Katholieke Universiteit Leuven
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Species and glycosylation dependency of PAI-1 inhibition

Britt VAN DE CRAEN

Doctoral thesis in Pharmaceutical Sciences

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Table of content

List of abbreviations

AMI	acute myocardial infarction
bFGF	basic fibroblast growth factor
cDNA	complementary DNA
CDR	complementarity determining region
C_H	constant domain of the heavy chain
C_L	constant domain of the light chain
ECM	extracellular matrix
E. coli	*Escherichia coli*
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
Fab	antigen-binding fragment
Fc	fragment, crystallizable
FDP	fibrin degradation products
glyc	glycosylated
HRP	horseradish peroxidase
hPAI-1	human PAI-1
Ig	immunoglobulin
K_A	equilibrium association constant
KO	knock-out
LB	luria broth base
LMW	low molecular weight
LPS	Lipopolysaccharide from *E.coli*
MA	monoclonal antibody
MMP	matrix metalloprotease
mPAI-1	mouse PAI-1
MW	molecular weight
NP-40	Tergitol-type NP-40 (detergent)
PAI-1	plasminogen activator inhibitor-1
PAI-$1^{-/-}$ mice	plasminogen activator inhibitor-1 deficient mice
PAI-1 stab	PAI-1-N150H-K154T-Q301P-Q319L-M354I

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
RCL	reactive center loop
ratPAI-1	rat PAI-1
SD	standard deviation
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
scFv	single chain variable fragment
SPR	surface plasmon resonance
TAFI	thrombin activatable fibrinolysis inhibitor
TGF-β	transforming growth factor-β
TF	tissue factor
TFPI	tissue factor pathway inhibitor
t-PA	tissue-type plasminogen activator
u-PA	urokinase-type plasminogen activator
V_H	variable domain of the heavy chain
V_L	variable domain of the light chain
wt	wild-type

List of publications

Dewilde M, **Van De Craen B**, Compernolle G, Madsen JB, Strelkov S, Gils A, Declerck PJ. Subtle structural differences between human and mouse PAI-1 reveal the basis for biochemical differences. *Journal of structural Biology* 2010; 171: 95-101.

Van De Craen B, Scroyen I, Abdelnabi R, Brouwers E, Lijnen HR, Declerck PJ, Gils A. Characterization of a panel of monoclonal antibodies toward mouse PAI-1 that exert a significant profibrinolytic effect *in vivo. Thrombosis Research* 2011, *in press*

Van De Craen B, Grymonprez K, Compernolle G, Declerck PJ, Gils A. Evaluating the reactivity of monoclonal antibodies toward various mouse and rat PAI-1 glycosylation knock-out mutants. *In preparation*

Van De Craen B, Abdelnabi R, Declerck PJ, Gils A. Comparison of the functional properties of stabilized glycosylated *versus* non-glycosylated human PAI-1. *In preparation*

Chapter 1

General introduction

1.1 The haemostatic system

Blood flow is maintained by the proper balance between haemostasis and fibrinolysis. Both biological processes are the consequence of a complex series of cascading enzymatic reactions (Figure 1.1). Haemostasis, the physiological cessation of bleeding, involves the interaction of vasoconstriction, platelet aggregation and coagulation. Fibrinolysis is the physiological breakdown of fibrin to limit and resolve redundant blood clots. Disruption of the delicate balance between haemostasis and fibrinolysis can results in pathological conditions of thrombosis (e.g. stroke, myocardial infarction or deep vein thrombosis) or abnormal bleeding [1].

1.1.1 The coagulation cascade

The concept of the coagulation cascade, being a series of sequential proteolytic reactions, was first introduced in 1964 [2]. The coagulation cascade is initiated when disruption of the endothelium exposes extravascular tissue factor (TF) to the plasma environment [3]. Tissue factor forms a complex with factor VIIa, the so-called extrinsic factor tenase complex, and activates the zymogens factor IX and factor X. Activated factor X then goes on to generate small amounts of thrombin (factor IIa). These small amounts of thrombin activate platelets and factors V, VIII and XI, feeding back into the cycle to increase the thrombin formation. In the amplification phase both the intrinsic tenase complex (factor IXa, factor VIIIa, Ca^{2+} and phospholipids) and the prothrombinase complex (factor Xa, factor Va, Ca^{2+} and phospholipids) accelerate the thrombin production and create a positive feed-back loop, in order to generate sufficient amounts of thrombin to form a stable clot [4]. The resulting 'thrombin burst' leads to the conversion of fibrinogen to fibrin by removing the fibrinopeptides A and B from fibrinogen [5].

Regulation of the coagulation cascade occurs at different levels. Antithrombin is the most important physiological inhibitor of the coagulation cascade. The predominance of its anticoagulant activity is focused on the regulation of thrombin, factor Xa and factor IXa [1]. The principle inhibitor of TF-initiated coagulation is tissue factor pathway inhibitor (TFPI), which binds and inhibits TF-factor VIIa complex. By targeting the initial procoagulant stimulus, TFPI ensures that a small stimulus does not result in uncontrolled

generation of thrombin [6]. A third inhibitor of the coagulation cascade is activated protein C, which catalyzes the inactivation of coagulation factors V(a) and VIII(a) in the presence of its cofactor protein S [7].

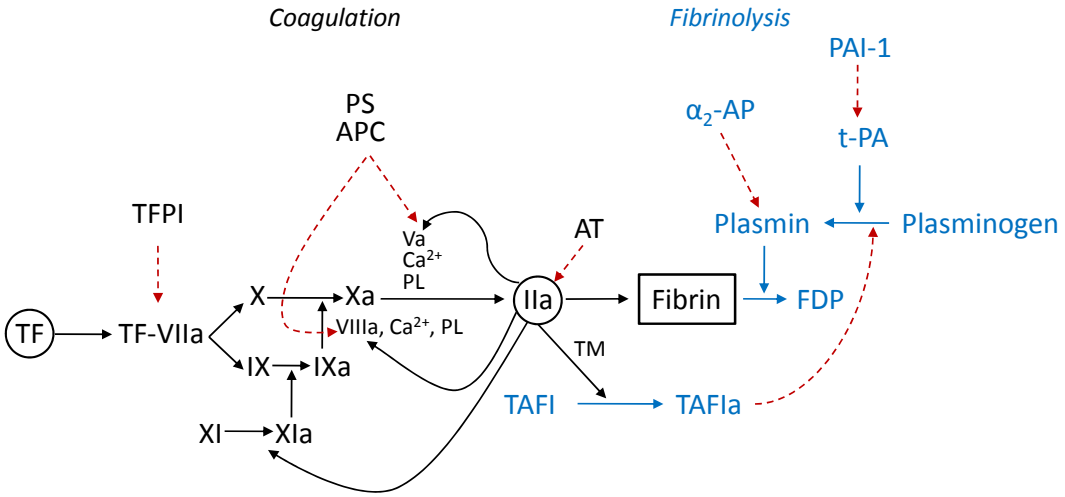

Figure 1.1: Schematic overview of the coagulation (black) and fibrinolysis (blue)

To improve the clarity of the figure, some zymogens and procoagulant surfaces are not depicted. A full line indicates activation; a dashed red line indicates inactivation or inhibition. The used abbreviations are: TF-VIIa: tissue factor-factor VIIa complex; TFPI: tissue factor pathway inhibitor; Xa-Va: factor Xa and Va (together with Ca^{2+} and phospholipids (PL) forming the prothrombinase complex); IXa-VIIIa: factor IXa-factor VIIIa (together with Ca^{2+} and phospholipids forming the intrinsic tenase complex); IIa: thrombin; TM: thrombomodulin; AT: antithrombin; APC: activated protein C; PS: protein S, XIa: factor XIa; TAFIa: activated thrombin activatable fibrinolysis inhibitor; t-PA: tissue-type plasminogen activator; α_2-AP: α_2-antiplasmin; PAI-1: plasminogen activator inhibitor-1; FDP: fibrin degradation products (adapted from [8]).

1.1.2 The fibrinolytic system

The fibrinolytic system consists of a proenzyme plasminogen that can be converted into the active enzyme plasmin. Plasmin is responsible for the dissolution of the fibrin network in redundant blood clots resulting in soluble fibrin degradation products and therefore plays a very important role in maintaining a patent vascular system [9]. Besides the degradation of fibrin, plasmin is also responsible for the conversion of latent metalloproteases (pro-MMPs) into active MMPs, which in turn degrade the extracellular matrix (ECM) [10].

The activation of plasminogen to plasmin can be mediated by two distinct plasminogen activators: tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen

activator (u-PA). t-PA mediated plasminogen activation is mostly involved in the dissolution of fibrin in the circulation whereas u-PA mediated plasminogen activation is mainly associated with pericellular proteolysis during e.g. tissue remodeling or tumor invasion [9]. All enzymes that are part of the plasminogen activator system (plasmin, t-PA and u-PA) are serine proteases, comprising a catalytic triad composed of histidine, aspartic acid and serine as active site [11].

Human plasminogen is synthesized by the liver as a 92 kDa, single chain glycoprotein and circulates in plasma with a concentration of 200 µg/ml. Plasminogen has a plasma half-life of approximately 2 days. All plasminogen activators convert plasminogen into plasmin by cleavage of a single Arg^{561}-Val^{562} peptide bond [9].

Tissue-type plasminogen activator (t-PA) is secreted by endothelial cells as a single polypeptide chain, resulting in plasma concentrations of about 5 ng/ml. This 68 kDa serine protease is rapidly cleared from the circulation with a plasma half-life of only 5 min [12]. T-PA is converted to a two-chain form (tct-PA) by plasmin by hydrolysis of the Arg^{275}-Ile^{276} peptide bond [9]. In contrast to the single chain precursor of most serine proteases, single chain t-PA (sct-PA) is enzymatically active. The catalytic efficiency of tct-PA is higher in solution with chromogenic substrate and with plasminogen but in the presence of templates, such as fibrinogen and native or cleaved fibrin, the difference between tct-PA en sct-PA disappears [9, 13]. In the presence of fibrin the activity of t-PA toward plasminogen is two orders of magnitude higher. Kinetic analysis suggests that the activation of plasminogen in the presence of fibrin occurs through the binding of the plasminogen activator molecule to the fibrin clot surface and the subsequent binding of a plasminogen molecule to form a cyclic ternary complex [14].

Urokinase-type plasminogen activator (u-PA) was originally isolated from urine [15] but later it was found to be secreted by a variety of cells. Single chain u-PA (scu-PA) is a 54 kDa glycoprotein that can be cleaved proteolytically by plasmin at the Lys^{158}-Ile^{159} peptide bond, resulting into two-chain u-PA (tcu-PA). A low molecular weight tcu-PA (33 kDa) is generated at high concentrations of plasmin by additional hydrolysis of the Lys^{135}-Lys^{136} peptide bond [9]. In certain cell cultures a low molecular weight form of scu-PA (32 kDa) occurs that is generated by hydrolysis of the Glu^{143}-Leu^{144} peptide bond and

as a result lacks the amino-terminal part of the high molecular weight form of scu-PA [16].

Thrombin hydrolyzes scu-PA at the Arg^{156}-Phe^{157} peptide bond, located two residues in the amino-terminal direction of the peptide bond sensitive to cleavage by plasmin. The two-chain molecule generated upon cleavage of scu-PA with thrombin is however functionally inactive and much less sensitive to activation with plasmin [17, 18].

The activity of the fibrinolytic system can be controlled at the level of plasmin, by α_2-antiplasmin, at the level of plasminogen activation by plasminogen activator inhibitors (e.g. PAI-1, PAI-2, PAI-3, protease nexin-1 and neuroserpin) or indirectly by thrombin activatable fibrinolysis inhibitor (TAFI).

α_2-antiplasmin is a 70 kDa glycoprotein that circulates in plasma at a concentration of 70 µg/ml. It is synthesized primarily in a plasminogen binding form that becomes partially converted to a less active form in circulating blood. α_2-antiplasmin forms an inactive 1:1 stoichiometric complex with plasmin, thereby inhibiting the activity of the enzyme [19].

Plasminogen activator inhibitor-1 (PAI-1) is the primary inhibitor of both t-PA and u-PA. Other plasminogen activator inhibitors include PAI-2 (placental type), PAI-3 (also known as activated protein C inhibitor), protease nexin-1 and the more recently discovered neuroserpin, that mainly regulates t-PA activity in the brain [20].

Thrombin activatable fibrinolysis inhibitor (TAFI) is a 60 kDa metallocarboxypeptidase produced by the liver and present in plasma. By the action of thrombin, thrombin/thrombomodulin complex or plasmin, the inactive zymogen is converted to the active enzyme TAFIa. Activated TAFI attenuates fibrinolysis by cleaving C-terminal lysines on partially degraded fibrin, thereby interfering with efficient plasminogen binding and activation [21].

1.1.3 Differences between the human and mouse fibrinolytic system

Lijnen *et al.* [22] performed an interesting study in which they purified the main components of the mouse fibrinolytic system and then studied their function *in vitro* in comparison to the human fibrinolytic system. While the biochemical properties of the individual compounds are largely overlapping between mice and humans, there are some important differences. Most importantly, the mouse fibrinolytic system seems to

be more resistant to activation (i.e. the generation of plasmin activity) than the human system. This effect seems to be mediated by a relative resistance of mouse plasminogen to activation by mouse t-PA and by a shorter half-life of mouse t-PA. The shorter half-life of mouse t-PA in comparison to human t-PA was suggested to result from a thus far unidentified mouse plasma inhibitor of t-PA other than PAI-1.

With regard to cross-species interactions of fibrinolytic components, mouse plasminogen seems to be activated more slowly by human t-PA than by mouse t-PA [22]. The concentration of some components of the fibrinolytic system also differs between mice and humans. For example, the concentration of PAI-1 in mouse plasma and platelets is lower in comparison to human plasma and platelets [23].

Overall, the differences between the mouse and human fibrinolytic system are minor, but they must be kept in mind when interpreting specific experiments. Furthermore, the human fibrinolytic system not only shows differences from the mouse fibrinolytic system but also in comparison to some other species used in laboratory experiments such as rabbit, rat, dogs and pigs [24].

1.2 Plasminogen activator inhibitor-1

1.2.1 Discovery and cloning of PAI-1

PAI-1 was first identified as a fibrinolytic inhibitor in the culture medium of bovine aortic endothelial cells [25], but later it was found to be produced by numerous tissues and cells. The origin of PAI-1 under normal and pathological conditions remains a source of speculation. Studies of human tissue suggest that the liver may be the primary source [26].

In blood two different pools of PAI-1 exist, one in plasma and another one in platelets. The amount of PAI-1 in plasma is rather low (0-60 ng/ml) but largely in the active conformation. Although platelets store high amounts of PAI-1 (200-300 ng/ml), they do not seem to contribute to plasma PAI-1 under normal conditions since only about 10% is in the active conformation [27, 28]. However, the release of PAI-1 from activated platelets may increase PAI-1 levels significantly during thrombosis.

In 1986 four groups independently described the isolation of full length cDNA encoding human PAI-1 (NCBI accession number M16006) [29-32]. The human PAI-1 gene is located on chromosome 7, spans approximately 12 200 base pairs and consists of nine exons and eight introns [33]. The expression of PAI-1 can be regulated by various growth factors (TGF-β, EGF, PDGF, bFGF), inflammatory cytokines (IL-1, TNF-α), hormones (insulin, corticosteroids) and endotoxins [34]. PAI-1 is secreted as a 45 kDa glycoprotein consisting of 379 or 381 amino acids (depending on N-terminal heterogeneity due to two possible cleavage sites for signal peptidases) and contains no disulphide bonds [31, 32, 35]. Three potential N-glycosylation sites have been identified in human PAI-1 (i.e. Asn^{209}, Asn^{265} and Asn^{329}) of which only two are utilized (i.e. Asn^{209}, Asn^{265}) [36]. Although glycosylation of PAI-1 has been shown not be essential for its activity [37], inactivation of PAI-1 by monoclonal antibodies or non-ionic detergents is often glycosylation dependent, suggesting that precautions should be taken when selecting the source of PAI-1 for the evaluation of PAI-1 inhibition [36].

1.2.2 PAI-1 structure

PAI-1 belongs to the serpin (serine protease inhibitor) superfamily of proteins. Serpins represent about 10% of all proteins in human plasma. Each serpin consists of 350-400 amino acids with a molecular mass of 38-70 kDa and a sequence homology of approximately 35%. Most serpins have the same highly ordered structure consisting of 3 β-sheets, 9 α-helices and a reactive center loop (RCL) containing residues P_{16} to $P_{10'}$. The reactive site (P_1-$P_{1'}$) is located in this reactive center loop approximately 30-40 amino acids from the carboxy-terminal end and mimics the normal substrate of the serine protease (Figure 1.2).

Two groups of serpins can be distinguished i.e. inhibitory serpins (comprising PAI-1) and non-inhibitory serpins. Inhibitory serpins form a covalent complex with the target protease by mimicking the normal substrate of this protease. This interaction involves the formation of an initial non-covalent Michaelis-like complex, followed by an acyl intermediate and finally by the formation of an ester bond between the carboxyl group of the P_1 residue of PAI-1 with the serine residue of the protease. With the formation of this covalent complex, the P_1-$P_{1'}$ bond of PAI-1 is cleaved and the target protease is translocated to the opposite pool of the PAI-1 molecule, thereby irreversibly inhibiting the activity of the protease [38, 39]. Non-inhibitory serpins do not form stable covalent complexes with their target proteases. Instead, their interaction results in the cleavage of the P_1-$P_{1'}$ bond in the serpin and the release of the protease. Consequently the activity of the protease is not inhibited. This type of interaction is also referred to as the "substrate" reaction of a serpin [38]

PAI-1 is a unique protein amongst the serpins because of its conformational flexibility. PAI-1 is secreted as an active protein that can form covalent complexes with u-PA and t-PA, thereby inhibiting both plasminogen activators. Active PAI-1 is not stable and under normal physiological conditions spontaneously converts to a stable non-reactive (latent) conformation with an apparent half-life of 1-2 hours at 37°C. This non-reactive form does not interact with target proteases, but it can be reactivated *in vitro* using denaturing agents or negatively charged phospholipids [40]. A third structural conformation of PAI-1, acting as a non-inhibitory substrate toward its target proteases has also been reported. This substrate form is cleaved by t-PA or u-PA but without the formation of a covalent complex [41, 42].

Several crystal structures of the different PAI-1 forms have been solved (figure 1.2). The first PAI-1 structure that was crystallographically characterized was latent PAI-1 in 1992 [43], followed by the structure of cleaved substrate PAI-1 in 1995 [44]. Later, the crystal structures of several stabilized active PAI-1 variants were solved [42, 45]. Elucidation of the three dimensional structure of active PAI-1 revealed that the N-terminal side of the reactive center loop is exposed for the target protease whereas the C-terminal part forms strand s1C in β-sheet C. Conversion to the latent conformation of PAI-1 involves the insertion of the N-terminal part of the reactive center loop in central β-sheet A forming the new β-strand s4A, the loss of strand s1C from β-sheet C and the formation of an extended loop by the C-terminal side of the reactive center loop resulting in the distortion of the P_1-$P_{1'}$ bond [42].

1.2.3 Functional stability of PAI-1

Although PAI-1 is synthesized in the active conformation, it spontaneously converts to the non-reactive latent conformation with a half-life of only 1-2 hours at 37°C.

Numerous studies have been dedicated to increase the stability of active PAI-1. It has been shown that external conditions or binding to other proteins can have a great impact on the stability of the PAI-1 structure. On the other hand a variety of mutants were constructed in order to increase the half-life of PAI-1.

1.2.3.1 External conditions

A low pH (≈ 5.5), a high salt concentration (1 M NaCl) and a low temperature (4°C) were shown to stabilize the structure of active PAI-1 [46, 47]. Furthermore binding to arginine has been shown to increase the stability 15-fold [48].

Since a decrease in pH is accompanied by a protonation of imidazole groups, it was speculated that one or several histidine residues contribute to the acid stabilization of PAI-1. Mangs *et al.* (2000) indeed demonstrated that His^{364} is responsible for the pH-dependent stability of PAI-1 [49].

The salt stabilization is ascribed to an anion binding site between β-strand s5A and the hinge domain. Electronegative ions form strong interactions with the partially positive nitrogens of the anion binding site, thereby increasing the energy barrier for the active to latent transition [50].

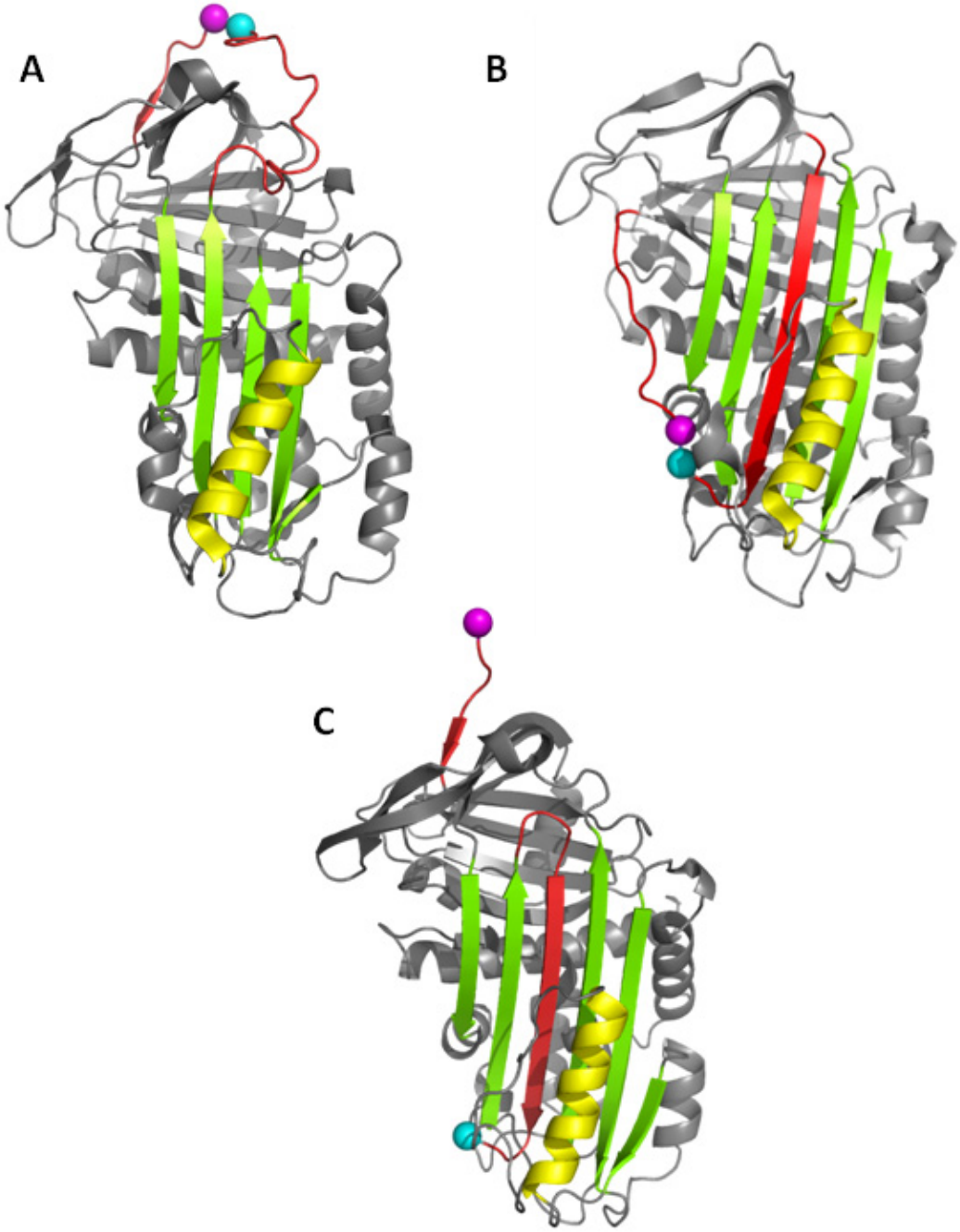

Figure 1.2: The structure of PAI-1 in the active (A), latent (B) and cleaved (C) conformation

ß-sheet A is indicated in green, α-helix F in yellow and the reactive center loop in red. The reactive site residues Arg^{346}-Met^{347} are indicated by blue and purple spheres respectively. Based on Nar et al. 2000 (A), Mottonen et al. 1992 (B) and Aertgeerts et al. 1995 (C) [42-44]

PAI-1 is also sensitive to oxidative inactivation and initial studies suggested that specific oxidation of Met^{347} at the $P_{1'}$ position in the RCL might be the cause of this inactivation [51]. However, more recent data indicate that after addition of an oxidant, PAI-1 undergoes a rapid conformational change to a distinct inactive conformation followed in a second step by the formation of precipitates. The exact nature of the oxidation-induced conformational change and the subsequent precipitation is not clear at present. Furthermore PAI-1 becomes more sensitive to oxidation in the presence of SDS, suggesting that SDS induces a conformational change that exposes oxidation-sensitive sites that are normally in the interior of the PAI-1 molecule [52].

1.2.3.2 Stabilization *in vivo*

Binding of PAI-1 to the plasma binding protein vitronectin has been shown to stabilize the PAI-1 molecule at least 2 to 3-fold [46, 53].

A second ligand able to stabilize the PAI-1 activity is α_1-acid glycoprotein, although the stabilizing effect is less pronounced than with vitronectin [54].

1.2.3.3 Stabilization by mutagenesis

Numerous mutants have been constructed in order to increase the stability of PAI-1. The need for these stable PAI-1 variants arose from the need to crystallize PAI-1 in the active conformation.

Berkenpas *et al*. (1995) identified a set of mutations that had significant stabilizing effects on the PAI-1 stability. Few single mutations displayed exceptional stability, only the mutation of the isoleucine residue at position 91 to a leucine resulted in a 9-fold stabilization of the PAI-1 activity ($t_{1/2} \approx 18$ hours). The mechanism by which this mutation stabilizes PAI-1 is still unknown. The most stable variant they identified turned out to be a quadruple mutant (N150H, K154T, Q319L, M354I) with a half-life of approximately 145 hours at 37°C, corresponding to a 72-fold stabilization in comparison to human PAI-1 wild-type [55]. A few years later Vleugels *et al*. described a variant of this quadruple mutant harboring a fifth mutation Q301P, which had very similar properties [56]. As described by Berkenpas *et al.* (1995), the mutations at positions 154, 319 and 354 have the most stabilizing effect on PAI-1 [55, 57].

Stoop *et al.* (2001) demonstrated that the combination of mutations at position 50, 56, 61, 70, 94, 150, 222, 223, 264 and 331 increased the half-life of PAI-1 245-fold [58].

A disulfide mutant with a more than 350-fold increased stability was described by Chorostowska-Wynimko *et al.* (2003). This mutant with targeted mutations at Q197C and G355C results in disulfide bond formation between residues at the top of s3A and s5A. This is suggested to stiffen the RCL and to prevent its backtracking into β-sheet A [59].

1.2.4 Target specificity of PAI-1

PAI-1 inhibits t-PA and u-PA very rapidly with second-order rate constants in the order of 10^7 $M^{-1}s^{-1}$ as well as thrombin, plasmin or activated protein C, yet with much lower rate constants (10^2-10^4 $M^{-1}s^{-1}$) [60-62].

It is generally accepted that the P_1 residue is the major determinant of the protease specificity of serpins [63]. Normally, the P_1 residue should be identical to the P_1 residue of the substrate and changes at the P_1 residue of serpins often result in altered target specificity. In PAI-1, a basic residue (Arg or Lys) is required at the P_1 position to obtain full inhibitory activity toward u-PA [64]. The presence of neutral or hydrophobic residues at this position leads to PAI-1 variants with more activity toward t-PA than u-PA, although none were as active as wild-type PAI-1 [65].

1.3 Role of PAI-1 in diverse pathologies

1.3.1 PAI-1 and cardiovascular diseases

There is substantial experimental and epidemiological evidence for a link between downregulation of the fibrinolytic system in human plasma and cardiovascular diseases. The first clinical evidence for a link between decreased fibrinolytic capacity, increased plasma PAI-1 levels and cardiovascular disease was reported in young survivors of a myocardial infarction [66]. Plasma PAI-1 activity was also increased in survivors of a myocardial infarction that go on to develop recurrent myocardial infarctions [67]. A more recent case-control study identified a strong association between elevated PAI-1 levels and an increased risk for myocardial infarction in middle-aged patients, an association that was independent of other conventional risk factors [68]. Strikingly, the occurrence of acute coronary events peaks in the early morning hours [69]. This may partly be explained by an inability to cope with a thrombus at that time due to a low fibrinolytic activity and has been linked to the strong diurnal variation in PAI-1 activity [70].

Aside from its role in ischemic heart disease, evidence was also provided for a potential role of PAI-1 in deep vein thrombosis [71, 72], neointima formation and restenosis [73, 74] and angiogenesis [75]. Furthermore, PAI-1 expression was found to be increased in atherosclerotic vessel walls and atherosclerotic lesions [76-78] which may facilitate thrombus formation in case of plaque rupture.

Despite the link observed in all previous studies, these results are in contradiction with observations from other studies. For example, the role of PAI-1 in deep vein thrombosis cannot be unambiguously determined, since some studies were not able to confirm a correlation between PAI-1 levels and deep vein thrombosis (reviewed in [79]). In addition, it was shown that associations between PAI-1 antigen and PAI-1 activity with risk of cardiovascular events disappeared after adjustment for parameters reflecting insulin resistance (i.e. body mass index, triglyceride and HDL cholesterol) [80]. The latter was also confirmed in the ARIC (Atherosclerosis Risk in Communities) study [81]. Eventually, these observations formed the basis for the hypothesis that PAI-1 forms a link between obesity, insulin resistance and the risk for cardiovascular events [82-84].

A deletion/insertion (4G/5G) polymorphism in the promotor region of PAI-1 has been associated with PAI-1 plasma levels. The 4G allele is associated with higher levels of PAI-1 and might therefore impose a higher risk for intravascular thrombosis. However, the contribution of this genetic variant to the risk for thrombosis, both arterial and venous, has not been clearly established [85].

Further evidence for a role of PAI-1 in thrombotic events was provided with the generation of transgenic mouse models. It was found that PAI-1 overexpressing mice developed necrotic tail tips and swollen hind feet as a result of venous occlusions [86] or suffered from age-dependent coronary artery thrombosis and myocardial infarction [87]. Disruption of the PAI-1 gene in mice was associated with increased resistance to thrombosis and a mild hyperfibrinolytic state, but did not impair haemostasis [88].

In addition to subjects with increased plasma levels of PAI-1, also patients afflicted with PAI-1 deficiency have been identified. In humans, deficiency of PAI-1, either by a functionally abnormal protein [89] or by a lack of plasma PAI-1 antigen [90, 91] results in a hyperfibrinolytic state. Moreover, patients with a complete PAI-1 deficiency caused by a homozygous frameshift mutation manifested abnormal bleeding after trauma or surgery but without any further abnormalities [92-94].

1.3.2 PAI-1 and cancer

The plasminogen activator system (especially u-PA) plays an important role in tumor progression, tumor invasion and metastasis formation. This is exemplified by a body of evidence showing a positive correlation between the expression of u-PA and the u-PA receptor (u-PAR) in several tumors and a poor prognosis for survival. It is generally assumed that such a “pro-malignant” effect of the u-PA/u-PAR system is mediated by increased local proteolysis and ECM matrix degradation thus favoring tumor invasion, by a pro-angiogenic effect of this system and also by u-PA/u-PAR signaling toward the tumor thereby shifting the tumor phenotype to a more malignant one. Paradoxically, high concentrations of PAI-1, an important inhibitor of the plasminogen activator system, also correlate with poor prognosis. It has indeed been demonstrated that high PAI-1 levels indicate a poor prognosis in breast cancer, ovarian cancer, urinary tract tumors and colorectal cancer. In fact, the measurement of PAI-1 in tumor tissue is

becoming an important prognostic parameter for a variety of malignant diseases [20, 75].

PAI-1 can influence the evolution of a tumor by regulating cell adhesion, migration and cell invasiveness. Three types of molecular interactions have been suggested for this regulatory effect of PAI-1: 1) direct inhibition of u-PA, 2) binding to vitronectin and 3) binding of u-PA/u-PAR/PAI-1 complexes to endocytosis receptors. The variability and the conflicting results obtained in different studies may be due to the fact that proteolytic and non-proteolytic actions of the u-PA system operate simultaneously in individual cells and that the net effect of PAI-1 depends on the relative importance of the proteolytic and non-proteolytic actions, the expression levels of u-PAR, integrins and endocytosis receptors, the abundance of vitronectin and the composition of the ECM in general [95].

