

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

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Abstract Plasminogen activator inhibitor-1 (PAI-1), an important risk factor for thrombotic diseases, is a member of the superfamily of serine proteinase inhibitors. To define structural rearrangements occurring during interaction between PAI-1 and its target proteinases we have raised monoclonal antibodies against the PAI-1/t-PA complex. Thirteen out of 401 monoclonal antibodies reacted preferentially with the PAI-1/t-PA complex as compared to free PAI-1 or free t-PA. Detailed characterization revealed the presence of two non-overlapping neoantigenic epitopes in the PAI-1/t-PA complex. Both neoantigenic epitopes were also exposed after complex formation between PAI-1 and either urokinase-type plasminogen activator, plasmin or thrombin as well as after cleavage of the reactive site loop of non-inhibitory substrate type PAI-1 variants. Thus, we have identified two neoantigenic epitopes, localized entirely in PAI-1, and commonly exposed after complex formation of active PAI-1 with various proteinases or after cleavage of substrate PAI-1. These monoclonal antibodies should facilitate further studies on the mechanism of interaction between various PAI-1 forms and its target proteinases.

Key words: Monoclonal antibody; Plasminogen activator inhibitor; PAI-1; Neoantigenic; Serpin

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1) is the main physiological inhibitor of tissue-type plasminogen activator (t-PA) in plasma [1]. Elevated levels of PAI-1 in plasma are associated with an increased risk of thromboembolism suggesting a critical role of PAI-1 in the *in vivo* regulation of fibrinolysis [2]. PAI-1 is a single-chain glycoprotein and is a member of the serine proteinase inhibitor (serpin) family [3–6]. PAI-1 inhibits both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) with second-order rate constants of $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ [7]. In addition, complex formation of PAI-1 with plasmin and thrombin has also been reported [8]. PAI-1 exhibits a unique conformational flexibility. It is synthesised as

an active form that spontaneously converts to a latent form that can be partially reactivated by denaturing agents such as sodium dodecyl sulfate, guanidinium chloride and urea [9]. Recently, a third conformation reacting as a non-inhibitory substrate has been identified [10].

The reactive site loop of serpins comprises 20 to 30 amino acids (P_{17} to P_{10}') at the carboxy terminal side of the protein. The bait peptide bond (P_1-P_1') in PAI-1 corresponds to the Arg³⁴⁶–Met³⁴⁷ bond [11]. Active serpins inhibit their target proteinases by formation of a 1:1 stoichiometric reversible complex followed by a covalent binding between the hydroxyl group of the active-site serine residue of the proteinase and the carboxyl group of the P_1 residue at the reactive center of the serpin [12]. Thus interaction between active PAI-1 and its target proteinases results in the formation of a stable covalent complex. Recent crystallographic data [13] have suggested that in latent PAI-1, the bait region and secondary binding sites are not accessible for interaction with its target proteinases. In contrast, the inactive substrate form reacts with its target proteinase resulting in a cleavage of the P_1-P_1' bond without formation of a stable complex [10,14]. Molecular details of the conformational differences responsible for the functional diversity of PAI-1 are not yet identified.

In the current study, a large panel of monoclonal antibodies was generated against the PAI-1/t-PA complex. Two neoantigenic epitopes could be identified in the PAI-1/t-PA complex that were also exposed in PAI-1 complexed with other proteinases as well as in cleaved PAI-1 generated from substrate PAI-1.

2. Materials and methods

2.1. Materials

96-well polystyrene microtiterplates were purchased from Costar (Cambridge, MA), RAM-IgG-HRP was from Nordic (Tilburg, The Netherlands). Dulbecco's modified Eagle's medium was from ICN Pharmaceuticals Inc. (Costa Mesa, CA). t-PA was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Urokinase-type plasminogen activator (u-PA) was kindly provided by Bournonville Pharma (Braine-l'Alleud, Belgium). Plasmin and thrombin were kind gifts from Dr. R. Lijnen (University of Leuven, Belgium). All other chemicals and reagents were of the highest quality available.

