Additivity in Effects of Vitronectin and Monoclonal Antibodies against α-Helix F of Plasminogen Activator Inhibitor-1 on Its Reactions with Target Proteinases*

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The serpin plasminogen activator inhibitor-1 (PAI-1) is a potential therapeutic target in cardiovascular and cancerous diseases. PAI-1 circulates in blood as a complex with vitronectin. A PAI-1 variant (N-(2-(iodoacetoxyl)ethyl)-N-methylamino-7-nitrobenz-2-oxa-3-diazole (NBD) P9 PAI-1) with a fluorescent tag at the reactive center loop (RCL) was used to study the effects of vitronectin and monoclonal antibodies (mAbs) directed against α-helix F (Mab-2 and MA-55F4C12) on the reactions of PAI-1 with tissue-type and urokinase-type plasminogen activators. Both mAbs delay the RCL insertion and induce an increase in the stoichiometry of inhibition (SI) to 1.4–9.5. Binding of vitronectin to NBD P9 PAI-1 does not affect SI but results in a 2.0–6.5-fold decrease in the limiting rate constant (k_{lim}) of RCL insertion for urokinase-type plasminogen activator at pH 6.2–8.0 and for tissue-type plasminogen activator at pH 6.2. Binding of vitronectin to the complexes of NBD P9 PAI-1 with mAbs results in a decrease in k_{lim} and in a 1.5–22-fold increase in SI. Thus, vitronectin and mAbs demonstrated additivity in the effects on the reaction with target proteases. The same step in the reaction mechanism remains limiting for the rate of RCL insertion in the absence and presence of Vn and mAbs. We hypothesize that vitronectin, bound to α-helix F on the side opposite to the epitopes of the mAbs, potentiates the mAb-induced delay in RCL insertion and the associated substrate behavior by selectively decreasing the rate constant for the inhibitory branch of PAI-1 reaction (k_i). These results demonstrate that mAbs represent a valid approach for inactivation of vitronectin-bound PAI-1 in vivo.

Plasminogen activator inhibitor-1 (PAI-1),† a member of the serpin superfamily of proteinase inhibitors (1–4), is involved in regulation of normal and pathological thrombolysis and fibrinolysis as well as in tumor invasion and metastasis (5–7). PAI-1 is a major endogenous regulator of tissue-type (tPA) and urokinase-type (uPA) plasminogen activators (8), which produce the active serine proteinase plasmin by cleavage of plasminogen. Similar to other serpins, PAI-1 employs the unique suicide mechanism of proteinase inactivation (Scheme 1). The enzyme (E) forms a Michaelis complex (EI) with PAI-1 (I), interacting with the reactive center loop (RCL) exposed to the solution in the active conformation (Fig. 1). However, cleavage of the scissile bond and formation of the acyl-enzyme (E−I') triggers "stressed-to-relaxed" transition of PAI-1, resulting in fast insertion of the C-terminal part of RCL as strand 4 of β-sheet A (Fig. 1), translocation of the proteinase from the initial binding site to the opposite pole of the PAI-1 molecule, and its inactivation, due to a mechanical distortion of the enzyme’s active site (9). An elegant hypothesis, proposing conservation of the free energy for proteinase distortion through reversible displacement of α-helix F, has been recently published (10). Since decylation of proteinase also occurs during the reaction, the PAI-1 mechanism includes inhibitory and substrate branches (Scheme 1), which yield either the final inhibitory complex (E−I*) or a cleaved inactive PAI-1 (I*) together with regenerated enzyme. The efficiency of the inhibitory reaction (Scheme 1) could be expressed quantitatively by the stoichiometry of inhibition (SI = 1 + k_{l}/k_{i}) (11, 12). Although the reaction mostly follows the inhibitory pathway (SI−1), the thermodynamically favorable products (E and I*) finally form (Scheme 1) through slow (k_{d} ≪ k_{i}) hydrolysis of the inhibitory complex (E−I*). Unlike other serpins, PAI-1 possesses an ability for spontaneous inactivation due to a "stressed-to-relaxed" transition via insertion of uncleaved RCL, resulting in the latent conformation of PAI-1 (13).

However, there is a physiological mechanism of stabilization of the active conformation of PAI-1. PAI-1 circulates in blood as a complex with vitronectin (Vn) (14, 15), a cell-adhesive glyco-

reactive center loop; SI, stoichiometry of inhibition; tPA, two-chain tissue-type plasminogen activator; uPA, urokinase type plasminogen activator; Vn, vitronectin; mAb, monoclonal antibody.

