Identification of functional synergism between monoclonal antibodies. Application to the enhancement of plasminogen activator inhibitor-1 neutralizing effects

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Abstract The serpin plasminogen activator inhibitor-1 (PAI-1), an important risk factor for thrombotic disease can be neutralized by distinct mechanisms. We hypothesized that the combination of two compounds, with PAI-1 neutralizing properties based on different mechanisms, may result in a synergistic effect. Therefore, seven monoclonal antibodies with PAI-1 neutralizing properties were pairwise evaluated for the possible presence of synergistic or antagonistic effects. Out of 21 combinations, three particular combinations, i.e. MA-33H1/MA-33B8, MA-33B8/MA-7D4B7, and MA-7D4B7/MA-33H1 exhibited strong synergistic effects in comparison with their properties when evaluated individually. The observed synergism resulted in a maximum enhancement between 2- and 5-fold ($P < 0.05$, vs. theoretically expected effect calculated based on additive effects). Strikingly, synergism was only observed between monoclonal antibodies directed against different epitopes and with different molecular mechanisms of PAI-1 neutralization. This phenomenon of synergism opens new perspectives in the design of therapeutic or preventive strategies aimed at enhancing endogenous fibrinolysis through modulation of PAI-1 activity.

Key words: Monoclonal antibody; PAI-1; Synergism; Serpin

1. Introduction

Plasminogen activator inhibitor-1 (PAI-1), a member of the superfamily of serine proteinase inhibitors (serpins), is the primary physiological inhibitor of both tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). PAI-1 exhibits a unique conformational flexibility and can occur as an active, latent or substrate conformation [1,2]. Since elevated levels of PAI-1 are associated with an increased risk of several important thrombotic deceases [3,4], development of strategies aimed at the inhibition of PAI-1 may be useful to prevent thrombosis or to enhance endogenous fibrinolysis. Several studies have indicated the potential clinical use of agents that inhibit PAI-1 activity. Enhancement of fibrinolysis, both in vivo and in vitro, has been reported by the inhibition of PAI-1 with polyclonal antibody fragments [5,6] or with monoclonal antibodies [7,8].

Antibodies might neutralize PAI-1 activity through at least three different mechanisms: (1) direct interaction with the reactive site loop; (2) enhancing the conversion of active to latent PAI-1; (3) switching active PAI-1 to substrate PAI-1. We hypothesized that combination of two monoclonal antibodies, with neutralizing properties towards PAI-1 based on two different mechanisms, might result in a synergistic activity. Therefore we evaluated seven monoclonal antibodies (with PAI-1 neutralizing properties) for possible synergistic interactions upon pairwise combination. Various methods, commonly used for evaluation of synergism or antagonism and well described in the pharmacological literature [9–11], such as effect summation, isoboles and the interaction index, were applied. Three monoclonal antibodies, each inhibiting PAI-1 by a distinct mechanism, were found to act strongly synergistically ($P < 0.05$) in the three possible combinations evaluated. These data confirm our hypothesis that, because of its unique conformational flexibility, synergism in neutralization of PAI-1 activity may play an important role and could be taken into account in the development of PAI-1 inhibitory compounds and/or in the design of therapeutic strategies for pharmacological suppression of PAI-1 activity.

2. Materials and methods

2.1. Materials

Human t-PA (predominantly in the single-chain form) was a kind gift from Boehringer Ingelheim (Brussels, Belgium). Chromogenic substrate S-2403 was obtained from Kabi Vitrum (Stockholm, Sweden). Recombinant human PAI-1 (expressed in *E. coli*) was prepared as described [12]. Monoclonal antibodies were raised against human PAI-1 as described previously: MA-7D4B7 [13,14], MA-7F5C12 [13,14], MA-8H9D4 [15], MA-33B8 [15], MA-33H1 [15], MA-55F4 and MA-56A7C10 [15]. This set of inhibitory monoclonal antibodies represent seven different epitopes [13–16] and exert their PAI-1 inhibitory properties through, at least, three distinct mechanisms [15,16]. 96-well polystyrene microtiter plates were purchased from Costar (Cambridge, MA).

2.2. PAI-1 activity assay

PAI-1 activity was determined using the method as described by Verheijen et al. [17]. In brief, PAI-1 containing samples (50 μl) in Tris-buffered saline (0.1 M NaCl, 0.05 M Tris/HCl, pH 7.4, containing 0.01% Tween 80; TBS) were mixed with an equal volume of t-PA (20 U/ml) in TBS and incubated for 15 min at 37°C. Subsequently, 100 μl of a solution containing plasminogen (1 μM), CNBr-digested fibrinogen (1 μM) and S-2403 (0.6 mM) were added. Residual t-PA was then quantitated by measuring the absorbance at 405 nm. One unit of PAI-1 is defined as the amount of PAI-1 required to neutralize one international unit of t-PA.