2.2. Preparation of recombinant proteins and of the purified plasminogen activator inhibitor-1/tissue-type plasminogen activator complex

Recombinant wt-PAI-1 and PAI-1 mutants were expressed in *E. coli* and purified as described previously [14]. PAI-1 activity was measured by the method described by Verheijen et al. [15] or by an immunofunctional method [16]. t-PA was calibrated versus the international reference preparation for t-PA (NIBSC 86/670; obtained from the National Institute for Biological Standards and Controls, London, UK). PAI-1

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Abbreviations: PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator; ELISA, enzyme linked immunosorbent assay; PBS, phosphate buffered saline; serpin, serine proteinase inhibitor; RAM, rabbit anti-mouse; HRP, horseradish peroxidase.

antigen was determined by ELISA [17] or spectrophotometrically at 280 nm using an absorbance coefficient $A_{1\%}^{1\text{cm}}$ of 10.

The PAI-1/t-PA complex used for immunization and selection of hybridomas was obtained as follows. Purified PAI-1 (~500000 U) was incubated with t-PA (~400000 U) for 25 min at 37°C. This mixture was then applied on a Sepharose 4B column to which a monoclonal antibody against t-PA (MA-62E8) was coupled. Bound antigen (i.e. PAI-1/t-PA complex) was eluted with 3 M KSCN. PAI-1/t-PA containing fractions were pooled and dialyzed against Tris 50 mM/Arginine 250 mM, pH 8.3. Concentration of PAI-1/t-PA complex was determined as described previously [16]. The purity of the preparations was assessed by SDS-PAGE using 10–15% gradient gels under non-reducing conditions with the Pharmacia Phast System (Uppsala, Sweden). Proteins were visualized by staining with Coomassie brilliant blue.

2.3. Preparation of enzyme–PAI-1 complexes and of enzyme-cleaved PAI-1

Complexes of PAI-1 with t-PA and u-PA were prepared by incubation (25 min at 37°C) of purified PAI-1 with a 2-fold molar excess of t-PA or u-PA, respectively. PAI-1/plasmin and PAI-1/thrombin complexes were prepared by incubation of PAI-1 for 2 h at 37°C with a 2-fold molar excess of plasmin and thrombin, respectively.

Cleaved PAI-1 (at P_1 – P_1') was prepared by incubation of the non-inhibitory PAI-1 mutants, PAI-1–A335P (alanine at position P_{12} substituted by a proline) and PAI-1–T333P (threonine at position P_{14} substituted by a proline), both exhibiting substrate characteristics [14,18], with a 2-fold molar excess of t-PA or u-PA for 25 min at 37°C.

Formation of complexes and cleavage products was verified by SDS-PAGE followed by staining with Coomassie brilliant blue.

2.4. Preparation of monoclonal antibodies against PAI-1/t-PA

Monoclonal antibodies against the purified PAI-1/t-PA complex were produced by the method of Galfré and Milstein [19]. PAI-1^{-/-} deficient mice [20] were immunized by subcutaneous injection of 10 µg PAI-1/t-PA complex in complete Freund's adjuvant, which was followed 2 weeks later by intraperitoneal injection of 10 µg PAI-1/t-PA complex in incomplete Freund's adjuvant. After an interval of at least 6 weeks, the mice were boosted intraperitoneally with 10 µg PAI-1/t-PA in saline on days 4 and 2 before the cell fusion. Spleen cells were isolated and fused with Sp2/0–Ag14 myeloma cells according to Fazekas de St Groth and Scheidegger [21]. After selection in hypoxanthine, aminopterin, thymidine medium, culture supernatants were screened for specific antibody production by ELISA, using microtiterplates coated with PAI-1/t-PA. The bound immunoglobulins were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (RAM-HRP). Supernatants yielding a positive response were subjected to a second screening (4-fold dilutions ranging from 1:10 to 1:10240) using microtiterplates coated with either PAI-1 or t-PA. Positive clones were used for the production of ascitic fluid in pristane-primed BALB/c mice [22]. The IgG fraction of the monoclonal antibodies was purified from ascites by affinity chromatography on protein A-sepharose [23]. HRP-conjugated monoclonal antibodies were produced as described by Nakane and Kawaoi [24].