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† The abbreviations used are: PAI-1, plasminogen activator inhibitor-1; NBD, N-(2-(iodoacetoxyl)ethyl)-N-methylamino-7-nitrobenz-2-oxa-3-diazole; NBD P9 PAI-1, S338C (position P9 of RCL) mutant variant of PAI-1 with NBD group attached to the cysteine residue; RCL, unique suicide mechanism of proteinase inactivation (Scheme 1). The enzyme (E) forms a Michaelis complex (EI) with PAI-1 (I), interacting with the reactive center loop (RCL) exposed to the solution in the active conformation (Fig. 1). However, cleavage of the scissile bond and formation of the acyl-enzyme (E−I') triggers "stressed-to-relaxed" transition of PAI-1, resulting in fast insertion of the C-terminal part of RCL as strand 4 of β-sheet A (Fig. 1), translocation of the proteinase from the initial binding site to the opposite pole of the PAI-1 molecule, and its inactivation, due to a mechanical distortion of the enzyme’s active site (9). An elegant hypothesis, proposing conservation of the free energy for proteinase distortion through reversible displacement of α-helix F, has been recently published (10). Since decylation of proteinase also occurs during the reaction, the PAI-1 mechanism includes inhibitory and substrate branches (Scheme 1), which yield either the final inhibitory complex (E−I*) or a cleaved inactive PAI-1 (I*) together with regenerated enzyme. The efficiency of the inhibitory reaction (Scheme 1) could be expressed quantitatively by the stoichiometry of inhibition (SI = 1 + k_{l}/k_{i}) (11, 12). Although the reaction mostly follows the inhibitory pathway (SI−1), the thermodynamically favorable products (E and I*) finally form (Scheme 1) through slow (k_{d} ≪ k_{i}) hydrolysis of the inhibitory complex (E−I*). Unlike other serpins, PAI-1 possesses an ability for spontaneous inactivation due to a "stressed-to-relaxed" transition via insertion of uncleaved RCL, resulting in the latent conformation of PAI-1 (13).
protein present in vivo in micromolar concentrations (16, 17). It has been shown that Vn interacts with PAI-1 with high affinity through its somatomedin B domain, which is composed of the 44 N-terminal amino acids (18). Binding of Vn induces conformational changes of the PAI-1 molecule (19), resulting in a delay in spontaneous inactivation of PAI-1 due to its conversion from the active to the latent form (15, 20). X-ray crystal structure analysis of the complex of PAI-1 with somatomedin B domain (21), together with random and site-directed mutagenesis (22–24), have demonstrated interaction of α-helices F and E as well as strand 1 of β-sheet A of PAI-1 with Vn (Fig. 1). On the other hand, binding of monoclonal antibodies (mAbs) to epitopes located on the side of α-helix F opposite to the Vn binding site side (Fig. 1) induces a decrease (Mab-2) (25) or even a complete loss (mAb CL2C8) (26) of the ability to bind Vn and an increase in the fraction of PAI-1 following the substrate pathway (for a review, see Ref. 2). Recent studies demonstrate that binding of Vn enhances PAI-1 neutralization by Mab-2 in reaction with uPA (25, 27). These observations strongly call for further studies of the mechanism by which Vn, alone and in combination with mAbs, affects the kinetics and stoichiometry of the reaction of PAI-1 with target proteinases.

To the best of our knowledge, there are no previously reported data on effects of Vn on the kinetics of insertion of RCL during the reaction of PAI-1 and its mAb complexes with target proteinases (Scheme 1). In the present study, we have investigated the effects of Vn on the reactions of tPA and uPA with Vn/(1-2-(iodoacetoxyethyl)-N-methylamino-7-nitrobenz-2-oxa-3-diazole (NBD) P9 PAI-1 and its complexes with mAbs, interacting with α-helix F. Effects on kinetics of RCL insertion and stoichiometry of the reaction of NBD P9 PAI-1 with target proteinases were determined for Vn, in combination with Mab-2, its Fab fragment (Fab-2), and MA-55F4C12, which have overlapping epitopes at or close to the N-terminal part of α-helix F of PAI-1 (Fig. 1). Since protonation of a histidine residue at the PAI-1/uPA interface induces a significant increase in the limiting rate of RCL insertion for NBD P9 PAI-1 and its complex with MA-55F4C12 (28), the effects of Vn were studied at normal physiological pH and at pH 8.0 and 6.2. The results obtained reveal the mechanism of modulation of PAI-1 activity by Vn and demonstrate additivity in the effects of Vn and mAbs directed against α-helix F on the reactions of PAI-1 with tPA and uPA.

