself. And what else do you need for a great adventure?

It was in June, 2004, I've heard the good news, I received a grant and it offered me the opportunity to come to Utrecht. I was so happy than never before, it was a huge step for me as a researcher and also for my private life. Two months later, after waving goodbye to my family and friends on the airport crying like a baby, I was thinking about what was going to happen in my new environment, research, and life. I was very scared. When I entered the lab, I found that in a funny way, people are similar to the characters I had around me in Hungary. These thoughts made my first weeks a bit easier, though my orientation was not really excellent in the lab. I often got lost in the corridors when I wanted to get back to the lab from the dark room, I couldn't find anything for my experiments and on top of it, I had to learn about thirty different names and memorize who Maarten, Martijn and Martin were. No mistakes were allowed, one of them was my boss. Soon I realized how nice my colleagues are; they tried their best to entertain me and were asking lovely questions such as: Are you hungary? Is Poland a nice country? Does your family have a television in Hungary? And surprisingly fast have some of them learned one of the most important Hungarian frames, egészségedre (cheers) so the language barriers were quickly broken. We had a beer (or two) on Fridays. I had many ups and downs during the three years, but these evenings with them became a kind of support for me. Fortunately, I soon got familiar with the lab, people, experiments, and work discussions, which were not always pleasant times for me. I learned how to switch on the old-fashioned radio in lab 2 and etiquettes such as when someone cries Bakki, we go downstairs to drink coffee and that in the afternouns we listen to Queens of the Stone Age really hard. After moving to Utrecht, a lovely period has started. I was finally free to stay in the lab a bit longer. From then on, platelet aggregation became my new passion. When everyone left, I was still peacefully aggregating through the evening and I analyzed the data within record time. The AIO room was a all-purpose place for me; a computer room, a chamber to have a chat, a place to drink beers after work, a space to practice Dutch language skills with Ronan, and a personal office for Coen to talk about his plans with everyone.

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Thank you very much! Köszönöm mindenkinek! Iedereen hartelijk bedankt!

Eszter

Curriculum Vitae

The author of this thesis was born on the 3rd of December, 1977 in Vác, Hungary. She studied biology at the Eötvös Loránd University, Faculty of Science in Budapest, Hungary from 1996 until 2002. During her university studies, she specialized in immunology. She completed her internship at the 3rd Department of Internal Medicine of Semmelweis University in Budapest in the laboratory of Prof. Dr. George Füst, under the supervision of Dr. László Cervenak. As a final year undergraduate student, she won the first prize at the National Academic Student Competition. After graduation, she worked for two years as a researcher at the Research Group of Metabolism, Genetics and Immunology, Hungarian Academy of Science in Budapest under the supervision of Dr. László Cervenak.

After receiving a scholarship from the International Visegrad Fund in 2004, she started to work in the Protein Misfolding group of Dr. Martijn Gebbink at the Laboratory of Thrombosis and Haemostasis, Department of Clinical Chemistry and Hematology in Utrecht, The Netherlands. Her research was awarded at the symposium of the Nederlandse Vereniging voor Trombose en Hemostase in March 2007. In the same year, she presented her work at two international conferences, at the XXIth Congress of the International Society on Thrombosis and Haemostasis in Geneva and at the UK-Netherlands Platelet Meeting in London.

From October, 2007, she is working as a postdoctoral researcher at Sanquin Research in Amsterdam under the supervision of Dr. Jan Voorberg.

List of Publications

Molecular and cellular aspects of protein misfolding and disease
E. Herczenik and M.F.B.G. Gebbink
Submitted for publication.

Protein kinase inhibitor induced endothelial cell cytotoxicity and its prediction based on calculated molecular descriptors
E. Herczenik, Z. Varga, D. Erős, M. Oroszlán, Sz. Rugonfalvi-Kiss, L. Romics, G. Füst, Gy. Kéri, L. Őrfi, L. Cervenak
Submitted for publication.

Activation of human platelets by misfolded proteins
E. Herczenik, B. Bouma, S.J.A. Korporaal, R.Strangi, Q. Zeng, P. Gros, M. Van Eck, T.J.C. Van Berkel, M.F.B.G. Gebbink and J.W.N. Akkerman
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