2.5. Determination of overlapping epitopes

The occurrence of overlapping epitopes in a selected panel of monoclonal antibodies ($n = 13$, see Discussion) was evaluated as follows. Microtiterplates were coated with the monoclonal antibodies and subsequently blocked with bovine serum albumin [17]. Samples (containing PAI-1/t-PA) were diluted in PBS containing Tween 80 (0.002%), EDTA (5 mM) and bovine serum albumin (1 g/l) (dilution buffer), and added to the wells. After incubation for 18 h at 4°C in a moist chamber, the wells were emptied and washed with PBS/Tween. Subsequently, the capacity to bind simultaneously another monoclonal antibody was evaluated by incubation (2 h at room temperature) of the wells with various HRP-conjugated monoclonal antibodies (1:4000). After washing the plates, 160 µl aliquots of a 0.1 M citrate, 0.2 M sodium phosphate buffer, pH 5.0, containing 300 µg/ml *o*-phenylenediamine and 0.003% hydrogen peroxide were added. After 30 to 60 min at room temperature the peroxidase reaction was stopped with 50 µl of 4 M H_2SO_4 . The absorbance was measured at 492 nm with a multiscan spectrophotometer (Spectra III/SLT, Salzburg, Austria). Binding of a conjugated antibody, as evidenced by a positive response in the above-mentioned procedure, indicates that the coated and the conjugated

antibody recognize a different epitope. Lack of binding indicates that both antibodies are directed against the same epitope or that a significant portion of the epitopes overlap. Each antibody was evaluated as coating antibody in combination with all antibodies conjugated to HRP ($n = 13 \times 13$).

2.6. Detection of neoantigenic epitopes in various other PAI-1 derivatives

The occurrence of common neoantigenic epitopes in PAI-1 derivatives other than the purified PAI-1/t-PA complex initially used for generation and screening of the antibodies was studied as follows. Two antibodies, i.e. MA-13C1 and MA-14D5-HRP, each representing a different neoantigenic epitope (see Results) in PAI-1/t-PA, were used to construct an ELISA as described above. Subsequently, the different PAI-1-related preparations were analyzed in various concentrations ranging from 500 ng/ml to 0.3 ng/ml and using serial 2-fold dilutions. Comparison of the reactivity of the PAI-1 derivatives studied in this ELISA then revealed either the presence or the absence of neoantigenic epitopes exposed in the various PAI-1 derivatives.

3. Results and discussion

3.1. Generation of monoclonal antibodies against the PAI-1/t-PA complex and detection of neoantigenic epitopes

Out of two fusions, 401 positive hybridomas were obtained producing a monoclonal antibody reactive with the PAI-1/t-PA complex. 285 and 103 clones reacted equally well with the PAI-1/t-PA complex and with t-PA or PAI-1, respectively, whereas 13 reacted strongly with the PAI-1/t-PA complex, not with free t-PA and only weakly with free PAI-1. Fig. 1 shows the dose–response curves for two monoclonal antibodies (e.g. MA-13C1 and MA-14D5) exhibiting a strong reactivity towards the complex, whereas their reactivity with free PAI-1 is 50 to 250-fold lower and no reactivity with free t-PA could be observed. These 13 monoclonal antibodies directed against a neoantigenic epitope were purified from ascites and were further subjected to a detailed epitope mapping as described in the methods. The data clearly revealed the presence of at least two non-overlapping, neoantigenic epitopes.

The results demonstrate that in the PAI-1/t-PA complex epitopes are present which were not previously occurring in the free reactants. These neoepitopes may have arisen in the PAI-1/t-PA complex consequent to (a) a combination (either three-dimensional or linear) of amino acid residues originating from PAI-1 and t-PA or (b) a rearrangement in either PAI-1 or t-PA resulting in the exposure of regions previously undetectable in either one of the free moieties. However, the monoclonal antibodies currently selected did not react with t-PA but did react

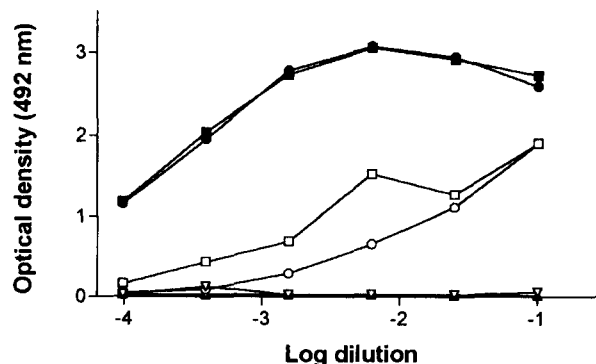


Fig. 1. Reactivity of culture supernatants (dilutions ranging from 1:10 to 1:10240) of MA-13C1 (■, □, ▽) and MA-14D5 (●, ○, △) towards various PAI-1 derivatives: PAI-1/t-PA (■, ●); free PAI-1 (□, ○) and free t-PA (▽, △).