**EXPERIMENTAL PROCEDURES**

**Proteins and Reagents**—Human Vn (2 mg/ml), purified from plasma, was from Promega (Madison, WI). Human recombinant tPA (Activase) was provided by Genentech (South San Francisco, CA). Human recombinant uPA was from Abbott. Analytical grade buffer reagents were from Sigma. The S338C (P9 Cys) PAI-1 mutant variant was purified, labeled with NBD, and characterized as described previously (13). The concentrations of NBD PAI-1, tPA, uPA, and antibodies were determined spectrophotometrically (29). To minimize buffer change effects, all experiments were performed in 50 mM phosphate buffer solutions with pH 7.4, 6.2, and 8.0 at 25 °C. A buffer with a certain pH was prepared directly by mixing solutions of 50 mM KH2PO4 with 50 mM K2HPO4. The concentration of buffer was selected to provide capacity for all stopped-flow data). Correlation coefficients (r²) calculated from curve fittings were also used as a parameter of goodness of fit (r² of fit exceeded 0.98 for all stopped-flow data).

**Measurements of Stoichiometry of Inhibition (SI)**—To determine the effect of Vn, mAbs, and both ligands on the distribution between inhibitory and substrate branches of PAI-1 reaction, the SI was determined. Values of SI for NBD P9 PAI-1-mAb complexes, with and without Vn, were measured directly by titration of complexes of NBD P9 PAI-1 with tPA or uPA, as described previously (29). Binary complexes of NBD P9 PAI-1 (10–40 nm) with mAbs, Fab-2 (50–200 nm), or Vn (20–80 nm) as well as ternary Vn/NBD P9 PAI-1-mAb (Fab-2) complexes were preformed and equilibrated at room temperature for at least 30 min. A Varian Cary Eclipse fluorescence spectrophotometer (excitation wavelength of 480 nm, emission at 530 nm; slit widths 5 and 10 nm for excitation and emission, respectively) was employed for titration of NBD P9 PAI-1 and NBD P9-1-mAb, NBD P9 PAI-1-Vn, or Vn/NBD P9 PAI-1-mAb complexes. The titration was performed by monitoring an increase in NBD fluorescence emission (13), resulting from interaction of NBD P9 PAI-1 or its complex with an aliquot of proteinase. After changes of fluorescence emission had reached the maximum, the next portion of the enzyme was added. At the end point of titration, the increase in fluorescence emission of the NBD group due to RCL insertion approached saturation, since all of the NBD P9 PAI-1 was cleaved. The SI values were calculated as the ratios between...
the numbers of moles of NBD P9 PAI-1 present and the numbers of moles of proteinase required for complete titration of uncomplexed NBD P9 PAI-1. The SI values were reported as averages of at least two independent experiments.

Fab Fragment of Mab-2—The Fab fragment of monoclonal anti-PAI-1 clone 2 antibody (Mab-2) (30) was obtained by digesting Mab-2 with 1:100 (w/w) papain for 18 h. After digestion was stopped with E64, Fab fragments were separated from the Fc domains using a Protein A-coupled Sepharose column and affinity-purified on a PAI-1-coupled Sepharose column. The affinity of Fab-2 to NBD P9 PAI-1 and NBD P9 PAI-1-Vn was estimated from the dependence of Km on the reaction with tPA on Fab-2 concentration, as described earlier for other anti-PAI-1 mAbs (29). Briefly, the reaction between tPA, taken at a concentration (1.5–2.0 μM) at least 2 times higher than the value of Km and NBD P9 PAI-1 or its preformed complex with Vn were carried out using SX-18MV stopped-flow reaction analyzer. The values of kobs, calculated by fitting the changes in the fluorescence of NBD group by a single exponential process. The values of Km were estimated from the midpoints of dependence of kobs versus the logarithm of Fab-2 concentration.

Additivity in Effects of Vn on the Reaction of NBD P9 PAI-1-mAb Complexes with Target Proteinases—The effects of Vn on the kinetics of RCL insertion and stoichiometry of the reaction of NBD P9 PAI-1-mAb complexes with target proteinases was tested through comparison of changes in the free energy of activation for the limiting step of the PAI-1 reaction (ΔG‡RCL), and partition activation free energy (ΔG‡partition). The values of ΔG‡RCL, were calculated from km, as ∆G‡RCL = RT ln(km/kmobs), kmobs is the limiting rate of RCL insertion for the reaction of NBD P9 PAI-1 with target proteinase in the absence of ligands; km values are the limiting rates of RCL insertion for NBD P9 PAI-1-mAb or ternary complexes VnNBD P9 PAI-1-mAb, T is the standard temperature 298 K, and R = 1.987 cal/mol·K is the gas constant. The values of ΔG‡partition (ΔG‡partition = RT ln(Km/Kmobs)) were calculated from the SI values for NBD P9 PAI-1-mAb complexes without and with Vn. The data were plotted as ∆G‡partition versus ∆G‡RCL. A line was fitted to the data, using SigmaPlot version 8.0 software.