Besides its effect on cell adhesion and migration PAI-1 can also influence tumor progression due to its effect on angiogenesis. For example, lack of PAI-1 completely abolished angiogenesis in aortic ring assays using vessels from gene inactivated mice, an effect that could be restored by exogenous PAI-1 and was dependent rather on the anti-proteolytic activity of PAI-1 than on its interaction with vitronectin [96]. Bajou *et al.* [97] reported that host PAI-1, at physiological concentrations, promotes *in vivo* tumor invasion and angiogenesis. In sharp contrast, inhibition of tumor vascularization was observed when PAI-1 was produced at supraphysiological levels, either by host cells or by tumor cells. This study clearly provided evidence for a dose-dependent effect of PAI-1 on tumor angiogenesis.

PAI-1 added to cell cultures also inhibits apoptosis in both normal vascular smooth muscle cells and tumor cells and thus may be another explanation for the contribution of PAI-1 to tumor proliferation [98].

In conclusion, the exact tumor biological functions of PAI-1 remain uncertain but PAI-1 seems to be multifunctional as it is expressed by multiple cell types and has multiple molecular interactions. The potential utilization of PAI-1 as a target for anti-cancer therapy depends on the further mapping of these functions.

1.3.3 PAI-1 and metabolic disturbances

Obesity is an increasingly important risk factor for the development of cardiovascular diseases. PAI-1 can be synthesized by adipocytes and it has been clearly established that plasma PAI-1 levels are increased in case of obesity and are reduced by weight loss.

The exact role of PAI-1 in adipose tissue development is still controversial at present (reviewed by Lijnen *et al.* [99]). In a nutritionally induced model of obesity, genetic deficiency of PAI-1 (PAI-$1^{-/-}$) resulted in accelerated body weight gain between 3 and 8 weeks of diet feeding, whereas after 8 weeks the difference in body weight gain between obese PAI-$1^{-/-}$ and obese PAI-$1^{+/+}$ mice became non-significant [100]. On the other hand, transgenic mice overexpressing PAI-1 had a lower body weight and lower adipose tissue mass in a model of nutritionally induced obesity [101]. [83]. A more recent study re-evaluating the role of PAI-1 in obesity using PAI-$1^{-/-}$ mice and true-littermate wild-type controls on a high fat diet revealed no significant difference in body weight and subcutaneous adipose tissue between both groups, whereas gonadal adipose tissue was increased in PAI-$1^{-/-}$ mice [102].

In contrast to the results obtained with nutritionally induced obesity models, PAI-1 deficiency in genetically obese (ob/ob) mice resulted in a significantly lower weight and improved hyperglycemia and hyperinsulinemia in comparison to wild-type ob/ob mice [83].

The metabolic syndrome is a concurrence of abdominal fat, disturbed glucose and insulin metabolism, dyslipidemia and hypertension and is strongly associated with subsequent development of diabetes type 2 and cardiovascular diseases. The link between PAI-1 and the metabolic syndrome associated with obesity was established many years ago and increased plasma PAI-1 levels can now be considered a true component of the syndrome [103]. PAI-1 gene expression is induced by many components of the metabolic syndrome, thus providing a link between elevated PAI-1, decreased fibrinolytic potential and increased risk for cardiovascular events [20]. However further experimental and clinical efforts are needed to better understand the complex interplay between PAI-1 and the metabolic syndrome.

1.4 PAI-1 inhibitors

As PAI-1 is considered to be a risk factor, especially for cardiovascular events, many research groups have attempted to develop selective PAI-1 inhibitors. Despite the extensive research that has been carried out regarding PAI-1 inhibition, no PAI-1 inhibitor is currently in clinical use.

1.4.1 Monoclonal antibodies

In 1986, the first PAI-1 neutralizing monoclonal antibodies were described by Nielsen *et al.* [104]. In the following years, a variety of monoclonal antibodies were raised against human PAI-1, human PAI-1/t-PA complex, mouse PAI-1, rat PAI-1 and porcine PAI-1 (reviewed in [35]). Monoclonal antibodies that inhibit PAI-1 activity can be subdivided into at least three different categories. Firstly, monoclonal antibodies can prevent the formation of the initial Michaelis complex formed between PAI-1 and its target protease. Because the epitopes of monoclonal antibodies ESPI-12 (residues 342-349), MAI-12 (residues 320-379), MA-42A2F6 (Lys^{243} and Glu^{350}), MA-44E4 (His^{185}, His^{186} and Arg^{187}) and MA-56A7C10 (Glu^{242}, Lys^{243}, Glu^{244}, Glu^{350}, Asp^{355} and Arg^{356}) were found to be located near the RCL of PAI-1, it was hypothesized that these antibodies most probably interfere directly with the PAI-1/protease interaction [105-107]. Epitope mapping of MA-124K1, a monoclonal antibody that inhibits rat PAI-1 activity but also simultaneously increases the binding of vitronectin to PAI-1, revealed major contribution of residues Glu^{212} and Glu^{220} localized on β-strands s1B and s2B [108].

A second category of PAI-1 inhibiting antibodies are the so-called 'switching antibodies'. Switching antibodies inhibit PAI-1 activity by inducing the substrate pathway of PAI-1, resulting in cleaved PAI-1 upon addition of protease and the subsequent release of active protease. MA-33H1F7, MA-55F4C12 [109], Mab2 [110] and CLB-2C8 [111] belong to this class of switching antibodies. Surprisingly, the epitopes of these antibodies are all located in the region around α-helix F and the turn connecting α-helix F with β-strand s3A. Further studies indicated that MA-33H1F7 and MA-55F4C12 interfere with the kinetics of RCL insertion, possibly through modulating the mobility of α-helix F. MA-8H9D4 also belongs to the group of switching antibodies but its epitope is located in another region i.e. the loop between α-helix I and β-strand s5A [112]. The substrate behavior of MA-8H9D4 is not correlated to its size, since studies with a smaller antibody

fragment and with a small synthetic molecule revealed the same substrate inducing properties as for the original antibody [113].

A third class of PAI-1 inhibiting antibodies can inhibit the PAI-1 activity by accelerating the conversion to the latent conformation of PAI-1 e.g. MA-33B8, MA-159M12 and MA-H4B3 [114-116]. The epitope of MA-33B8 was identified in the turn connecting α-helix D with β-strand s2A (Lys^{88} and Asp^{89}) and the top of β-strand s2B (His^{229}). Simultaneously another group reported their results regarding the epitope of MA-33B8. Although they identified approximately the same binding region, they found some extra amino acid residues contributing to the epitope of MA-33B8 (i.e. Asn^{87}, Gln^{174}, Gly^{230}, Thr^{232} and residues Asn^{329} and Ser^{331} located on s4A/s5A) [117, 118]. MA-159M12 induces latency transition in rat PAI-1 only. Quite surprisingly the epitope of this monoclonal antibody is located on the amino-terminal part of α-helix A (Pro^{2}, Leu^{3}, Pro^{4} and Glu^{5}), which is difficult to explain in view of the current understanding of PAI-1 stability [115]. More recently, Dupont et al. reported the epitope of MA-H4B3 to be located on residues Tyr^{210}, Glu^{212}, Tyr^{241} and Arg^{271}. MA-H4B3 binds to the pre-latent conformation of PAI-1 [119].

1.4.2 Peptides

Previous studies indicated that is possible to inhibit the activity of a serpin by preventing the insertion of the RCL. Eitzman *et al.* followed a similar approach and designed a 14-amino acid peptide corresponding to residues P_{14} to P_1 of the RCL of PAI-1. This peptide inhibited PAI-1 activity by accelerating the conversion from active to non-reactive PAI-1 and enhanced *in vitro* lysis of both platelet-rich and platelet-poor clots containing recombinant PAI-1 by three to five-fold respectively. However, the effect of this peptide was drastically reduced when it was tested toward vitronectin-bound PAI-1 [120]. Another synthetic peptide corresponding to residues P_{14}-P_7 of the RCL of PAI-1 induced increased substrate behavior. It was speculated that this peptide inserted into β-sheet A as strand s4A [121]. Later this hypothesis was confirmed by the elucidation of the crystal structure of PAI-1 in complex with another 'substrate inducing' peptide corresponding to part of the reactive center loop (P_{14}-P_{10}). In this structure the first peptide occupied the same space as the residues P_{14}-P_{10} in latent and cleaved PAI-1, whereas the other peptide occupied the same space as residues P_6-P_2 in cleaved PAI-1. Insertion of these

peptides into β-sheet A prevents insertion of the RCL loop and therefore inhibits the activity of PAI-1 [122]. More recently, a 17-amino acid peptide, based on domain 5 of high molecular weight kininogen was shown to inhibit the binding of PAI-1 to vitronectin. Although the peptide had no effect on the activity of PAI-1 itself, displacement of PAI-1 from its stabilizing molecule vitronectin resulted in reduction of PAI-1 activity and subsequently accelerated clot lysis [123].

1.4.3 Low molecular weight inhibitors

Different low molecular weight (LMW) compounds that inhibit PAI-1 activity have been described. The first compounds with PAI-1 inhibiting activity were two diketopiperazines produced by *Streptomyces* species that were identified as part of a screening program of microbial metabolites [124]. Based on these diketopiperazine templates, new PAI-1 inhibitory compounds have been constructed (i.e. XR1853, XR5118 and XR11211) displaying IC_{50} values between 0.2 and 62 µM in *in vitro* plasmin generation assays [125-127]. XR5118 enhanced the rate and extent of blood clot lysis in an *ex vivo* clot lysis assay after intravenous administration of the compound (5 mg/kg). In a rat model of arterial thrombosis, intravenous infusion of XR5118 significantly prolonged the time to blood vessel occlusion [126]. In addition, systemic infusion of XR5118 induced a significant reduction in plasma PAI-1 activity levels, increased endogenous thrombolysis and attenuated thrombus growth [128]. These studies demonstrated the feasibility of using small molecular weight compounds for the *in vivo* inhibition of PAI-1 activity.

Björquist *et al.* developed another low molecular weight inhibitor, AR-H029953XX. Flufenamic acid was used as template for the development of AR-H029953XX since it had been reported that flufenamic acid and its derivatives enhanced plasma clot lysis *in vitro* [129]. In a plasmin generation assay, the IC_{50} value was determined to be 54 ± 8 µM [130], whereas the binding site of AR-H029953XX was suggested to be located on residues Arg^{76}, Arg^{115} and Arg^{118} on the surface of PAI-1 [129].

Also Triton X-100, a detergent widely used in buffer for biochemical analyses, was shown to inhibit the PAI-1 activity. Experiments with a variety of other non-ionic amphiphilic compounds revealed that the amphiphilic character of the compound is, at least in part, responsible for the observed effects [131]. Screening of 20 000 synthetic compounds

indicated another LMW PAI-1 inhibitor, fendosal, with an IC_{50} value of 36 ± 4 μM in a plasmin generation assay [130].

A recently discovered indole derivative, PAI-039 or tiplaxtinin, seemed a very promising PAI-1 inhibitor [132]. It has been examined in several animal models that suggested a putative value of PAI-1 inhibition for therapeutic purposes [133-135]. However, in another study, tiplaxtinin was not able to inhibit PAI-1 activity in rat plasma 15 minutes after i.v. injection, which was suggested to be due to the fact that tiplaxtinin is unable to inhibit vitronectin-bound PAI-1 because of the overlapping epitopes of vitronectin and tiplaxtinin on PAI-1 [136, 137].

S35225 has been shown to act as a direct inhibitor of PAI-1 activity *in vitro* in rat and human plasma, where vitronectin is constitutively present, as well as in blood after intravenous administration to the rat [136].

Cale *et al.* [138] described a novel class of polyphenolic PAI-1 inhibitors that showed IC_{50} values for PAI-1 between 10 and 200 nM. This represents an enhanced potency of 10–1000-fold over previously reported PAI-1 inactivators. Inhibition of PAI-1 by these compounds was reversible, and their primary mechanism of action was to block the initial association of PAI-1 with a protease. Consistent with this mechanism and in contrast to some previously described PAI-1 inactivators, these compounds inactivate PAI-1 in the presence of vitronectin. Two of the compounds showed efficacy in plasma and one blocked PAI-1 activity *in vivo* in mice. The authors suggested that the known cardiovascular benefits of dietary polyphenols may derive in part from their inactivation of PAI-1.

More recently, Izuhara *et al.* described a PAI-1 inhibiting low molecular weight compound, TM5275 that displayed an antithrombotic effect equivalent to that of clopidogrel in a rat arterial thrombosis model. TM5275 did not affect the activated partial thromboplastin time and prothrombin time and did not prolong the bleeding time. Combined administration of TM5275 and t-PA improved the therapeutic efficacy of the latter, without additional adverse effects in comparison to t-PA alone. These data suggest that appropriate combination of oral TM5275 and t-PA might prove valuable in clinical emergencies without inducing severe bleeding disorders. The antithrombotic benefits of TM5275 were also confirmed in a monkey model of photochemically induced

arterial thrombosis, in which TM5275 again displayed the same antithrombotic effect as clopidogrel, without enhanced bleeding tendency [139].

1.4.4 Inhibition of PAI-1 synthesis

Another approach to stimulate endogenous fibrinolysis is to inhibit PAI-1 synthesis. Some currently marketed drugs e.g. thiazolidinediones or fibrates have been reported to decrease PAI-1 levels in plasma [140, 141]. A butadiene derivative, T-686, attenuated the augmentation of PAI-1 antigen accumulation induced by TGF-β in cultured endothelial cells. Furthermore, T-686 also attenuated the increase in PAI-1 activity seen in rabbits with aortic atherosclerosis. This was accompanied by the reduction in aortic PAI-1 mRNA expression and a reduction in the development of atherosclerotic lesions [142]. Pawlowska *et al.* showed that an oligonucleotide anti-sense to PAI-1 mRNA (MPO-16R) reduced PAI-1 activity in rat plasma and platelets. In an experimental model of rat arterial thrombosis, low doses of MPO-16R caused a significant delay in the occlusion time [143]. Recently, a new panel of antisense oligonucleotides was described by Jiang *et al.* that decreased the PAI-1 antigen and activity induced in HUVEC cells by TGF-β [144].

1.5 Antibodies

The production of antibodies (also referred to as immunoglobulins or Ig) by B lymphocytes is part of the humoral immune system of vertebrates. Immunoglobulins circulate in the blood and other body fluids where they are able to bind, neutralize and eliminate foreign antigens, such as viruses, bacteria or toxins.

1.5.1 Structure of antibodies

The basic structure of an IgG antibody has been elucidated as a symmetric monomer consisting of two identical heavy chains and two identical light chains, connected via disulfide bonds. A typical antibody molecule can be represented by a Y-shape (Figure 1.3) with two identical antigen binding sites at the tips of the Y-shape (Fab or antigen-binding fragment). The tail of the Y-shape, called the Fc (crystallizable fraction), is responsible for the biological effector functions such as complement activation, phagocytosis and binding to cellular Fc receptors.

Five classes of immunoglobulins can be distinguished based on their heavy chains: IgA, IgD, IgE, IgG and IgM (α, δ, ε, γ and μ heavy chains respectively). Furthermore, each antibody contains one type of light chain, kappa or lambda (κ or λ). A light chain consists of one variable (V_L) and one constant (C_L) domain, whereas a heavy chain has one variable (V_H) and three or four constant domains (C_H). Both heavy and light chains harbor a variable region of about 110 amino acids at the amino-terminus with three hypervariable segments called complementarity determining regions (CDRs). These hypervariable loops form the two antigen binding sites of an immunoglobulin molecule and determine its specificity.

The two domains of an antibody (i.e. the Fab and Fc) are connected with each other by the hinge region. This hinge region contains the principal disulfide linkages between the heavy chains and is susceptible to proteolysis by papain or pepsin. Fragmentation with papain, which cuts above the disulfide linkage results in two monovalent Fab fragments and a single Fc part, whereas digestion with pepsin, which cleaves below the first disulfide bond in the hinge region, results in one $F(ab')_2$ fragment and a partially digested Fc fragment [145]. scFv fragments are the smallest molecular units which can retain the binding affinity of the parental antibody and they comprise the V_H and V_L domains which are connected by a linker [146].

Immunoglobulins display a dual function since they are either exposed on the membrane surface of B lymphocytes as antigen receptors or are secreted by plasma cells. Transmembrane and secreted forms of an antibody are the result of differential splicing of a primary transcript RNA. In the course of an immune response specific B lymphocytes undergo isotype switching, which is the transition from production of IgM phenotype to IgG surface or secreted antibodies. In addition, point mutations of the rearranged heavy and light chain variable regions can occur, giving rise to somatic hypermutation necessary to increase antibody affinity (affinity maturation).

Immunoglobulin G (150 kDa) is the most abundant in serum, accounting for up to 80% of all secreted antibodies. There are four different IgG isotypes in human serum (IgG1, IgG2, IgG3 and IgG4). Immunoglobulin M accounts for approximately 10% of serum antibodies. It is expressed as a monomer on the surface of B lymphocytes as antigen receptor, but the secreted form consist of a pentamer held together by a J (joining) chain. Immunoglobulin A, constitutes about 10-15% of serum antibodies and also represents the major antibody class in secretions (e.g. saliva, tears or mucus), where it is covalently connected via J chains together with an additional polypeptide called the secretory component. Immunoglobulins D and M are the major membrane bound forms of antibodies, expressed on mature B cells. Finally immunoglobulin E is responsible for the immediate hypersensitivity and causes symptoms such as hay fever, asthma and anaphylactic shock.

The key feature of monoclonal antibodies is their unique specificity. Antibodies recognize only one particular antigenic determinant (called the epitope) on a given molecule. All antibodies secreted by a given hybridoma represent identical immunoglobulins that display identical binding strengths to their antigen (affinity) and have identical physicochemical properties (isotype, stability). In principle, monoclonal antibodies can be produced in unlimited quantities, since the hybridoma cell can be stored by cryopreservation at least up to decades. [145].

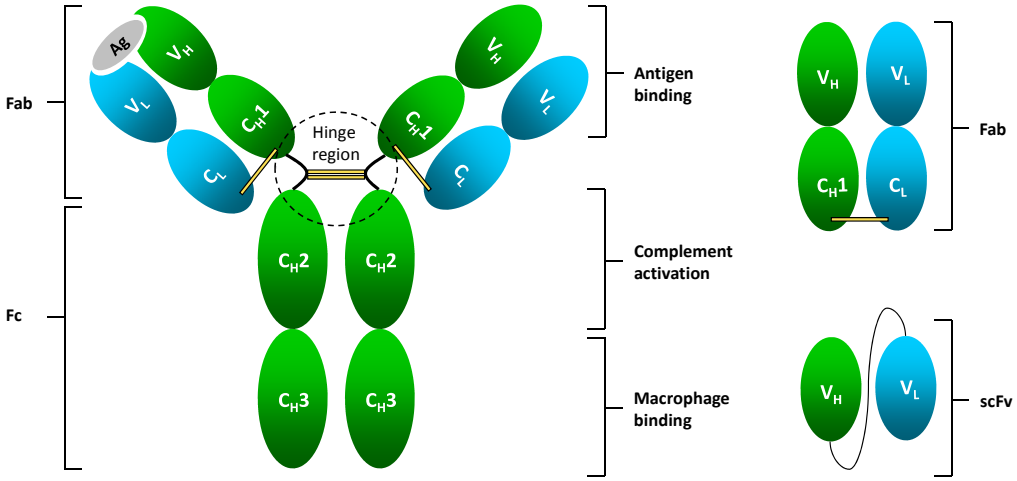

Figure 1.3: IgG structure and function

An IgG antibody is composed of four polypeptide chains, which comprise two heavy (represented in green) and two light (represented in blue) chains linked by disulfide bonds (yellow bars). A light chain consists of one variable (V_L) and one constant (C_L) domain, whereas the heavy chain has one variable (V_H) and three constant domains (C_H). Parts of the Fab region and Fc region responsible for antigen binding and effector functions are shown (adapted from [147])

1.5.2 Therapeutic antibodies

1.5.2.1 Production of mouse monoclonal antibodies

In 1975 Cesar Milstein and Georg Köhler developed a technique allowing the production of monoclonal antibodies in mouse cell cultures (hybridoma technology), for which they were honored with the Nobel Prize in 1984 [148]. After immunization of a mouse with a selected antigen, antibody producing B-lymphocytes are isolated from the spleen and fused with immortal mouse myeloma cells. This fusion results in immortal, antibody-producing cells (hybridomas) that can each produce one unique mouse monoclonal antibody of choice. Once the hybridoma celline is established, large quantities of antibodies can be obtained employing modern cell culture devices for long-term propagation. Harvest of the antibody enriched medium can be performed several times until the productivity ceases. The antibody yield throughout is comparable to the formerly favored ascites production in mice that is now prohibited in most western countries by animal protection laws [145, 149].

1.5.2.2 Therapeutic applications of monoclonal antibodies

Therapeutic application of mouse monoclonal antibodies in clinical trials is hampered because of the short serum half-life, inefficient human effector functions and because of the production of human anti-mouse antibodies (HAMA-response) [150]. The emergence of antibodies as therapeutics was made possible by the advent of core technologies designed to overcome these problems associated with mouse monoclonal antibodies i.e. chimerization and humanization of mouse antibodies. Chimerization involves the joining of mouse variable domains to the constant domains of a human monoclonal antibody. Generation of a humanized antibody involves grafting of the CDRs from a mouse antibody to a human IgG antibody (CDR grafting) [151].
However, nowadays many monoclonal antibodies entering clinical trials are completely human [152]. Screening of large recombinant antibody libraries is exploited to build human antibodies with high specificity and affinity [153]. Transgenic mice expressing human immunoglobulin genes will respond after immunization with the production of entirely human antibodies. After fusion with mouse myelomas, these human antibodies are secreted by the resulting hybridomas [154].

The therapeutic application of monoclonal antibodies has increased dramatically in recent years. At current 24 monoclonal antibodies have been approved for clinical use by the Food and Drug Administration of which two-thirds are either chimeric or humanized products [155]. One may expect that the amount of approved antibodies will increase drastically during the following years since more than 20% of all biopharmaceuticals currently being evaluated in clinical trials are full-length antibodies, antibody fragments or antibody conjugates [156]. Antibody derived drugs posses a number of desirable pharmaceutical characteristics, including good solubility and stability, a long serum half-life, high specificity and limited off-target cytotoxicity. However, antibody preparations also possess some attributes that complicate drug development, including poor oral availability and high production costs [157]. Most antibody based therapeutics were developed for the treatment of cancer or immunological disorders. Besides these traditional indications, monoclonal antibodies are also being studied to treat infectious diseases and conditions like osteoporosis, respiratory disorders, Alzheimer's disease and pain [155]. Additionally, monoclonal

antibodies have a broad *in vitro* application, including western blotting analysis, immunohistochemistry, enzyme-linked immunosorbent assay (ELISA), affinity chromatography, *etc.*

1.6 Goal of the study

Plasminogen activator inhibitor-1 is the most important direct inhibitor of tissue-type and urokinase-type plasminogen activator and therefore PAI-1 has an important anti-fibrinolytic effect.

Rodent models are commonly used to study the inhibition of PAI-1 because they are readily available and relatively inexpensive. Disease pathogenesis in these animals generally tends to approximate disease progression in humans, although there is some divergence in cellular mechanisms and pathogenesis [158] and animal proteins are often not identical to their human counterparts.

Most PAI-1 inhibitory antibodies were generated and evaluated toward human PAI-1 and have not been thoroughly characterized for their reactivity toward PAI-1 from other species. Even if these antibodies showed reactivity toward non-glycosylated PAI-1 from different species, their reactivity toward the glycosylated variants often was not investigated. Therefore the first goal of this study was the generation and characterization of monoclonal antibodies reacting with glycosylated mouse PAI-1 that can be used to study the role of PAI-1 in different mouse models (**Chapter 2**).

Although there are only slight differences in the biochemical properties of glycosylated and non-glycosylated PAI-1 [37], the reactivity and inhibitory capacity of monoclonal antibodies toward PAI-1 can be glycosylation dependent [36]. The second goal of the study was to explore the differential reactivity of PAI-1 inhibitory antibodies toward non-glycosylated *versus* glycosylated mouse and rat PAI-1 variants (**Chapter 3**).

Due to the intrinsic instability of PAI-1, the expression of glycosylated PAI-1 in eukaryotic cells only yields inactive protein. A 72-fold stabilized form of non-glycosylated human PAI-1, comprising five point mutations, was previously expressed in *E. coli* cells. In the third part of the study we introduced these 5 mutations in the cDNA of human PAI-1 cloned into a eukaryotic expression vector. After expression of glycosylated human PAI-1 in HEK293T cells we compared the functional properties of stabilized glycosylated *versus* non-glycosylated human PAI-1 (**Chapter 4**).

Chapter 2

Characterization of a panel of monoclonal antibodies toward mouse PAI-1 that exert a significant profibrinolytic effect *in vivo*

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2.1 Introduction

The plasminogen activator system consists of a proenzyme, plasminogen that has to be converted to the active enzyme plasmin. The activation of plasminogen to plasmin is catalyzed by the serine proteases tissue-type plasminogen activator (t-PA) or urokinase-type plasminogen activator (u-PA). t-PA mediated plasminogen activation is mainly responsible for the dissolution of fibrin in the circulation, whereas u-PA mediated plasminogen activation is thought to be involved in pericellular proteolysis during tissue remodeling, tissue repair and tumor invasion [9, 20, 159-161]. The activity of the plasminogen activator system can be modulated directly at the level of plasmin by α_2-antiplasmin or at the level of plasminogen activation by thrombin-activatable fibrinolysis inhibitor (TAFI) or plasminogen activator inhibitors. Plasminogen activator inhibitor-1 (PAI-1) is the most important physiological inhibitor of both t-PA and u-PA [9].

Plasminogen activator inhibitor-1 (PAI-1) belongs to the serpin (serine protease inhibitor) superfamily of proteins [29]. Active PAI-1 forms a stable, covalent complex with its target proteases, which leads to the irreversible inactivation of both t-PA and u-PA. PAI-1 is synthesized as an active molecule but has a very short half-life ($t_{1/2}$ = 2 hours at 37°C) [162, 163]. Active PAI-1 spontaneously converts to an inactive latent conformation that can be partially reactivated by denaturing agents [164]. In plasma, active PAI-1 can be stabilized by binding to vitronectin [53]. A third structural conformation of PAI-1, acting as a non-inhibitory substrate toward target proteases has also been reported [41].

A potential role of PAI-1 in the regulation of fibrinolysis was first demonstrated by the observation that elevated levels of PAI-1 are associated with increased tendency toward thrombosis [163]. Further evidence for a role of PAI-1 in fibrinolysis was reported with the generation of PAI-1 overexpressing mice that suffered from venous thrombosis in tails and hind feet [86], whereas PAI-1 knock-out mice exhibited an increased tendency toward spontaneous fibrinolysis [88, 165]. Therapeutic targeting of PAI-1 became even more promising after clinical observations showing increased PAI-1 activity in diverse acute disorders such as myocardial infarction, deep vein thrombosis and unstable angina [161, 166-168]. All these data indicate that targeting PAI-1 by pharmacological inhibition represents a promising target to promote *in vivo* clot dissolution.

A functional role for PAI-1 has also been suggested in diverse physiological processes like adipogenesis, apoptosis, wound healing or vascular remodeling and in some pathological conditions like the metabolic syndrome, obesity, tumor development and tumoral angiogenesis [20, 95, 160, 161]. However, for these disease states, it is not always clear if the effect of PAI-1 depends on its antifibrinolytic potential or on its interference with cell migration or extracellular matrix remodeling.

The effect of PAI-1 inhibition on diverse thrombotic and non-thrombotic diseases can be studied in mouse models, when the appropriate immunological tools are available. The majority of the available monoclonal antibodies have been raised against human PAI-1 [104, 105, 169, 170]. Most of these antibodies have not been thoroughly characterized regarding their reactivity toward PAI-1 from other species. And even if the antibodies showed reactivity toward non-glycosylated PAI-1 from different species, their reactivity toward glycosylated mouse PAI-1 often has not been determined. Indeed, two previous studies provided evidence that the inactivation of PAI-1 by some monoclonal antibodies is largely glycosylation dependent [36, 119]. This glycosylation dependent recognition suggests that precautions should be taken when selecting the source of PAI-1 for the evaluation of PAI-1 inhibitors. Since PAI-1 is expected to be bound to vitronectin in plasma and especially in the extracellular matrix, it is important that potentially interesting mouse PAI-1 inhibitors have a good inhibitory effect, not only to free glycosylated PAI-1 but also to glycosylated PAI-1 in its vitronectin-bound state. For MA-33H1F7, a monoclonal antibody generated toward human PAI-1 but cross-reacting with rat and mouse PAI-1, a decreased inhibitory capacity was observed in the presence of vitronectin [171]. This reduced inhibitory effect toward vitronectin-bound PAI-1 limits the *in vivo* potential of MA-33H1F7. These data clearly indicate that thorough evaluation of the reactivity of an antibody toward recombinant glycosylated PAI-1 and even vitronectin-bound PAI-1 must precede further *in vivo* testing.

Our goal was the generation and characterization of monoclonal antibodies reacting with vitronectin-bound glycosylated mouse PAI-1 that can be used to study the role of PAI-1 in different mouse models.

2.2 Materials and methods

2.2.1 Materials

Human t-PA (Actilyse®) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Human u-PA was kindly provided by Bournonville Pharma (Brain l'Alleud, Belgium). The pcDNA3.1(+) vector was purchased from Invitrogen (Merelbeke, Belgium). Chromogenic substrate S2403 for the plasminogen-coupled chromogenic assay was from Nodia/Chromogenix (Antwerp, Belgium). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (GAM-HRP) was obtained from Bio-Rad (Hercules, California, USA). Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (RAM-HRP) was ordered from Nordic Immunological Laboratories (Tilburg, The Netherlands). 96-well polystyrene microtiter plates were obtained from Costar (Cambridge, Massachusetts, USA). SDS-PAGE for proteins was performed with the PhastSystem™ using PhastGel™ gradient 10-15 % gels and analyzed by densitometric scanning with Imagemaster™ software (GE healthcare, Uppsala, Sweden). Mouse multimeric vitronectin was from Molecular Innovations (Novi, Michigan, USA). Human embryonic kidney 293T cells (HEK293T) were obtained from ATTC-LGC standards (Teddington, Middlesex, UK). Protein A-sepharose™ 4 fast flow and SP-sepharose™ (fast flow) were purchased from GE healthcare Bio-Sciences (Uppsala, Sweden). DMEM/F-12 medium was from GIBCO (Invitrogen) and hybridoma supplement was obtained from ThromboGenics (Leuven, Belgium). Thromboplastin (Dade® Innovin®, recombinant human tissue factor) was purchased from Siemens Healthcare, Diagnostics Products (Marburg, Germany). Oligonucleotides for mutagenesis and sequencing were from Sigma-Aldrich (St. Louis, Missouri, USA). dNTP polymerization mix was obtained from Stratagene (La Jolla, California, USA). Mutagenesis was performed according to the QuickChange™ XL site-directed mutagenesis kit from Stratagene. *Pfx50*™ DNA polymerase was purchased from Invitrogen. The pIGE2 vector was kindly provided by Innogenetics (Gent, Belgium). PCR reactions were carried out using the Mastercycler Gradient from Eppendorf (Hamburg, Germany). DNA was sequenced by LGC genomics (Berlin, Germany). Mice were obtained from Charles River Laboratories (USA) or Janvier (France). Figures and statistical analyses were obtained with GraphPad Prism 5.03 software (GraphPad Software Inc, San Diego, California, USA). All analytical reagents used were of analytical grade.

2.2.2 Expression of glycosylated and non-glycosylated PAI-1 from different species

In order to generate recombinant glycosylated mouse, rat and human PAI-1, PAI-1 cDNA was cloned into the expression vector pcDNA3.1(+) by use of standard techniques and the recombinant glycosylated PAI-1 proteins were transiently expressed in HEK293T cells [172]. Glycosylated mouse PAI-1 was purified in a latent form from the media of these HEK293T cells by ion-exchange chromatography on SP-sepharose^{TM}. As running buffer, 0.15 M phosphate pH=5.7 was used and the column was washed with the same phosphate buffer containing 0.2 M NaCl. Elution was performed with 0.15 M phosphate buffer pH=5.7 and a gradient of NaCl from 0.2 M to 1.3 M. Fractions containing glycosylated PAI-1 were identified by SDS-PAGE analysis and dialyzed against 0.15 M phosphate buffer pH=6 containing 0.5 M NaCl to obtain the same buffer conditions as for non-glycosylated mouse PAI-1.

The same purification procedure was used to purify recombinant glycosylated rat and human PAI-1, only buffer conditions differed slightly. For glycosylated rat PAI-1 0.15 M phosphate buffer pH=5.7 + 0.15 M NaCl was used as a wash buffer and the gradient for elution was set from 0.15 M to 1.3 M NaCl. For glycosylated human PAI-1 purified fractions were dialyzed against 0.15 M phosphate buffer pH=6 containing 0.7 M NaCl.

