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*

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The serpin plasminogen activator inhibitor-1 (PAI-1) has a dual function: 1) it plays an important role as a direct inhibitor of the plasminogen activation system, and 2) its interaction with the adhesive glycoprotein vitronectin suggests a role in tissue remodeling and metastasis, independent from its proteinase inhibitory properties. Unique to this serpin is the close association between its conformational and functional properties. Indeed, PAI-1 can occur in an active and a latent conformation, but both functions are exclusively present in the active conformation. We report here the epitope localization and functional effects of a monoclonal antibody (MA-124K1) that inhibits rat PAI-1 activity and simultaneously increases the binding of inactive PAI-1 to vitronectin (the affinity constant of PAI-1 for vitronectin is $2 \times 10^7$ M$^{-1}$ in the absence of MA-124K1 and $160 \times 10^7$ M$^{-1}$ in the presence of MA-124K1). To the best of our knowledge, this is the first monoclonal antibody dissociating the proteinase inhibitory properties from the vitronectin binding properties in PAI-1. Mutation of Glu$^{212}$ and/or Glu$^{220}$ in rat PAI-1 to Ala results in a strongly reduced affinity or absence of binding to MA-124K1. The three-dimensional structure of PAI-1 reveals that these residues constitute a conformational epitope close to the reactive-site loop and compatible with the effect of MA-124K1 on the inhibitory properties of PAI-1. However, the vitronectin binding site is localized at the opposite site of the molecule, indicating that the effect of MA-124K1 involves an allosteric modulation of the vitronectin binding site. Cell culture experiments revealed a significant reduction of cell attachment and migration in the presence of MA-124K1, providing evidence for the functional relevance of this antibody-mediated up-regulation of the vitronectin binding properties of PAI-1. In conclusion, a novel mechanism for interference with PAI-1 functions has been identified and is of importance in the modulation of cell migration and related events (e.g. tumor metastasis).

The plasminogen/plasmin system plays an important role in the hemostatic balance through dissolution of fibrin and in a variety of pericellular events, including cell migration and angiogenesis, through activation of matrix metalloproteinases, cytokines, and growth factors (1, 2). Plasminogen activator inhibitor-1 (PAI-1)$^1$ is the primary inhibitor of tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Because of its antiproteinolytic properties, elevated levels of PAI-1 are associated with several thrombotic diseases such as myocardial infarction, deep vein thrombosis, and obesity (3, 4). In addition, PAI-1 is generally assumed to play a role in tissue remodeling and tumor metastasis (5). Importantly, its proteinase inhibitory role at the extracellular matrix is further stressed by the observation that the active form of PAI-1 is bound to and stabilized by vitronectin, a major constituent of the extracellular matrix, thereby resulting in a mechanism allowing directed control of proteolytic events in the extracellular environment.

Vitronectin belongs to the group of adhesive glycoproteins playing an important role in the attachment of cells to their surrounding matrix and may participate in the regulation of cell differentiation, proliferation, and morphogenesis. Its cell binding properties are mediated through interactions between cellular integrins and the arginine-glycine-aspartic acid (RGD) sequence in the N-terminal region of vitronectin (6). The vitronectin binding properties of PAI-1 have reinforced the role of PAI-1 in these pericellular processes and extended its role beyond its proteinase inhibitory properties. Indeed, PAI-1 has been shown to inhibit cell migration through interference with the binding of the integrin $\alpha_i\beta_j$ to vitronectin (7). This interference is most likely due to the fact that the PAI-1 binding region in vitronectin is also located in the N-terminal region (8, 9). Thus, PAI-1 plays a dual role in extravascular processes, on the one hand via its capacity to inhibit plasminogen activators, and on the other hand via its capacity to prevent cell adhesion. Currently, the relative contribution of both functions to the effects of PAI-1 in tumor development, metastasis, and angiogenesis remains elusive. Whereas the prevention of tumor invasion and neovascularization due to the absence of host PAI-1 have been suggested to be due to an excess of proteolytic activity (10), high levels of PAI-1 have been suggested to prevent cell migration (and subsequent metastasis) consequent to its vitronectin binding properties (7, 8). Whereas its proteinase inhibitory properties reside in the reactive-site loop, the vitronectin binding properties have been suggested to reside on the opposite side of the molecule (i.e. a region comprising $\alpha hC$,

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$^1$ The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; t-PA, tissue-type plasminogen activator; u-PA, urokinase-type plasminogen activator.
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ohE, and β1A). In active PAI-1, both properties are present, whereas in latent PAI-1, both properties are absent consequent to conformational changes.