RESULTS

Effects of Vn and mAbs, Directed against α-helix F, on the Kinetics of RCL Insertion during the Reaction of NBD P9 PAI-1 with tPA and uPA—To determine the effects of Vn on the kinetics of RCL insertion, the dependencies of the observed rate constant (kobs) of increase in the NBD-fluorescence emission on the proteinase concentration (Fig. 2) were determined at normal physiologic pH 7.4 (25 °C). The dependences of kobs on proteinase concentration always demonstrated saturation (Fig. 2) for both target proteinases. Therefore, tPA and uPA interact with complexes of NBD P9 PAI-1 with mAb or Vn in a manner similar to that described for uncomplexed NBD P9 PAI-1 (31).

The values of the limiting rate of RCL insertion (klim) and the concentration of proteinase at half-saturation (Km) for the reactions of NBD P9 PAI-1 and its mAb or Vn complexes with tPA and uPA were calculated (Table I). Vn, on its own, induced an almost 7-fold decrease in klim for the reaction of NBD P9 PAI-1 with uPA, but only a 1.4-fold decrease in klim for the reaction with tPA (Table I). Mab-2 alone induced a 4.5-fold decrease in klim for uPA and a 17-fold decrease in klim for tPA (Table I). The effect of Mab-2 on the reaction of NBD P9 PAI-1 with target proteinases was similar to that observed earlier for MA-55F4C12 (29). Both mAbs have epitopes at the N terminus of α-helix F (32). Binding of Vn did not change dramatically the specificity (klim/Km) for the reaction with uPA; the decrease in klim, observed for the reaction of NBD P9 PAI-1 with uPA in the presence of Vn, was accompanied by similar changes in Km (Table I). In contrast, binding of mAbs induced significant decreases in klim for the reaction with tPA, without changes in Km (Table I), which resulted in a corresponding decrease in klim/Km. The different effects of Vn and mAbs on the kinetics of the reaction of PAI-1 with uPA and tPA could indicate different mechanisms of modulation of the PAI-1 reaction by Vn and mAbs.

Vn affected the kinetics of RCL insertion for the reactions of NBD P9 PAI-1-mAb complexes with tPA and uPA in a manner similar to the reactions of the uncomplexed serpin (Table I). In the presence of Vn, klim for the reaction of complexes of NBD P9 PAI-1 with Mab-2 or MA-55F4C12 with uPA decreased by almost an order of magnitude (Table I). Similar to the effects on uncomplexed NBD P9 PAI-1, a decrease in klim for the reaction with uPA due to binding of Vn to NBD P9 PAI-1-mAb was accompanied by a 5.0- and 7.5-fold decrease in Km for Mab-2 and MA-55F4C12, respectively (Table I). In contrast to the reaction with uPA, Vn caused a 1.8–2.0-fold decrease in klim for the reaction of complexes with tPA with a corresponding decrease in the specificity of the reaction (Table I). The similarity in the effects of Vn on the reactions of free NBD P9 PAI-1 and its complexes with mAbs could indicate additivity in the effects of the two ligands on kinetics of RCL insertion during reaction with target proteinases.

Effects of Vn and mAbs on SI for the Reactions of NBD P9 PAI-1 with Target Proteinases—To determine the effect of Vn on partitioning between inhibitory and substrate branches (Scheme I) of the PAI-1-mAb mechanism, the stoichiometry of inhibition (SI) for the reactions of tPA and uPA with NBD P9 PAI-1-mAb complexes was measured with and without Vn (Fig. 3). Vn did not affect the SI for the reactions of NBD P9 PAI-1 with target proteinases, which agrees with the data obtained.
earlier (26). However, the fraction of the substrate reaction for NBD P9 PAI-1 complexes with Mab-2 and MA-55F4C12 dramatically increases in the presence of Vn, from 30–70 to 85–95% (Table II). Similar to its effects on the kinetics of RCL insertion, Vn affected reactions of tPA and uPA differently; the values of SI for the reaction of NBD P9 PAI-1-mAb complexes with uPA increased approximately by an order of magnitude from 1.5 ± 0.1 to 16 ± 1 for Mab-2 and from 2.5 ± 0.3 (29) to 18 ± 2 for MA-55F4C12 (Table II). These results were in agreement with the data obtained earlier for wild type PAI-1 (25, 27). The SI for the reaction with tPA increased by 2.8- and 4.5-fold for complexes with Mab-2 and MA-55F4C12, respectively. Therefore, at physiological pH, binding of Vn to complexes of NBD P9 PAI-1 with mAbs induced a decrease in \( k_{\text{lim}} \) and an increase in the fraction of the substrate reaction for both target proteinases, but this effect was more pronounced for the reactions with uPA.