The purified latent form of PAI-1 was partially converted to its active conformation by denaturation of a 20 µg/ml solution of PAI-1 in 6 M guanidinium chloride and refolding by extensive dialysis against 50 mM NaAcetate, 2 mM glutathione, 1 M NaCl, 0.01% Tween 80, pH=5.5 at 4°C (10-20% active PAI-1 could be obtained) [47]. Non-glycosylated mouse, rat and human PAI-1 were expressed in *E. coli* and purified, largely in the active conformation, in a similar way as described for human PAI-1 [109].

2.2.3 Generation of monoclonal antibodies toward mouse PAI-1

Monoclonal antibodies (MA) against recombinant non-glycosylated and glycosylated mouse PAI-1 (mPAI-1) were produced by the method developed by Galfré and Milstein [173]. PAI-1$^{-/-}$ deficient mice were immunized by subcutaneous injection of 10-50 µg of glycosylated mPAI-1 or 55 µg of non-glycosylated mPAI-1 in complete Freund's adjuvant. This was followed 2 weeks later by intraperitoneal injection of 10-50 µg of glycosylated or 55 µg of non-glycosylated mPAI-1 in incomplete Freund's adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with either 10-50 µg of

glycosylated mPAI-1 or 55 µg of non-glycosylated mPAI-1 in physiological saline on days 4 and 2 before cell fusion. Cell fusion, selection of positive hybridoma cells and purification of MA was performed as described in [174].

2.2.4 Cross-reactivity of monoclonal antibodies with non-glycosylated and glycosylated PAI-1 from different species.

Cross-reactivity of the monoclonal antibodies with non-glycosylated and glycosylated PAI-1 from different species (mouse, rat and human) was evaluated by comparative analysis of the reactivity of the purified antibodies in an ELISA system using microtiter plates coated for 72 hours with 4 µg/ml (200 µl/well) of the respective antigens. As a negative control 4 µg/ml of TAFI protein was coated. Plates were blocked by a 2 hour incubation with PBS containing 1% bovine serum albumin (BSA) (200 µl/well). Serial 4-fold dilutions (2.5 µg/ml to 0.002 µg/ml, in PTA buffer (PBS, 0.1% BSA, 0.002% Tween 80)) of the monoclonal antibodies were applied to the wells (180 µl/well) of each microtiter plate and incubated for 18 hours at 4°C. For determination of blank values, PTA buffer was used as a sample instead of a monoclonal antibody dilution. For detection of bound immunoglobulins, RAM-HRP was used. When the optical density at a concentration of 0.625 µg/ml of monoclonal antibody was lower than 20 times the blank value, this was considered negative reactivity.

Absence of reactivity of the monoclonal antibodies toward vitronectin was evaluated in a one-side ELISA assay using a similar ELISA protocol as described above. Plates were coated for 72 hours with 2 µg/ml of mouse vitronectin and recombinant (non-) glycosylated mouse PAI-1 (positive control). Serial 10-fold dilutions (10 µg/ml to 0.0001 µg/ml, in PTA buffer) of the antibodies were applied to the wells of the microtiter plates (180 µl/well) and incubated for 18 hours at 4°C.

2.2.5 PAI-1 neutralization assay

The inhibitory capacity of the monoclonal antibodies toward recombinant non-glycosylated and glycosylated mPAI-1 was determined using a plasminogen-coupled chromogenic method described by Verheijen et al. [175]. A range of molar ratio of antibody over PAI-1 between 32 and 0.0625 was tested, as described previously [176].

To evaluate the effect of vitronectin binding on the inactivation of recombinant glycosylated mPAI-1 by monoclonal antibodies, mPAI-1 was pre-incubated with different concentrations of vitronectin (equimolar or 33-fold molar excess of vitronectin over mPAI-1) for 30 min at 37°C.

2.2.6 Functional distribution assay

The functional distribution of PAI-1 after incubation with the different monoclonal antibodies was determined as described previously in [169].

2.2.7 Immunohistochemistry

The applicability of the monoclonal antibodies in immunohistochemistry was evaluated on intra-abdominal gonadal fat tissue, inguinal subcutaneous fat tissue and on *de novo* formed fat pads from PAI-1 overexpressing mice and control mice as described previously [177]. Briefly, *de novo* formed fat pads were obtained by subcutaneous injection of 3T3-F442A preadipocytes in the back of athymic Balb/c NUDE mice. As a negative control gonadal fat tissue from PAI-1 knock-out mice was used. Paraffin sections (10 µm) were prepared for immunohistochemistry. Staining for PAI-1 in adipose tissue sections was performed with the MP-antibodies as primary antibodies, and RAM-biotin as secondary antibody. Bound antibodies were detected using streptavidin-HRP followed by signal amplification with the Tyramide Signal Amplification system (Perkin Elmer, Boston, Massachusetts, USA) and DAB/H_2O_2 staining. Paraffin sections were counterstained with hematoxylin/eosin. All pictures were made on a Zeiss Axioplan 2 imaging microscope (magnification 40x) with AxioVision rel.4.6 software (Carl Zeiss, Oberkochen, Germany). At least three pictures from at least three sections were made. Analysis was performed with homemade macros on the KS300 or AxioVision rel.4.6 Software from Carl Zeiss.

2.2.8 Mouse thromboembolism model

A panel of five PAI-1 inhibiting antibodies, reacting with glycosylated mouse PAI-1 *in vitro* and MA-33H1F7, were evaluated for their inhibitory potential in a mouse thromboembolism model. In this model, 10 mg/kg of monoclonal antibody was administered via the tail vein to Swiss mice of approximately 20 g. Five minutes after the

antibody injection, a suboptimal concentration of t-PA (0.1 mg/kg) was injected i.v., immediately followed by i.v. injection of thromboplastin (3.3 µg/kg) to evoke thromboembolisms. Mice were evaluated after 15 minutes and gained a positive score if they displayed normal physical activity. A negative score was obtained in case of paralysis of one or more of the limbs or in case of death. MA-T30E5A2, a non-inhibitory antibody reacting with human TAFI, but not with mouse TAFI was used as a negative control antibody [178]. All animals received care in compliance with the European conventions on Animal care and all experiments were approved by the local ethical committee of the KULeuven.

2.2.9 Affinity measurements and epitope mapping

Affinity constants for binding of the monoclonal antibodies to PAI-1 wild-type and mutants were determined by surface plasmon resonance (SPR) analysis on a Biacore™ 3000 instrument (Biacore™AB, Uppsala, Sweden) as described in [179]. Mouse PAI-1 mutants were produced as described previously [180].

2.2.10 Statistical analysis

Data are expressed as mean ± SD. Statistical significance between proportions was analyzed using a Fisher's exact test. Two-tailed P values < 0.05 were considered statistically significant.

2.3 Results

2.3.1 Generation of monoclonal antibodies toward mouse PAI-1

When mice were immunized with non-glycosylated mPAI-1, one fusion yielded 21 positive hybridomas producing MA toward mPAI-1. All antibodies (designated as MA-MP antibodies) were successfully purified from conditioned medium by affinity chromatography on a Protein A column. When mice were immunized with glycosylated mPAI-1, 4 different fusions yielded only 3 MA with very low affinities toward both non-glycosylated and glycosylated mPAI-1 (i.e. MA-GMP32E8, MA-GMP47B4 and MA-GMP50E11). The reason for this unsuccessful production of MA toward glycosylated mPAI-1 is unclear. Because of their low affinities toward mPAI-1 these 3 antibodies will not be discussed further in the remainder of the chapter.

2.3.2 Cross-reactivity of monoclonal antibodies with glycosylated and non-glycosylated PAI-1 from different species.

Cross-reactivity of all 21 MA with glycosylated and non-glycosylated mouse, rat and human PAI-1 was first analyzed in an ELISA assay (Table 2.1 and figure 2.1). Based on the cross-reactivity data, antibodies could be subdivided in 7 different groups. Since the goal of this study is the generation of a panel of MA to further elucidate the role of PAI-1 in different mouse models, we will mainly focus on those groups including antibodies reacting with both non-glycosylated and glycosylated mPAI-1 (groups 1-4) in the remainder of the chapter.

MA belonging to group 1 cross-react with glycosylated and non-glycosylated PAI-1 from all species under investigation. A second interesting group is composed of MA cross-reacting with glycosylated and non-glycosylated mouse and rat PAI-1, with non-glycosylated human PAI-1 but not with glycosylated human PAI-1. A third group consists of MA cross-reacting with glycosylated and non-glycosylated mouse and rat PAI-1, but not with human PAI-1. And finally group 4 comprises one antibody cross-reacting only with glycosylated and non-glycosylated mouse PAI-1. Four representative graphs for the respective groups are given in figure 2.1.

Monoclonal antibodies belonging to groups 1-4 were also evaluated for their binding to mouse vitronectin. In a one-side ELISA assay most antibodies displayed no binding

toward mouse vitronectin. Only for the non-inhibitory antibody MA-MP22E2 binding toward mouse vitronectin could be observed, suggesting that mouse vitronectin displays a region very similar to the epitope of MA-MP22E2 (results not shown).

Table 2.1: Cross-reactivity of monoclonal antibodies toward non-glycosylated (non-glyc) and glycosylated (glyc) mouse, rat and human PAI-1

MA	Group	Mouse PAI-1		Rat PAI-1		Human PAI-1	
		Non-glyc	Glyc	Non-glyc	Glyc	Non-glyc	Glyc
MP2D2	1						
MP6H6	1						
MP11A11	1						
MP21F3	1						
MP25H3	1						
MP8B1	2						
MP22E2	2						
MP26H2	2						
MP2D9	3						
MP8H11	3						
MP11C2	3						
MP21F7	3						
MP2H2	4						
MP1H12	5						
MP10F9	5						
MP12F10	5						
MP13H7	5						
MP16E5	5						
MP21B5	5						
MP18E10	6						
MP13G10	7						

Cross-reactivity data were obtained from one-side ELISA assays. When the optical density at a concentration of 0.625 µg/ml of monoclonal antibody was lower than 20 times the blank value, this was considered negative reactivity (grey highlight = cross-reactivity).

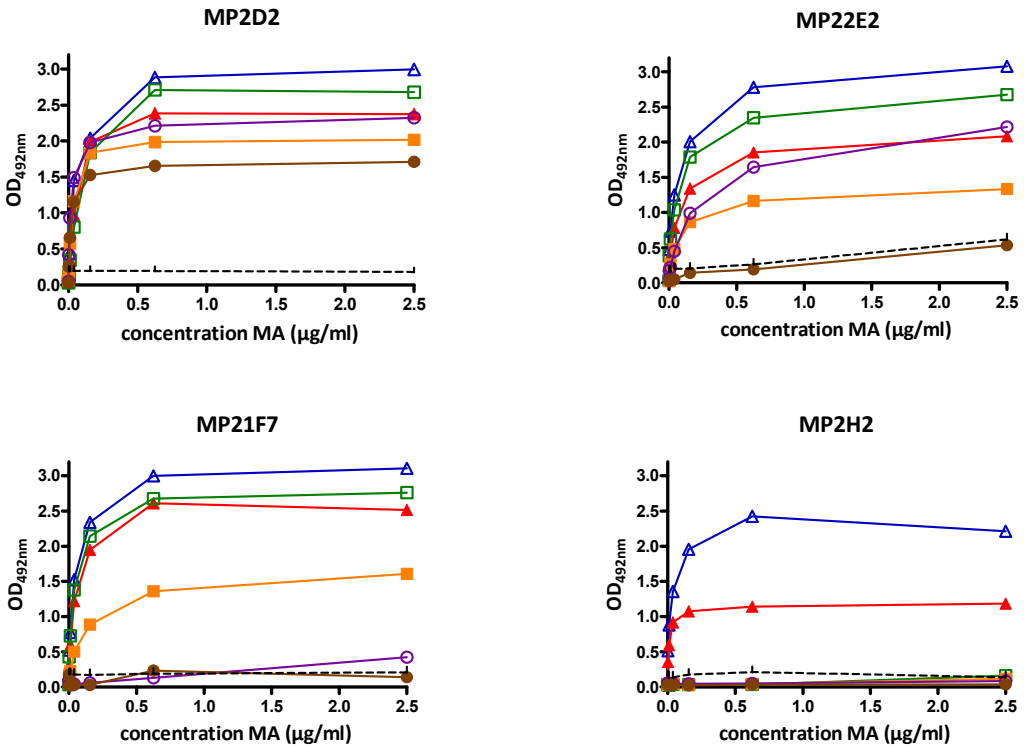

Figure 2.1: Cross-reactivity of monoclonal antibodies toward recombinant PAI-1 from different species.

Cross-reactivity of monoclonal antibodies toward non-glycosylated mouse PAI-1 (Δ, blue), glycosylated mouse PAI-1 (▲, red), non-glycosylated rat PAI-1 (□, green), glycosylated rat PAI-1 (■, orange), non-glycosylated human PAI-1 (○, purple), glycosylated human PAI-1 (●, brown) and a negative control (i.e. TAFI, dotted line, ׀, black).

2.3.3 PAI-1 neutralization assay

Five MA (MA-MP2D2, MA-MP6H6, MA-MP11A11, MA-MP8B1 and MA-MP26H2), belonging to the first four groups, exerted a strong inhibitory effect (i.e. 58-80 % inhibition of PAI-1 activity at a 32-fold molar excess of MA) toward both non-glycosylated and glycosylated mPAI-1 activity in the PAI-1 neutralization assays with t-PA (Table 2.2). For three MA (MA-MP21F3, MA-MP11C2 and MA-MP2H2) only a minor inhibitory effect was seen (i.e. 25-56% inhibition of PAI-1 activity).

Inhibitory activity of the antibodies toward glycosylated mPAI-1 after pre-incubation of PAI-1 with mouse vitronectin was evaluated in the same PAI-1 neutralization assay (Table 2.2). Berry et al. previously demonstrated that MA-33H1F7, generated toward human PAI-1 but cross-reacting with mouse and rat PAI-1, is no longer able to inhibit vitronectin-bound rat PAI-1 [169, 171]. In our study, the inhibitory activity of MA-33H1F7

(IC_{50}-values for MA-33H1F7 in the PAI-1 neutralization assay were 6.9 ± 1.5 nM and 4.3 ± 0.70 nM toward non-glycosylated and glycosylated mPAI-1 respectively) was reduced after incubation with vitronectin with almost total abolishment of PAI-1 inhibition at a 33-fold molar excess of vitronectin over mouse PAI-1, thereby confirming the binding of vitronectin to mouse PAI-1.

However, all inhibitory MP-antibodies evaluated in the PAI-1 neutralization assay keep their full PAI-1 inhibitory potential in the presence of a 33-fold molar excess of mouse vitronectin (Table 2.2), except for MA-MP11C2 where a slight reduction in the percentage inhibition of PAI-1 was seen in the presence of vitronectin. Dose response curves from the PAI-1 neutralization assay in the presence of vitronectin obtained for monoclonal antibodies MA-MP2D2 and MA-33H1F7 are shown in figure 2.2.

Table 2.2: Inhibitory capacity of monoclonal antibodies (groups 1 to 4) toward non-glycosylated mouse PAI-1 and toward glycosylated mouse PAI-1 in the absence or presence of mouse vitronectin

MA	Group	Non-glycosylated mouse PAI-1		Glycosylated mouse PAI-1		Glycosylated mouse PAI-1 + vitronectin	
		% inhibition¥	IC_{50} (nM)†	% inhibition¥	IC_{50} (nM)†	% inhibition¥	IC_{50} (nM)†
MP2D2	1	79 ± 5.5	1.6 ± 0.5	79 ± 8.2	0.9 ± 0.2	77 ± 4.5	1.0 ± 0.2
MP6H6	1	75 ± 0.9	2.5 ± 0.5	62 ± 0.6	1.4 ± 0.2	64 ± 13	0.9 ± 0.1
MP11A11	1	80 ± 3.8	1.5 ± 0.2	58 ± 4.5	1.2 ± 0.2	62 ± 12	1.0 ± 0.3
MP21F3	1	31 ± 5.7	6.6 ± 1.5	30 ± 11	35 ± 9.7	37 ± 6.7	162 ± 96
MP25H3	1	< 3	/	< 3		< 3	/
MP8B1	2	79 ± 7.5	2.1 ± 0.4	61 ± 5.8	1.2 ± 0.2	64 ± 11	1.1 ± 0.2
MP22E2	2	7.6 ± 4.7	/	< 3	/	< 3	/
MP26H2	2	78 ± 6.3	2.8 ± 0.4	65 ± 12	1.1 ± 0.2	60 ± 15	0.6 ± 0.2
MP2D9	3	< 3	/	< 3	/	< 3	/
MP8H11	3	< 3	/	< 3	/	< 3	/
MP11C2	3	25 ± 6.1	44 ± 18	35 ± 12	859 ± 332	11 ± 2.3	ND
MP21F7	3	< 3	/	< 3	/	< 3	/
MP2H2	4	48 ± 12	62 ± 17	56 ± 2.7	246 ± 57	49 ± 16	175 ± 78

¥ Values represent the percentage inhibition of PAI-1 activity (mean ± SD; n ≥ 3) as calculated from plasminogen-coupled chromogenic assays with t-PA. One hundred percent PAI-1 activity is the PAI-1 activity in the absence of monoclonal antibody. The percentage inhibition by the monoclonal antibody was calculated from the residual PAI-1 activity in the presence of a 32-fold molar excess of monoclonal antibody over PAI-1.

† The IC_{50}-values (mean ± SD; n ≥ 3) were determined from one-site binding hyperbola plots of the percentage inhibition of PAI-1 activity against the antibody concentration used in the assay mixture using GraphPad prism software (ND: IC_{50} values could not be determined due to the very low inhibitory capacity of MA-MP11C2 toward vitronectin-bound PAI-1).

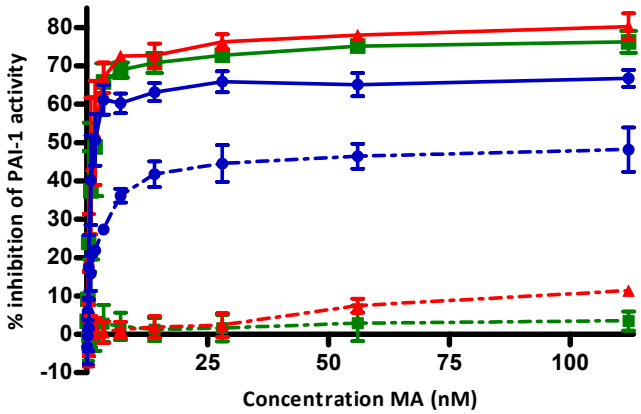

Figure 2.2: Inhibition of glycosylated mouse PAI-1 by monoclonal antibodies MA-MP2D2 and MA-33HF7 in the presence of an equimolar or a 33-fold molar excess of mouse vitronectin.

Mouse PAI-1 was incubated with buffer (●, blue), an equimolar concentration (▲, red) or a 33-fold (■, green) molar excess of mouse vitronectin before the PAI-1 neutralization assay. Curves show the percentage inhibition of PAI-1 activity (mean ± SD; n ≥ 3) by MA-MP2D2 (full line) and MA-33H1F7 (dotted line).

2.3.4 Functional distribution assay

Table 2.3 illustrates the reaction products generated after interaction of non-glycosylated mouse PAI-1 with a two-fold molar excess of t-PA in the presence or absence of a three-fold molar excess of the different MA (comparable results were obtained with u-PA, results not shown). Incubation of non-glycosylated mPAI-1 with a two-fold molar excess of t-PA in the absence of any antibody (= control) resulted in the formation of a stable complex (53 ± 5.2%), corresponding to the fraction active PAI-1, small amounts of cleaved PAI-1 (11 ± 2.9%), originating from the substrate pathway of PAI-1, and residual non-reactive PAI-1, originating from latent PAI-1 (36 ± 4.0%).

For MA-MP2D2, MA-MP6H6, MA-MP8B1, MA-MP11A11 and MA-MP26H2 a clear conversion from an active to a substrate pathway of PAI-1 was observed. MA-MP21F3 also induces the substrate pathway of PAI-1, although to a much lesser extent. For MA-MP22E2, MA-MP11C2 and MA-MP2H2 the functional distribution of PAI-1 was not significantly different from the control sample.

Table 2.3: Functional distribution assay

MA	Group	% Complexed	% Non-reactive	% Cleaved
Control		***53 ± 5.2***	***36 ± 4.0***	***11 ± 2.9***
MP2D2	1	7.6 ± 5.6	49 ± 21	43 ± 26
MP6H6	1	9.1 ± 6.6	37 ± 3.4	54 ± 7.3
MP11A11	1	10 ± 6.7	48 ± 19	42 ± 25
MP21F3	1	46 ± 0.9	36 ± 4.1	18 ± 4.1
MP25H3	1	48 ± 3.3	39 ± 3.7	13 ± 2.9
MP8B1	2	8.5 ± 9.7	49 ± 12	42 ± 22
MP22E2	2	48 ± 2.1	38 ± 5.1	14 ± 4.7
MP26H2	2	6.8 ± 1.0	37 ± 2.7	56 ± 2.0
MP2D9	3	50 ± 1.9	38 ± 3.1	12 ± 3.4
MP8H11	3	52 ± 3.1	39 ± 2.2	8.6 ± 3.6
MP11C2	3	49 ± 3.7	38 ± 1.2	13 ± 4.9
MP21F7	3	57 ± 4.9	34 ± 4.3	9.3 ± 0.9
MP2H2	4	50 ± 4.6	43 ± 3.5	7.3 ± 1.2

Quantification of reaction products formed during incubation of non-glycosylated mPAI-1 with a two-fold molar excess of t-PA in the presence of a three-fold molar excess of monoclonal antibody or PBS (= control). Incubation of PAI-1 with t-PA results in the formation of a stable covalent complex, originating from the inhibitory pathway, cleaved PAI-1, originating from the substrate pathway and residual non-reactive PAI-1 (Results are expressed as percentage of PAI-1 protein; mean ± SD; n≥ 3).

2.3.5 Immunohistochemistry

Three MA with good reactivity toward glycosylated mPAI-1 (i.e. MA-MP2D2, MA-MP6H6 and MA-MP21F7) were evaluated in immunohistochemistry experiments. Paraffin sections from gonadal and subcutaneous fat tissue and from *de novo* formed fat pads derived from systemic PAI-1 overexpressing mice and control mice were stained with DAB/H_2O_2 and Harris hematoxylin counterstaining. As a negative control, fat tissue from PAI-1 KO mice was stained following the same procedures.

All three MA could be used to detect native mPAI-1 on fat tissue sections. A difference in response could be seen between sections from PAI-1 overexpressing mice and fat tissue sections from control mice (Figure 2.3). Computer-assisted image analysis of *de novo* formed fat pads revealed 6.3 ± 3.4% positively stained area with fat tissue from PAI-1 overexpressing mice in comparison to 2.1 ± 1.1% for control tissues when MA-MP2D2 was used as primary antibody ($p = 0.003$). For gonadal fat tissue, data were 1.2 ± 0.5%

versus 0.6 ± 0.6% (p = 0.029) for overexpressing and control tissue respectively. For subcutaneous fat tissue, percentage positively stained area was 3.0 ± 4.3% versus 0.8 ± 1.0% (p = 0.019) for overexpressing and control tissue respectively. No response was observed with fat tissue from PAI-1 KO mice.

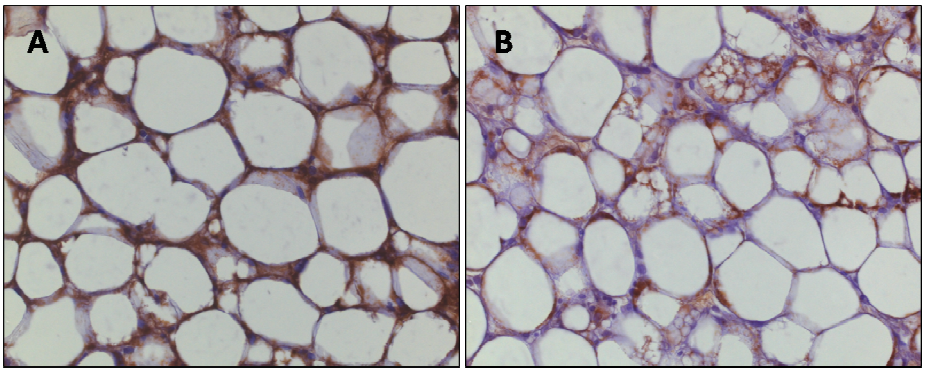

Figure 2.3: Immunostaining for mouse PAI-1.

MA-MP2D2 was used as primary antibody on de novo formed fat pads of PAI-1 overexpressing (A) and control mice (B).

2.3.6 Mouse thromboembolism model

To evaluate the PAI-1 inhibitory potential of the MA *in vivo*, a mouse thromboembolism model was used. The 5 antibodies exerting a strong inhibitory effect (i.e. >58% inhibition) toward vitronectin-bound glycosylated mPAI-1 in the *in vitro* PAI-1 neutralization assay and MA-33H1F7 were evaluated in this model. For all five antibodies evaluated, a statistically significant increase in the percentage of mice with normal physical activity could be detected after a 15 min observation period compared to control mice to which MA-T30E5A2 was administered (Table 2.4). MA-33H1F7 [169] was also evaluated in the same thromboembolism model because of the differential reactivity MA-33H1F7 has to vitronectin-bound PAI-1 compared to the newly generated MP-antibodies (Figure 2.2). Also after injection of MA-33H1F7 an increase in the percentage of mice with normal physical activity was seen (i.e. 47 % *versus* 29 % for MA-33H1F7 and MA-T30E5A2 respectively). However this increase is less pronounced as for the MP-antibodies and therefore did not reach statistical significance. These results suggest that reactivity toward vitronectin-bound PAI-1 might be an important aspect to achieve stronger PAI-1 inhibition *in vivo.*

Table 2.4: In vivo evaluation of the PAI-1 inhibitory potential of monoclonal antibodies in a mouse thromboembolism model

MA	Mice with normal physical activity (%)	n	P*
MA-T30E5A2†	***29%***	***45***	
MP2D2	54%	50	0.022
MP6H6	56%	54	0.009
MP8B1	51%	47	0.035
MP11A11	50%	50	0.039
MP26H2	59%	49	0.004
MA-33H1F7	47%	49	0.091

† Negative control antibody

**A two-tailed P-value < 0.05 in comparison to the negative control antibody was considered statistically significant (Fisher's exact test).*

2.3.7 Affinity measurements and epitope mapping

Affinity constants for binding between the antibodies and recombinant PAI-1 were determined by surface plasmon resonance (SPR) analysis. SPR analyses were only performed for the strong inhibitory antibodies also reacting with glycosylated mPAI-1 (i.e. MA-MP2D2, MA-MP6H6, MA-MP8B1, MA-MP11A11 and MA-MP26H2). The results obtained for these affinity measurements are summarized in table 2.5.

All MP-antibodies evaluated show high affinity toward both non-glycosylated and glycosylated mPAI-1 (K_A values ranging from 10^8 to 10^9 M^{-1}). For non-glycosylated and glycosylated rat PAI-1 the K_A values measured were very similar to the corresponding values obtained for mPAI-1 (results not shown). The affinity of the MP-antibodies toward non-glycosylated human PAI-1 was clearly reduced in comparison to the murine values (in the range of 10^7 M^{-1}). Binding constants toward glycosylated human PAI-1 could not be determined using SPR analysis (i.e. apparent $K_A < 10^6$ M^{-1}). The reason for this differential reactivity toward glycosylated human PAI-1 in one-side ELISA (Table 2.1) and SPR analysis (Table 2.5) is unclear, but such differences have been reported previously [181].

Using the alanine scanning method, attempts were made to unravel the epitope of the MA. Eight single amino acid residues present in mouse PAI-1 were mutated to alanine (mPAI-1-E128A, mPAI-1-V129A, mPAI-1-E130A, mPAI-1-R131A, mPAI-1-K154A, mPAI-1-R142A, mPAI-1-D150A and mPAI-1-E159A) and the affinity of the MP-antibodies toward

the resulting mutant proteins was evaluated by SPR analysis. For all five evaluated MP-antibodies a clear reduction in affinity (K_A) could be detected with mPAI-1-E130A, mPAI-1-K154A and mPAI-1-E159A compared to mPAI-1 wt. For mPAI-1-R131A only a mild reduction in affinity was seen (Table 2.5).

Table 2.5: Affinity measurements and epitope mapping

	MP2D2	**MP6H6**	**MP8B1**	**MP11A11**	**MP26H2**
hPAI-1 wt	0.097 ± 0.0065	0.032 ± 0.014	0.084 ± 0.068	0.074 ± 0.017	0.036 ± 0.014
Glyc hPAI-1 wt	NB	NB	NB	NB	NB
mPAI-1 wt	3.0 ± 0.95	1.1 ± 0.26	0.97 ± 0.27	2.2 ± 0.64	1.2 ± 0.34
Glyc mPAI-1 wt	2.0 ± 1.0	0.75 ± 0.42	0.72 ± 0.41	1.4 ± 0.66	0.84 ± 0.44
mPAI-1-E128A	6.9 ± 1.8	2.8 ± 0.75	2.2 ± 0.45	5.0 ± 1.1	2.5 ± 0.50
mPAI-1-V129A	1.9 ± 0.56	1.3 ± 0.38	0.97 ± 0.30	2.4 ± 0.78	1.1 ± 0.36
mPAI-1-E130A	0.045 ± 0.018	0.013 ± 0.011	NB	0.033 ± 0.033	NB
mPAI-1-R131A	1.5 ± 0.54	0.51 ± 0.20	0.46 ± 0.12	0.93 ± 0.39	0.51 ± 0.14
mPAI-1-K154A	0.17 ± 0.052	0.11 ± 0.051	0.073 ± 0.024	0.16 ± 0.11	0.052 ± 0.013
mPAI-1-R142A	5.6 ± 1.8	1.8 ± 0.44	1.5 ± 0.36	4.0 ± 0.99	1.6 ± 0.39
mPAI-1-D150A	4.9 ± 1.5	1.8 ± 0.37	1.4 ± 0.31	3.5 ± 0.77	1.6 ± 0.33
mPAI-1-E159A	0.23 ± 0.086	0.081 ± 0.065	0.044 ± 0.033	0.086 ± 0.019	0.045 ± 0.031

Affinity constants ($K_A\ 10^9\ M^{-1}$) of monoclonal antibodies toward PAI-1 wild-type (wt) and mutants were determined by SPR-analysis on a Biacore™3000 instrument (Results are expressed as mean ± SD, n ≥ 3; NB: $K_A < 10^6\ M^{-1}$).

2.4 Discussion

The aim of this study was the generation of a panel of well characterized inhibitory monoclonal antibodies reacting with both non-glycosylated and glycosylated mouse PAI-1, that can be used to study the effect of pharmacological PAI-1 inhibition in diverse mouse models.

Previously, panels of monoclonal antibodies toward recombinant non-glycosylated human, rat and mouse PAI-1 have been generated [116, 169, 182]. However, most of these antibodies do not show sufficient reactivity toward glycosylated mouse PAI-1. MA-H4B3 and MA-124K1 are inhibitory monoclonal antibodies originally generated toward non-glycosylated mouse and rat PAI-1 respectively [108, 116]. MA-H4B3 was already used in certain *in vivo* studies on PAI-1 function but some recent results demonstrated that this antibody has no reactivity toward glycosylated mouse PAI-1, making the negative conclusions of these *in vivo* studies not fully valid [36, 119, 183]. Recently we observed that MA-124K1 has a strongly reduced reactivity toward glycosylated mouse PAI-1, which might explain the absence of a clear effect of PAI-1 inhibition in preliminary *in vivo* studies (unpublished results). Monoclonal antibody MA-33H1F7 was generated toward non-glycosylated human PAI-1 and does show reactivity and inhibitory potential toward both non-glycosylated and glycosylated mouse PAI-1. However, the percentage inhibition of PAI-1 activity by MA-33H1F7 reaches a plateau at 40-50%, making this antibody no perfect candidate for *in vivo* evaluation of PAI-1 inhibition [169, 176].

These results stress the need for a panel of well characterized antibodies that can be used to further unravel the role of PAI-1 in thrombotic and non-thrombotic mouse models.

In this study PAI-$1^{-/-}$ mice were immunized with non-glycosylated mouse PAI-1, resulting in 21 monoclonal antibody producing hybridoma cell lines. Cross-reactivity of the newly generated antibodies toward non-glycosylated and glycosylated mouse, rat and human PAI-1 was evaluated using one-side ELISA assays (Table 2.1) and SPR analysis (Table 2.5). Thorough characterization of the cross-reactivity of PAI-1 inhibiting antibodies is important because significant species-dependent differences in reactivity toward PAI-1 have been observed for PAI-1 inhibitory compounds [180, 184, 185]. Thirteen of the examined MP-antibodies showed good reactivity toward both non-glycosylated and glycosylated mouse PAI-1, which makes them potentially useful for the evaluation of

PAI-1 inhibition in mouse models. Although PAI-1 expressed in HEK293T cells is definitely glycosylated, we cannot exclude that there might be differences with the glycosylation profile of the native protein. However, the sugar composition and the number and size of branches in the sugar tree is known to vary among glycans bound to a protein, among glycoproteins, and among cell types, tissues, and species [186]. Glycosylation of human and rat PAI-1 was shown to be largely tissue-dependent, with expression of non-glycosylated PAI-1 in liver and platelets and of glycosylated PAI-1 in adipose tissue [187, 188]. Especially obese subjects with elevated PAI-1 levels that are at risk for cardiovascular events have plasma PAI-1 with a similar glycosylation pattern as adipose tissue [188]. These results indicate the importance of PAI-1 inhibitory compounds that can inhibit both non-glycosylated and glycosylated variants of PAI-1.