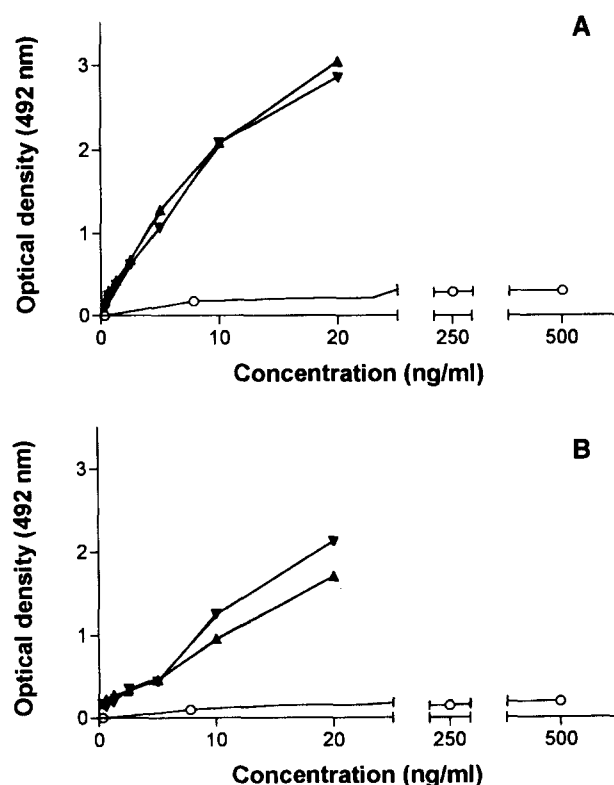


Fig. 2. Exposure of neoantigenic epitopes in PAI-1 complexed to various serine proteinases. Dose-response curves in the MA-13C1/MA-14D5-HRP ELISA are shown for free PAI-1 (○) and (A) PAI-1 complexed with t-PA (▲) or u-PA (▼); and (B) PAI-1 complexed with plasmin (▲) or thrombin (▼).

weakly with free PAI-1. The latter observation is suggestive for a specific rearrangement that would have occurred in PAI-1.

3.2. Detection of common neoantigenic epitopes in various PAI-1 derivatives

Two monoclonal antibodies (i.e. MA-13C1 and MA-14D5) each representing a distinct neoantigenic epitope, were used as capture and tagging antibody, respectively, to evaluate whether these neoantigenic epitopes might occur in various other PAI-1 derivatives.

Fig. 2 shows typical dose-response curves for free PAI-1 and for PAI-1/t-PA, PAI-1/u-PA, PAI-1/plasmin and PAI-1/thrombin complexes in the MA-13C1/MA-14D5-HRP ELISA. From these data it appears that all complexed forms of PAI-1 exhibit an increased reactivity compared to free PAI-1 (>160- to >200-fold). The similarly enhanced reactivity towards complexed PAI-1 irrespective of the serine proteinase used substantiates the hypothesis that the neoantigenic epitopes are located entirely in the PAI-1-moiety of the complex, originating from the interaction between PAI-1 and its target proteinases, i.e. t-PA, u-PA, plasmin or thrombin.

Dose-response curves before and after incubation (with t-PA or u-PA) of the inactive variants PAI-1-A335P and PAI-1-T333P, both exerting a substrate behaviour [14,18], revealed that these neoantigenic epitopes are also generated after cleavage (at the P_1 - P_1' bond) of the inactive substrate PAI-1 form. PAI-1-T333P cleaved with t-PA was 102 ± 23 ($n = 7$) times more reactive compared to intact PAI-1-T333P; cleavage with

u-PA resulted in an increased reactivity of 184 ± 48 ($n = 7$) fold. PAI-1-A335P cleaved with t-PA or u-PA was 43 ± 17 ($n = 9$) and 101 ± 54 ($n = 8$), respectively, more reactive as compared to intact PAI-1-A335P.