**Effect of pH on the Response of the Kinetics of RCL Insertion to Vn and mAbs**—Protonation of a histidine at the PAI-1/PA interface (28) alters the pattern of the exosite interactions and results in an increase of both \( k_{\text{lim}} \) and \( K_m \), probably the limiting step of the reaction remaining the same (31). To detect possible changes in the mechanism of the PAI-1 reaction (Scheme 1) due to binding of Vn and mAbs, their effects on \( k_{\text{lim}} \) and \( K_m \) were studied at pH 6.2 (Fig. 4). The reactions with uPA (no exosite interactions through histidine) and studies at pH 8.0 (where more than 95% of histidines with normal pK\(_a\) are deprotonated) were used as controls. At pH 8, Vn caused no effect on \( k_{\text{lim}} \) for the reaction of NBD P9 PAI-1 with tPA in the absence of mAbs (Table III), indicating the presence of a fraction of the protonated species at pH 7.4. However, binding of Vn resulted in a 1.5–1.6-fold decrease in \( k_{\text{lim}} \) for NBD P9 PAI-1-tPA complexes without significant changes in \( K_m \) (Table III). Similarly to the results observed at pH 7.4, Vn decreased significantly both \( k_{\text{lim}} \) and \( K_m \) for the reaction between uPA and NBD P9 PAI-1 or its complexes with mAb at pH 8.0 (Table III). Therefore, at the physiological pH 7.4 (Table I) and at the slightly alkaline pH 8.0 (Table III), Vn selectively affected the kinetics of the reaction with uPA. In contrast, when the experiments were performed at pH 6.2 (Fig. 4), the effects of Vn on the reaction with tPA were dramatically different (Table III). At this pH, Vn induced a 3.5-fold decrease in \( k_{\text{lim}} \) for the reaction with free NBD P9 PAI-1 and an almost 5-fold decrease in \( k_{\text{lim}} \) for the reaction with its mAb complexes (Table III). The effects of Vn on the \( k_{\text{lim}} \) for the reaction with uPA were similar to that observed at the other pH values. As a result, protonation of the histidine at the PAI-1/PA interface resulted in almost the same values of \( k_{\text{lim}} \) and \( K_m \) (0.34–0.36 s\(^{-1}\) and 0.21–0.28 \( \mu \)M, respectively) for the reactions of both ternary complexes with both target proteinases (Table III). Therefore, the similarity in the effects of Vn alone, mAb alone, and both ligands together on the reaction of NBD P9 PAI-1 with tPA and uPA at pH 6.2 supports the hypothesis that a deprotonated histidine at the PAI-1/PA interface participates in interactions controlling different kinetics of inhibition of tPA and uPA by PAI-1, observed at neutral pH (28).

### Table 1

| Ligand(s) | \( k_{\text{lim}} \) | \( K_m \) | \( k_{\text{lim}} \) | \( K_m \) |
|-----------|----------------|---------|----------------|---------|
| None      | 1.7 ± 0.1      | 0.09 ± 0.02 | 5.8 ± 0.4      | 0.05 ± 0.02 |
| Vn        | 1.0 ± 0.05     | 5.1 ± 0.01  | 6.1 ± 0.1      | 0.01 ± 0.01  |
| MA-55F4C12 | 0.15 ± 0.04   | 0.04 ± 0.00  | 0.07 ± 0.04    | 0.01 ± 0.00  |
| Mab-2     | 0.08 ± 0.01    | 0.04 ± 0.00  | 0.35 ± 0.01    | 0.01 ± 0.01  |
| MA-55F4C12 + Vn | 0.09 ± 0.00 | 0.15 ± 0.00  | 1.0 ± 0.00     | 0.01 ± 0.00  |

**Effects of pH on the SI for the Reactions of NBD P9 PAI-1 with Target Proteinases in the Absence or Presence of Vn and mAbs**—The values of SI for the reactions of uPA and tPA with Vn and mAbs complexes of NBD P9 PAI-1 as well as the ternary complexes (Table IV) were determined (Fig. 3) at pH 6.2 and 8.0. The effects of mAbs on SI were different for tPA and uPA. With uPA, the fractions of the substrate reaction for NBD P9 PAI-1-mAb complexes with Mab-2 and MA-55F4C12 were similar at both pH values. The SI values observed at pH 6.0 for MA-55F4C12 were approximately 2 times higher than those at pH 8.0 (Table IV). On the other hand, there was no significant difference in SI at different pH for the reaction of NBD P9 PAI-1-Mab-2 complex with tPA. As expected, all of the values of SI observed at pH 8.0 (Table IV) were close to those obtained at pH 7.4 (Table II). The SI values obtained for the NBD P9 PAI-1-Vn complex with uPA and tPA were close to unity at both pH 6.2 and 8.0 (data not shown). Studying the combined effect of both Vn and mAbs, it was found that Vn always enhanced the antibody-induced redirection of the PAI-1 reaction to the substrate branch (Scheme I and Table IV). There was no significant pH dependence for SI for the reaction with uPA either for NBD P9 PAI-1-mAb (SI = 1.4–2.6) or for Vn/NBD P9 PAI-1-mAb complexes (SI = 15–18) (Tables II and IV). Therefore, an increase