Vitronectin-bound PAI-1 is the dominant form of PAI-1 in plasma and in the extracellular matrix [53, 189]. It was already demonstrated that some LMW compounds that had a very potent PAI-1 inhibitory effect *in vitro* were no longer able to inhibit PAI-1 in a plasma environment. This discrepancy between the obtained data might be attributed in part to the fact that the LMW compounds were no longer able to inhibit PAI-1 when it was associated with vitronectin [136, 137, 190]. Also for MA-33H1F7, a species-dependent reduction in inhibitory capacity was detected toward PAI-1 bound to vitronectin [171]. In the presence of a 33-fold molar excess of vitronectin, the inhibitory effect of MA-33H1F7 toward human PAI-1 was reduced by 44%, whereas for rat PAI-1 the inhibitory effect of MA-33H1F7 was almost completely abolished [171]. The reduced inhibitory potential of MA-33H1F7 toward vitronectin-bound mouse PAI-1 could be confirmed in this study using the PAI-1 neutralization assay. These observations imply that potentially interesting PAI-1 inhibitory compounds must have a good inhibitory capacity not only to free PAI-1 but also to PAI-1 bound to vitronectin.

Of the thirteen monoclonal antibodies cross-reacting with recombinant glycosylated mouse PAI-1, five showed good inhibitory capacity toward both non-glycosylated and glycosylated mouse PAI-1 (i.e. 58 to 80 % inhibition of PAI-1 activity). More important, in the presence of a 33-fold molar excess of mouse vitronectin over PAI-1 these antibodies kept their inhibitory effect toward glycosylated mouse PAI-1. So in contrast to MA-33H1F7 and many previously generated LMW PAI-1 inhibitors, the MP-antibodies are clearly able to bind to vitronectin-bound PAI-1.

In the functional distribution assay it was demonstrated that all strong inhibitory antibodies inhibit PAI-1 activity by inducing a conversion from the active to the substrate pathway of PAI-1 (switching antibodies). Previously, the epitopes of two other substrate inducing antibodies, namely MA-33H1F7 and MA-55F4C12, were allocated to the region around α-helix F (epitopes: Glu^{130}-Arg^{131}-Lys^{154} and Glu^{128}-Val^{129}-Glu^{130}-Arg^{131}-Lys^{154} for MA-33H1F7 and MA-55F4C12 respectively). For this reason, epitope mapping of the MP-antibodies was started in the same region. SPR-analysis of various mPAI-1 mutants indicated that the epitope of the MP-antibodies is located in the region around α-helix F and the turn connecting α-helix F with β-sheet s3A, again stressing the importance of this region for the functional properties of PAI-1. Although the MP-antibodies share some epitope residues (E130 and K154) with the anti-human PAI-1 antibody MA-33H1F7 (Table 2.5), their epitopes are clearly not the same. MA-33H1F7 has a high affinity toward non-glycosylated human PAI-1 (K_A= 3.2 ± 0.6 x10^9 M^{-1}) whereas for the MP-antibodies the affinity toward human PAI-1 was about 100-fold lower (10^7 M^{-1}). In addition to residues E130 and K154, we identified E159 in mouse PAI-1 as a major residue in the epitope of the MP-antibodies. Residue E159 is not present in human PAI-1 which is in accordance with decreased binding of the MP-antibodies to human PAI-1. The localization of the epitope around α-helix F is at the opposite side of the PAI-1 protein compared to the potential N-glycosylation sites (N209, N265 and N329), situated more closely to the reactive center loop. These specific positions explain why attached glycans do not interfere with the binding of these antibodies to PAI-1.

Binding between PAI-1 and vitronectin is largely due to interactions between the somatomedin B domain of vitronectin and residues R101, M110 and Q123 of the flexible joint region of PAI-1 [191]. Lack of inhibitory capacity of MA-33H1F7 toward vitronectin bound mouse PAI-1 suggests that binding of vitronectin to PAI-1 partially shields the epitope of MA-33H1F7, whereas for most MP-antibodies the epitope remains completely available in the PAI-1-vitronectin complex. Residue E159, present in mouse PAI-1, is part of the epitope of the MP-antibodies, whereas for MA-33H1F7 this residue did not contribute to the epitope. The results indicate that the binding orientation of the MP-antibodies to mouse PAI-1 is indeed different than for MA-33H1F7, possibly explaining the different reactivity toward vitronectin-bound PAI-1.

To evaluate the inhibitory effect of the MP-antibodies *in vivo*, a mouse thromboembolism model was used. For all five newly generated antibodies (i.e. MA-MP2D2, MA-MP6H6, MA-MP8B1, MA-MP11A11 and MA-MP26H2) a statistically significant increase in the percentage of mice with normal physical activity was detected compared to a control group that had been treated with a negative control antibody. The lower percentage of PAI-1 inhibition (max 40-50%) and the lack of reactivity toward vitronectin-bound PAI-1 observed with MA-33H1F7 might explain the slighter increase in percentage of mice with normal physical activity in comparison with MP-antibody treated mice (Table 2.4).

To the best of our knowledge, this is the first report on the generation of monoclonal antibodies that inhibit recombinant glycosylated mouse PAI-1 even in the presence of vitronectin. The monoclonal antibodies generated in this study could therefore be useful to study the effect of pharmacological PAI-1 inhibition in different *in vitro* and *in vivo* models in order to further unravel the complex function of PAI-1 in diverse physiological and pathological conditions.

Chapter 3

Evaluating the reactivity of monoclonal antibodies toward various mouse and rat PAI-1 glycosylation knock-out mutants

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3.1 Introduction

Tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) are serine proteases that cleave the circulating proenzyme plasminogen, resulting in the active protease plasmin. Activation of plasminogen by t-PA is mainly responsible for the lysis of fibrin in a blood clot, whereas u-PA mediated activation of plasminogen plays a major role in pericellular proteolysis. The plasminogen activator system is involved in various physiological processes and therefore its activity must be tightly controlled. On the one hand the activity of plasmin can be inhibited directly by α_2-antiplasmin, and on the other hand the conversion of plasminogen to plasmin can be inhibited by thrombin activatable fibrinolysis inhibitor (TAFI) or by plasminogen activator inhibitors. The most important direct physiological inhibitor of t-PA and u-PA is plasminogen activator inhibitor-1 with second order rate constants for the inhibition being in the order of 10^7 $M^{-1}s^{-1}$ [9, 161].

Plasminogen activator inhibitor-1 is a member of the serpin (serine protease inhibitor) superfamily of proteins [42] and is expressed by many different cell types in a variety of tissues [26]. After secretion in its active conformation, PAI-1 can form stable covalent complexes with t-PA and u-PA and thereby irreversibly blocks the activity of both plasminogen activators [162]. If active PAI-1 does not interact with its target proteases, it rapidly converts to the inactive latent conformation with a half-live of approximately 2 hours at 37°C [42]. In blood, active PAI-1 can bind to vitronectin, resulting in a PAI-1-vitronectin complex that is 2-3-fold more stable at 37°C than free PAI-1 [53].

Increased plasma PAI-1 levels have been implicated in diverse vascular disorders such as myocardial infarction and venous thrombo-embolism. In addition to its role in fibrinolysis, a potential role of PAI-1 has also been suggested in a variety of other pathological processes, such as tumor growth and metastasis [95-97]. Because of the suggested role of PAI-1 in these diverse physiological pathways PAI-1 has been intensively investigated as a potential therapeutic target.

It is hypothesized that PAI-1 circulates both in a glycosylated and non-glycosylated state *in vivo*, depending on the cell type that expresses the protein [188]. PAI-1 has 3 potential N-linked glycosylation sites at asparagines N209, N265 and N329 which are conserved in all species investigated so far (human, mouse, rat, pig, rabbit, cow and mink) [36]. Although there are only slight differences in the biochemical properties of glycosylated

and non-glycosylated PAI-1 [37], the reactivity and inhibitory capacity of monoclonal antibodies toward PAI-1 can be glycosylation dependent [36]. It has been demonstrated previously that human PAI-1 displays a heterogeneous glycosylation pattern at positions N209 and N265 while position N329 is not utilized [36]. However to the best of our knowledge no information is available about the glycosylation pattern present in rat and mouse PAI-1. Since PAI-1 inhibiting antibodies are often evaluated in mouse or rat models, differential reactivity of these inhibitors toward glycosylated and non-glycosylated rat and mouse PAI-1 must be determined before proceeding to *in vivo* experiments. In the present study we compared the reactivity of antibodies toward non-glycosylated and glycosylated mouse and rat PAI-1 and toward specific glycosylation knock-out mutants harboring only one potential glycosylation site.

3.2 Materials and methods

3.2.1 Materials

The pcDNA3.1(+) vector was purchased from Invitrogen (Merelbeke, Belgium). SDS-PAGE for proteins was performed with the PhastSystem™ using PhastGel™ gradient 10-15% gels (GE healthcare, Uppsala, Sweden). Human embryonic kidney 293T cells (HEK293T) were obtained from ATTC-LGC standards (Teddington, Middlesex, UK). Lipofectamine™ 2000 Reagent for transfection was purchased from Invitrogen (Merelbeke, Belgium). SP-sepharose™ (fast flow) was purchased from GE healthcare Bio-Sciences (Uppsala, Sweden). Horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody (RAM-HRP) was ordered from Nordic Immunological Laboratories (Tilburg, The Netherlands). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (GAM-HRP) was obtained from Bio-Rad (Hercules, California, USA). Oligonucleotides for mutagenesis and sequencing were from Sigma-Aldrich (St. Louis, Missouri, USA). dNTP polymerization mix was obtained from Stratagene (La Jolla, California, USA). *Pfx50*™ DNA polymerase was purchased from Invitrogen. PCR reactions were carried out using the Mastercycler Gradient from Eppendorf (Hamburg, Germany). Plasmid DNA purifications were performed using the Nucleobond® PC 500 kit (Macherey-Nagel, Düren, Germany). DNA was sequenced by LGC genomics (Berlin, Germany). ECL Plus Western Blotting Detection Reagent was purchased from GE healthcare (Uppsala, Sweden). The unstained low range protein marker was from Bio-Rad (Hercules, California, USA). Vivaspin-4 columns (30,000 MWCO) were purchased from Sartorius Stedim Biotech S.A. (Aubagne, France). All analytical reagents used were of analytical grade.

3.2.2 Expression and purification of non-glycosylated and glycosylated mouse and rat PAI-1

Mouse and rat PAI-1 cDNA fragments were cloned into expression vector pcDNA3.1(+) by use of standard techniques. The resulting plasmids were transiently transfected into HEK293T cells [172] according to the lipofectamine™ 2000 protocol (Invitrogen). Presence of PAI-1 proteins in the conditioned medium of HEK293T cells was verified by western blotting analysis using MA-33H1F7 [169] as primary antibody and GAM-HRP as secondary antibody. Detection of bound antibodies was performed with ECL Plus

Western Blotting Detection Reagent. Glycosylated mouse PAI-1 was purified from the media of HEK293T cells (± 75 ml) by ion-exchange chromatography on SP-sepharose™. A 0.15 M phosphate buffer pH=5.7 was used as running buffer. The column was washed with 0.15 M phosphate buffer containing 0.2 M NaCl. Elution was performed with 0.15 M phosphate buffer pH=5.7 and a gradient of NaCl from 0.2 M to 1.3 M. Fractions containing glycosylated mouse PAI-1 were identified by SDS-PAGE analysis and dialyzed against 0.15 M phosphate buffer pH=6 containing 0.5 M NaCl to obtain the same buffer conditions as for non-glycosylated mouse PAI-1. The same purification protocol was used to purify glycosylated rat PAI-1, with slightly different buffer conditions. For glycosylated rat PAI-1 0.15 M phosphate buffer pH=5.7 + 0.15 M NaCl was used as a wash buffer and the gradient for elution was set from 0.15 M to 1.3 M NaCl.

Non-glycosylated mouse and rat PAI-1 were expressed in *E. coli* and purified in a similar way as described for non-glycosylated human PAI-1 [109].

Molecular mass of non-glycosylated and glycosylated PAI-1 samples was compared on SDS-PAGE analysis. PAI-1 samples were diluted in PBS to appropriate concentrations. Proteins were denaturated by adding SDS (final concentration of 1%) and heating for 30s at 100°C. Electrophoretic mobility was analyzed by SDS-PAGE performed under non-reducing conditions using 10-15% gradient gels with the PhastSystem™. Proteins were visualized by staining with Coomassie Brilliant Blue.

3.2.3 Evaluation of the binding of monoclonal antibodies to PAI-1 variants

Cross-reactivity of monoclonal antibodies with non-glycosylated and glycosylated rat and mouse PAI-1 and with the respective glycosylation knock-out mutants was evaluated in an ELISA system using microtiter plates coated with 4 µg/ml of the respective antigens. As a negative control 4 µg/ml of TAFI protein was coated. Plates were blocked by 2 hour incubation with PBS containing 1% bovine serum albumin (BSA) (200 µl/well). Serial 4-fold dilutions (10 µg/ml to 0.002 µg/ml, in PTA buffer (PBS, 0.1% BSA, 0.002% Tween 80)) of the monoclonal antibodies were applied to the wells (180 µl/well) of each microtiter plate and incubated for 18 hours at 4°C. For determination of blank values, PTA buffer was used as a sample instead of a monoclonal antibody dilution. For detection of bound immunoglobulins, RAM-HRP was used. The absorbance was read at 492 nm using an ELx808 Absorbance Microplate Reader from BioTek (Winooski, Vermont, USA).

3.2.4 Prediction of potential N-glycosylation sites

We used the prediction server NetNGlyc 1.0 of the Technical University of Denmark (Gupta, Jung and Brunak) to identify the Asn-X-Ser/Thr sequons present in mouse, rat and human PAI-1 (http://www.cbs.dtu.dk/services/NetNGlyc/).

3.2.5 Generation of mouse and rat PAI-1 glycosylation knock-out mutants

The pcDNA3.1(+) vector containing the cDNA fragments for mouse PAI-1 (mPAI-1) and rat PAI-1 (ratPAI-1) were used as a template for site-directed mutagenesis. Mutagenesis was performed according to the QuickChange™ XL site-directed mutagenesis protocol from Stratagene (La Jolla, California, USA). Using the appropriate primers, potential N-glycosylation sites in rat and mouse PAI-1 were removed by mutating an asparagine (N) residue to a glutamine (Q) residue. Following mutants were constructed using this procedure: 3 double mutants (mPAI-1-N209Q-N265Q, mPAI-1-N209Q-N329Q, mPAI-1-N265Q-N329Q), 5 triple mutants (mPAI-1-N209Q-N265Q-N329Q, ratPAI-1-N209Q-N265Q-N329Q, ratPAI-1-N65Q-N265Q-N329Q, ratPAI-1-N65Q-N209Q-N329Q, ratPAI-1-N65Q-N209Q-N265Q) and 1 quadruple mutant (ratPAI-1-N65Q-N209Q-N265Q-N329Q). Resulting plasmids were transiently transfected in HEK293T cells using the lipofectamine™ 2000 protocol (Invitrogen) and expressed proteins were purified from the media of the conditioned cells by ion-exchange chromatography on SP-sepharose™ as described for glycosylated mouse and rat PAI-1 wild-type (cfr. 3.2.2). In case of low concentrations after purification, proteins were upconcentrated using Vivaspin-4 (30,000 MWCO) columns.

3.2.6 Deglycosylation experiments

Deglycosylation experiments were performed using the enzymatic deglycosylation kit from Prozyme (Hayward, California, USA) according to the protocol provided by the manufacturer. Briefly 30 µl solution containing PAI-1 variants was denatured at 100°C during 5 min after addition of 10 µl of incubation buffer (0.25 M sodium phosphate, pH=7.0) and 2.5 µl of the provided denaturation buffer (2% SDS and 1 M β-mercaptoethanol). After addition of 2.5 µl detergent solution (15% NP-40), 1 µl of N-glycanase solution was added and the mixture was incubated for 3 hours at 37°C. After deglycosylation, samples were subjected to SDS-PAGE analysis performed under non-

reducing conditions using 10-15% gradient gels with the PhastSystem™. Proteins were visualized by western blotting using MA-33H1F7 as primary antibody and GAM-HRP as secondary antibody followed by ECL staining.

3.3 Results

3.3.1 Purification and characterization of non-glycosylated and glycosylated mouse and rat PAI-1

Glycosylated mouse PAI-1 (mPAI-1) and glycosylated rat PAI-1 (ratPAI-1) could be expressed in HEK293T cells as was verified by western blotting analysis of conditioned medium. Expression yields after purification were 15.6 ± 6.4 and 13.1 ± 8.9 µg PAI-1 per ml of HEK293T cell medium for glycosylated mouse and glycosylated rat PAI-1 respectively. When proteins were analyzed using SDS-PAGE analysis a clear difference in molecular masses was observed between non-glycosylated and glycosylated mouse PAI-1 (Figure 3.1, lanes 1 and 2 respectively) and between non-glycosylated and glycosylated rat PAI-1 (Figure 3.1, lanes 4 and 5 respectively). The higher molecular masses observed for the glycosylated proteins could be attributed to the presence of glycan chains. Indeed, deglycosylation of glycosylated PAI-1 with N-glycanase resulted in PAI-1 forms with a similar molecular mass as the non-glycosylated equivalents (Figure 3.1 lane 3 *versus* lane 1 and lane 6 *versus* lane 4).

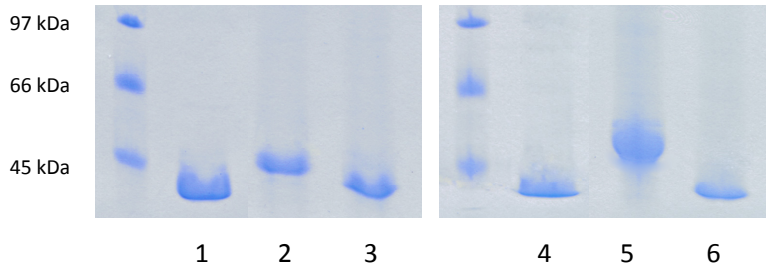

Figure 3.1: Comparison of the molecular mass of glycosylated and non-glycosylated mouse and rat PAI-1

Non-glycosylated mPAI-1 (lane 1), glycosylated mPAI-1 (lane 2), glycosylated mPAI-1 deglycosylated with N-glycanase (lane 3), non-glycosylated ratPAI-1 (lane 4), glycosylated ratPAI-1 (lane 5) and glycosylated ratPAI-1 deglycosylated with N-glycanase (lane 6) were analyzed by SDS-PAGE analysis followed by coomassie staining. (Protein marker: Unstained low range marker (Bio-Rad)).

3.3.2 Reactivity of monoclonal antibodies toward non-glycosylated and glycosylated mouse and rat PAI-1

We measured the binding of 5 PAI-1 inhibiting monoclonal antibodies (i.e. MA-MP2D2, MA-33H1F7, MA-124K1, MA-H4B3 and MA-159M12) toward non-glycosylated *versus* glycosylated PAI-1 immobilized on a solid phase (Figures 3.2 and 3.3 for mouse and rat PAI-1 respectively).

MA-H4B3 was generated toward non-glycosylated mouse PAI-1 but cross-reacts with non-glycosylated rat and human PAI-1 [36, 116]. It has been demonstrated previously that binding of MA-H4B3 to human PAI-1 is hampered by the presence of a glycan chain at position N265 [36, 119]. MA-124K1 and MA-159M12 were originally generated toward non-glycosylated rat PAI-1 [108, 192]. MA-MP2D2 and MA-33H1F7 were generated toward non-glycosylated mouse and human PAI-1 respectively [169].

3.3.2.1 Mouse PAI-1

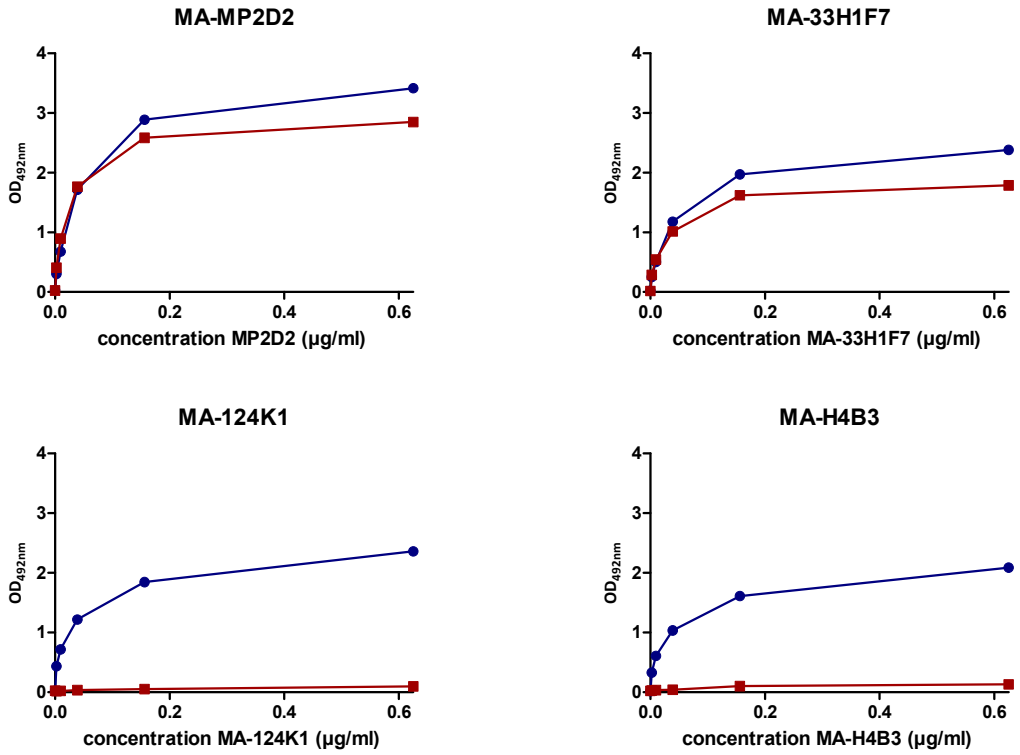

Figure 3.2: Binding of monoclonal antibodies toward non-glycosylated and glycosylated mouse PAI-1

Binding of monoclonal antibodies MA-MP2D2, MA-33H1F7, MA-124K1 and MA-H4B3 toward non-glycosylated mPAI-1 (%, blue) and glycosylated mPAI-1 (■, red).

Under the conditions used, MA-MP2D2 and MA-33H1F7 reacted very similarly with non-glycosylated and glycosylated mouse PAI-1.

For MA-124K1 and MA-H4B3 good binding toward non-glycosylated mouse PAI-1 was detected, whereas virtually no reactivity toward glycosylated mouse PAI-1 was observed (Figure 3.2).

3.3.2.2 Rat PAI-1

As observed for mouse PAI-1, MA-MP2D2 and MA-33H1F7 react very well with both non-glycosylated and glycosylated rat PAI-1 (Figure 3.3).

For MA-124K1 there was some residual binding to glycosylated rat PAI-1, but this binding was less pronounced as for non-glycosylated rat PAI-1. Virtually no binding of MA-H4B3 toward glycosylated rat PAI-1 could be detected.

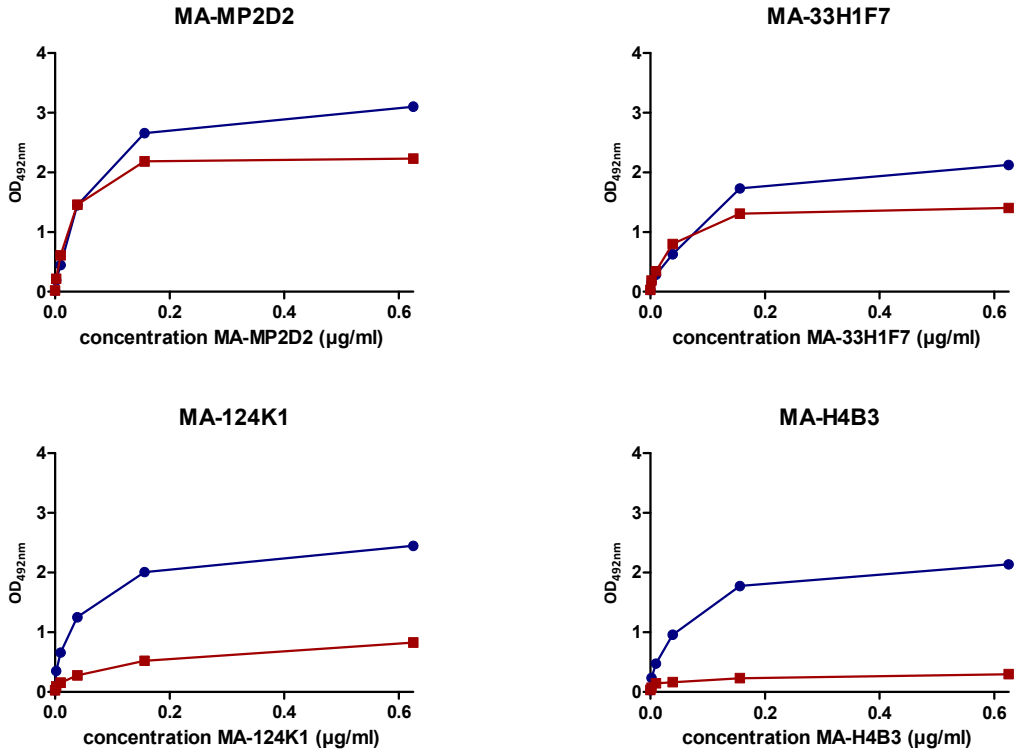

Figure 3.3: Binding of monoclonal antibodies toward non-glycosylated and glycosylated rat PAI-1

Binding of monoclonal antibodies MA-MP2D2, MA-33H1F7, MA-124K1 and MA-H4B3 toward non-glycosylated ratPAI-1 (●, blue) and glycosylated ratPAI-1 (■, red).

3.3.3 Prediction of potential N-glycosylation sites

The NetNGlyc 1.0 prediction server was used to identify Asn-X-Ser/Thr (N-X-S/T) sequons potentially utilized for N-linked glycosylation.

For human PAI-1 this server confirmed the presence of 3 potential N-linked glycosylation sites at asparagines N209, N265 and N329 as was described previously [36]. For mouse PAI-1 the same three glycosylation sequons were predicted. However, for rat PAI-1, an additional N-linked glycosylation site was predicted at position N65 in addition to the three glycosylation sequons at position N209, N265 and N329.

3.3.4 Generation of mouse and rat PAI-1 glycosylation knock-out mutants

To identify potential glycosylation positions attributing to the differential reactivity of monoclonal antibodies toward non-glycosylated *versus* glycosylated mouse and rat PAI-1, glycosylation knock-out mutants were constructed.

The cDNA of all mouse and rat PAI-1 glycosylation knock-out mutants was successfully produced using site-directed mutagenesis. Although western blotting analysis of conditioned medium showed expression of all mutants (results not shown), not all mutants could be purified successfully.

3.3.4.1 Mouse PAI-1

For mouse PAI-1 mutants, combined introduction of mutations N209Q and N265Q resulted in very low PAI-1 concentrations and a low yield of purified PAI-1 (Table 3.1). The total PAI-1 yield that was obtained for mPAI-1-N209Q-N265Q and mPAI-1-N209Q-N265Q-N329Q was 37 and 14 µg respectively. Due to these very low yields, mutants mPAI-1-N209Q-N265Q and mPAI-1-N209Q-N265Q-N329Q could not be characterized thoroughly and were included only in the deglycosylation experiments.

Table 3.1: PAI-1 concentration and total PAI-1 yield for mouse PAI-1 mutants

Mutant	Concentration (mg/ml)	Total PAI-1 yield (µg)
mPAI-1-N209Q-N265Q	0.023	37
mPAI-1-N209Q-N329Q	0.160	216
mPAI-1-N265Q-N329Q	0.338	439
mPAI-1-N209Q-N265Q-N329Q	0.035	14

Mouse PAI-1 mutants were expressed in HEK293T cells and purified on a SP-sepharose™ column.

Double glycosylation knock-out mutants of mouse PAI-1 have the feature of harboring only one residual N-glycosylation site. Therefore a shift in electrophoretic mobility or changes in antibody binding characteristics can be attributed to this residual asparagine residue.

3.3.4.2 Rat PAI-1

Similar as observed for mouse PAI-1, simultaneous introduction of N65Q, N209Q and N265Q resulted in low yields for rat PAI-1 variants (Table 3.2). Therefore ratPAI-1-N65Q-N209Q-N265Q and ratPAI-1-N65Q-N209Q-N265Q-N329Q were only evaluated in the deglycosylation experiments.

Table 3.2: PAI-1 concentration and total PAI-1 yield for rat PAI-1 mutants

Mutant	Concentration (mg/ml)	Total PAI-1 yield (µg)
ratPAI-1-N209Q-N265Q-N329Q	0.059	71
ratPAI-1-N65Q-N209Q-N265Q	0.012	8
ratPAI-1-N65Q-N209Q-N329Q	0.094	94
ratPAI-1-N65Q-N265Q-N329Q	0.084	101
ratPAI-1-N65Q-N209Q-N265Q-N329Q	0.011	15

Rat PAI-1 mutants were expressed in HEK293T cells and purified on a SP-sepharose™ column.

Since rat PAI-1 harbors four potential N-glycosylation sites, triple mutants needed to be constructed to ensure the presence of only one putative N-glycosylation site.

3.3.5 Deglycosylation experiments

Using western blotting analysis, the molecular mass of different mouse and rat PAI-1 variants was compared before and after N-deglycosylation to verify the presence of N-linked sugar chains (Figure 3.4 and figure 3.5 for mouse and rat PAI-1 variants respectively). If N-glycosylation is present, a reduction in molecular mass and hence increased electrophoretic mobility is expected after deglycosylation.

3.3.5.1 Mouse PAI-1

A clear decrease in molecular mass after deglycosylation was detected for glycosylated mouse PAI-1 (Figure 3.4, lanes 1-2) and for glycosylated mPAI-1-N209Q-N329Q (Figure 3.4, lanes 9-10), indicating the presence of an N-linked sugar chain at position N265. For the other double mutants no clear shift in mobility was seen after deglycosylation. These

results suggest that no or very short sugar chains are present at position N209 and N329. For the triple mutant mPAI-1-N209Q-N265Q-N329Q (Figure 3.4, lanes 11-12), the molecular mass before and after deglycosylation was comparable, thereby confirming that no other N-glycosylation sites are utilized in the mouse PAI-1 protein.

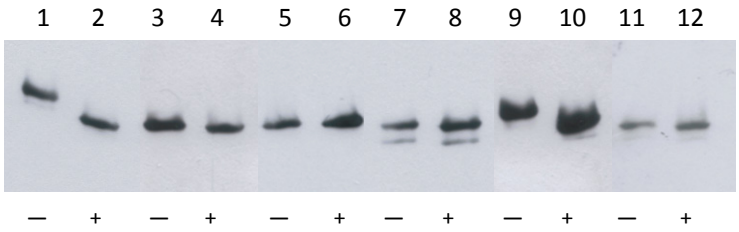

Figure 3.4: N-deglycosylation of mouse PAI-1 glycosylation knock-out mutants

Glycosylated mPAI-1 (lanes 1-2), glycosylated mPAI-1-N209Q-N265Q (lanes 3-4), non-glycosylated mPAI-1 (lanes 5-6), glycosylated mPAI-1-N265Q-N329Q (lanes 7-8), glycosylated mPAI-1-N209Q-N329Q (lanes 9-10) and glycosylated mPAI-1-N209Q-N265Q-N329Q (lanes 11-12) were separated by gel electrophoresis before (—) and after (+) N-deglycosylation with N-glycanase and identified by western blotting using ECL staining.

3.3.5.2 Rat PAI-1

For rat PAI-1 a shift in molecular mass was observed for glycosylated rat PAI-1 (Figure 3.5, lanes 1-2), glycosylated ratPAI-1-N209Q-N265Q-N329Q (Figure 3.5, lanes 7-8) and glycosylated ratPAI-1-N65Q-N209Q-N329Q (Figure 3.5, lanes 9-10). For the other mutants and for non-glycosylated rat PAI-1 produced in *E. coli* no decrease in molecular mass was seen after deglycosylation.