In the present study, we present a monoclonal antibody (MA-124K1) that can inhibit rat PAI-1 activity and simultaneously increase the binding of inactivated PAI-1 to vitronectin. elucidation of the exact binding site (epitope) for MA-124K1 is indispensable to unravel the molecular mechanism of its functional effects on PAI-1 and reveals how the two functions can be dissociated from each other and can be modulated in the opposite direction. The obtained data illustrate the feasibility of an allosteric up-regulation of the vitronectin binding properties of PAI-1.

EXPERIMENTAL PROCEDURES

Materials—Recombinant rat PAI-1, human PAI-1, and rat/human PAI-1 chimeras were produced as described previously (11–13). Human, rat, and murine vitronectin were purchased from Molecular Innovations. Human t-PA (predominantly in the single-chain form) was a kind gift from Boehringer Ingelheim (Brussels, Belgium), and human u-PA (consisting of a mixture of high and low molecular weight u-PA in a 75:25 ratio) was a kind gift from Bourvonnais Pharma (Braine l’Alleud, Belgium). Monoclonal antibodies against rat and human PAI-1 were produced as described previously (14, 15). Restriction enzymes were obtained from Amersham Pharmacia Biotech, Life Technologies, Inc., and Stratagene (La Jolla, CA). Pfu Turbo™ DNA polymerase was purchased from Stratagene; synthetic oligonucleotides (for mutagenesis and DNA sequencing) were synthesized by Amersham Pharmacia Biotech. Cell culture medium (RPMI 1640) was purchased from Life Technologies, Inc.

General DNA Techniques—DNA manipulation techniques were carried out according to standard procedures and following the instructions of the manufacturers. Plasmid DNA was isolated using Nucleobond® cartridges (Macherey-Nagel). DNA was sequenced with the Autoread Sequencing® kit and the Automated Laser Fluorescent ALF® apparatus (both from Amersham Pharmacia Biotech). Polymerase chain reaction was performed using the GeneAmp® 2400 (PerkinElmer Life Sciences).

Construction of Rat PAI-1 Mutants—Mutants were created using a method based on the QuickChange™ site-directed mutagenesis kit from Stratagene.

Therefore, pIGE2-rat-PAI-1 was used as template to generate pIGE2-rat-PAI-1-E220 (Glu220 to Ala), pIGE2-rat-PAI-1-E242 (Glu242 to Ala), pIGE2-rat-PAI-1-K243 (Lys243 to Ala), pIGE2-rat-PAI-1-D222 (Asp222 to Ala), pIGE2-rat-PAI-1-H219 (His219 to Ala), pIGE2-rat-PAI-1-E257 (Glu257 to Ala), and pIGE2-rat-PAI-1-R260 (Arg260 to Ala) using the appropriate oligonucleotides containing the desired mutations as primer. Polymerase chain reaction was performed essentially as described previously (16), with slight adaptations of the temperatures used for annealing. Subsequently, the amplified DNA was subjected to a high performance liquid chromatography analysis to confirm the mutations. Sequencing analysis was performed as described previously (12), using 10-μl aliquots of the purified DNA. The sequences were analyzed with the BLAST program (http://www.ncbi.nlm.nih.gov/blast) to confirm the mutations.

Determination of the Conformational Distribution of Rat PAI-1 and Its Mutants—PAI-1 samples were diluted to a concentration of 200 μg/ml in phosphate-buffered saline buffer and incubated with a 2-fold molar excess of t-PA or u-PA at 37 °C for 15 min. The reaction products were analyzed by SDS-polyacrylamide gel electrophoresis using 10–15% gels followed by Coomassie Brilliant Blue staining. The reaction products formed were quantified by densitometric scanning of the gels (11) using the Image Master™ system (Amersham Pharmacia Biotech).