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**Fig. 3.** Titration of mAb and Vn complexes of NBD P9 PAI-1 with tPA. Aliquots of 1.5 \( \mu \)M tPA (0.5–3.0 \( \mu \)M) were added to 0.8 ml of 20 nm solution of NBD P9 PAI-1 (●) preincubated (30 min in 0.05 M phosphate buffer, pH 7.4) with 40 nm Vn (○), 0.1 \( \mu \)M Mab-2 (□), or 0.1 \( \mu \)M Vn and 40 nm Vn (□). The increase in NBD fluorescence emission (excitation and emission wavelengths are 480 and 530 nm, respectively) resulting from RCL insertion due to the reaction of NBD P9 PAI-1 with the proteinase \( (F - F_0) \) was measured. Relative changes in NBD fluorescence emission \( (F - F_0)/(F_{\text{max}} - F_0) \) were plotted as a function of the moles of proteinase added.
TABLE II

|                  | Vn  | Fab-2 | Vn + Fab-2 | MA-55F4C12 | MA-55F4C12 + Fab-2 |
|------------------|-----|-------|------------|------------|--------------------|
| **Vn**           | 1.00 ± 0.05 | 3.0 ± 0.3 | 8.5 ± 0.5 | 4.2 ± 0.3 (4.7 ± 0.5) | 19 ± 2 |
| **Fab-2**        | 1.05 ± 0.05 | 1.5 ± 0.1 | 16 ± 1    | 2.5 ± 0.3 | 18 ± 2 |

*Measured in 0.1 M Hepes, pH 7.4, with 0.1 M NaCl (29).*

**DISCUSSION**

Previously, it was shown that Vn and Fab-2, when added together, strongly increase the SI for the reaction of PAI-1 with tPA, whereas any of the ligands alone had little or no effect on the fraction of the substrate pathway (25, 27). This observation is highly relevant in relation to the potential clinical use of monoclonal antibodies as PAI-1-inactivating agents, since most, if not all, PAI-1 is expected to be bound to the highly abundant Vn in vivo (14, 15). Here, we report studies into the mechanisms of the combined effect of Vn and mAbs (Fab-2 and MA-55F4C12) on PAI-1. We observed additivity of the effects of Vn and mAbs directed against α-helix F on the reactions of PAI-1 with the target proteinases. The epitopes of both of the mAbs and the Vn binding site (Fig. 1) are placed distantly from the exposed RCL, where the initial proteinase docking Michaelis complex, are acyl-enzyme and acyl-enzyme (31). The values of k_{lim}, reported by changes in the fluorescence of the P9 NBD group, reflect burying of RCL and formation of the final inhibitory complex (E-I*, Scheme 1). Differences in k_{lim} could conceivably result from (i) changes in the rate of one and the same rate-limiting step or (ii) another step in the reaction mechanism (Scheme 1) becoming rate-limiting. The significantly lower k_{lim} value for the reaction of PAI-1 with tPA than for the reaction with tPA at physiological pH has been attributed to a contribution of exosite interactions between positively charged residues of the 37-loop of tPA and the P4' and P5' glutamates of PAI-1 (33–36). This exosite interaction stabilizes the Michaelis complex and the acyl-enzyme intermediate (EI and E-I*, Scheme 1) (31), increasing the free activation energy of the rate-limiting step of the reaction (ΔG_{RCL}). Disruption of this exosite interaction (31, 36) induces an increase in both k_{lim} and K_{lim} but the rate-limiting step remains the same (31). This is also true for the effect of protonation of an as yet unidentified histidine residue at PAI-1/PAI interface (28). Vn, when bound to NBD P9 PAI-1, causes a decrease in both k_{lim} and K_{lim} for the reaction with uPA, which is reciprocal to that observed when the exosite interactions are disrupted. In addition, neither
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Changes in the pattern of the interactions at the PAI-1/tPA interface (28, 31, 36) or binding of Vn to uPA (Tables II and IV) significantly affect the SI value, which remains close to unity. Therefore, the effects of Vn on $k_{\text{lim}}$ and $K_m$ for uPA are likely to reflect stabilization of $E\cdot I$ and/or $EI$ (Scheme 1), similar to that observed for the exosite interactions of tPA. In contrast to its effect on the reaction with uPA, Vn has almost no effect on $k_{\text{lim}}$, $K_m$, or SI for the reaction with tPA at pH 7.4 and 8.0. However, at pH 6.2, when the histidine at the PAI-1/tPA interface is protonated, Vn affects the reaction with tPA in a manner similar to that with uPA (Tables I and III). Therefore, under conditions where the exosite interactions at PAI-1/tPA interface are altered, the effects of Vn become the same for both tPA and uPA. These results further support the hypothesis that the difference in the kinetics of RCL insertion between tPA and uPA observed at the normal physiological pH is partly due to the contribution of a deprotonated histidine to the exosite interactions (28). Since the pH dependence of $k_{\text{lim}}$ and $K_m$ for the reaction of NBD P9 PAI-1-Vn with tPA was similar to that observed for the uncomplexed inhibitor, the binding of Vn is unlikely to result in another step becoming rate-limiting for the RCL insertion.