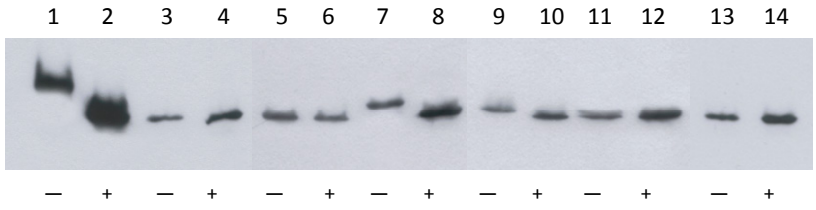

Figure 3.5: N-deglycosylation of rat PAI-1 glycosylation knock-out mutants

Glycosylated ratPAI-1 (lanes 1-2), glycosylated ratPAI-1-N65Q-N209Q-N265Q (lanes 3-4), non-glycosylated ratPAI-1 (lanes 5-6), glycosylated ratPAI-1-N209Q-N265Q-N329Q (lanes 7-8), glycosylated ratPAI-1-N65Q-N209Q-N329Q (lanes 9-10), glycosylated ratPAI-1-N65Q-N265Q-N329Q (lanes 11-12) and glycosylated ratPAI-1-N65Q-N209Q-N265Q-N329Q (lanes 13-14) were separated by gel electrophoresis before (—) and after (+) N-deglycosylation with N-glycanase and identified by western blotting using ECL staining.

From these results it can be concluded that there is N-glycosylation present at positions N65 and N265 in rat PAI-1, whereas the other potential N-glycosylation sequons are not utilized or harbor only very short sugar chains.

The western blotting analysis confirmed the identity of the observed bands since a highly specific anti-PAI-1 antibody (i.e. MA-33H1F7) is used for detection. For mPAI-1-N265Q-N329Q an additional band was detected on western blot (Figure 3.4, lanes 7-8). This fragment probably corresponds to a truncated product of the mutant protein. It is unclear at present why this band appears only for the mPAI-1-N265Q-N329Q mutant and not for any other variants.

3.3.6 Reactivity of monoclonal antibodies toward PAI-1 variants

We compared the binding of monoclonal antibodies to non-glycosylated PAI-1, glycosylated PAI-1 and PAI-1 glycosylation knock-out mutants all immobilized on a solid phase (Figures 3.6 and 3.7 for mouse and rat PAI-1 respectively).

3.3.6.1 Mouse PAI-1

The reactivity of MA-MP2D2 and MA-33H1F7 toward the glycosylation knock-out mutants of mouse PAI-1 is comparable to the binding they display toward non-glycosylated and glycosylated wild-type mouse PAI-1 (Figure 3.6). These data confirm proper folding and binding characteristics for the mutant mouse PAI-1 variants.

As indicated in figure 3.2, MA-124K1 and MA-H4B3 display good binding toward non-glycosylated mouse PAI-1, whereas virtually no reactivity toward glycosylated mouse PAI-1 could be detected. By simultaneous removal of the asparagines at positions N265 and N329 binding of both MA-124K1 and MA-H4B3 could be partially restored, although not to the same extend as toward non-glycosylated mouse PAI-1. For mPAI-1-N209Q-N329Q binding was similar as for glycosylated mouse PAI-1 (Figure 3.6). These findings correspond with the data from the deglycosylation of mouse PAI-1 mutants indicating that the presence of a glycan chain at position N265 interferes with the binding of MA-H4B3 and MA-124K1. Unfortunately, due to the low purification yield of mPAI-1-N209Q-N265Q we cannot rule out an effect of position N329 on antibody binding. However, deglycosylation of mPAI-1-N209Q-N265Q does not result in a major shift in

electrophoretic mobility and therefore the presence of large sugar chains at this position and consequently interference with antibody binding is highly unlikely.

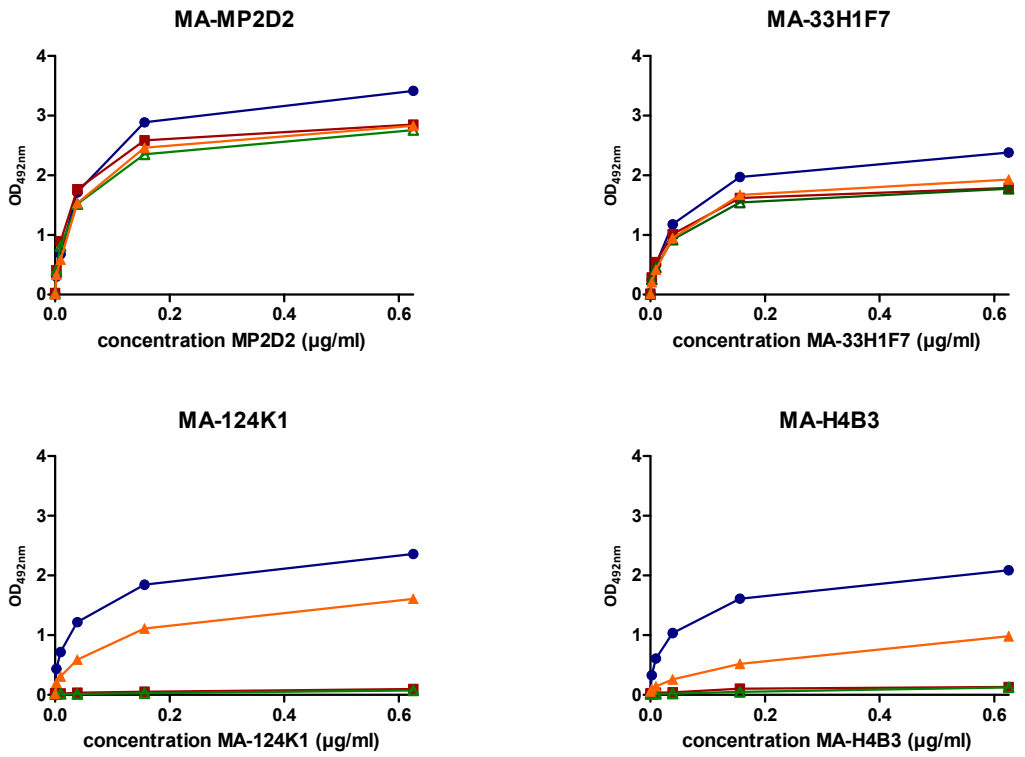

Figure 3.6: Binding of monoclonal antibodies toward mouse PAI-1 wild-type and glycosylation knock-out mutants.

Binding of monoclonal antibodies MA-MP2D2, MA-33H1F7, MA-124K1 and MA-H4B3 toward non-glycosylated mPAI-1 (●, blue), glycosylated mPAI-1 (■, red), mPAI-1-N209Q-N329Q (Δ, green) and mPAI-1-N265Q-N329Q (▲, orange).

3.3.6.2 Rat PAI-1

MA-MP2D2 and MA-33H1F7 display good binding toward all rat PAI-1 glycosylation knock-out mutants, indicating proper reactivity of the mutant variants in ELISA.

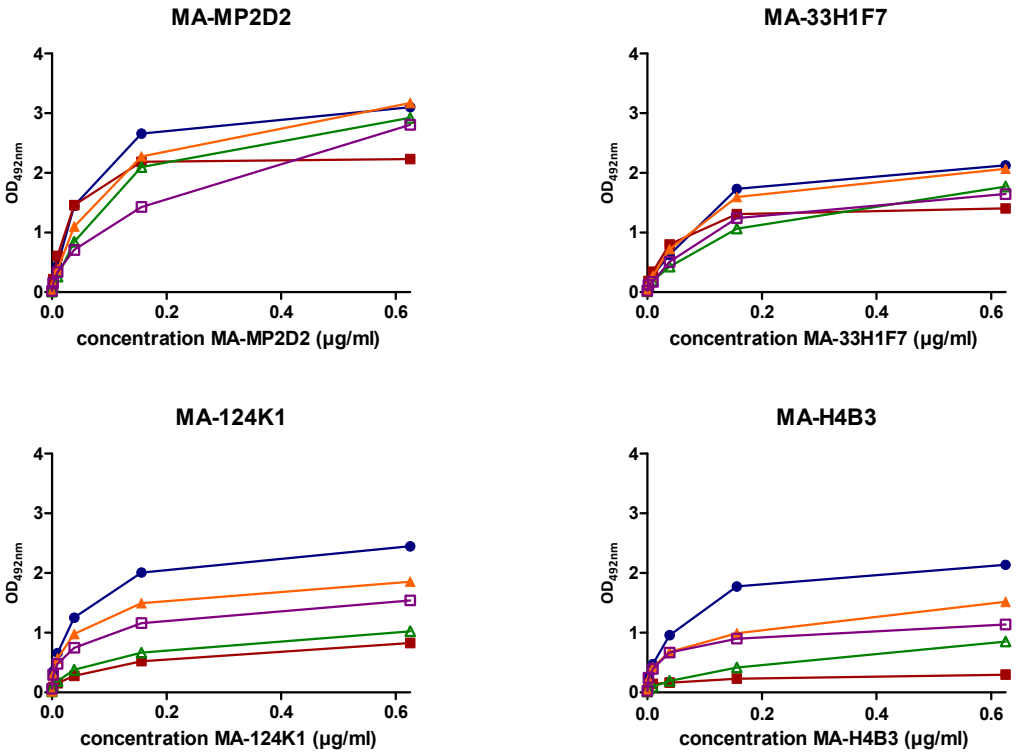

Figure 3.7: Binding of monoclonal antibodies toward rat PAI-1 wild-type and glycosylation knock-out mutants.

Binding of monoclonal antibodies MA-MP2D2, MA-33H1F7, MA-124K1 and MA-H4B3 toward non-glycosylated ratPAI-1 (●, blue), glycosylated ratPAI-1 (■, red), ratPAI-1-N65Q-N209Q-N329Q (Δ, green), ratPAI-1-N65Q-N265Q-N329Q (▲, orange) and ratPAI-1-N209Q-N265Q-N329Q (□, purple).

For MA-124K1 there was residual binding to glycosylated rat PAI-1, but this binding was less pronounced as for non-glycosylated rat PAI-1 (Figure 3.7). Binding of MA-124K1 to ratPAI-1-N65Q-N209Q-N329Q was comparable to the binding of glycosylated ratPAI-1, suggesting that the glycan chain attached to N265 is mainly responsible for the reduced reactivity of MA-124K1 to glycosylated rat PAI-1. Binding of MA-124K1 to ratPAI-1-N209Q-N265Q-N329Q and toward ratPAI-1-N65Q-N265Q-N329Q was higher in comparison to glycosylated ratPAI-1, but since binding could not totally equal binding to

non-glycosylated PAI-1 we cannot conclude contribution of only N265 to antibody interference.

It was demonstrated previously that binding of MA-H4B3 to human PAI-1 is hampered by the presence of a glycan chain at position N265 [36, 119]. For rat PAI-1 we could confirm the very low binding of MA-H4B3 to glycosylated rat PAI-1 and to ratPAI-1-N65Q-N209Q-N329Q. Binding of MA-H4B3 was increased toward glycosylation knock-out mutants ratPAI-1-N209Q-N265Q-N329Q and ratPAI-1-N65Q-N265Q-N329Q in comparison to glycosylated ratPAI-1. As observed for human PAI-1, these data clearly indicate that the presence of N-linked glycan chains hampers the interaction of MA-H4B3 with glycosylated rat PAI-1 (Figure 3.7).

We also evaluated the reactivity of monoclonal antibody MA-159M12 toward the rat PAI-1 variants (Figure 3.8). Reactivity of MA-159M12 toward mouse PAI-1 variants was not examined since the antibody does not react with neither non-glycosylated nor glycosylated mouse PAI-1. Although MA-159M12 only shows reactivity toward non-glycosylated rat PAI-1 and not toward glycosylated rat PAI-1, the binding could not be restored by selective elimination of potential N-glycosylation sites. The differential reactivity of MA-159M12 toward glycosylated and non-glycosylated rat PAI-1 could thus not be explained by the N-glycosylation status of the protein.

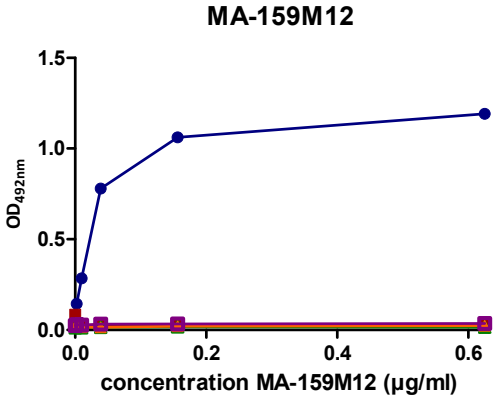

Figure 3.8: Binding of MA-159M12 toward rat PAI-1 wild-type and glycosylation knock-out mutants.

Binding of monoclonal antibody MA-159M12 toward non-glycosylated ratPAI-1 (●, blue), glycosylated ratPAI-1 (■, red), ratPAI-1-N65Q-N209Q-N329Q (Δ, green), ratPAI-1-N65Q-N265Q-N329Q (▲, orange) and ratPAI-1-N209Q-N265Q-N329Q (□, purple).

3.4 Discussion

Recombinant PAI-1 has been purified using several eukaryotic cell lines such as Chinese hamster ovary (CHO) cells, HEK293T cells, insect cells and the yeast *Pichia Pastoris*. Natural PAI-1 could be obtained from HT1080 cells or HUVEC cells [36, 104, 193-195]. Besides expression by eukaryotic cells, non-glycosylated PAI-1 has also been purified from prokaryotic cells like *Escherichia coli* [196]. Most biochemical studies have been performed with non-glycosylated PAI-1 produced in *Escherichia coli*, whereas PAI-1 derived from natural cell lines and PAI-1 expressed in eukaryotic cells is glycosylated [36]. Although glycosylation of proteins can have a major influence on the biochemical properties of a protein, non-glycosylated and glycosylated PAI-1 display very similar biochemical characteristics [37]. However, recent papers reported minor differences between glycosylated and non-glycosylated PAI-1 [197, 198]. Especially the reactivity and inhibitory capacity of monoclonal antibodies toward PAI-1 can be glycosylation dependent [36]. This glycosylation dependent recognition suggests that precautions should be taken when selecting the source of PAI-1 for the evaluation of PAI-1 inhibitors. Mouse and rat models are often used to study the physiological effect of PAI-1 inhibition by monoclonal antibodies or low molecular weight compounds [171, 192, 199]. Disease pathogenesis in these rodents is usually comparable to the disease progression in humans, although small differences in physiological pathways and pathogenesis have been reported [22, 158]. Animal proteins also differ from their human counterparts in amino acid sequence and structure. As a result, data obtained in one particular animal model fail to be reproduced in other animal models or in humans. Comparative evaluation of therapeutic targets from different species is therefore of importance and could explain obtained inter-species differences.

Presence of glycosylation in PAI-1 was shown to affect the reactivity of two PAI-1 inhibiting monoclonal antibodies evaluated in this study i.e. MA-H4B3 and MA-124K1. MA-H4B3 was generated toward non-glycosylated mouse PAI-1 but cross-reacts with non-glycosylated rat and human PAI-1 [36, 116]. This monoclonal antibody inhibits PAI-1 activity by stabilizing the prelatent form of PAI-1 and thereby accelerates the latency transition [119]. MA-H4B3 was already used in certain *in vivo* studies on PAI-1 function but since this antibody has no reactivity toward glycosylated mouse PAI-1, the negative conclusions of these studies are not fully reliable [119, 183]. MA-124K1 was originally

generated toward non-glycosylated rat PAI-1 and inhibits activity of both non-glycosylated rat and mouse PAI-1 [108]. Absence of reactivity of MA-124K1 toward glycosylated mouse PAI-1 might explain the absence of a clear effect of PAI-1 inhibition with this antibody in preliminary *in vivo* studies (unpublished results). An explanation for the differential reactivity of MA-H4B3 and MA-124K1 toward non-glycosylated and glycosylated PAI-1 seems trivial since their epitopes (Tyr^{210}, Glu^{212}, Tyr^{241}, Glu^{242}, Arg^{271}, Glu^{220} and Glu^{212}, Glu^{220} for MA-H4B3 and MA-124K1 respectively [108, 119]) are located only about 10 Å from glycosylation site N265. Therefore the glycans present at this position are likely to hinder the access of the monoclonal antibodies to their respective epitopes.

It is not clear at present why the reactivity toward glycosylation knock-out mutants could not equal the reactivity of both antibodies toward non-glycosylated PAI-1, but the most readily explanation is that expression in eukaryotic cells results in small conformational differences in comparison to the expression in *E. coli* that might affect antibody binding. In addition, the presence of much smaller O-linked glycan chains can still interfere with the epitopes of MA-H4B3 and MA-124K1 on rat and mouse PAI-1 and explain the differential reactivity between glycosylation knock-out mutants produced in eukaryotic cells and *E. coli* PAI-1.

MA-159M12 accelerates the active to latent conversion of rat PAI-1 and its epitope is composed of residues Pro^2, Lys^3, Pro^4 and Glu^5 located in α-helix A in rat PAI-1 [184]. For MA-159M12, lack of reactivity toward glycosylated rat PAI-1 could not be restored by removal of N-glycosylation sites, so lack of reactivity toward glycosylated rat PAI-1 cannot be attributed to the presence of glycan chains. Another possible explanation is the N-terminal difference in amino acid sequence of both proteins. For non-glycosylated rat PAI-1 the first amino acid (+1) in the mature protein sequence was mutated from a serine to an alanine residue during cloning in the expression vector [200], whereas for the glycosylated protein the original serine residue is still present at position +1. Substitution from the hydrophilic serine residue to the hydrophobic alanine residue might result in subtle conformational changes leading to a distortion in the MA-159M12 epitope region.

PAI-1 has 3 potential N-linked glycosylation sites at asparagines N209, N265 and N329 which have been conserved in all species investigated so far (human, mouse, rat, pig, rabbit, cow and mink). It was demonstrated that human PAI-1 displays a heterogeneous

glycosylation pattern of asparagines N209 and N265, while N329 is not utilized [36]. Analysis of the glycosylation pattern of natural human PAI-1 derived from different cell sources confirmed the actual use of only two N-linked glycosylation sites, but also showed a heterogeneous, tissue-specific glycosylation pattern [188].

While the glycosylation status of human PAI-1 has been characterized thoroughly, to date no information is available about the glycosylation pattern of mouse and rat PAI-1. Three potential N-glycosylation sites were predicted for mouse PAI-1 (i.e. N209, N265 and N329) whereas for rat PAI-1 an additional N-glycosylation position was predicted at asparagine N65. To verify which potential N-glycosylation sites are indeed utilized after expression of PAI-1 in HEK293T cells, glycosylation knock-out mutants were deglycosylated and electrophoretic mobility was compared before and after deglycosylation. Since the glycosylation knock-out mutants harbor only one residual glycosylation site, differences in molecular mass after deglycosylation can be attributed to one particular asparagine residue. An asparagine to glutamine mutation was chosen to eliminate the N-glycosylation site since this is a very common method to study the effect of glycans and results in a very modest amino acid substitution effect [201-203]. For mouse PAI-1, combined removal of asparagines N209 and N265 resulted in very low yields. The reason for this low yield is not clear but might result from the fact that the introduced mutations lead to conformational changes thereby hampering a correct folding of the PAI-1 protein. The same low yield was seen for rat PAI-1 mutants that combined mutations N65Q, N209Q and N265Q, but again no straightforward explanation can be given. Electrophoretic mobility of the mouse PAI-1 mutants before and after deglycosylation indicated presence of glycan chains at position N265, whereas N209 and N329 do not seem to be glycosylated. For rat PAI-1, the additional N-glycosylation site at asparagine N65 is indeed glycosylated as is asparagine N265. Positions N209 and N329 do not seem to be utilized for N-glycosylation in the rat PAI-1 protein. We cannot ensure that the glycosylation profile obtained for mouse and rat PAI-1 expressed in HEK293T cells is identical to the glycosylation profile of the native PAI-1 proteins, but it was demonstrated previously that human PAI-1 expressed in HEK293T cells has the same glycosylation profile as human PAI-1 produced by dexamethasone treated HT-1080 cells. Moreover glycosylation is a very heterogeneous process, that is highly cell and tissue specific [186, 188]. However, these data clearly indicate that glycosylation might differ between species, even when expressed in the same eukaryotic

cell type. Therefore it is important to evaluate the reactivity of putative therapeutic targets from different species.

In conclusion, comparative evaluation of the reactivity of PAI-1 inhibiting monoclonal antibodies toward rat and mouse PAI-1 wild-type and glycosylation knock-out mutants revealed a clear glycosylation dependent reactivity profile. These data stress the importance of selecting the proper source of PAI-1 when evaluating PAI-1 inhibitors *in vitro*. Moreover, extrapolation of data between species should be done with care, since reactivity of inhibitors toward putative therapeutic targets is highly species dependent.

Chapter 4

Comparison of the functional properties of stabilized glycosylated *versus* non-glycosylated human PAI-1

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Manuscript in preparation

4.1 Introduction

Plasminogen activator inhibitor-1 (PAI-1) inhibits the activity of both tissue-type and urokinase-type plasminogen activator (t-PA & u-PA) and therefore is an important regulator of the plasminogen activator system [9]. Because of the suggested role in diverse physiological pathways PAI-1 has been investigated intensively as a potential therapeutic target [20, 161]. PAI-1 is a member of the serpin (serine protease inhibitor) superfamily of proteins. The tertiary structure of active PAI-1 shares motifs common to most serpins, like the solvent exposed reactive center loop (RCL), designated P_{16} to $P_{10'}$. The RCL provides a 'bait' peptide bond (P_1-$P_{1'}$) that mimics the normal substrate of the target protease. In PAI-1, the Arg^{346}-Met^{347} bond has been identified as the P_1-$P_{1'}$ bond [42]. In its active conformation the P_1-$P_{1'}$ bond is cleaved upon reaction with the target protease, followed by insertion of the amino terminal part of the RCL into β-sheet A, hereby relocating the protease to the opposite pole of PAI-1. This interaction between PAI-1 and the protease results in the formation of a 1:1 SDS-stable covalent complex, thereby distorting the active site of the protease [39, 42]. If RCL insertion during cleavage is hampered in one way or another, PAI-1 is more likely to be released from the protease, thus acting as a substrate and not as an inhibitor [41]. Under physiological conditions active PAI-1 is rapidly converted to the latent conformation with an apparent half life of 1-2 hours at 37°C [204]. Upon latency transition, the amino terminal part of the reactive center loop is inserted into β-sheet A, forming the new β-strand s4A and the carboxy terminal portion stretches across the surface of the protein [43]. Low pH (pH=5.5), high salt concentration (1 M NaCl) and low temperature (4°C) significantly reduce the rate of latency transition [47]. In addition, stabilized PAI-1 mutants have been constructed, ultimately resulting in a PAI-1 variant with a half-live of about 145 h (= humPAI-1 stab) [55, 56, 205]. However, all these stabilized mutants were expressed in *Escherichia coli* (*E. coli*) and therefore resulted in non-glycosylated proteins. PAI-1 is expected to circulate both in a glycosylated and non-glycosylated state *in vivo*, depending on the cell type that expresses the protein [188]. Since inhibition of PAI-1 activity by monoclonal antibodies is often glycosylation dependent [36, 119], it is important to evaluate the inhibitory potential of new PAI-1 inhibitors not only toward non-glycosylated but also toward glycosylated PAI-1.

Glycosylated PAI-1 can be produced in eukaryotic cells, cultured at 37°C. Due to the thermal instability of human PAI-1 wild-type at 37°C, virtually all PAI-1 wild-type is converted to the latent conformation prior to purification. Glycosylated PAI-1 can be reactivated *in vitro* using denaturing agents, but the efficiency of these reactivation procedures remains rather low. In the current study we investigated whether stabilizing mutations previously reported for non-glycosylated human PAI-1 (i.e. N150H, K154T, Q301P, Q319L and M354I) [205] could also stabilize glycosylated human PAI-1, thereby circumventing the need for reactivation of glycosylated PAI-1 *in vitro*.

4.2 Materials and methods

4.2.1 Materials

Human t-PA (Actilyse®) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Polystyrene microtiter plates were obtained from Costar (Cambridge, Massachusetts, USA). The pcDNA3.1(+) vector was purchased from Invitrogen (Merelbeke, Belgium). SDS-PAGE of proteins was performed with the PhastSystem™ using PhastGel™ gradient 10-15% gels and analyzed by densitometric scanning with Imagemaster™ software (GE healthcare, Uppsala, Sweden). Human embryonic kidney 293T (HEK293T) cells were obtained from ATTC-LGC standards (Teddington, Middlesex, UK). Lipofectamin 2000 Reagent for transfection was obtained from Invitrogen (Merelbeke, Belgium). Oligonucleotides for mutagenesis and sequencing were from Sigma-Aldrich (St. Louis, Missouri, USA). dNTP polymerization mix was obtained from Stratagene (La Jolla, California, USA). *Pfx50*™ DNA polymerase was purchased from Invitrogen. Plasmid DNA purifications were performed using the Nucleobond® PC 500 kit (Macherey-Nagel, Düren, Germany). DNA was sequenced by LGC genomics (Berlin, Germany). Horseradish peroxidase-conjugated goat anti-mouse IgG antibody (GAM-HRP) was obtained from Bio-Rad (Hercules, California, USA). ECL Plus Western Blotting Detection Reagent was purchased from GE healthcare (Uppsala, Sweden). The chromogenic plasmin substrate S2403 was obtained from Nodia/chromogenix (Antwerp, Belgium). Heparin sepharose™ was purchased from GE healthcare Bio-Sciences (Uppsala, Sweden). Regression and statistical analyses were obtained with GraphPad Prism 5.03 software (GraphPad Software Inc, San Diego, California, USA). All analytical reagents used were of analytical grade.

4.2.2 Construction of glycosylated human PAI-1 stab cDNA

Five mutations (N150H, K154T, Q301P, Q319L and M354I) that are known to stabilize the structure of non-glycosylated human PAI-1 72-fold [56] were introduced successively by site-directed mutagenesis. Site-directed mutagenesis was carried out according to the QuickChange™ XL site-directed mutagenesis protocol from Stratagene. Mutants were constructed using the appropriate primers and the pcDNA3.1(+) vector containing the cDNA fragment encoding human PAI-1 (pcDNA3.1(+)-humPAI-1) as a template.

Thermocycling was conducted in an Eppendorf Mastercycler™ gradient thermal cycler (Hamburg, Germany) and included an initial denaturation step of 4 min at 94°C followed by 24 cycles of denaturation (1 min at 94°C), annealing (1 min at an appropriate temperature) and elongation (14 min at 72°C). The reaction was ended with a final elongation step of 10 min at 72°C. Following successful amplification, 1 µl of *Dpn*I (20 U/µl) was added to digest (1 hour at 37°C) the methylated parental template DNA. Subsequently the PCR product was transformed into TOP10F' *E. coli* cells (Invitrogen), followed by overnight selection on agar plates containing ampicilline (100 µg/ml). For large-scale DNA purification, a single colony was grown overnight in 150 ml LB (Luria Broth Base, Invitrogen) containing ampicilline (100 µg/ml) and the DNA was isolated using the Nucleobond® PC 500 kit. The concentration and purity was determined spectrophotometrically. The presence of the introduced mutations as well as the absence of any unwanted mutations was verified by nucleotide sequencing.

4.2.3 Expression and purification of glycosylated human PAI-1 stab

pcDNA3.1(+)-humPAI-1-N150H-K154T-Q301P-Q319L-M354I plasmid DNA was transiently transfected in HEK293T cells using the lipofectamin 2000 protocol (Invitrogen). Expression of glycosylated human PAI-1-N150H-K154T-Q301P-Q319L-M354I protein (= glycosylated humPAI-1 stab) was first verified by western blotting analysis of the cell medium using MA-31C9 [206, 207], reacting with human PAI-1, as primary antibody and GAM-HRP as secondary antibody. Detection of bound antibodies was performed with the ECL Plus Western Blotting Detection kit. Antigen levels of glycosylated human PAI-1 stab (24, 48, 72 and 96 hours post transfection) were analyzed by an in-house developed ELISA assay (i.e. MA-31C9/MA-56A7C10-HRP). Activity levels in the medium samples (24, 48, 72 and 96 hours post transfection) were analyzed using the plasminogen coupled chromogenic assay (cfr. 4.2.4). Cell medium was harvested after 48 hours and the glycosylated humPAI-1 stab protein was purified on a heparin sepharose™ column. Briefly, cell medium was dialyzed against 0.15 M phosphate buffer pH=5.2 and loaded on a heparin sepharose™ column using the same buffer as running buffer. The column was washed with 0.15 M phosphate buffer pH=5.2 and elution of PAI-1 protein was performed with 0.15 M phosphate buffer pH=5.2 and a gradient of NaCl from 0 to 1.3 M. Fractions containing glycosylated humPAI-1 stab were identified by SDS-PAGE analysis

and dialyzed against 0.15 M phosphate buffer pH=6 containing 0.7 M NaCl to obtain the same buffer conditions as for non-glycosylated human PAI-1 wild-type (= humPAI-1 wt) and non-glycosylated humPAI-1 stab.

Glycosylated humPAI-1 wt was purified using the same protocol as for glycosylated humPAI-1 stab. Non-glycosylated humPAI-1 wt and non-glycosylated humPAI-1 stab were produced and purified in a similar way as described previously [109].

4.2.4 Determination of the specific inhibitory activity against t-PA

PAI-1 activity toward t-PA was determined using a plasminogen coupled chromogenic assay as described previously [175]. Briefly, 50 µl of PAI-1 sample was incubated in a microtiter plate with 50 µl of t-PA (20 U/ml) for 15 min at 37°C. Afterwards 100 µl of a solution containing plasminogen (1 µM), CNBr-digested fibrinogen (1 µM) and S2403 (0.6 mM) was added to the wells of the microtiter plate. Residual active t-PA will convert plasminogen into plasmin that will subsequently hydrolyze the chromogenic substrate S2403. Plasminogen activator activity can be calculated based on the comparison of the absorbance at 405 nm between samples to be analyzed and a standard curve constructed with known concentrations of t-PA. T-PA was calibrated using the international reference preparation for t-PA (NIBSC 86/670). All PAI-1 activity data are expressed as a percentage of the theoretical maximum activity i.e. 745,000 U/mg (t-PA inhibitory units) calculated on the basis of a specific activity for t-PA of 500 000 U/mg and molecular masses of 67 kDa and 45 kDa for t-PA and PAI-1 respectively.

4.2.5 Functional distribution assay

PAI-1 samples were diluted to a final concentration of maximum 400 µg/ml in PBS pH=7.4 (140 mM NaCl, 8 mM $Na_2HPO_4.2H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4) and incubated for 25 min at 37°C with a 2-fold molar excess of t-PA. The reaction was terminated by adding SDS (final concentration of 1 %) and heating for 30s at 100°C. Reaction products were analyzed by SDS-PAGE (non-reducing conditions) using 10-15% gradient gels with the PhastSystem™. Proteins were visualized by staining with Coomassie Brilliant Blue and quantified by densitometric scanning with the Imagemaster™ software. Incubation of PAI-1 with t-PA results in the formation of a stable covalent complex, corresponding to the fraction active PAI-1, small amounts of

cleaved PAI-1, originating from the substrate pathway of PAI-1, and residual non-reactive PAI-1, originating from latent PAI-1.

4.2.6 Functional stability of PAI-1 variants

PAI-1 samples were dialyzed against PBS pH=7.4 to obtain buffer conditions and pH near to physiological conditions. After dialysis, samples were incubated at 37°C and aliquots were collected after various time points. To calculate the half-life of active PAI-1 variants, residual PAI-1 activity in the aliquots was measured using the plasminogen coupled chromogenic assay and the functional distribution assay. The amount of residual active PAI-1 for each time point was expressed as percentage of the initial amount of active PAI-1. The decay in PAI-1 activity was used for the calculation of half-life using one phase decay or linear regression curves (GraphPad Prism 5.03 software).

4.2.7 Statistical analysis

Data are expressed as mean ± SD. Data were compared using an unpaired student's t-test. Two-tailed P values < 0.05 were considered statistically significant.

4.3 Results

4.3.1 Expression and purification of glycosylated human PAI-1 stab

The pcDNA3.1(+)-humPAI-1-N150H-K154T-Q301P-Q319L-M354I plasmid was constructed successfully using site-directed mutagenesis. Glycosylated humPAI-1 stab could be expressed in HEK293T cells as was verified by western blotting analysis of cell medium. PAI-1 antigen and activity levels were verified 24, 48, 72 and 96 hours post transfection. The activity/antigen ratio gradually decreased in function of time after transfection (Figure 4.1), even though the antigen levels kept on increasing with time. In order to obtain purified glycosylated humPAI-1 stab with a maximal percentage activity, medium needs to be harvested 24 or 48 hours post transfection. Because antigen levels after 24 hours were rather low and therefore complicated purification, medium of HEK293T cells was harvested 48 hours after the transient transfection of HEK293T cells. Purification on heparin sepharose™ yielded an average of 21 ± 11 µg/ml glycosylated humPAI-1 stab protein per ml of conditioned medium.