Affinity of Antibodies with PAI-1, PAI-1 Mutants, and Vitronectin—Affinity constants for the binding between proteins were determined using the BIAcore™ 3000 analytical system equipped with the CM5 sensor chip (BIAcore® AB) as described previously (17). In brief, coupling of antibodies to the sensor chip was done according to the instructions of the manufacturer at 10 μg/ml (50-μl fractions). Purifications of the analyte were in 20 mM HEPES, pH 7.2, containing 1 mM EDTA. PAI-1 was perfused at a concentration of 50 nM at a flow rate of 10 μl/min. When indicated, in a subsequent step vitronectin (50 nM) was perfused at a flow rate of 10 μl/min. Rate constants were determined using BIAevaluation™ 3.0 software, and affinity constants were calculated as k₅/k₇. PAI-1 activity was measured as described previously (15). Antibody-induced inactivation of PAI-1 was measured through quantification of residual PAI-1 activity after incubation of PAI-1 with antibody. Briefly, 20 ng/ml rat PAI-1 (~50–60% active), in either the presence or absence of vitronectin (10 μg/ml), was incubated (25 min, 37 °C) with monoclonal antibody at concentrations ranging from 8 μg/ml to 60 ng/ml using serial 2-fold dilutions.

Interaction between PAI-1 and Vitronectin—Binding of PAI-1 to immobilized vitronectin was measured by enzyme-linked immunosorbent assay using plates coated with rat, murine, or human vitronectin (1 μg/ml) for capture and MA-8M7-HRP (against rat PAI-1) (15) for detection. PAI-1 (100 ng/ml) was incubated with monoclonal antibody (2-fold serial dilutions, concentrations ranging from 5 μg/ml to 40 ng/ml) or buffer at 37 °C for 25 min before application to the wells. The obtained data illustrate the feasibility of an allosteric up-regulation of the vitronectin binding properties of PAI-1.

RESULTS

Interaction between PAI-1 and Vitronectin in the Absence or Presence of Monoclonal Antibodies—Preliminary experiments by enzyme-linked immunosorbent assay confirmed that the active form of rat PAI-1 binds to vitronectin, whereas the latent form does not (data not shown).

Incubation of rat PAI-1 with the inhibitory monoclonal antibody MA-124K1 (19) resulted in a dose-dependent increase of the binding of PAI-1 to vitronectin (Fig. 1A). A virtually identical dose-dependent effect was observed with Fab fragments of MA-124K1. A noninhibitory control anti-PAI-1 antibody (MA-32K3) did not affect the binding. No binding was observed when vitronectin was replaced with bovine serum albumin. Interestingly, latent rat PAI-1 and preformed complexes between rat PAI-1 and t-PA or u-PA also exhibited strongly

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increased binding to vitronectin in the presence of MA-124K1 (Fig. 1B).

To exclude possible artifacts as a result of the use of immobilized vitronectin, comparable experiments were performed using soluble vitronectin (see “Experimental Procedures”) that yielded similar observations (Fig. 1A).

Evaluation of the affinity of the binding between PAI-1 and vitronectin in the absence ($K_a = 2 \times 10^7 \text{M}^{-1}$) or presence of MA-124K1 ($K_a = 160 \times 10^7 \text{M}^{-1}$) or Fab fragments thereof ($K_a = 170 \times 10^7 \text{M}^{-1}$) revealed that interaction between PAI-1 and the antigen binding site of MA-124K1 resulted in an 80-fold increased affinity of PAI-1 for vitronectin.

**Inhibitory Effect of Monoclonal Antibodies toward PAI-1 Activity in the Absence or Presence of Vitronectin**—As shown in Fig. 2, the PAI-1 neutralizing properties of MA-124K1 were potentiated in the presence of vitronectin.

**Localization of the Binding Region of MA-124K1**—Evaluation of the differential binding of MA-124K1 to various rat/human PAI-1 chimeras revealed that the epitope was localized in the region from amino acid 187 to amino acid 277 (Fig. 3). Subsequently, differences in binding properties of MA-124K1 to PAI-1 from different species (human, rat, porcine, rabbit, and murine) and sequence differences between PAI-1 from different species within the amino acid 187–277 region, combined with localization in the three-dimensional structure of PAI-1, resulted in the initial selection of the following five residues as possible candidates to contribute to the epitope: Glu220, Asp244, Asp255, Glu257, and Arg260 (all residues were charged, located at the surface, and nonconserved between rat PAI-1 and PAI-1 from non-cross-reacting species). In addition, residues Glu181, His185, and Arg187 (charged, surface-localized, and not necessarily fully nonconserved) were also considered in this first round because these are located in the vicinity of other selected residues and may therefore potentially contribute to the epitope (16).