The effects of mAbs on the kinetics of RCL insertion are different from those observed for Vn. The mAbs decrease $k_{\text{lim}}$ for tPA by more than about an order of magnitude and $k_{\text{lim}}$ for uPA by 1.5–2-fold (Tables I and III) (29). The very similar effects of Mab-2 and MA-55F4C12 are in agreement with the immediate expectations from the fact that their epitopes are overlapping (Fig. 1) (25, 32). However, unlike Vn, the mAbs affect $k_{\text{lim}}$ of tPA without changes in $K_m$ and always resulted in an increase in the SI value (Tables II and IV). Therefore, Vn and mAbs affect the kinetics and the stoichiometry of the interaction of NBD P9 PAI-1 with target proteinases through different mechanisms. However, similar to the results observed for NBD P9 PAI-1-Vn, a decrease in pH to 6.2 resulted in a considerable increase in both $k_{\text{lim}}$ and $K_m$ for the reaction of tPA with NBD P9 PAI-1-mAb (Tables I and III), indicating that the rate of RCL insertion for NBD P9 PAI-1 and its complexes with the mAbs are limited by the same step of the reaction.

Vn and mAbs act additively when bound to the same PAI-1 molecule. Similarly to uncomplexed NBD P9 PAI-1, the interaction of Vn with NBD P9 PAI-1-mAb complex results in a decrease in both $k_{\text{lim}}$ and $K_m$ for the reaction with uPA (Tables I and III). The effect of Vn on $k_{\text{lim}}$ for the reaction of NBD P9 PAI-1 with tPA at pH 7.4 and 8.0 is also much less pronounced than that for uPA (Tables I and III). Moreover, protonation of the histidine at the PAI-1/tPA interface affects the kinetics of the RCL insertion for ternary complexes of NBD P9 PAI-1 with Vn and mAb in a manner similar to that observed for binary complexes and results in similar kinetics of RCL insertion for the reactions with tPA and uPA at pH 6.2 (Table III). Therefore, in contrast to Vn, avidity effects are unlikely to contribute to the mechanism of modulation of PAI-1 activity.

### TABLE III

| pH | mAb               | tPA  | tPA (+Vn) | uPA  | uPA (+Vn) |
|----|-------------------|------|-----------|------|-----------|
| 8.0| None              | 1.50±0.05 | 0.04±0.01 | 23.4±1.66 | 4.7±0.3 | 0.84±0.11 |
|     | Mab-2             | 0.15±0.02 | 0.02±0.01 | 6.4±0.8 | 0.52±0.08 | 0.60±0.03 | 0.14±0.03 |
| MA-55F4C12 | 0.50±0.02 | 0.03±0.01 | 5.5±0.57 | 0.09±0.3 | 0.46±0.02 | 0.15±0.02 |
| 6.2| None              | 8.1±0.59 | 0.9±0.39 | 10.3±1.9 | 1.8±0.06 | 5.3±1.2 | 1.7±0.6 |
|     | Mab-2             | 1.6±0.1 | 0.42±0.07 | 0.34±0.03 | 0.26±0.09 | 4.4±0.2 | 1.6±0.1 | 0.35±0.02 | 0.21±0.03 |
| MA-55F4C12 | 1.8±0.1 | 0.53±0.08 | 0.34±0.02 | 0.24±0.03 | 2.0±0.2 | 0.28±0.08 | 0.36±0.02 | 0.28±0.04 |

*Values taken from Ref. 28.

### TABLE IV

| pH | mAb               | tPA  | tPA (+Vn) | uPA  | uPA (+Vn) |
|----|-------------------|------|-----------|------|-----------|
| 8.0| Mab-2             | 3.0±0.5 | 4.5±0.5 | 1.4±0.2 | 15±1 |
| MA-55F4C12 | 5.5±0.5 | 11±1 | 2.6±0.4 | 15±1 |
| 6.2| Mab-2             | 3.7±0.4 | 8.0±20 | 1.8±0.2 | 15±2 |
| MA-55F4C12 | 9.5±1.0 | 110±30 | 1.6±0.2 | 15±1 |