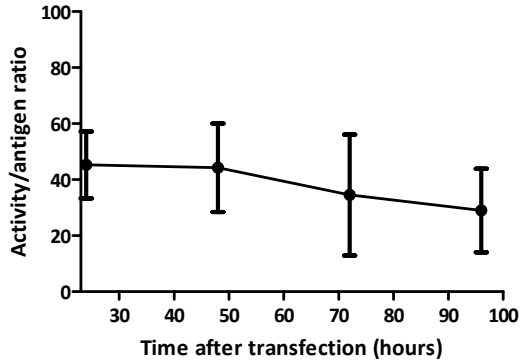

Figure 4.1: Activity/antigen ratio of glycosylated humPAI-1 stab post transfection

Cell medium was collected 24, 48, 72 and 96 hours after transfection of HEK293T cells with glycosylated humPAI-1 stab cDNA. PAI-1 antigen and activity levels were determined using an ELISA and plasminogen coupled chromogenic assay respectively. Results are expressed as activity/antigen ratio (mean ± SD; n = 4).

4.3.2 Specific inhibitory activity against t-PA

PAI-1 activity toward t-PA was determined using the plasminogen coupled chromogenic assay. The specific activity of purified glycosylated humPAI-1 stab was 168,000 ± 9,800 U/mg, corresponding to 23 ± 1.3 % of the theoretical maximum value (i.e. 745,000 U/mg). In contrast, for glycosylated humPAI-1 wt virtually no activity could be detected after purification (i.e. 4,900 ± 0,180 U/mg or 0.66 ± 0.024 % of the theoretical maximum activity).

In comparison, the t-PA inhibitory activity of non-glycosylated humPAI-1 stab and non-glycosylated humPAI-1 wt, expressed in *E. coli,* was 277,000 ± 64,000 and 270,000 ± 57,000 U/mg, corresponding to 37 ± 8.6 % and 36 ± 7.7 % of the theoretical maximum activity respectively.

4.3.3 Functional distribution of PAI-1 variants

Quantitative evaluation of the reaction products formed during the incubation of PAI-1 variants with a 2-fold molar excess of t-PA are shown in table 4.1.

Under these conditions, glycosylated humPAI-1 stab resulted in the formation of a stable covalent complex (48 ± 4.1%), corresponding to the fraction active PAI-1, small amounts of cleaved PAI-1 (17 ± 4.2 %), originating from the substrate pathway of PAI-1, and residual non-reactive PAI-1, originating from latent PAI-1 (35 ± 1.4 %).

Table 4.1: Functional distribution of PAI-1 variants

	% Complexed	% Non-reactive	% Cleaved
Glycosylated humPAI-1 stab	**48 ± 4.1**	**35 ± 1.4**	**17 ± 4.2**
Glycosylated humPAI-1 wt	< 1	79 ± 4.9	21 ± 5.0
Non-glycosylated humPAI-1 stab	57 ± 1.4	11 ± 3.5	32 ± 2.4*
Non-glycosylated humPAI-1 wt	47 ± 3.8	40 ± 1.3	13 ± 2.5

Results are expressed as percentage of PAI-1 protein (mean ± SD; n ≥ 3)

**P < 0.05 in comparison to non-glycosylated humPAI-1 wt, glycosylated humPAI-1 stab and glycosylated humPAI-1 wt (unpaired t-test)*

Non-glycosylated humPAI-1 stab displayed a significantly increased substrate behavior in comparison to non-glycosylated humPAI-1 wt [205], glycosylated humPAI-1 stab and glycosylated humPAI-1 wt. In line with its full latency, no complex formation was detected when glycosylated humPAI-1 wt was incubated with t-PA. When active PAI-1 was incubated at 37°C, the decrease in activity was for all variants associated with a concomitant increase in latent conformation (Figure 4.2).

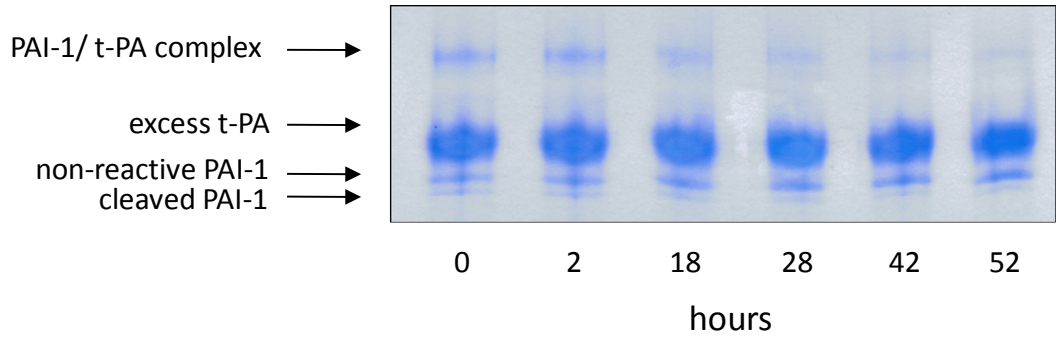

Figure 4.2: Functional distribution of glycosylated humPAI-1 stab upon incubation at 37°C

Glycosylated humPAI-1 stab was dialyzed against PBS and incubated at 37°C. Aliquots were collected at certain time points and incubated with a 2-fold molar excess of t-PA for 25 min at 37°C. Reaction products were analyzed by SDS-PAGE analysis, coomassie staining and densitometric scanning.

4.3.4 Functional stability of PAI-1 variants

Two different methods were used to determine the functional stability of the active PAI-1 variants. In both methods, PAI-1 samples were incubated at 37°C and aliquots were collected at various time points. The percentage of active PAI-1 present after different time intervals at 37°C was calculated either by the functional distribution assay or by the plasminogen coupled chromogenic assay. The residual PAI-1 activity for each aliquot was expressed as percentage of the initial amount of active PAI-1 and the decay in PAI-1 activity was used to calculate the half-live (Figure 4.3). The half-lives calculated for all active PAI-1 variants are reported in table 4.2.

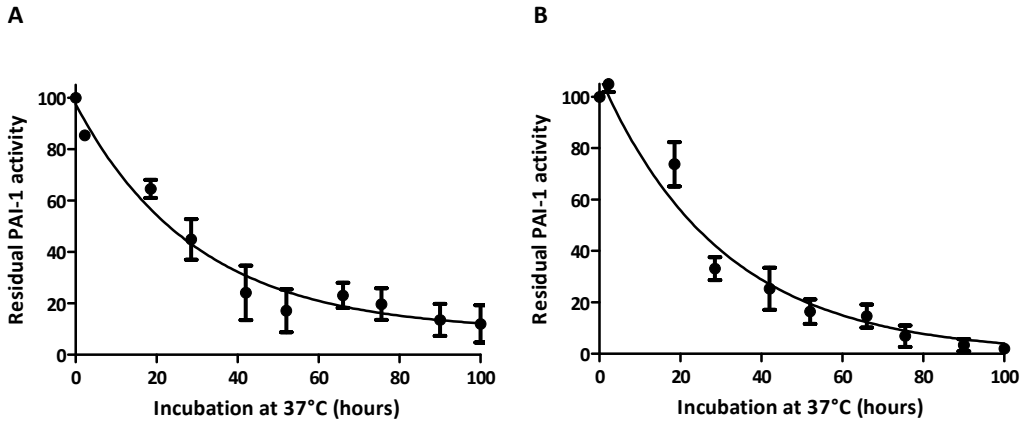

Figure 4.3: Decay of glycosylated humPAI-1 stab activity upon incubation at 37°C

PAI-1 samples were incubated at 37°C and aliquots were collected after various time points. Residual PAI-1 activity was determined in the functional distribution assay (A) or the plasminogen coupled chromogenic assay (B). Half-life was calculated using one phase decay regression analysis (mean ± SD; n = 4)

Glycosylated humPAI-1 stab exhibited a half-life of 21-28 hours (Table 4.2). This half-life is significantly shorter than the half-life of non-glycosylated humPAI-1 stab (i.e. 185-195 hours) but significantly increased in comparison to the half-life observed for non-glycosylated humPAI-1 wt (i.e. 1.2-1.8 hours). The half-lives observed for non-glycosylated humPAI-1 stab and non-glycosylated humPAI-1 wt are in the same range as values reported previously [55, 56].

Table 4.2: Functional stability of PAI-1 variants

	Half-life (Based on the functional distribution assay)	**Half-life (Based on the plasminogen coupled chromogenic assay)**
Glycosylated humPAI-1 stab	**28 ± 6.8***	**21 ± 5.3***
Glycosylated humPAI-1 wt	NA	NA
Non-glycosylated humPAI-1 stab	185 ± 22	195 ± 62
Non-glycosylated humPAI-1 wt	1.8 ± 0.48	1.2 ± 0.20

Results are expressed in hours (mean ± SD; n ≥ 3)
**P < 0.001 in comparison to non-glycosylated humPAI-1 wt and non-glycosylated humPAI-1 stab*
NA: not applicable

4.4 Discussion

Relative to other serpins, PAI-1 is the least stable in its active conformation. At 37°C it converts very rapidly to the latent conformation with a half-live of about 1-2 hours. Berkenpas *et al*. (1995) identified a set of mutations that had significant stabilizing effects on the PAI-1 stability. The most stable variant they identified turned out to be a quadruple mutant (N150H, K154T, Q319L, M354I) with a half-life of approximately 145 hours at 37°C, corresponding to a 72-fold stabilization in comparison to human PAI-1 wild-type [55]. A few years later Vleugels *et al*. described a variant of this quadruple mutant harboring a fifth mutation Q301P (= PAI-1 stab), which had very similar properties [56]. The first crystal structures of non-glycosylated human PAI-1 stab [42, 45] explained the contribution of mutations N150H, K154T and Q319L to the PAI-1 stability enhancement. These mutations are located in the region of the PAI-1 protein expected to change position during the transition of intact to latent or cleaved PAI-1 (Figure 4.4). When the lysine (K) residue at position 154 is mutated to a threonine (T) residue, the T154 side chain becomes partially buried, resulting in the formation of a 3_{10}-helix [42]. This helical structure leads to the formation of a network of hydrogen bonds that interconnect the top face of β-sheet A to the helix F-loop moiety. The N150H and Q319L mutations presumably also favor the formation of the 3_{10}-helix and resulting hydrogen bonds through avoidance of steric conflict and through burial of hydrophobic surface respectively [45]. It was hypothesized by Sharp *et al.* (1999) that these hydrogen bonds in the 3_{10}-helix conformation need to be broken up upon insertion of the RCL into β-sheet A, thereby explaining the higher stability of the PAI-1 stab mutant. Dewilde *et al.* hypothesized that the stabilizing effect of the fourth mutation M354I is due to the substitution of the methionine to a more hydrophobic residue, isoleucine. This gain in hydrophobicity is important since in the active structure this side chain points toward a hydrophobic pocket (residues L224, I237, A239, L273, L275, F278 and I352). Due to the stronger interaction of I354 with this pocket, this mutation will anchor the C-terminal part of the RCL to the top of the molecule, thereby stabilizing PAI-1 [57]. In the current study we set out to produce a glycosylated variant of the previously reported human PAI-1 stab protein [56] and compared its functional properties with the properties of non-glycosylated human PAI-1 stab.

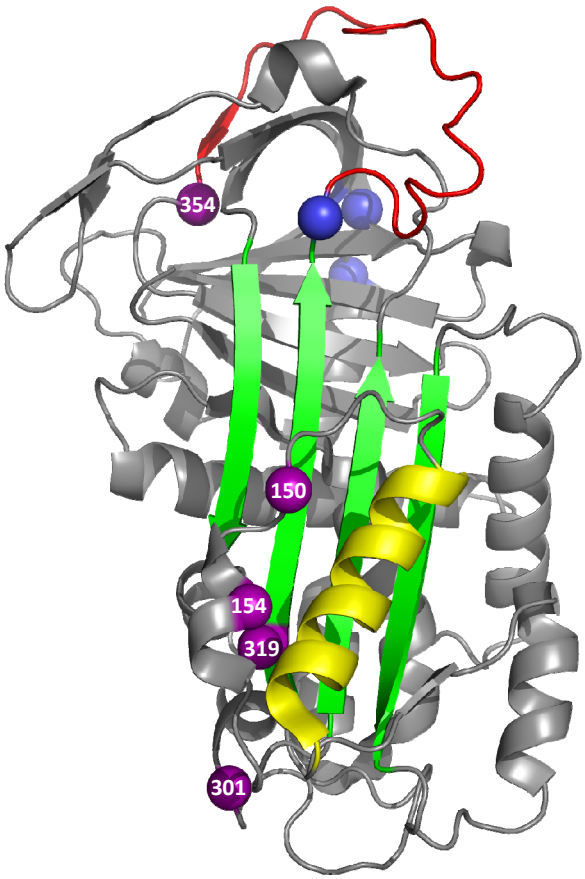

Figure 4.4: structure of non-glycosylated human PAI-1 stab in the active conformation

β-sheet A is shown in green, α-helix F is shown in yellow and the reactive center loop is shown in red. The five mutations, N150H, K154T, Q301P, Q319L and M354I, stabilizing the structure of PAI-1 are represented by pink spheres. The three potential N-glycosylation sites in human PAI-1 are represented by blue spheres [36] (Based on Nar et al. (2000) [42]).

Glycosylated human PAI-1 stab could be successfully expressed in HEK293T cells, revealing an optimum harvest time 48 hours post transfection. The t-PA inhibitory activity of purified glycosylated human PAI-1 stab (168,000 ± 9,800 U/mg, corresponding to 23 ± 1.3 % of the theoretical maximum value), measured in the plasminogen coupled chromogenic assay, was about 1.6-fold lower than the activity of non-glycosylated human PAI-1 stab (=277,000 ± 64,000 U/mg or 37 ± 8.6% of the theoretical maximum activity). The somewhat lower inhibitory activity for the glycosylated protein could be expected due to the 48 hour incubation of the PAI-1 producing HEK293T cells at 37°C prior to harvesting the medium. In contrast, during expression of non-glycosylated

human PAI-1 stab by *E.coli* cells, cells are incubated for 3 hours at 42°C before purification.

Using the functional distribution assay an initial activity of 48 ± 4.1 % could be determined, an only 1.2-fold reduction in comparison to the activity of non-glycosylated human PAI-1 stab (57 ± 1.4 %). It is not entirely clear why the different assays yield different PAI-1 activities but this discrepancy was already observed previously for other PAI-1 variants (unpublished results) and therefore these differences in activity are most probably just a result of the different experimental set-up of the assays.

The half-life of glycosylated human PAI-1 stab was determined using a plasminogen coupled chromogenic assay and a functional distribution assay yielding values of 21 ± 5.3 and 28 ± 6.8 hours respectively. The half-lives of non-glycosylated human PAI-1 wt and non-glycosylated human PAI-1 stab were calculated using the same assays and corresponded to values reported previously for both proteins [55, 205]. Although the stability of glycosylated human PAI-1 stab was significantly increased in comparison to the stability of non-glycosylated human PAI-1 wt, this increased stability was not as pronounced as for non-glycosylated human PAI-1 stab. It is not clear at present why introduction of the same mutations does not result in a similar stabilizing effect in glycosylated *versus* non-glycosylated human PAI-1. The presence of glycan chains seems to interfere with the stabilizing effect of one or more of the five mutations on the PAI-1 structure. Mutations N150H, K154T, Q301P and Q319L are located in the region around α-helix F and are thus not located in proximity of the potential N-glycosylation sites in human PAI-1 (Figure 4.4). Direct interference of glycan chains with the stabilizing interactions of the mutations is therefore unlikely. It is more reasonable that presence of one or more glycan chains results in small conformational changes in the PAI-1 structure. Due to these subtle structural differences some mutations might not be able to exert the same stabilizing interactions as in the non-glycosylated structure, resulting in a reduced stability in comparison to the non-glycosylated protein. Only for mutation M354I that lies in closer proximity to potential N-glycosylation sites (Figure 4.4) both direct interference of glycans with stabilizing interactions or indirect conformational changes might be an explanation for the reduced stability in comparison to non-glycosylated PAI-1. It has been shown previously that small differences in the crystal structure of PAI-1 might indeed result in larger biochemical differences such as changes in the calculated half-live. Indeed, the introduction of the same five stabilizing mutations in non-

glycosylated mouse PAI-1 did not have the same effect as for human PAI-1. The mouse PAI-1 stab variant displayed an only 2-fold increased stability in comparison to wild-type mouse PAI-1, whereas for human PAI-1 introduction of the same 5 mutations resulted in a 72-fold increased half-life [179].

In conclusion, we expressed successfully a stabilized form of glycosylated human PAI-1 that displays measurable activity after purification i.e. 34-fold higher activity than glycosylated human PAI-1 wt. Strikingly the half-life of glycosylated human PAI-1 stab was about 8-fold lower than that of the non-glycosylated stabilized variant. Nonetheless the increased stability in comparison to human PAI-1 wt will facilitate further investigation of the glycosylated human PAI-1 structure and function and provides the additional benefit that active glycosylated PAI-1 is readily available for evaluation of PAI-1 inhibitory compounds that should react with glycosylated PAI-1 *in vivo.*

Chapter 5

General discussion

Hemostasis is a very important physiological process, responsible for maintaining the integrity of our circulatory system after vascular damage. Vessel wall injury and extravasation of blood rapidly initiate a series of events that help to seal off the disrupted vessel wall. Circulating platelets are recruited to the site of injury and the blood coagulation cascade, initiated by tissue factor, culminates in the generation of thrombin and fibrin. Under normal conditions, regulatory mechanisms constrain thrombus formation both temporally and spatially. However, when pathologic processes overwhelm the regulatory mechanisms of hemostasis, excessive quantities of thrombin form, initiating thrombosis [208].

Resolution of redundant blood clots in circulation results mainly from the enzymatic action of plasmin, the central enzyme of the fibrinolytic cascade. Strict control of the fibrinolytic system is important since excessive fibrinolysis will result in a bleeding tendency, whereas impairment of fibrinolysis is associated with thrombosis [209].

To situate this research in a larger context it is important to keep in mind the current strategies for thrombolytic therapy and their major drawbacks. Current thrombolytic drugs used for the treatment of acute myocardial infarction or ischemic stroke are based on the dissolution of blood clots by infusion of plasminogen activators.

The first generation of thrombolytic drugs (e.g. streptokinase) consisted of systemic activators of plasminogen, thus activating both fibrin-bound and circulating plasminogen. Streptokinase is a bacterial protein, originally isolated from beta-hemolytic *Streptococcus* species. It is the least expensive fibrinolytic compound and is administered by short-term infusions. This enzyme however is antigenic and leads to the development of anti-streptococcal antibodies, which precludes readministration. The drawbacks observed for streptokinase led to the development of the second generation of thrombolytic drugs, including recombinant t-PA (alteplase). The development of t-PA was encouraged by the theory that since t-PA has to bind to fibrin to express significant activity, administered t-PA would be targeted to the clot and systemic plasmin generation would be limited. Following on t-PA, the so-called third generation of thrombolytics was engineered. The main goal was to improve their properties in terms of longer plasma half-life, fibrin specificity or resistance to natural inhibitors such as PAI-1. This third generation of thrombolytic drugs includes reteplase (recombinant non-glycosylated t-PA with decreased plasma clearance), tenecteplase (a mutant of t-PA with

a triple-site substitution that increases plasma half-life, fibrin binding and resistance to PAI-1) and lanoteplase (a variant of t-PA with a greater fibrinolytic activity and slower plasma clearance). Despite all detailed investigations on the biochemical properties of plasminogen activators, the newer fibrinolytic agents are no vast therapeutic improvement compared to the first generation thrombolytics. However, their ease of administration paved the way for these bolus agents to become the standard for thrombolytic therapy [210, 211].

Currently some new thrombolytic therapies are under investigation. Staphylokinase is produced by certain strains of *Staphylococcus aureus* and has good fibrin selectivity. It has successfully completed phase II clinical trials and is currently evaluated in phase III trials for treatment of acute myocardial infarction. In terms of efficacy, staphylokinase has been shown to be comparable to t-PA, while potentially being available at a much lower cost. Microplasmin is a truncated and stable form of plasmin that has recently been evaluated in phase II clinical trials for the treatment of stroke, even after the 3 hour timeframe in which conventional thrombolytics need to be administered. Another interesting thrombolytic drug under development for the treatment of ischemic stroke is desmoteplase, a compound isolated from the saliva of bats [211, 212].

Despite all progress made, current thrombolytic therapy is still associated with significant drawbacks including the need for large therapeutic doses, limited fibrin specificity and the risk for either severe bleeding complications or reocclusion [212]. These drawbacks could be circumvented by developing new thrombolytics or by improving current thrombolytic therapy, which will go hand in hand with an improved understanding of the regulation of the fibrinolytic system.

One of the major inhibitors of the fibrinolytic system is PAI-1. Since its discovery in 1983, several studies have examined the role of PAI-1 levels in cardiovascular diseases. Today there is substantial evidence indicating a link between high plasma PAI-1 levels and the risk for arterial and venous thrombosis [35, 71, 72, 167]. Inhibition of PAI-1 might therefore be an interesting approach for either prevention of cardiovascular events or as adjuvant to improve the current thrombolytic treatment regimes. Izuhara *et al.* (2010) recently identified a PAI-1 inhibiting LMW compound that could improve the therapeutic efficacy of t-PA and could reduce its adverse effects in a non-human primate model.

These results are very promising for the further development of PAI-1 inhibitors as adjuvant therapy in thrombolysis [139].

Aside from its role in cardiovascular diseases, high plasma PAI-1 concentrations were reported in case of obesity or the metabolic syndrome and constitute a strong marker for poor prognosis in cancer (cfr chapter 1). Besides subjects with increased plasma levels of PAI-1, also patients with PAI-1 deficiency have been identified. In humans, deficiency of PAI-1, results in abnormal bleeding but only after trauma or surgery and without any further abnormalities [92-94].

Although a lot of research has focused on the role of PAI-1 in diverse pathologies, these studies often generated conflicting results. Before one can consider targeting PAI-1 therapeutically, the exact outcome of long-term PAI-1 inhibition in humans will need to be considered. Therefore the exact function of PAI-1 in diverse physiological processes definitely needs further clarification.

Preclinical evaluation of potential therapeutic targets often involves *in vivo* testing in rodent models. Although a lot of anti-human PAI-1 antibodies have been generated, most of them do not cross-react sufficiently with mouse PAI-1. This differential reactivity of monoclonal antibodies toward human *versus* mouse PAI-1 could be expected due to the amino acid and conformational differences observed for both proteins.

The amino acid sequence homology between human PAI-1 and mouse PAI-1 is 78,9% [179]. Although the stability and functional properties of both species are comparable [213], important species differences have been observed with respect to their sensitivity to PAI-1 inhibitory agents [179, 185]. Recently, we elucidated the crystal structure of latent mouse PAI-1, revealing subtle conformational differences between the human and mouse PAI-1 structure. The position of α-helix A was similar in rat and mouse PAI-1, but different compared to that of α-helix A in human PAI-1, consequently resulting in different functional effects of monoclonal antibodies targeting this region. Structural differences between mouse and human PAI-1 were also observed in the gate region (the loop connecting β-strand s3C to β-strand s4C) [179]. This gate region plays a role in the active to latent transition [44] and displayed much more flexibility in the mouse PAI-1 structure in comparison to human PAI-1. The increased flexibility of the gate region in mouse PAI-1 was related to the observation that five mutations known to stabilize human PAI-1 72-fold do not have the same stabilizing effect for mouse PAI-1. The third

region with structural differences between human and mouse PAI-1 comprises the RCL. In the N-terminal part of the RCL of mouse PAI-1, the last residue of s4A is F341, while in latent human PAI-1 s4A stretches till residue V343. C-terminally, the RCL is situated above the C-terminus in latent mouse PAI-1, whereas this is the other way around in latent human PAI-1 [179].

Besides inter-species differences one also needs to take into account variation in the glycosylation pattern of PAI-1, when PAI-1 is expressed by different cell types. Most antibodies were generated toward non-glycosylated human PAI-1, expressed in *E. coli* cells. Even if these antibodies showed cross-reactivity with non-glycosylated mouse PAI-1, they often do not cross-react sufficiently with glycosylated mouse PAI-1. Human PAI-1 displays a heterogeneous and tissue-dependent glycosylation pattern in plasma and presumably this also applies for PAI-1 in mouse plasma [188]. Therefore, thorough *in vitro* characterization of PAI-1 inhibitory antibodies toward both non-glycosylated and glycosylated mouse PAI-1 is a prerequisite for further *in vivo* assessment of mouse PAI-1 inhibition.

Vitronectin-bound PAI-1 is the dominant form of PAI-1 in plasma and in the extracellular matrix. Nevertheless, the interaction between active PAI-1 and vitronectin is often not taken into account when evaluating PAI-1 inhibiting compounds *in vitro.* It was already demonstrated that some LMW compounds that had a very potent effect *in vitro* were no longer able to inhibit PAI-1 in plasma environment and this discrepancy was attributed in part to the lack of reactivity toward vitronectin-bound PAI-1 [136, 137, 190]. These observations imply that potentially interesting PAI-1 inhibitory compounds must have a good inhibitory capacity not only to free PAI-1 but also to PAI-1 bound to vitronectin.

In the first part of the study (chapter 2) we generated a new panel of monoclonal antibodies with good reactivity toward vitronectin-bound glycosylated mouse PAI-1. PAI-1 deficient mice were immunized with either non-glycosylated or glycosylated mouse PAI-1. Unfortunately, only immunization with non-glycosylated mouse PAI-1 resulted in the generation of antibodies with high binding affinity. It is not clear at present why immunization with glycosylated mouse PAI-1 did not yield high affinity anti-PAI-1

antibodies. It is possible that glycosylation masks highly immunogenic epitopes otherwise exposed in recombinant proteins derived from *E. coli* thereby resulting in reduced immunogenicity of the glycosylated variant [214]. On the other hand, presence of glycan chains might confer small conformational changes to the structure of glycosylated *versus* non-glycosylated mouse PAI-1 that result in a reduced immunogenicity.

Immunization with non-glycosylated mouse PAI-1 resulted in five monoclonal antibodies with high affinity and a strong inhibitory effect toward glycosylated mouse PAI-1. Similar inhibitory effects were seen in the presence of a 33-fold molar excess of vitronectin. The PAI-1 inhibitory potential of the antibodies *in vivo* was demonstrated in a mouse thromboembolism model, in which the evaluated antibodies significantly increased the percentage of mice with normal physical activity in comparison to mice treated with negative control antibody.

Recently these monoclonal antibodies toward mouse PAI-1 were used in a second *in vivo* study in which mice were injected with LPS to increase PAI-1 antigen and activity levels. Preliminary results indicate that i.v. administration of MA-MP2D2 and MA-MP26H2 significantly decreased PAI-1 activity levels in plasma again revealing the *in vivo* inhibitory effect of the newly generated antibodies.

To the best of our knowledge this is the first panel of monoclonal antibodies that can inhibit mouse PAI-1 in the presence of vitronectin and that show a profibrinolytic effect *in vivo.* Therefore these antibodies provide excellent immunological tools to further investigate the role of PAI-1 in mouse models.

The use of intact antibodies is not always the method of choice since their long half-life and large size can reduce their efficacy. The Fc-mediated receptor functions of full-size antibodies are often unwanted and may lead to severe side effects (e.g. massive cytokine release and associated toxic effects). However, these intact antibodies can serve as a starting point for the generation of smaller antibody fragments. Two popular antibody formats that might retain the high binding affinity of the parental IgG are the antigen-binding fragment or Fab and the single-chain variable fragment or scFv. In general, these antibody fragments have some advantages such as an exquisite binding specificity and a low toxicity. Because of their smaller size, fragments can more easily penetrate into tissues. Another advantage is that fragments can be produced in *E. coli*

rather than mammalian cells, more than halving the production costs. Antibody fragments are also cleared from the body much faster, although their half-life can be lengthened to some degree by PEGylation [215]. Despite early excitement regarding these antibody fragments, not many have been commercialized. This is mainly due to the fact that in practice, antibody fragments rarely retain equal binding affinity in comparison with the parental antibody, were often poorly soluble and very prone to aggregation [146]. Interest in antibody fragments was revived more recently when it was discovered that at least two types of organisms, the camelids and cartilaginous fish (sharks) have not only conventional antibodies circulating in their blood, but have also evolved high affinity heavy-chain antibodies [216, 217]. As their name suggests, these heavy-chain antibodies consist of only two heavy chains and lack the light chains. It is possible to express only the variable fragment of a heavy chain antibody (called VHH in camelids and V-NAR in sharks). An advantage of these VHH and V-NAR domains is that they display long surface loops, often larger than for conventional antibodies and are therefore better able to penetrate cavities in target antigens. However, for *in vivo* administration humanization might be crucial to reduce immunogenicity, although VHH fragments have been claimed to be only minimally immunogenic [146, 218]. For some applications it might be desirable to engineer monovalent Fab, scFv, VHH or V-NAR fragments into multivalent molecules (e.g. diabodies). These multivalent antibody fragments can show significant increases in functional affinity, can be made multispecific when sub-parts target different antigens and can have improved retention properties in comparison with the parent IgG [146].

Although antibodies are highly specific, non-antibody based strategies to inhibit PAI-1 are therapeutically more easily applicable. The major advantages of LMW inhibitors over monoclonal antibodies is that they can be administered orally, are easier and cheaper to produce on an industrial scale and can be stored with much fewer difficulties in comparison to monoclonal antibodies. Therefore a lot of pharmaceutical companies have focused on the development of LMW PAI-1 inhibitors [130, 132, 219, 220]. Unfortunately, several of the developed compounds were associated with different drawbacks such as poor physicochemical properties, toxicity and strong aspecific binding to serum albumin. Although extensive research has been performed regarding the

development of a LMW PAI-1 inhibitor no compound is currently being evaluated in (pre-)clinical trials.

Monoclonal antibodies might be a good starting point for the rational development of new PAI-1 inhibitory LMW compounds. Structure-based drug design is today an integral part of most industrial drug discovery programs. For such a structural based drug design, excellent knowledge of the structure of the target protein is essential. The recently described crystal structure of mouse PAI-1 and the observed subtle differences with the human PAI-1 structure provide valuable additional information [179]. Besides knowledge on the structure of the therapeutic target it is also important to know which region of the protein should be targeted in a rational drug design approach. Is this respect the highly specific and well characterized panel of monoclonal antibodies with their described epitopes might provide an ideal starting point for a rational drug design approach. All strong inhibitory antibodies described in chapter 2 targeted the region around α-helix F and the turn connecting α-helix F with β-sheet s3A. It has been suggested before that in serpins α-helix F, together with β-strands s1-3A can move relative to the remaining part of the molecule and that this movement is a prerequisite for the inhibitory capacity [221]. Binding of the antibodies to this particular region can be expected to either restrict the conformational flexibility of α-helix F and β-strands s1-3A or to reposition this region relative to the remainder part of the PAI-1 molecule. Consequently, the rate or the extent of RCL insertion into β-sheet A will be reduced, resulting in a shift from the inhibitory to the substrate pathway of PAI-1 [109].

Unfortunately, all strong inhibitory antibodies that showed high affinity toward both non-glycosylated and glycosylated mouse PAI-1 share the same epitope region, thereby restricting the diversity of the antibody panel. The fact that these antibodies show no differential reactivity depending on the glycosylation status is most likely due to the fact that their epitope is located at the opposite side of the PAI-1 molecule compared to the potential N-glycosylation sites, situated more closely to the RCL. These specific positions might explain why glycosylation had no influence on the reactivity of the antibodies, but also suggests why no antibodies with epitope regions around the RCL were identified to bind both glycosylated and non-glycosylated mouse PAI-1. Although PAI-1 is not heavily glycosylated, the presence of glycan chains can be a restricting factor for antibody binding. As shown in chapter 3, removal of specific N-linked glycan chains can partially restore binding of antibodies that show differential reactivity toward glycosylated *versus*

non-glycosylated mouse and rat PAI-1. Especially for antibodies with an epitope location near the potential N-glycosylation sites of PAI-1, this differential reactivity needs to be considered.

Mouse PAI-1 contains the same three potential glycosylation sites that were described for human PAI-1 (i.e. N209, N265 and N329) and seems to be primarily glycosylated at the asparagine residue at position N265. For rat PAI-1 a fourth glycosylation site could be predicted at position N65. We recently found that this position N65, together with N265, is N-glycosylated in rat PAI-1.