2.3. PAI-1 neutralization assay

Based on previous experiments carried out to evaluate the influence of monoclonal antibodies on the activity of human PAI-1 [14,15], seven monoclonal antibodies were selected to study possible synergistic effects. Neutralization of PAI-1 activity by monoclonal antibodies was determined as described [15], and based on quantitation of residual PAI-1 activity after preincubation of PAI-1 with different concentrations of monoclonal antibodies. Briefly, 100 μl PAI-1 (50 ng/ml)

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were incubated either with 100 μl buffer (TBS) or with 100 μl of a solution containing purified monoclonal antibodies at the indicated concentration. After incubation of this mixture for 25 min at 37°C, residual PAI-1 activity was quantitated with the PAI-1 activity assay as described above. The percentage inhibition (i.e. neutralization of PAI-1 activity) was then calculated based on the ratio of the PAI-1 activity in the presence versus the absence of antibodies.

2.4. Evaluation of synergism

Two methods were applied for evaluation of synergism [9–11], effect summation and isoboles in combination with calculation of the interaction index.

Firstly, if E_1, E_2 and E_{1+2} represent the effect (i.e. extent of PAI-1 neutralization) of monoclonal antibody 1, monoclonal antibody 2 and a combination of both, respectively, then synergism is present when $E_{1+2} > E_1+E_2$. The effect summation approach was applied as follows. A theoretical dose-response curve ('E_1+E_2'), was constructed based on the two respective individual dose-response curves and was subsequently compared with the experimental dose-response curves obtained for the equimolar combination of the two antibodies. Alternatively, an experimental dose-response curve obtained by varying the concentration of one antibody in the presence of a fixed concentration of another antibody was compared with the corresponding theoretical dose response based on the respective concentrations of the two antibodies used.

Secondly, the concentration, $[\text{MA } 1]_e$ and $[\text{MA } 2]_e$, of monoclonal antibody 1 and monoclonal antibody 2, respectively, that produce a predefined effect (i.e. 50% neutralization of PAI-1) was determined from individual dose-response curves. On the other hand dose-response curves were constructed using particular combinations of two antibodies MA 1 and MA 2 (with the ratio MA 1:MA 2 ranging between 0.02 and 22) and the concentrations ($[\text{MA } 1]_c$ and $[\text{MA } 2]_c$) of the respective antibodies in a particular combination required to produce the same effect were deduced. These data were then used to construct isoboles and to calculate the interaction index

$$\left(\text{Int Ind} = \frac{[\text{MA } 1]_c}{[\text{MA } 1]_e} + \frac{[\text{MA } 2]_c}{[\text{MA } 2]_e}\right).$$

2.5. Other methods

Affinity constants of antibodies for PAI-1 or for PAI-1 saturated with another monoclonal antibody were determined as described previously using Biacore (Pharmacia, Upsala, Sweden) [15].

3. Results and discussion

In an initial phase of the study seven monoclonal antibodies, MA-7D4B7, MA-7F5C12, MA-8H9D4, MA-33B8, MA-33H1, MA-55F4 and MA-56A7C10 were evaluated, individually and as equimolar mixtures (i.e. 21 combinations) for their capacity to neutralize PAI-1 activity. The presence or absence of synergism was then deduced from the constructed dose-response curves as analysed by the effect summation approach.

Based on these data three pairs (i.e. MA-33B8/MA-33H1, MA-33B8/MA-7D4B7, MA-33H1/MA-7D4B7) clearly showed synergism as illustrated in Fig. 1. All other combinations exhibited marginal synergism or no synergism (data not shown). Antagonism was never observed. It is interesting to note that these antibodies are directed against different epitopes. This observation is in keeping with the general view that synergism can only be observed between compounds exerting similar biological/pharmacological effects but through interaction with different targets.

These three combinations were then subjected to a more detailed analysis.

For each of these antibodies, dose-response curves were determined either in the absence or in the presence of a constant amount of another antibody. The data represented in