Generation and preliminary characterization of the corresponding alanine mutants (i.e. respective residue replaced by alanine) (small scale expression, not purified) by enzyme-linked immunosorbent assays (data not shown) revealed that only rat-PAI-1-E220A had a strongly reduced binding to MA-124K1. Therefore, in a second selection round, the surface around position 220 was scanned (on the three-dimensional structure) for other residues possibly contributing to the epitope. Based upon distance measurements, charge, and surface localization, the residues at positions Glu212, His219, Asp222, Glu242, and Lys243 and either one of these residues in combination with residue 220 were mutated to an alanine and evaluated for their binding properties to MA-124K1. Preliminary evaluation of the binding of the combination mutant rat-PAI-1-E212A/E220A exhibited a decreased binding to MA-124K1. Further scanning of the area around position 212 revealed the absence of any other charged residues. It was there-
fore concluded that Glu\textsuperscript{220} and Glu\textsuperscript{212} constitute the main molecular determinants of the epitope for MA-124K1. The corresponding mutants (rat-PAI-1-E212A, rat-PAI-1-E220A, and rat-PAI-1-E212A/E220A) were then purified on a large scale for further detailed characterization and evaluation of their binding affinity to MA-124K1.

Characterization of Rat-PAI-1-E212A, Rat-PAI-1-E220A, and Rat-PAI-1-E212A/E220A—All three mutants exhibited inhibitory activity toward t-PA and u-PA similar to that observed for rat PAI-1 (Table I). Incubation of rat PAI-1 or the mutants for 24 h at 37 °C resulted in a complete loss of inhibitory activity, predominantly due to conversion of the active conformation to the latent conformation (data not shown). However, slight differences were observed when comparing the rate of the active transition to that of the latent transition (Table II). Rat-PAI-1-E212A and rat-PAI-1-E212A/E220A are characterized by a 2.5-fold longer half-life compared with that of rat PAI-1. The half-life of rat-PAI-1-E220A was nearly identical to that of rat PAI-1.

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**DISCUSSION**

The current study describes a PAI-1-neutralizing monoclonal antibody, MA-124K1, which upon binding to PAI-1 increases the vitronectin binding properties of PAI-1, thereby enhancing its inhibitory effect on cell migration.

To the best of our knowledge, this is the first report describing a monoclonal antibody (or any other compound) exerting an opposite effect on the two functions of PAI-1. Previous studies describing other PAI-1 inhibitory monoclonal antibodies revealed either no interference with the vitronectin binding properties (20) or a decreased binding to vitronectin (21). In view of the dual biological effects of PAI-1 associated with these two biochemical properties, the specific functional effects of this antibody open new perspectives in further exploring the relative role of these two biological functions.

Binding of PAI-1 to vitronectin has been demonstrated in various species (15, 22–25) and is an exclusive property of the active, inhibitory conformation of PAI-1 (22, 23). The vitronectin binding properties of PAI-1 have therefore led to the generally accepted hypothesis that localization of PAI-1 activity plays a regulatory role in pericellular proteolysis and related events such as tissue remodeling (26, 27). Absence of this function in PAI-1 knockout mice has been shown to result in an increased degradation of the extracellular matrix, resulting in impaired neovascularization and prevention of cancer invasion (10). Recently, it was observed that the PAI-1 binding site and the integrin binding site of vitronectin are localized in the same region and that PAI-1 prevents α₅β₃ integrin binding to vitronectin and therefore inhibits integrin- and vitronectin-mediated cell migration, independent from its proteinase inhibitory functions (7, 8). These antiadhesive properties of PAI-1 have thus been considered to possibly form the basis for anti-
tumoral strategies. Based on these data, we hypothesize that neutralization of PAI-1 activity, together with prevention of cell migration, could form a useful, synergistic antitumoral strategy because both act at different levels. The effect of MA-124K1 on cell attachment and migration further substantiates the biological relevance of our observations. These data also support our hypothesis that up-regulation of the vitronectin binding properties of PAI-1 eventually results in an enhanced impairment of the cell binding properties of vitronectin. This is in full agreement with the underlying molecular mechanism of the PAI-1 antiadhesive properties, i.e. both binding sites on vitronectin (i.e. for PAI-1 and for integrins) are adjacent to each other (7–9), thereby excluding simultaneous binding of both ligands.