Presence of glycan chains can not only influence antibody binding but also induces small biochemical differences between glycosylated and non-glycosylated PAI-1. The latency transition of non-glycosylated, but not of glycosylated human PAI-1 was strongly accelerated by a non-ionic detergent i.e. Triton X-100 [197]. In our study, the presence of glycan chains seems to interfere with the stabilizing effect of five mutations previously reported for human PAI-1 (chapter 4). Non-glycosylated human PAI-1-N150H-K154T-Q301P-Q319P-M354I has a 72-fold increased half-life in comparison to non-glycosylated human PAI-1 wild-type [55]. Strikingly, the half-life of glycosylated human PAI-1-N150H-K154T-Q301P-Q319P-M354I was much lower than that of the non-glycosylated stabilized variant. Four mutations (i.e. N150H, K154T, Q301P and Q319) are situated in the region around α-helix F and are thus not located in proximity of potential N-glycosylation sites. Most likely the subtle structural differences that are inferred by the presence of glycan chains hamper the stabilizing effect exerted by one or more of these PAI-1 mutations. It has been shown previously that small conformational differences can have a great impact on the stability of PAI-1 e.g. introduction of N150H, K154T, Q301P and Q319P and M354I (mutations stabilizing non-glycosylated human PAI-1 72-fold) in mouse PAI-1 resulted in an only 2-fold increased half-life in comparison to mouse PAI-1 wild-type [179].

These findings again indicate that data obtained from non-glycosylated PAI-1, expressed in *E. coli* and most commonly used for *in vitro* PAI-1 investigation, cannot simply be extrapolated to glycosylated PAI-1 or to *in vivo* experiments.

In conclusion, evaluation of a new panel of monoclonal antibodies toward mouse PAI-1 highlighted the importance of species- and glycosylation-dependent recognition of PAI-1

by potential therapeutic inhibitors and provided us with a stabilized form of glycosylated human PAI-1 that will facilitate biochemical and structural analyses of glycosylated PAI-1.

Future prospects:

Despite the fact that many *in vivo* studies showed a beneficial effect of PAI-1 inhibition, no LMW- or antibody-based PAI-1 inhibitor is currently available. This study may accelerate the rational development of such inhibitors and provides interesting immunological tools to further unravel the effect of PAI-1 inhibition in mouse models.

Further *in vivo* experiments of the most potent monoclonal antibodies will be performed in mice injected with LPS. Dose-response curves will reveal any differential PAI-1 inhibitory capacity of the various antibodies.

The generated antibodies also serve as the starting point for the development of smaller antibody derivatives. These fragments retain the targeting specificity of whole monoclonal antibodies but can be produced more economically and posses other unique and superior properties for a range of diagnostic and therapeutic applications. Currently scFv fragments of MA-MP2D2 and MA-MP26H2 are being produced as part of another PhD project. The PAI-1 inhibitory properties of these antibody fragments will be evaluated and compared with the properties of the parental antibody both *in vitro* and *in vivo*.

Evaluation of the consequences of PAI-1 inhibition should not be limited to thrombotic disease models but should also focus on the sometimes contradictory results obtained for PAI-1 inhibition in conditions like angiogenesis, tumor development, obesity and the metabolic syndrome. Fundamental research explaining these conflicting results obtained with PAI-1 inhibitors is necessary before (pre-)clinical studies with a PAI-1 inhibitor can be considered.

Summary

Cardiovascular diseases can result from an imbalance between plasminogen activators (mainly t-PA) and plasminogen activator inhibitors (mainly PAI-1). Restoration of this imbalance in the plasminogen activator system is possible via two approaches i.e. either by increasing the levels of circulating t-PA or by decreasing the levels of active PAI-1 through PAI-1 inhibition. Current thrombolytic therapy is mainly focused on increasing the circulating t-PA concentration but it remains associated with severe side effects such as bleeding complications. Co-administration of a PAI-1 inhibitor together with current thrombolytic drugs might help to reduce these complications and improve the thrombolytic efficacy and treatment outcome.

Besides its role in thrombotic disorders plasma PAI-1 levels are also indicative for poor prognosis in cancer and are highly elevated in some non-thrombotic conditions, like obesity and the metabolic syndrome. Also for these pathologies, interference with PAI-1 activity might have a positive impact on disease progression and prognosis.

A lot of research has focused on the development of PAI-1 inhibitors, yet to date no commercial PAI-1 inhibitor is available. Although the role of PAI-1 in cardiovascular events is well documented, its role in non-thrombotic conditions such as cancer definitely requires further validation before potential (pre-)clinical studies with a PAI-1 inhibitor.

Preclinical evaluation of putative therapeutic targets *in vivo* is often started in rodent models since disease pathogenesis in mice or rats tends to approximate disease progression in humans. However, most animal proteins differ substantially from their human counterparts. As a result, PAI-1 inhibitory antibodies generated toward human PAI-1 often do not cross-react with PAI-1 from other species. In **chapter 2** we describe the generation and *in vitro* and *in vivo* characterization of a new panel of monoclonal antibodies reacting with vitronectin-bound glycosylated mouse PAI-1. These antibodies provide interesting tools to further study the complex role of PAI-1 in different thrombotic and non-thrombotic mouse models.

In **chapter 3**, comparative evaluation of the reactivity of PAI-1 inhibiting monoclonal antibodies toward rat and mouse PAI-1 wild-type and glycosylation knock-out mutants

revealed a clear glycosylation dependent reactivity profile. Electrophoretic mobility of mouse PAI-1 glycosylation knock-out mutants before and after deglycosylation indicates the presence of glycan chains at position N265, whereas N209 and N329 do not seem to be glycosylated. For rat PAI-1, N-glycosylation is observed at asparagine N65 and at asparagine N265. These data stress the importance of selecting the proper source of PAI-1 when evaluating PAI-1 inhibitors *in vitro*, since reactivity of inhibitors toward putative therapeutic targets is species and glycosylation dependent.

In the last part of the study (**chapter 4**) we compared the functional properties of stabilized glycosylated *versus* non-glycosylated human PAI-1. A stabilized form of human PAI-1, comprising five point mutations, was expressed in eukaryotic cells and the stability of this purified protein was compared with the stability of the non-glycosylated human PAI-1 mutant.

In conclusion, this study provides good immunological tools to further unravel the complex role of PAI-1 in various physiological processes using mouse models and adds to our knowledge about the biochemical and functional properties of glycosylated *versus* non-glycosylated PAI-1 from different species.

Samenvatting

Cardiovasculaire aandoeningen kunnen het gevolg zijn van een onevenwicht tussen plasminogeen activatoren (vooral t-PA) en plasminogeen activator inhibitoren (vooral PAI-1). Het herstellen van dit onevenwicht in het plasminogeen activator systeem is mogelijk op twee verschillende manieren, i.e. door het verhogen van de circulerende t-PA waarden in plasma of door verlaging van de concentratie actief PAI-1 door middel van PAI-1 inhibitoren. De huidige trombolytische therapie is vooral gericht op de verhoging van de t-PA concentratie in plasma, maar kan gepaard gaan met levensbedreigende neveneffecten zoals ernstige bloedingen. Coadministratie van PAI-1 inhibitoren en de huidige trombolytica zou deze complicaties kunnen verminderen en op die manier de trombolytische efficiëntie en het resultaat van de behandeling kunnen verbeteren. Naast de rol van PAI-1 in cardiovasculaire aandoeningen, zijn verhoogde plasma PAI-1 concentraties ook een indicatie voor slechte prognose in kanker en worden sterk verhoogde PAI-1 waarden gemeten in bloed van patiënten met obesitas en metabool syndroom. Ook voor deze aandoeningen kan interferentie met de activiteit van PAI-1 een impact hebben op het ziekteverloop en de prognose.
Er is reeds veel onderzoek gebeurd naar de ontwikkeling van PAI-1 inhibitoren, maar tot op heden is er nog geen commerciële PAI-1 inhibitor beschikbaar. Want hoewel de rol van PAI-1 in cardiovasculaire aandoeningen zeer uitgebreid is beschreven, moet de exacte functie van PAI-1 in niet-trombotische aandoeningen zeker nog verder onderzocht worden vooraleer een PAI-1 inhibitor voor humaan gebruik kan worden getest in klinische studies.

Preklinische evaluatie van vermeende therapeutische eiwitten *in vivo* gebeurt vaak eerst in knaagdieren omdat het ziekteverloop in muizen en ratten vaak veel gelijkenissen vertoont met het humane ziekteverloop. Nochtans vertonen rat en muis eiwitten vaak substantiële verschillen in vergelijking met hun humane tegenhanger. Als gevolg van deze inter-species verschillen reageren PAI-1 inhiberende antilichamen opgewekt tegen humaan PAI-1 vaak niet met PAI-1 van andere species. In **hoofdstuk 2** wordt de aanmaak en *in vitro* en *in vivo* karakterisering van een nieuw panel monoklonale antilichamen beschreven die reageren met vitronectine gebonden geglycosyleerd muis PAI-1. Deze

antilichamen zijn veelbelovende instrumenten om de rol van PAI-1 in verschillende trombotische en niet-trombotische aandoeningen verder uit te diepen.

Een vergelijkende studie van de reactiviteit van PAI-1 inhiberende antilichamen tegenover rat en muis PAI-1 wild-type en glycosylatie knock-out mutanten wees op een duidelijk glycosylatie afhankelijk bindingspatroon. Eerder onderzoek naar het glycosylatiepatroon van humaan PAI-1 toonde aan dat er drie potentiële N-glycosylatie plaatsen aanwezig zijn in de humaan PAI-1 sequentie, namelijk asparagine N209, N265 en N329. Van deze drie posities bleken alleen asparagine residuen N209 en N265 ook effectief suiker ketens te dragen. Hoewel het glycosylatiepatroon van humaan PAI-1 grondig bestudeerd werd was er nog geen informatie beschikbaar over het glycosylatiepatroon van muis en rat PAI-1. Elektroforetische mobiliteit van muis PAI-1 glycosylatie knock-out mutanten voor en na deglycosylatie duidt op de aanwezigheid van suiker ketens op positie N265, terwijl posities N209 en N329 niet geglycosyleerd zijn. Voor rat PAI-1 werd N-glycosylatie aangetoond op posities N65 en N265. Deze gegevens tonen duidelijk aan dat men de juiste bron van PAI-1 moet selecteren voor de *in vitro* evaluatie van PAI-1 inhiberende compounds en antilichamen zodat men optimaal rekening houdt met de inter-species en glycosylatie verschillen (**Hoofdstuk 3**)

In het laatste deel van dit onderzoek (**Hoofdstuk 4**) werden de functionele eigenschappen van gestabiliseerd geglycosyleerd *versus* niet-geglycosyleerd humaan PAI-1 vergeleken. Een gestabiliseerde vorm van humaan PAI-1, met vijf aminozuur mutaties in de proteïne sequentie, werd tot expressie gebracht in eukaryote cellen en de stabiliteit van dit geglycosyleerde eiwit werd vergeleken met de stabiliteit van de niet-geglycosyleerde humane PAI-1 mutant.

Samengevat levert deze studie goede immunologische instrumenten om de complexe rol van PAI-1 inhibitie en stimulatie verder te onderzoeken in muis modellen en levert ze een bijdrage aan onze kennis over de biochemische en functionele eigenschappen van geglycosyleerd *versus* niet-geglycosyleerd PAI-1 van verschillende species.

References

1 Rau JC, Beaulieu LM, Huntington JA, Church FC. Serpins in thrombosis, hemostasis and fibrinolysis. *Journal of Thrombosis and Haemostasis*. 2007; **5**: 102-15.

2 Macfarlane RG. Enzyme cascade in blood clotting mechanism + its function as biochemical amplifier. *Nature*. 1964; **202**: 498-9.

3 Mackman N, Tilley RE, Key NS. Role of the extrinsic pathway of blood coagulation in hemostasis and thrombosis. *Arteriosclerosis Thrombosis and Vascular Biology*. 2007; **27**: 1687-93.

4 Adams RLC, Bird RJ. Review article: Coagulation cascade and therapeutics update: Relevance to nephrology. Part 1: Overview of coagulation, thrombophilias and history of anticoagulants. *Nephrology*. 2009; **14**: 462-70.

5 Wolberg AS. Thrombin generation and fibrin clot structure. *Blood Reviews*. 2007; **21**: 131-42.

6 Crawley JTB, Lane DA. The haemostatic role of tissue factor pathway inhibitor. *Arteriosclerosis Thrombosis and Vascular Biology*. 2008; **28**: 233-42.

7 Castellino FJ, Ploplis VA. The protein C pathway and pathologic processes. *Journal of Thrombosis and Haemostasis*. 2009; **7**: 140-5.

8 Egberg N. Schematic presentation of the hemostatic system. In: Blombäck M, Antovic J, eds. *Essential guide to blood coagulation*, 1 edn. Singapore: Wiley-Blackwell, 2009.

9 Rijken DC, Lijnen HR. New insights into the molecular mechanisms of the fibrinolytic system. *Journal of Thrombosis and Haemostasis*. 2009; **7**: 4-13.

10 Lijnen HR, Collen D. Matrix metalloproteinase system deficiencies and matrix degradation. *Thrombosis and Haemostasis*. 1999; **82**: 837-45.

11 Polgar L. The catalytic triad of serine peptidases. *Cellular and Molecular Life Sciences*. 2005; **62**: 2161-72.

12 Collen D, Lijnen HR. New approaches to thrombolytic therapy. *Arteriosclerosis*. 1984; **4**: 579-85.

13 Thelwell C, Longstaff C. The regulation by fibrinogen and fibrin of tissue plasminogen activator kinetics and inhibition by plasminogen activator inhibitor 1. *Journal of Thrombosis and Haemostasis*. 2007; **5**: 804-11.

14 Hoylaerts M, Rijken DC, Lijnen HR, Collen D. Kinetics of the activation of plasminogen by human tissue type plasminogen activator - role of fibrin. *Journal of Biological Chemistry*. 1982; **257**: 2912-9.

15 Williams J. The fibrinolytic activity of urine. *British journal of experimental pathology*. 1951; **32**: 530-7.

16 Stump DC, Lijnen HR, Collen D. Purification and characterization of a novel low molecular weight form of single-chain urokinase-type plasminogen activator. *Journal of Biological Chemistry*. 1986; **261**: 7120-6.

17 Lijnen HR, Vanhoef B, Collen D. Activation with plasmin of two-chain urokinase-type plasminogen activator derived from single-chain urokinase-type plasminogen activator by treatment with thrombin. *European Journal of Biochemistry*. 1987; **169**: 359-64.

18 Demunk GAW, Groeneveld E, Rijken DC. Acceleration of the thrombin inactivation of single chain urokinase-type plasminogen activator (pro-urokinase) by thrombomodulin. *Journal of Clinical Investigation*. 1991; **88**: 1680-4.

19 Coughlin PB. Antiplasmin - The forgotten serpin? *Febs Journal*. 2005; **272**: 4852-7.

20 Dellas C, Loskutoff DJ. Historical analysis of PAI-1 from its discovery to its potential role in cell motility and disease. *Thrombosis and haemostasis*. 2005; **93**: 631-40.

21 Willemse JL, Heylen E, Nesheim ME, Hendriks DF. Carboxypeptidase U (TAFIa): a new drug target for fibrinolytic therapy? *Journal of Thrombosis and Haemostasis*. 2009; **7**: 1962-71.
22 Lijnen HR, Vanhoef B, Beelen V, Collen D. Characterization of the murine plasma fibrinolytic system. *European Journal of Biochemistry*. 1994; **224**: 863-71.
23 Kawasaki T, Dewerchin M, Lijnen HR, Vermylen J, Hoylaerts MF. Vascular release of plasminogen activator inhibitor-1 impairs fibrinolysis during acute arterial thrombosis in mice. *Blood*. 2000; **96**: 153-60.
24 Korninger C, Collen D. Studies on the specific fibrinolytic effect of human extrinsic (tissue-type) plasminogen activator in human blood and in various animal species in vitro. *Thrombosis and Haemostasis*. 1981; **46**: 561-5.
25 Loskutoff DJ, Vanmourik JA, Erickson LA, Lawrence D. Detection of an unusually stable fibrinolytic inhibitor produced by bovine endothelial cells. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences*. 1983; **80**: 2956-60.
26 Simpson AJ, Booth NA, Moore NR, Bennett B. Distribution of plasminogen activator inhibitor (PAI-1) in tissues. *Journal of Clinical Pathology*. 1991; **44**: 139-43.
27 Booth NA, Simpson AJ, Croll A, Bennett B, Macgregor IR. Plasminogen activator inhibitor (PAI-1) in plasma and platelets. *British Journal of Haematology*. 1988; **70**: 327-33.
28 Kruithof EKO, Nicolosa G, Bachmann F. Plasminogen activator inhibitor-1 - development of a radioimmunoassay and observations on its plasma concentration during venous occlusion and after platelet aggregation. *Blood*. 1987; **70**: 1645-53.
29 Pannekoek H, Veerman H, Lambers H, Diergaarde P, Verweij CL, Vanzonneveld AJ, Vanmourik JA. Endothelial plasminogen activator inhibitor (PAI): a new member of the Serpin gene family. *Embo Journal*. 1986; **5**: 2539-44.
30 Ny T, Sawdey M, Lawrence D, Millan JL, Loskutoff DJ. Cloning and sequence of a cDNA coding for the human beta-migrating endothelial cell type plasminogen activator inhibitor. *Proceedings of the National Academy of Sciences of the United States of America*. 1986; **83**: 6776-80.
31 Ginsburg D, Zeheb R, Yang AY, Rafferty UM, Andreasen PA, Nielsen L, Dano K, Lebo RV, Gelehrter TD. cDNA cloning of human plasminogen activator inhibitor from endothelial cells. *Journal of Clinical Investigation*. 1986; **78**: 1673-80.
32 Andreasen PA, Riccio A, Welinder KG, Douglas R, Sartorio R, Nielsen LS, Oppenheimer C, Blasi F, Dano K. Plasminogen activator inhibitor type-1 - Reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing. *Febs Letters*. 1986; **209**: 213-8.
33 Loskutoff DJ, Linders M, Keijer J, Veerman H, Vanheerikhuizen H, Pannekoek H. Structure of the human plasminogen activator inhibitor-1 gene - nonrandom distribution of introns. *Biochemistry*. 1987; **26**: 3763-8.
34 Irigoyen JP, Munoz-Canoves P, Montero L, Koziczak M, Nagamine Y. The plasminogen activator system: biology and regulation. *Cellular and Molecular Life Sciences*. 1999; **56**: 104-32.
35 Gils A, Declerck PJ. The structural basis for the pathophysiological relevance of PAI-l in cardiovascular diseases and the development of potential PAI-l inhibitors. *Thrombosis and Haemostasis*. 2004; **91**: 425-37.
36 Gils A, Pedersen KE, Skottrup P, Christensen A, Naessens D, Deinum J, Enghild JJ, Declerck PJ, Andreasen PA. Biochemical importance of glycosylation of plasminogen activator inhibitor-I. *Thrombosis and Haemostasis*. 2003; **90**: 206-17.
37 Lawrence D, Strandberg L, Grundstrom T, Ny T. Purification of active human plasminogen activator inhibitor-1 from Escherichia coli-comparison with natural and

recombinant forms purified from eukaryotic cells. *European Journal of Biochemistry*. 1989; **186**: 523-33.

38 Law RHP, Zhang QW, McGowan S, Buckle AM, Silverman GA, Wong W, Rosado CJ, Langendorf CG, Pike RN, Bird PI, Whisstock JC. An overview of the serpin superfamily. *Genome Biology*. 2006; **7**. 10.1186/gb-2006-7-5-216.

39 Huntington JA, Read RJ, Carrell RW. Structure of a serpin-protease complex shows inhibition by deformation. *Nature*. 2000; **407**: 923-6.

40 Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plasminogen activators that can be activated by denaturants. *Journal of Biological Chemistry*. 1985; **260**: 1581-7.

41 Declerck PJ, Demol M, Vaughan DE, Collen D. Identification of a conformationally distinct form of plasminogen activator inhibitor-1, acting as a noninhibitory substrate for tissue-type plasminogen activator. *Journal of Biological Chemistry*. 1992; **267**: 11693-6.

42 Nar H, Bauer M, Stassen JM, Lang D, Gils A, Declerck PJ. Plasminogen activator inhibitor 1. Structure of the native serpin, comparison to its other conformers and implications for serpin inactivation. *Journal of Molecular Biology*. 2000; **297**: 683-95.

43 Mottonen J, Strand A, Symersky J, Sweet RM, Danley DE, Geoghegan KF, Gerard RD, Goldsmith EJ. Structural basis of latency in plasminogen activator inhibitor-1. *Nature*. 1992; **355**: 270-3.

44 Aertgeerts K, Debondt HL, Deranter CJ, Declerck PJ. Mechanisms contributing to the conformational and fucntional flexibility of plasminogen activator inhibitor-1. *Nature Structural Biology*. 1995; **2**: 891-7.

45 Sharp AM, Stein PE, Pannu NS, Carrell RW, Berkenpas MB, Ginsburg D, Lawrence DA, Read RJ. The active conformation of plasminogen activator inhibitor 1, a target for drugs to control fibrinolysis and cell adhesion. *Structure*. 1999; **7**: 111-8.

46 Lindahl TL, Sigurdardottir O, Wiman B. Stability of plasminogen activator inhibitor-1 (PAI-1). *Thrombosis and Haemostasis*. 1989; **62**: 748-51.

47 Sancho E, Tonge DW, Hockney RC, Booth NA. Purification and characterization of active and stable recombinant plasminogen activator inhibitor accumulated at high levels in *Escherichia coli*. *European Journal of Biochemistry*. 1994; **224**: 125-34.

48 Keijer J, Linders M, Ehrlich H, Gebbink RK, Pannekoek H. Stabilization of plasminogen activator inhibitor type-1 (PAI-1) activity by arginine - Possible implications for the interaction of PAI-1 with vitronectin. *Fibrinolysis*. 1990; **4**: 153-9.

49 Mangs H, Sui GC, Wiman B. PAI-1 stability: the role of histidine residues. *Febs Letters*. 2000; **475**: 192-6.

50 Stout TJ, Graham H, Buckley DI, Matthews DJ. Structures of active and latent PAI-1: A possible stabilizing role for chloride ions. *Biochemistry*. 2000; **39**: 8460-9.

51 Lawrence DA, Loskutoff DJ. Inactivation of plasminogen-activator inhibitor by oxidants. *Biochemistry*. 1986; **25**: 6351-5.

52 Strandberg L, Lawrence DA, Johansson LBA, Ny T. The oxidative inactivation of plasminogen activator inhibitor type-1 results from a conformational change in the molecule and does not require the involvement of the P1' methionine. *Journal of Biological Chemistry*. 1991; **266**: 13852-8.

53 Declerck PJ, Demol M, Alessi MC, Baudner S, Paques EP, Preissner KT, Mullerberghaus G, Collen D. Purification and characterization of a plasminogen activator inhibitor-1 binding protein from human plasma-Identification as a multimeric form of S-protein (vitronectin). *Journal of Biological Chemistry*. 1988; **263**: 15454-61.

54 Boncela J, Papiewska I, Fijalkowska I, Walkowiak B, Cierniewski CS. Acute phase protein alpha(1)-acid glycoprotein interacts with plasminogen activator inhibitor type 1 and stabilizes its inhibitory activity. *Journal of Biological Chemistry*. 2001; **276**: 35305-11.

55 Berkenpas MB, Lawrence DA, Ginsburg D. Molecular evolution of plasminogen activator inhibitor-1 functional stability. *Embo Journal*. 1995; **14**: 2969-77.

56 Vleugels N, Gils A, Mannaerts S, Knockaert I, Declerck PJ. Evaluation of the mechanism of inactivation of plasminogen activator inhibitor-1 by monoclonal antibodies using a stable variant. *Fibrinolysis & Proteolysis*. 1998; **12**: 277-82.

57 Dewilde M, Strelkov SV, Rabijns A, Declerck PJ. High quality structure of cleaved PAI-1-stab. *Journal of Structural Biology*. 2009; **165**: 126-32.

58 Stoop AA, Eldering E, Dafforn TR, Read RJ, Pannekoek H. Different structural requirements for plasminogen activator inhibitor 1 (PAI-1) during latency transition and proteinase inhibition as evidenced by phage-displayed hypermutated PAI-1 libraries. *Journal of Molecular Biology*. 2001; **305**: 773-83.

59 Chorostowska-Wynimko J, Swiercz R, Skrzypczak-Jankun E, Wojtowicz A, Selman SH, Jankun J. A novel form of the plasminogen activator inhibitor created by cysteine mutations extends its half-life: Relevance to cancer and angiogenesis. *Molecular Cancer Therapeutics*. 2003; **2**: 19-28.

60 Ehrlich HJ, Gebbink RK, Keijer J, Linders M, Preissner KT, Pannekoek H. Alteration of serpin specificity by a protein cofactor-vitronectin endows plasminogen activator inhibitor-1 with thrombin inhibitory properties. *Journal of Biological Chemistry*. 1990; **265**: 13029-35.

61 Keijer J, Linders M, Wegman JJ, Ehrlich HJ, Mertens K, Pannekoek H. On the target specificity of plasminogen activator inhibitor-1 - the role of heparin, vitronectin, and the reactive site. *Blood*. 1991; **78**: 1254-61.

62 Rezaie AR. Vitronectin functions as a cofactor for rapid inhibition of activated protein C by plasminogen activator inhibitor-1 - Implications for the mechanism of profibrinolytic action of activated protein C. *Journal of Biological Chemistry*. 2001; **276**: 15567-70.

63 Carrell R, Travis J. Alpha-1-antitrypsin and the serpins - variation and countervariation. *Trends in Biochemical Sciences*. 1985; **10**: 20-4.

64 Sherman PM, Lawrence DA, Yang AY, Vandenberg ET, Paielli D, Olson ST, Shore JD, Ginsburg D. Saturation mutagenesis of the plasminogen activator inhibitor-1 reactive center. *Journal of Biological Chemistry*. 1992; **267**: 7588-95.

65 Sherman PM, Lawrence DA, Verhamme IM, Paielli D, Shore JD, Ginsburg D. Identification of tissue-type plasminogen activator-specific plasminogen activator inhibitor-1 mutants - evidence that 2nd sites of interaction contribute to target specificity. *Journal of Biological Chemistry*. 1995; **270**: 9301-6.

66 Hamsten A, Wiman B, Defaire U, Blomback M. Increased plasma levels of a rapid inhibitor of tissue plasminogen activator in young survivors of myocardial infraction. *New England Journal of Medicine*. 1985; **313**: 1557-63.

67 Hamsten A, Defaire U, Walldius G, Dahlen G, Szamosi A, Landou C, Blomback M, Wiman B. Plasminogen activator inhibitor in plasma - risk factor for recurrent myocardial infarction. *Lancet*. 1987; **2**: 3-8.

68 Thogersen AM, Jansson JH, Boman K, Nilsson TK, Weinehall L, Huhtasaari F, Hallmans G. High plasminogen activator inhibitor and tissue plasminogen activator levels in plasma precede a first acute myocardial infarction in both men and women - Evidence for the fibrinolytic system as an independent primary risk factor. *Circulation*. 1998; **98**: 2241-7.

69 Ridker PM, Manson JE, Buring JE, Muller JE, Hennekens CH. Circadian variation of acute myocardial infarction and the effect of low-dose aspirin in a randomized trial of physicians. *Circulation*. 1990; **82**: 897-902.

70 Andreotti F, Kluft C. Circadian variation of fibrinolytic activity in blood. *Chronobiology International*. 1991; **8**: 336-51.

71 Juhan-Vague I, Valadier J, Alessi MC, Aillaud MF, Ansaldi J, Philip-Joet C, Holvoet P, Serradimigni A, Collen D. Deficient t-PA release and elevated PA inhibitor levels in

patients with spontaneous or recurrent deep venous thrombosis. *Thrombosis and Haemostasis*. 1987; **57**: 67-72.

72 Meltzer ME, Lisman T, de Groot PG, Meijers JCM, le Cessie S, Doggen CJM, Rosendaal FR. Venous thrombosis risk associated with plasma hypofibrinolysis is explained by elevated plasma levels of TAFI and PAI-1. *Blood*. 2010; **116**: 113-21.

73 Garg N, Fay WP. Plasminogen activator inhibitor-1 and Restenosis. *Current Drug Targets*. 2007; **8**: 1003-6.

74 Konstantinides S, Schafer K, Loskutoff DJ. Do PAI-1 and vitronectin promote or inhibit neointima formation? The exact role of the fibrinolytic system in vascular remodeling remains uncertain. *Arteriosclerosis Thrombosis and Vascular Biology*. 2002; **22**: 1943-5.

75 Binder BR, Mihaly J. The plasminogen activator inhibitor "paradox" in cancer. *Immunology Letters*. 2008; **118**: 116-24.

76 Schneiderman J, Sawdey MS, Keeton MR, Bordin GM, Bernstein EF, Dilley RB, Loskutoff DJ. Increased type-1 plasminogen activator inhibitor gene expression in atherosclerotic human arteries. *Proceedings of the National Academy of Sciences of the United States of America*. 1992; **89**: 6998-7002.

77 Lupu F, Bergonzelli GE, Heim DA, Cousin E, Genton CY, Bachmann F, Kruithof EKO. Localization and production of plasminogen activator inhibitor-1 in human healthy and atherosclerotic arteries. *Arteriosclerosis and Thrombosis*. 1993; **13**: 1090-100.

78 Fay WP, Garg N, Sunkar M. Vascular functions of the plasminogen activation system. *Arteriosclerosis Thrombosis and Vascular Biology*. 2007; **27**: 1231-7.

79 Prins MH, Hirsh J. A critical review of the evidence supporting a relationship between impaired fibrinolytic activity and venous thromboembolism. *Archives of Internal Medicine*. 1991; **151**: 1721-31.

80 Juhan-Vague I, Pyke SDM, Alessi MC, Jespersen J, Haverkate F, Thompson SG. Fibrinolytic factors and the risk of myocardial infarction or sudden death in patients with angina pectoris. *Circulation*. 1996; **94**: 2057-63.

81 Folsom AR, Aleksik N, Park E, Salomaa V, Juneja H, Wu KK. Prospective study of fibrinolytic factors and incident coronary heart disease - The Atherosclerosis Risk in Communities (ARIC) Study. *Arteriosclerosis Thrombosis and Vascular Biology*. 2001; **21**: 611-7.

82 Alessi MC, Peiretti F, Morange P, Henry M, Nalbone G, JuhanVague I. Production of plasminogen activator inhibitor 1 by human adipose tissue - Possible link between visceral fat accumulation and vascular disease. *Diabetes*. 1997; **46**: 860-7.

83 Schafer K, Fujisawa K, Konstantinides S, Loskutoff DJ. Disruption of the plasminogen activator inhibitor-1 gene reduces the adiposity and improves the metabolic profile of genetically obese and diabetic ob/ob mice. *Faseb Journal*. 2001; **15**: 1840-2.

84 Nordt TK, Sawa H, Fujii S, Bode C, Sobel BE. Augmentation of arterial endothelial cell expression of the plasminogen activator inhibitor type-1 (PAI-1) gene by proinsulin and insulin in vivo. *Journal of Molecular and Cellular Cardiology*. 1998; **30**: 1535-43.

85 Tsantes AE, Nikolopoulos GK, Bagos PG, Bonovas S, Kopterides P, Vaiopoulos G. The effect of the plasminogen activator inhibitor-1 4G/5G polymorphism on the thrombotic risk. *Thrombosis Research*. 2008; **122**: 736-42.

86 Erickson LA, Fici GJ, Lund JE, Boyle TP, Polites HG, Marotti KR. Development of venous occlusions in mice transgenic for the plasminogen activator inhibitor-1 gene. *Nature*. 1990; **346**: 74-6.

87 Eren M, Painter CA, Atkinson JB, Declerck PJ, Vaughan DE. Age-dependent spontaneous coronary arterial thrombosis in transgenic mice that express a stable form of human plasminogen activator inhibitor-1. *Circulation*. 2002; **106**: 491-6.

88 Carmeliet P, Stassen JM, Schoonjans L, Ream B, Vandenoord JJ, Demol M, Mulligan RC, Collen D. Plasminogen activator inhibitor-1 gene deficient mice. II. Effects on

hemostasis, thrombosis and thrombolysis. *Journal of Clinical Investigation*. 1993; **92**: 2756-60.

89 Schleef RR, Higgins DL, Pillemer E, Levitt LJ. Bleeding diathesis due to decreased fucntional activity of type 1 plasminogen activator inhibitor. *Journal of Clinical Investigation*. 1989; **83**: 1747-52.

90 Dieval J, Nguyen G, Gross S, Delobel J, Kruithof EKO. A lifelong bleeding disorder associated with a deficiency of plasminogen activator inhibitor type-1. *Blood*. 1991; **77**: 528-32.

91 Lee MH, Vosburgh E, Anderson K, McDonagh J. Deficiency of plasma plasminogen activator inhibitor-1 results in hyperfibrinolytic bleeding. *Blood*. 1993; **81**: 2357-62.

92 Fay WP, Parker AC, Condrey LR, Shapiro AD. Human plasminogen activator inhibitor-1 (PAI-1) deficiency: Characterization of a large kindred with a null mutation in the PAI-1 gene. *Blood*. 1997; **90**: 204-8.

93 Minowa H, Takahashi Y, Tanaka T, Naganuma K, Ida S, Maki I, Yoshioka A. Four cases of bleeding diathesis in children due to congenital plasminogen activator inhibitor-1 deficiency. *Haemostasis*. 1999; **29**: 286-91.