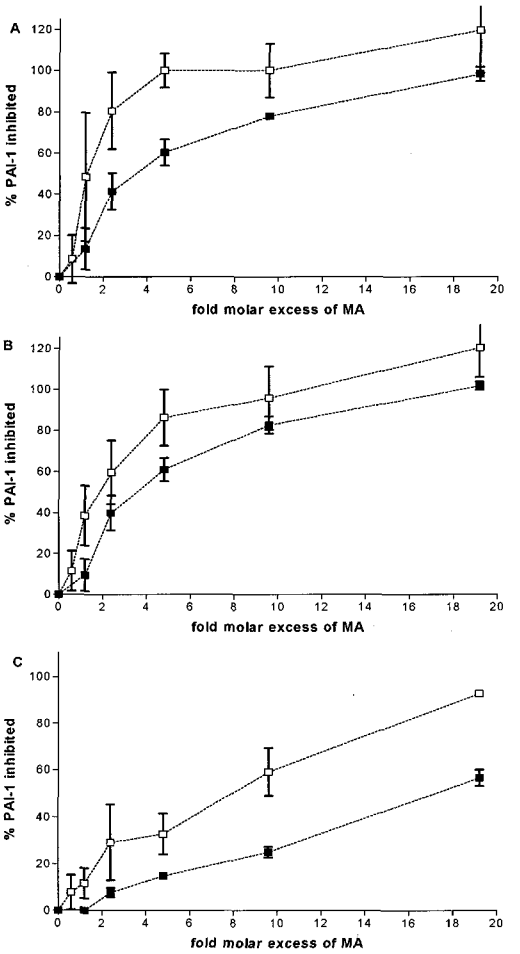

Fig. 1. Inhibition of PAI-1 activity by an equimolar mixture of monoclonal antibodies. Theoretical dose-response curve (closed symbol) and experimental dose-response curve (open symbol) of mixtures of two monoclonal antibodies (at equimolar ratio). A: MA-33H1/MA-7D4B7; B: MA-33B8/MA-33H1; C: MA-7D4B7/MA-33B8. Mean ± S.D., $n = 3$.

Fig. 2 show the pronounced synergy of the mixtures of a fixed amount of one antibody with different amounts of the other antibody. In the majority of the cases the difference between theoretically expected and experimentally observed effect is statistically significant ($P < 0.05$) and reaches up to 5-fold (e.g. MA-33H1 and MA-7D4B7). Following the construction of various dose-response curves using these combinations at different ratios the isoboles as shown in Fig. 3 were obtained. From their concave shape it can again be concluded that these combinations are characterized by a functional synergism with respect to PAI-1 neutralization. The respective interaction indices (Fig. 3 insets) are consistently lower than 1, further confirming the phenomena of synergism between these antibodies. From the obtained data it is clear that these particular pairs of antibodies act synergistically on the neutralization of PAI-1 activity. A possible explanation for this synergism could be that binding of one antibody to PAI-1 would increase the affinity of PAI-1 for the other antibody, thereby resulting in a steeper dose-response curve. To evaluate this possibility, the affinity constants of the individual antibody for binding to PAI-1 as such and to PAI-1 saturated with another antibody were determined. As can be deduced from

the data in Table 1 no major influence occurs, indicating that the observed synergism is not related to induced increases in affinities.

Importantly all three antibodies inactivate PAI-1 by different molecular mechanisms. MA-33H1 switches PAI-1 from an inhibitory molecule to a substrate-like molecule [15] and the epitope has been found to be located between amino acid Glu-128 and Ala-156 of PAI-1 [18]. MA-33B8 most likely neutralizes PAI-1 activity through an enhanced conversion of active to latent PAI-1 ([15]; Shore, J. and Declerck, P.J., in preparation). MA-33B8 is directed against a conformational epitope, not localized in the direct area of the reactive site loop. In contrast, MA-7D4B7 would inhibit PAI-1 through a direct interaction with the reactive site loop [14,16]. In this view and based on the known conformational flexibility of PAI-1 and its capacity to adopt various conformations with different functional properties, it could be speculated that the conformational change induced upon binding of one antibody makes PAI-1 more vulnerable to inhibition with the other antibody.

In conclusion, combining different monoclonal antibodies that exhibit neutralizing properties towards PAI-1 through distinct molecular mechanisms, results in synergistic functional effects. Even though the underlying molecular mechanism responsible for this observation is not clear yet, the possibility of synergism between various PAI-1 modulating compounds should be considered in the design of therapeutic or preventive strategies to interfere with PAI-1 activity. Indeed our observation also opens new perspectives in the search for low molecular weight compounds with PAI-1 inhibitory prop-