These findings confirm that the monoclonal antibodies represent two newly exposed epitopes that are exclusively located in the PAI-1 moiety of the reaction product formed between PAI-1, either reacting as an inhibitor or as a substrate, and the serine proteinases. It is important to note that a weak but significant reactivity is also observed for the intact substrate PAI-1 variants, indicating that the epitopes, covered by these 2 monoclonal antibodies, are partially expressed in the intact PAI-1 mutants. Most likely the distortion of the reactive site loop induced by the introduction of a proline results in a partial exposure of these epitopes.

Similar studies have been performed with the serpins antithrombin III [25–27] and C1-inhibitor [28–30]. Asakura et al. reported the preparation and characterization of five monoclonal antibodies against neoantigenic epitopes in the thrombin/antithrombin III complex out of 18 antibodies directed against the complex. One out of these five monoclonal antibodies was found to interfere with the inhibition of thrombin by antithrombin III. However, in our current study, none of the monoclonal antibodies against the neoantigenic epitopes did affect the functional behaviour of PAI-1 i.e. more than 80% of the PAI-1 activity was recovered after incubation of PAI-1 with a ten-fold molar excess of monoclonal antibody. This indicates that the epitopes recognized by the antibodies are not located in the near vicinity of the active site residues Arg³⁴⁶-Met³⁴⁷ of PAI-1. de Agostini et al. [28,29] nor Nuijens et al. [30] provided information on the functional effects of their monoclonal antibodies.

From our current data it is clear that the two distinct neoantigenic epitopes are entirely located in PAI-1 and become accessible only after conformational changes specifically induced by interaction with its target proteinases. None of the 13 monoclonal antibodies can detect the PAI-1/t-PA complex or cleaved PAI-1 in Western blots (data not shown) strongly suggesting that the neoantigenic epitopes are discontinuous epitopes [31] generated by the assembly of residues that were not adjacent in free intact PAI-1.

It is well-known that covalent complex conformation between serpins and their target proteinases is associated with a cleavage of the bait peptide bond P_1 - P_1' [32–34]. Therefore, in view of the observation that both neoantigenic epitopes are exposed in complexed as well as in cleaved PAI-1, it is most likely that cleavage of the P_1 - P_1' bond is a major event responsible for the generation of these epitopes. Recent structural studies on PAI-1 have shown that in cleaved PAI-1 [35] the aminoterminal portion of the reactive site loop (i.e. P_{16} - P_1) forms a new β -strand in s4A by insertion into β -sheet A. This particular feature is similar to that observed in latent PAI-1 [13]. Since the antibodies react with cleaved but not with latent PAI-1 it can be concluded that rearrangement of P_{16} - P_1 in PAI-1 does not play an important role, if any, for the generation of the neoepitopes. This is in contrast to the observation of Asakura et al. [27] identifying a neoantigenic epitope, in the thrombin/antithrombin III complex, mainly composed of the P_{12} - P_8 region located at the NH_2 -terminal part of the cleavage site of antithrombin III. However, one of the major differences between cleaved PAI-1 and intact latent PAI-1 is the formation of a new β -strand s1C involving P_6' to P_{13}' of the reactive site

loop [35] indicating that this region might contribute significantly to the differential reactivity of our antibodies. This hypothesis is also compatible with the observation that these antibodies do not interfere with PAI-1 activity whereas those reported by Asakura et al. [26,27] do neutralize antithrombin III activity.

In conclusion, we succeeded to raise and identify monoclonal antibodies against two distinct neoantigenic epitopes in the PAI-1/t-PA complex. These epitopes were found to be localized entirely in the PAI-1 moiety and evidence was obtained that the conformational rearrangements in the C-terminal portion of the reactive site loop contribute significantly to their exposure. Such antibodies have not been described for PAI-1 yet and comparison with data for other serpins shows that the current neoantigenic epitopes are most likely located in different regions than those reported for other serpins.

These monoclonal antibodies may constitute useful tools for further studies on the conformational and functional flexibility of PAI-1 and on the mechanism of interaction between various PAI-1 forms and its target proteinases.

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