94 Mehta R, Shapiro AD. Plasminogen activator inhibitor type 1 deficiency. *Haemophilia*. 2008; **14**: 1255-60.

95 Durand MK, Bodker JS, Christensen A, Dupont DM, Hansen M, Jensen JK, Kjelgaard S, Mathiasen L, Pedersen KE, Skeldal S, Wind T, Andreasen PA. Plasminogen activator inhibitor-1 and tumour growth, invasion, and metastasis. *Thrombosis and Haemostasis*. 2004; **91**: 438-49.

96 Devy L, Blacher S, Grignet-Debrus C, Bajou K, Masson R, Gerard RD, Gils A, Carmeliet G, Carmeliet P, Declerck PJ, Noel A, Foidart JM. The pro- or antiangiogenic effect of plasminogen activator inhibitor 1 is dose dependent. *Faseb Journal*. 2002; **16**: 147-54.

97 Bajou K, Maillard C, Jost M, Lijnen RH, Gils A, Declerck P, Carmeliet P, Foidart JM, Noel A. Host-derived plasminogen activator inhibitor-1 (PAI-1) concentration is critical for in vivo tumoral angiogenesis and growth. *Oncogene*. 2004; **23**: 6986-90.

98 Schneider DJ, Chen Y, Sobel BE. The effect of plasminogen activator inhibitor type 1 on apoptosis. *Thrombosis and Haemostasis*. 2008; **100**: 1037-40. 10.1160/th08-04-0234.

99 Lijnen HR, Juhan-Vague I. The fibrinolytic system and obesity. *Thrombosis and Haemostasis*. 2002; **88**: 882.

100 Morange PE, Lijnen HR, Alessi MC, Kopp F, Collen D, Juhan-Vague I. Influence of PAI-1 on adipose tissue growth and metabolic parameters in a murine model of diet-induced obesity. *Arteriosclerosis Thrombosis and Vascular Biology*. 2000; **20**: 1150-4.

101 Lijnen HR, Maquoi E, Morange P, Voros G, Van Hoef B, Kopp F, Collen D, Juhan-Vague I, Alessi MC. Nutritionally induced obesity is attenuated in transgenic mice overexpressing plasminogen activator inhibitor-1. *Arteriosclerosis Thrombosis and Vascular Biology*. 2003; **23**: 78-84.

102 Lijnen HR. Effect of plasminogen activator inhibitor-l deficiency on nutritional ly-ind uced obesity in mice. *Thrombosis and Haemostasis*. 2005; **93**: 816-9.

103 Alessi MC, Juhan-Vague I. PAI-1 and the metabolic syndrome - Links, causes, and consequences. *Arteriosclerosis Thrombosis and Vascular Biology*. 2006; **26**: 2200-7.

104 Nielsen LS, Andreasen PA, Grondahlhansen J, Huang JY, Kristensen P, Dano K. Monoclonal antibodies to human 54,000 molecular weight plasminogen activator inhibitor from fibrosarcoma cells-inhibitor neutralization and one-step affinity purification. *Thrombosis and Haemostasis*. 1986; **55**: 206-12.

105 Keijer J, Linders M, Vanzonneveld AJ, Ehrlich HJ, Deboer JP, Pannekoek H. The interaction of plasminogen activator inhibitor-1 with plasminogen activators (tissue-type and urokinase-type) and fibrin - Localization of interaction sites and physiological relevance. *Blood*. 1991; **78**: 401-9.

106 Bijnens AP, Gils A, Stassen JM, Komissarov AA, Knockaert I, Brouwers E, Shore JD, Declerck PJ. The distal hinge of the reactive site loop and its proximity - A target to modulate plasminogen activator inhibitor-1 activity. *Journal of Biological Chemistry*. 2001; **276**: 44912-8.
107 Perrie AM, Macgregor IR, Booth NA. Definition of epitopes within plasminogen activator inhibitor type-1 (PAI-1) using multiple peptide synthesis. *Fibrinolysis*. 1993; **7**: 257-63.
108 Ngo TH, Hoylaerts MF, Knockaert I, Brouwers E, Declerck PJ. Identification of a target site in plasminogen activator inhibitor-1 that allows neutralization of its inhibitory properties concomitant with an allosteric up-regulation of its antiadhesive properties. *Journal of Biological Chemistry*. 2001; **276**: 26243-8.
109 Bijnens AP, Gils A, Knockaert I, Stassen JM, Declerck PJ. Importance of the hinge region between alpha-helix F and the main part of serpins, based upon identification of the epitope of plasminogen activator inhibitor type 1 neutralizing antibodies. *Journal of Biological Chemistry*. 2000; **275**: 6375-80.
110 Wind T, Jensen MA, Andreasen PA. Epitope mapping for four monoclonal antibodies against human plasminogen activator inhibitor type-1 - Implications for antibody-mediated PAI-1-neutralization and vitronectin-binding. *European Journal of Biochemistry*. 2001; **268**: 1095-106.
111 Stoop AA, Jespers L, Lasters I, Eldering E, Pannekoek H. High-density mutagenesis by combined DMA shuffling and phage display to assign essential amino acid residues in protein-protein interactions: Application to study structure-function of plasminogen activation inhibitor 1 (PAI-I). *Journal of Molecular Biology*. 2000; **301**: 1135-47.
112 Naessens D, Gils A, Compernolle G, Declerck PJ. Elucidation of a novel epitope of a substrate-inducing monoclonal antibody against the serpin PAI-1. *Journal of Thrombosis and Haemostasis*. 2003; **1**: 1028-33.
113 Debrock S, Sironi L, Declerck PJ. Cloning of a single-chain variable fragment (scFv) switching active plasminogen activator inhibitor-1 to substrate. *Gene*. 1997; **189**: 83-8.
114 Verhamme I, Kvassman JO, Day D, Debrock S, Vleugels N, Declerck PJ, Shore JD. Accelerated conversion of human plasminogen activator inhibitor-1 to its latent form by antibody binding. *Journal of Biological Chemistry*. 1999; **274**: 17511-7.
115 Ngo TH, Zhou Y, Stassen JM, Declerck PJ. Importance of N-terminal residues in plasminogen activator inhibitor 1 on its antibody induced latency transition. *Thrombosis and Haemostasis*. 2002; **88**: 288-93.
116 Declerck PJ, Verstreken M, Collen D. Immunoassay of murine t-PA, u-PA and PAI-1 using monoclonal antibodies raised in gene-inactivated mice. *Thrombosis and Haemostasis*. 1995; **74**: 1305-9.
117 Naessens D, Gils A, Compernolle G, Declerck PJ. Elucidation of the epitope of a latency-inducing antibody: identification of a new molecular target for PAI-1 inhibition. *Thrombosis and Haemostasis*. 2003; **90**: 52-8.
118 Gorlatova NV, Elokdah H, Fan K, Crandall DL, Lawrence DA. Mapping of a conformational epitope on plasminogen activator inhibitor-1 by random mutagenesis - Implications for serpin function. *Journal of Biological Chemistry*. 2003; **278**: 16329-35.
119 Dupont DM, Blouse GE, Hansen M, Mathiasen L, Kjelgaard S, Jensen JK, Christensen A, Gils A, Declerck PJ, Andreasen PA, Wind T. Evidence for a pre-latent form of the serpin plasminogen activator inhibitor-1 with a detached beta-strand 1C. *Journal of Biological Chemistry*. 2006; **281**: 36071-81.
120 Eitzman DT, Fay WP, Lawrence DA, Francischmura AM, Shore JD, Olson ST, Ginsburg D. Peptide-mediated inactivation of recombinant and platelet plasminogen activator inhibitor-1 in vitro. *Journal of Clinical Investigation*. 1995; **95**: 2416-20.

121 Kvassman JO, Lawrence DA, Shore JD. The acid stabilization of plasminogen activator inhibitor-1 depends on protonation of a single group that affects loop insertion into beta-sheet A. *Journal of Biological Chemistry*. 1995; **270**: 27942-7.
122 Xue YF, Bjorquist P, Inghardt T, Linschoten M, Musil D, Sjolin L, Deinum J. Interfering with the inhibitory mechanism of serpins: crystal structure of a complex formed between cleaved plasminogen activator inhibitor type 1 and a reactive-centre loop peptide. *Structure with Folding & Design*. 1998; **6**: 627-36.
123 Chavakis T, Pixley RA, Isordia-Salas I, Colman RW, Preissner KT. A novel antithrombotic role for high molecular weight kininogen as inhibitor of plasminogen activator inhibitor-1 function. *Journal of Biological Chemistry*. 2002; **277**: 32677-82.
124 Bryans J, Charlton P, ChicarelliRobinson I, Collins M, Faint R, Latham C, Shaw I, Trew S. Inhibition of plasminogen activator inhibitor-1 activity by two diketopiperazines, XR330 and XR334 produced by Streptomyces sp. *Journal of Antibiotics*. 1996; **49**: 1014-21.
125 Charlton PA, Faint RW, Bent F, Bryans J, ChicarelliRobinson I, Mackie I, Machin S, Bevan P. Evaluation of a low molecular weight modulator of human plasminogen activator inhibitor-1 activity. *Thrombosis and Haemostasis*. 1996; **75**: 808-15.
126 Charlton P, Faint R, Barnes C, Bent F, Folkes A, Templeton D, Mackie I, Machin S, Bevan P. XR5118, a novel modulator of plasminogen activator inhibitor-1 (PAI-1), increases endogenous tPA activity in the rat. *Fibrinolysis & Proteolysis*. 1997; **11**: 51-6.
127 Folkes A, Roe MB, Sohal S, Golec J, Faint R, Brooks T, Charlton P. Synthesis and in vitro evaluation of a series of diketopiperazine inhibitors of plasminogen activator inhibitor-1. *Bioorganic & Medicinal Chemistry Letters*. 2001; **11**: 2589-92.
128 Friederich PW, Levi M, Biemond BJ, Charlton P, Templeton D, vanZonneveld AJ, Bevan P, Pannekoek H, tenCate JW. Novel low-molecular-weight inhibitor of PAI-1 (XR5118) promotes endogenous fibrinolysis and reduces postthrombolysis thrombus growth in rabbits. *Circulation*. 1997; **96**: 916-21.
129 Bjorquist P, Ehnebom J, Inghardt T, Hansson L, Lindberg M, Linschoten M, Stromqvist M, Deinum J. Identification of the binding site for a low-molecular-weight inhibitor of plasminogen activator inhibitor type 1 by site-directed mutagenesis. *Biochemistry*. 1998; **37**: 1227-34.
130 Gils A, Stassen JM, Nar H, Kley JT, Wienen W, Ries UJ, Declerck PJ. Characterization and comparative evaluation of a novel PAI-1 inhibitor. *Thrombosis and Haemostasis*. 2002; **88**: 137-43.
131 Gils A, Declerck PJ. Modulation of plasminogen activator inhibitor 1 by Triton X-100 - Identification of two consecutive conformational transitions. *Thrombosis and Haemostasis*. 1998; **80**: 286-91.
132 Elokdah H, Abou-Gharbia M, Hennan JK, McFarlane G, Mugford CP, Krishnamurthy G, Crandall DL. Tiplaxtinin, a novel, orally efficacious inhibitor of plasminogen activator inhibitor-1: Design, synthesis, and preclinical characterization. *Journal of Medicinal Chemistry*. 2004; **47**: 3491-4.
133 Lijnen HR, Alessi MC, Frederix L, Collen D, Juhan-Vague I. Tiplaxtinin impairs nutritionally induced obesity in mice. *Thrombosis and Haemostasis*. 2006; **96**: 731-7.
134 Crandall DL, Quinet EM, Ayachi S, Hreha AL, Leik CE, Savio DA, Juhan-Vague I, Alessi MC. Modulation of adipose tissue development by pharmacological inhibition of PAI-1. *Arteriosclerosis Thrombosis and Vascular Biology*. 2006; **26**: 2209-15.
135 Baxi S, Crandall DL, Meier TR, Wrobleski S, Hawley A, Farris D, Elokdah H, Sigler R, Schaub RG, Wakefield T, Myers D. Dose-dependent thrombus resolution due to oral plaminogen activator inhibitor (PAI)-I inhibition with tiplaxtinin in a rat stenosis model of venous thrombosis. *Thrombosis and Haemostasis*. 2008; **99**: 749-58.

136 Rupin A, Gaertner R, Mennecier P, Richard I, Benoist A, De Nanteuil G, Verbeuren TJ. S35225 is a direct inhibitor of Plasminogen Activator Inhibitor type-1 activity in the blood. *Thrombosis Research*. 2008; **122**: 265-70.
137 Gorlatova NV, Tale JM, Elokdah H, Li DH, Fan's K, Warnock M, Crandall DL, Lawrence DA. Mechanism of inactivation of plasminogen activator inhibitor-1 by a small molecule inhibitory. *Journal of Biological Chemistry*. 2007; **282**: 9288-96.
138 Cale JM, Li SH, Warnock M, Su EJ, North PR, Sanders KL, Puscau MM, Emal CD, Lawrence DA. Characterization of a Novel Class of Polyphenolic Inhibitors of Plasminogen Activator Inhibitor-1. *Journal of Biological Chemistry*. 2010; **285**: 7892-902.
139 Izuhara Y, Yamaoka N, Kodama H, Dan T, Takizawa S, Hirayama N, Meguro K, de Strihou CV, Miyata T. A novel inhibitor of plasminogen activator inhibitor-1 provides antithrombotic benefits devoid of bleeding effect in nonhuman primates. *Journal of Cerebral Blood Flow and Metabolism*. 2010; **30**: 904-12.
140 Derosa G, D'Angelo A, Ragonesi PD, Ciccarelli L, Piccinni MN, Pricolo F, Salvadeo S, Montagna L, Gravina A, Ferrari I, Galli S, Paniga S, Cicero AFG. Effects of rosiglitazone and pioglitazone combined with metformin on the prothrombotic state of patients with type 2 diabetes mellitus and metabolic syndrome. *Journal of International Medical Research*. 2006; **34**: 545-55.
141 Fujii S, Sawa H, Sobel BE. Inhibition of endothelial cell expression of plasminogen activator inhibitor type-1 by gemfibrozil. *Thrombosis and Haemostasis*. 1993; **70**: 642-7.
142 Vinogradsky B, Bell SP, WoodcockMitchell J, Ohtani A, Fujii S. A new butadiene derivative, T-686, inhibits plasminogen activator inhibitor type-1 production in vitro by cultured human vascular endothelial cells and development of atherosclerotic lesions in vivo in rabbits. *Thrombosis Research*. 1997; **85**: 305-14.
143 Pawlowska Z, Chabielska E, Kobylanska A, Maciaszek A, Swiatkowska M, Buczko W, Stec WJ, Cierniewski CS. Regulation of PAI-1 concentration in platelets by systemic administration of antisense oligonucleotides to rats. *Thrombosis and Haemostasis*. 2001; **85**: 1086-9.
144 Jiang QS, Wang SQ. Design and screening of antisense oligodeoxynucleotides against PAI-1 mRNA in endothelial cells in vitro. *Acta Pharmacologica Sinica*. 2006; **27**: 1018-23.
145 Moldenhauer G. Selection strategies: Monoclonal antibodies. In: Dübel S, ed. *Handbook of Therapeutic Antibodies*. Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA, 2007.
146 Holliger P, Hudson PJ. Engineered antibody fragments and the rise of single domains. *Nature Biotechnology*. 2005; **23**: 1126-36.
147 Carter PJ. Potent antibody therapeutics by design. *Nature Reviews Immunology*. 2006; **6**: 343-57.
148 Kohler G, Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. 1975; **256**: 495-7.
149 Trebak M, Chong JM, Herlyn D, Speicher DW. Efficient laboratory-scale production of monoclonal antibodies using membrane-based high-density cell culture technology. *Journal of Immunological Methods*. 1999; **230**: 59-70.
150 Khazaeli MB, Conry RM, Lobuglio AF. Human immune-response to monoclonal antibodies. *Journal of Immunotherapy*. 1994; **15**: 42-52.
151 Carter P. Improving the efficacy of antibody-based cancer therapies. *Nature Reviews Cancer*. 2001; **1**: 118-29.
152 Reichert JM, Rosensweig CJ, Faden LB, Dewitz MC. Monoclonal antibody successes in the clinic. *Nature Biotechnology*. 2005; **23**: 1073-8.
153 Hoogenboom HR. Selecting and screening recombinant antibody libraries. *Nature Biotechnology*. 2005; **23**: 1105-16.
154 Lonberg N. Human antibodies from transgenic animals. *Nature Biotechnology*. 2005; **23**: 1117-25.

155 Nelson AL, Dhimolea E, Reichert JM. Development trends for human monoclonal antibody therapeutics. *Nature Reviews Drug Discovery*. 2010; **9**: 767-74.
156 Reich G. Pharmaceutical Formulation and Clinical Application. In: Dübel S, ed. *Handbook of Therapeutic Antibodies*. Weinheim: WYLEY-VCH Verlag GmbH & Co. KGaA, 2007.
157 Wang W, Wang EQ, Balthasar JP. Monoclonal Antibody Pharmacokinetics and Pharmacodynamics. *Clinical Pharmacology & Therapeutics*. 2008; **84**: 548-58.
158 Mestas J, Hughes CCW. Of mice and not men: Differences between mouse and human immunology. *Journal of Immunology*. 2004; **172**: 2731-8.
159 McMahon B, Kwaan HC. The Plasminogen Activator System and Cancer. *Pathophysiology of Haemostasis and Thrombosis*. 2007; **36**: 184-94.
160 Stefansson S, McMahon GA, Petitclerc E, Lawrence DA. Plasminogen activator inhibitor-1 in tumor growth, angiogenesis and vascular remodeling. *Current Pharmaceutical Design*. 2003; **9**: 1545-64.
161 Lijnen HR. Pleiotropic functions of plasminogen activator inhibitor-1. *Journal of Thrombosis and Haemostasis*. 2005; **3**: 35-45.
162 Gils A, Declerck PJ. Structure-function relationships in serpins: Current concepts and controversies. *Thrombosis and Haemostasis*. 1998; **80**: 531-41.
163 Kruithof EKO. Plasminogen activator inhibitor type 1: Biochemical, biological and clinical aspects. *Fibrinolysis*. 1988; **2**: 59-70.
164 Hekman CM, Loskutoff DJ. Endothelial cells produce a latent inhibitor of plamsinogen activators that can be activated by denaturants. *Journal of Biological Chemistry*. 1985; **260**: 1581-7.
165 Carmeliet P, Kieckens L, Schoonjans L, Ream B, Vannuffelen A, Prendergast G, Cole M, Bronson R, Collen D, Mulligan RC. Plasminogen activator inhibitor-1 gene deficient mice. I. Generation by homologeous recombination and characterization. *Journal of Clinical Investigation*. 1993; **92**: 2746-55.
166 Westrick RJ, Eitzman DT. Plasminogen activator inhibitor-1 in vascular thrombosis. *Current Drug Targets*. 2007; **8**: 996-1002.
167 Vaughan DE. PAI-1 and atherothrombosis. *Journal of Thrombosis and Haemostasis*. 2005; **3**: 1879-83.
168 Juhan-Vague I, Valadier J, Alessi M, Aillaud M, Ansaldi J, Philip Joet C, Holvoet P, Serradimigni A, Collen D. Deficient t-PA release and elevated PA inhibitor levels in patients with spontaneous or recurrent deep venous thrombosis. *Thrombosis and haemostasis*. 1987; **57**: 67-72.
169 Debrock S, Declerck PJ. Neutralization of plasminogen activator inhibitor-1 inhibitory properties: Identification of two different mechanisms. *Biochimica Et Biophysica Acta-Protein Structure and Molecular Enzymology*. 1997; **1337**: 257-66.
170 Declerck PJ, Alessi MC, Verstreken M, Kruithof EKO, Juhanvague I, Collen D. Measurement of plasminogen activator inhibitor-1 in biological fluids with a murine monoclonal antibody-based enzyme-linked immunosorbent assay. *Blood*. 1988; **71**: 220-5.
171 Berry CN, Lunven C, Lechaire I, Girardot C, O'Connor SE. Antithrombotic activity of a monoclonal antibody inducing the substrate form of plasminogen activator inhibitor type 1 in rat models of venous and arterial thrombosis. *British Journal of Pharmacology*. 1998; **125**: 29-34.
172 Hansen M, Busse MN, Andreasen PA. Importance of the amino-acid composition of the shutter region of plasminogen activator inhibitor-1 for its transitions to latent and substrate forms. *European Journal of Biochemistry*. 2001; **268**: 6274-83.
173 Galfré G, Milstein C. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol*. 1981; **73**: 3-46.

174 Gils A, Alessi MC, Brouwers E, Peeters M, Marx P, Leurs J, Bouma B, Hendriks D, Juhan-Vague I, Declerck PJ. Development of a genotype 325-specific proCPU/TAFI ELISA. *Arteriosclerosis Thrombosis and Vascular Biology*. 2003; **23**: 1122-7.
175 Verheijen JH, Chang GTG, Kluft C. Evidence for the occurence of a fast-acting inhibitor for tissue-type plasminogen activator in human plasma. *Thrombosis and Haemostasis*. 1984; **51**: 392-5.
176 Verbeke K, Gils A, Declerck PJ. Inhibition of plasminogen activator inhibitor-1: antibody fragments and their unique sequences as a tool for the development of profibrinolytic drugs. *Journal of Thrombosis and Haemostasis*. 2004; **2**: 298-305.
177 Scroyen I, Jacobs F, Cosemans L, De Geest B, Lijnen HR. Effect of plasminogen activator inhibitor-1 on adipogenesis in vivo. *Thrombosis and Haemostasis*. 2009; **101**: 388-93.
178 Gils A, Ceresa E, Macovei AM, Marx PF, Peeters M, Compernolle G, Declerck PJ. Modulation of TAFI function through different pathways - implications for the development of TAFI inhibitors. *Journal of Thrombosis and Haemostasis*. 2005; **3**: 2745-53.
179 Dewilde M, Van De Craen B, Compernolle G, Madsen JB, Strelkov S, Gils A, Declerck PJ. Subtle structural differences between human and mouse PAI-1 reveal the basis for biochemical differences. *Journal of Structural Biology*. 2010; **171**: 95-101.
180 Dewilde M, Van De Craen B, Compernolle G, Madsen JB, Strelkov S, Gils A, Declerck PJ. Subtle structural differences between human and mouse PAI-1 reveal the basis for biochemical differences. *Journal of Structural Biology*. 2010.
181 Hillmayer K, Vancraenenbroeck R, De Maeyer M, Compernolle G, Declerck PJ, Gils A. Discovery of novel mechanisms and molecular targets for the inhibition of activated thrombin activatable fibrinolysis inhibitor. *Journal of Thrombosis and Haemostasis*. 2008; **6**: 1892-9.
182 Ngo TH, Verheyen S, Knockaert I, Declerck PJ. Monoclonal antibody-based immunoassays for the specific quantitation of rat PAI-1 antigen and activity in biological samples. *Thrombosis and Haemostasis*. 1998; **79**: 808-12.
183 Scroyen I, Christiaens V, Lijnen HR. No functional role of plasminogen activator inhibitor-1 in murine adipogenesis or adipocyte differentiation. *Journal of Thrombosis and Haemostasis*. 2007; **5**: 139-45.
184 Gils A, Meissenheimer LM, Compernolle G, Declerck PJ. Species-dependent molecular drug targets in plasminogen activator inhibitor-1 (PAI-1). *Thrombosis and Haemostasis*. 2009; **102**: 609-10.
185 Cale JM, Li S-H, Warnock M, Su EJ, North PR, Sanders KL, Puscau MM, Emal CD, Lawrence DA. Characterization of a novel class of polyphenolic inhibitors of plasminogen activator inhibitor-1. *Journal of Biological Chemistry*. 2010.
186 Helenius A, Aebi M. Intracellular functions of N-linked glycans. *Science*. 2001; **291**: 2364-9.
187 Serrano R, Barrenetxe J, Orbe J, Rodriguez JA, Gallardo N, Martinez C, Andres A, Paramo JA. Tissue-specific PAI-1 gene expression and glycosylation pattern in insulin-resistant old rats. *American Journal of Physiology-Regulatory Integrative and Comparative Physiology*. 2009; **297**: R1563-R9.
188 Brogren H, Sihlbom C, Wallmark K, Lonn M, Deinum J, Karlsson L, Jern S. Heterogeneous glycosylation patterns of human PAI-1 may reveal its cellular origin. *Thrombosis Research*. 2008; **122**: 271-81.
189 Wiman B, Almquist A, Sigurdardottir O, Lindahl T. Plasminogen activator inhibitor-1 (PAI) is bound to vitronectin in plasma. *Febs Letters*. 1988; **242**: 125-8.
190 Egelund R, Einholm AP, Pedersen KE, Nielsen RW, Christensen A, Deinum J, Andreasen PA. A regulatory hydrophobic area in the flexible joint region of plasminogen activator

inhibitor-1, defined with fluorescent activity-neutralizing ligands - Ligand-induced serpin polymerization. *Journal of Biological Chemistry*. 2001; **276**: 13077-86.

191 Jensen JK, Durand MKV, Skeldal S, Dupont DM, Bodker JS, Wind T, Andreasen PA. Construction of a plasminogen activator inhibitor-1 variant without measurable affinity to vitronectin but otherwise normal. *Febs Letters*. 2004; **556**: 175-9.

192 Ngo TH, Declerck PJ. Suppression of plasminogen activator inhibitor 1 (PAI-1) activity levels in rats by monoclonal antibodies. *Fibrinolysis & Proteolysis*. 1998; **12**: 335-9.

193 Kjoller L, Martensen PM, SottrupJensen L, Justesen J, Rodenburg KW, Andreasen PA. Conformational changes of the reactive-centre loop and beta-strand 5A accompany temperature-dependent inhibitor-substrate transition of plasminogen-activator inhibitor 1. *European Journal of Biochemistry*. 1996; **241**: 38-46.

194 Alessi MC, Declerck PJ, Demol M, Nelles L, Collen D. Purification and characterization of natural and recombinant human plasminogen activator inhibitor-1 (PAI-1). *European Journal of Biochemistry*. 1988; **175**: 531-40.

195 Rodenburg KW, Kjoller L, Petersen HH, Andreasen PA. Binding of urokinase-type plasminogen activator plasminogen activator inhibitor-1 complex to the endocytosis receptors alpha(2)-macroglobulin receptor low-density lipoprotein receptor-related protein and very-low-density lipoprotein receptor involves basic residues in the inhibitor. *Biochemical Journal*. 1998; **329**: 55-63.

196 Gils A, Knockaert I, Declerck PJ. Substrate behavior of plasminogen activator inhibitor-1 is not associated with a lack of insertion of the reactive site loop. *Biochemistry*. 1996; **35**: 7474-81.

197 Gils A, Knockaert I, Brouwers E, Declerck PJ. Glycosylation dependent conformational transitions in plasminogen activator inhihitor-1: evidence for the presence of two active conformations. *Fibrinolysis & Proteolysis*. 2000; **14**: 58-64.

198 Andreasen PA, Egelund R, Jensen S, Rodenburg KW. Solvent effects on activity and conformation of plasminogen activator inhibitor-1. *Thrombosis and Haemostasis*. 1999; **81**: 407-14.

199 Hennan JK, Morgan GA, Swillo RE, Antrilli TM, Mugford C, Vlasuk GP, Gardell SJ, Crandall DL. Effect of tiplaxtinin (PAI-039), an orally bioavailable PAI-1 antagonist, in a rat model of thrombosis. *Journal of Thrombosis and Haemostasis*. 2008; **6**: 1558-64.

200 Ngo TH, Bijnens AP, Knockaert I, Declerck PJ. Expression, purification, and characterization of recombinant rat plasminogen activator inhibitor-1. *Fibrinolysis & Proteolysis*. 1997; **11**: 37-43.

201 Javed R, Yarimizu K, Pelletier N, Li C, Knowles AF. Mutagenesis of lysine 62, asparagine 64, and conserved region 1 reduces the activity of human ecto-ATPase (NTPDase 2). *Biochemistry*. 2007; **46**: 6617-27.

202 Kern A, Agoulnik AI, Bryant-Greenwood GD. The low-density lipoprotein class a module of the relaxin receptor (leucine-rich repeat containing G-protein coupled receptor 7): Its role in signaling and trafficking to the cell membrane. *Endocrinology*. 2007; **148**: 1181-94.

203 Tansky MF, Pothoulakis C, Leeman SE. Functional consequences of alteration of N-linked glycosylation sites on the neurokinin 1 receptor. *Proceedings of the National Academy of Sciences of the United States of America*. 2007; **104**: 10691-6.

204 Levin EG. Quantitation and properties of the active and latent plasminogen activator inhibitors in cultures of human endothelial cells. *Blood*. 1986; **67**: 1309-13.

205 Vleugels N, Leys J, Knockaert I, Declerck PJ. Effect of stabilizing versus destabilizing interactions on plasminogen activator inhibitor-1. *Thrombosis and Haemostasis*. 2000; **84**: 871-5.

206 Debrock S, Declerck PJ. Characterization of common neoantigenic epitopes generated in plasminogen activator inhibitor-1 after cleavage of the reactive center loop or after complex formation with various serine proteinases. *Febs Letters*. 1995; **376**: 243-6.
207 Bijnens AP, Ngo TH, Gils A, Dewaele J, Knockaert I, Stassen JM, Declerck PJ. Elucidation of the binding regions of PAI-1 neutralizing antibodies using chimeric variants of human and rat PAI-1. *Thrombosis and Haemostasis*. 2001; **85**: 866-74.
208 Furie B, Furie BC. Mechanisms of disease: Mechanisms of thrombus formation. *New England Journal of Medicine*. 2008; **359**: 938-49.
209 Collen D. The plasminogen (fibrinolytic) system. *Thrombosis and Haemostasis*. 1999; **82**: 259-70.
210 Kunadian V, Gibson M. Thrombolytics and myocardial infarction. *Cardiovascular Therapeutics*. 2010; **00**: 1-8.
211 Longstaff C, Thelwell C. Understanding the enzymology of fibrinolysis and improving thrombolytic therapy. *Febs Letters*. 2005; **579**: 3303-9.
212 Collen D, Lijnen HR. Thrombolytic agents. *Thrombosis and Haemostasis*. 2005; **93**: 627-30.
213 Xu Z, Balsara RD, Gorlatova NV, Lawrence DA, Castellino FJ, Ploplis VA. Conservation of critical functional domains in murine plasminogen activator inhibitor-1. *Journal of Biological Chemistry*. 2004; **279**: 17914-20.
214 Li HJ, d'Anjou M. Pharmacological significance of glycosylation in therapeutic proteins. *Current Opinion in Biotechnology*. 2009; **20**: 678-84.
215 Baker M. Upping the ante on antibodies. *Nature Biotechnology*. 2005; **23**: 1065-72.
216 Muyldermans S. Single domain camel antibodies: current status. *Reviews in Molecular Biotechnology*. 2001; **74**: 277-302.
217 Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. *Nature*. 1995; **374**: 168-73.
218 Vincke C, Loris R, Saerens D, Martinez-Rodriguez S, Muyldermans S, Conrath K. General Strategy to Humanize a Camelid Single-domain Antibody and Identification of a Universal Humanized Nanobody Scaffold. *Journal of Biological Chemistry*. 2009; **284**: 3273-84.
219 Liang A, Wu F, Tran K, Jones SW, Deng G, Ye B, Zhao ZC, Snider RM, Dole WP, Morser J, Wu QY. Characterization of a small molecule PAI-1 inhibitor, ZK4044. *Thrombosis Research*. 2005; **115**: 341-50.
220 Crandall DL, Herman JK, Elokdah H, Krishnamurthy G, Antrilli TM, Bauer JS, Morgan GA, Swillo RE. WAY-140312 reduces plasma PAI-1 while maintaining normal platelet aggregation. *Biochemical and Biophysical Research Communications*. 2003; **311**: 904-8.
221 Carrell RW, Stein PE. The biostructural pathology of the serpins: Critical function of sheet opening mechanism. *Biological Chemistry Hoppe-Seyler*. 1996; **377**: 1-17.

Curriculum Vitae

Britt Van De Craen was born on the 14th of February 1983 in Turnhout, Belgium. She graduated from Koninklijk Atheneum Mol in 2001. Following high school graduation she enrolled at the Faculty of Pharmaceutical Sciences of the Katholieke Universiteit Leuven, where she achieved the degree of Master in Pharmaceutical Sciences in 2006. In the same academic year she carried out a research project in the Laboratory for Cell Metabolism entitled '*Initial characterization of mice with a muscle specific knock-out of Pex5'.*

In 2006 she initiated here PhD project in the Laboratory for Pharmaceutical Biology at the Faculty of Pharmaceutical Sciences of the Katholieke Universiteit Leuven with Prof. Ann Gils as promotor and Prof. Paul J. Declerck as co-promotor. Between 2007 and 2011 she was research assistant of the Research Foundation-Flanders (FWO-Vlaanderen).