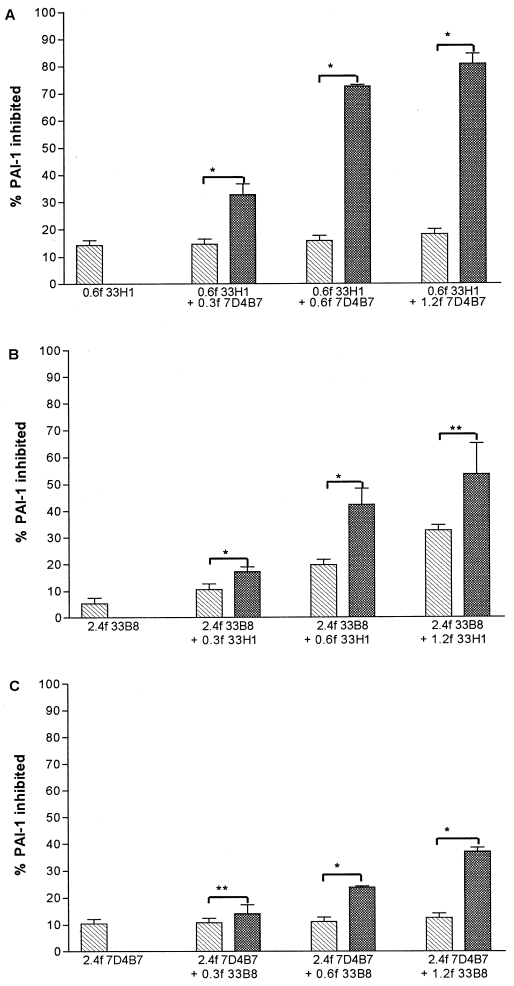

Fig. 2. Inhibition of PAI-1 activity by various concentrations of one monoclonal antibody in the presence of a fixed dose of another antibody. The hatched bars represent the theoretically expected value and the filled bars represent the experimental value. A: MA-33H1/MA-7D4B7; B: MA-33B8/MA-33H1; C: MA-7D4B7/MA-33B8. *: $P<0.05$, **: n.s., mean ± S.D., $n=3$.

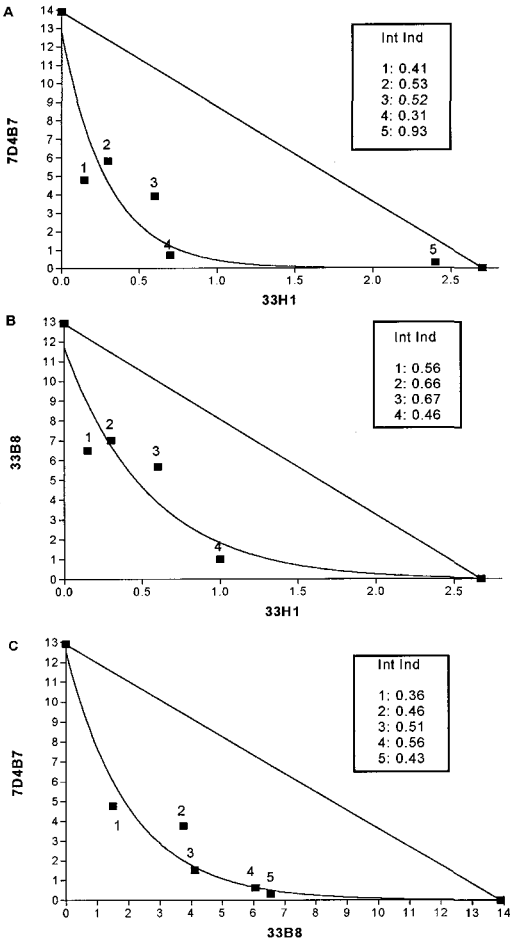

Fig. 3. Isoboles and interaction index of different combinations (at various ratios) of monoclonal antibodies. The graphs represent the molar excess of each antibody in a combination with a particular ratio MA 1:MA 2 (cfr methods), required to inhibit 50% of PAI-1 activity. The insets show the obtained interaction index (Int Ind). A: MA-33H1/MA-7D4B7; B: MA-33B8/MA-33H1; C: MA-7D4B7/MA-33B8. The straight line represents the graph that would have been obtained in case of the absence of synergism.

Table 1
Affinity constants (10^8 M^{-1}) of the monoclonal antibodies for binding to PAI-1 (either pretreated or not)

Pretreatment	Monoclonal antibody		
	MA-33H1	MA-33B8	MA-7D4B7
None	15.1	9.0	2.5
MA-33H1	NA	6.4	1.4
MA-33B8	11.0	NA	1.6
MA-7D4B7	2.2	2.5	NA

NA: not applicable.
Data represent mean value of two experiments.

erties: therapeutic application of such compounds may well benefit from a possible synergism since simultaneous administration of two synergistically acting compounds will allow to obtain the desired pharmacological effect at a much lower dose. Consequently a higher specificity may be obtained with much less pronounced side-effects.

It is also important to note that, in contrast to the generally observed mechanism of synergism (i.e. involving interaction of two compounds with different target molecules), this is, to the best of our knowledge, the first report on synergism occurring through interaction of two compounds with the same target molecule. In the current study, the existence of this phenomenon, for which we propose the name 'intra-molecular synergism', is most likely associated with the flexibility of the target molecule, i.e. PAI-1. Yet, it is interesting to speculate that a similar approach of 'intra-molecular synergism' may be applicable for interference with the activity of other putative target proteins.

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