For a number of reasons (e.g. the inevitable association between its antiproteinase activity and its vitronectin binding properties, and the observation that the active site and the vitronectin binding site are at opposite site of the molecule), it seemed irrational to search for a single compound exhibiting both effects on PAI-1. However, our current observation with MA-124K1 provides evidence that it is possible to develop strategies achieving efficient inactivation of PAI-1 with a concomitant significant increase of its vitronectin binding properties. The loss of affinity of the mutants (rat PAI-1-E212A, rat PAI-1-E220A, and rat PAI-1-E212A/E220A) for the antibody provides evidence for the localization of the epitope. Although residues 212 and 220 are not close in the linear sequence, they are situated close to each other in the three-dimensional structure of PAI-1.

In view of the epitope localization and the observed effects of MA-124K1, it is important to note that even though the reactive-site loop (responsible for the antiproteinase properties) and the vitronectin binding region are situated at opposite poles of PAI-1 (28, 29) (Fig. 6), both have been suggested to be conformationally linked (30). Localization of particular secondary structural elements in the three-dimensional structure may reconcile the observed dual effects with the remote distance of both functional sites and form the molecular basis for the action of MA-124K1. Whereas the binding of MA-124K1 to its epitope (in the vicinity of the reactive-site loop) is most likely to be responsible for the neutralization of the proteinase inhibitory activity, the particular localization of the epitope relative to the vitronectin binding site suggests a transduction of interactions throughout the molecule, corresponding to an allosteric modulation. It is worth mentioning that comparison of the dose-response curves (i.e. Fig. 1 versus Fig. 4) reveals that whereas a 1-fold molar excess of MA-124K1 over PAI-1 results in a maximal effect on the vitronectin binding, a 5-fold or higher excess is required to achieve a maximal inhibition of its proteinase inhibitory properties. This apparent discrepancy further supports the concept that the dual effect of MA-124K1 on both PAI-1 functions is the consequence of two distinct molecular mechanisms.

Strikingly, the epitope and the vitronectin binding site are connected through the presence of αA, αD, and αH (Fig. 6). Because allosteric regulation of binding properties often involves α-helical secondary structures (31–33), it is tempting to speculate that the above-mentioned α-helices participate in the observed intramolecular signal transduction, ultimately result-
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ing in an increased exposure of the vitronectin binding site. Interestingly, MA-124K1 also induces an increased vitronectin binding in latent PAI-1 and in complexes of PAI-1 with t-PA or u-PA. This observation is compatible with the molecular explanation of the MA-124K1 effects because the contribution of $\alpha$hA, $\alpha$hD, and $\alpha$hH to the allosteric effect can be considered to be unaffected by latency or complex formation. Importantly, the binding properties as well as the functional effects of Fab fragments of MA-124K1 are identical to those observed with the intact antibody, demonstrating that the direct interaction with the epitope plays an important role in the functional effects and excluding the possibility that any of the observed effects are an indirect consequence of the bivalency of the antibody.

A very similar increase in binding between rat PAI-1 and murine or human vitronectin was also observed (data not shown). This observation is in agreement with previous observations suggesting that the domain for vitronectin binding is conserved in PAI-1 from different species and that the binding domain for PAI-1 in the vitronectin molecule is also highly conserved among vitronectins from different species (21, 28, 29, 34, 35).

In conclusion, the current study identifies a novel mechanism for simultaneous but opposite interference with both functional properties of PAI-1. The observed unique, allosteric effects of MA-124K1 on PAI-1 and the epitope localization open new perspectives for the rational design of compounds exerting a dual effect on PAI-1 and therefore having putative antitumoral properties.

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