### TABLE V

| pH | Parameter | tPA  | tPA (+Vn) | uPA  | uPA (+Vn) |
|----|-----------|------|-----------|------|-----------|
| 8.0| $k_{\text{lim}}$ (s$^{-1}$) | 0.20±0.06 | 0.15±0.05 | 8.5±0.5 | 0.75±0.03 |
|   | $K_m$ (µM) | 0.06±0.02 | 0.05±0.03 | 1.2±0.1 | 0.14±0.02 |
| 6.2| SI        | 1.8±0.2 | 2.7±0.2 | 1.4±0.2 | 8.6±0.2 |
|   | $k_{\text{lim}}$ (s$^{-1}$) | 1.2±0.2 | 0.35±0.05 | 4.8±0.8 | 0.46±0.05 |
|   | $K_m$ (µM) | 0.54±0.16 | 0.23±0.04 | 2.3±0.7 | 0.15±0.02 |
|   | SI        | 2.6±0.2 | 6.5±0.5 | 1.5±0.3 | 2.5±0.2 |

*Values taken from Ref. 28.

Effects of Vn on the SI for the reactions of NBD P9 PAI-1-mAb complexes with tPA and uPA at pH 8.0 and 6.2. SI was measured by titration of fixed amounts of NBD P9 PAI-1 or its mAb complexes with proteinase as described under "Experimental Procedures." SI values ± S.E. are averages of at least two titrations.
The observed increase in \( \Delta G_{\text{RCL}} \) caused by binding Vn to NBD P9 PAI-1 could be explained if Vn selectively affects \( k_i \). In contrast to the effects on the reactions of free NBD P9 PAI-1, binding of Vn to the NBD P9 PAI-1-mAb complex always induced an increase in SI (Tables II and IV). An increase in SI due to interaction of PAI-1 with ligands corresponds to a decrease in \( \Delta G_{\text{RCL}} = -RT \ln(k_i/k_k) = -RT \ln(\text{SI} - 1) \), the difference in the free activation energy between substrate and inhibitory branches of the PAI-1 mechanism (Scheme 1). Indeed, the decrease in \( \Delta G_{\text{RCL}}^\ddagger \) resulting from a selective effect on \( k_i \) will be detectable only when \( k_i > k_k \) are comparable (i.e. when Vn binds to preformed NBD P9 PAI-1-mAb complex). The correlation \( (r^2 = 0.83) \) between \( \Delta G_{\text{RCL}}^\ddagger \) and the decrease in \( \Delta G_{\text{RCL}} \) observed for NBD P9 PAI-1-mAb and for ternary complexes (Fig. 5) supports this suggestion. This dependence illustrates an additivity in the effects of Vn on the kinetics of RCL insertion and distribution between the substrate and inhibitory pathways for the reaction of PAI-1 with target proteinases at different pH. In the analysis, we disregarded the data at pH 6.2, since conformational changes of tPA at slightly acidic pH (37, 38) would be expected to confound the interpretation of the data and could explain an observed slight deviation from the correlation for the tPA data obtained at pH 6.2 (not shown).

PAI-1 and Vn have the potential for multiple molecular interactions, each of which may have distinct physiological effects. The data obtained in the present study demonstrate that Vn will potentiate the PAI-1-neutralizing effect of a ligand, which makes \( k_i \) and \( k_k \) comparable. The high physiological concentration of Vn and its ability to form oligomeric deposits in the extracellular matrix provide a possibility for control of the activity of PAI-1 up to a micromolar range of PAI-1 concentration. Indeed, it is known that Vn modulates PAI-1 specificity. The PAI-1-Vn complex efficiently inhibits thrombin (39) and activated protein C (40); also, its binding to Vn increases the fraction of the substrate reaction with thrombin from \( \sim 30\% \) to more than \( 80\% \) (41). On the other hand, thrombin competes with mAb CLB-2C8, which has an epitope overlapping with \( \alpha \)-helix F (26). Therefore, the observed increase in clot lysis by thrombin and factor Xa (42) in the presence of Vn could be a physiological outcome of modulation of PAI-1 inactivation by Vn through an increase in the fraction of the substrate reaction (SI), caused by selective decrease in \( k_i \) (Scheme 1). Indeed, if Vn affects the reaction with thrombin and factor Xa in a manner similar to that observed for mAbs (i.e. decreases \( k_i \) and increases SI), clot lysis would be stimulated due to increased activity of plasminogen activators.

Conclusively, the results obtained in this study agree with the data obtained previously (24, 25, 27), and clearly demonstrate a new mechanism of PAI-1 inactivation governed by mutual action of Vn, an endogenous stabilizer of active PAI-1, and mAbs binding to \( \alpha \)-helix F. The observed effect of Vn on PAI-1 neutralization by mAbs elucidates an attractive strategy for the design of novel inhibitors of PAI-1 and should be considered in studies aiming at therapeutic modulation of PAI-1 activity. Moreover, the observed potentiation by Vn of substrate behavior by PAI-1 in the presence of mAbs could mimic a physiological mechanism of modulation of PAI-1 activity.

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