Functional Characterization of Monoclonal Antibodies Directed against Fibrin Binding Domains of Tissue-type Plasminogen Activator*

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Johann Wojta†, Renate Beckmann‡, Liselotte Turcu§, Oswald F. Wagner†‡, Anton-Jan van Zonneveld§, and Bernd R. Binder†¶

From the †Laboratory for Clinical-Experimental Physiology, Department of Medical Pathology, University of Vienna, A-1090 Vienna, Austria and the §Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam 1006 AK, The Netherlands

Fibrin interacts with tissue-type plasminogen activator (tPA) via the finger and the kringle 2 domains. Three monoclonal antibodies against tPA, designated MPW3VPA, MPW6VPA, and MPW7VPA, which react with epitopes in the tPA molecule involved in fibrin binding, were characterized. The IgM monoclonal antibody MPW6VPA, directed against an epitope close to the finger and epidermal growth factor domains, stimulated plasminogen activation only in the absence of CNBr-fibrinogen fragments by increasing kcat in a dose-dependent fashion, an effect which was not restricted to the intact molecule. These results suggest that MPW6VPA mimics the initial effect of fibrin bound to the tPA molecule, which results in a change of kcat values. The MPW6VPA effect was reversed by another antibody, MPW3VPA, also directed against epidermal growth factor and finger domains. The latter antibody also inhibited plasminogen activation by tPA in the presence of CNBr-fibrinogen fragments. MPW7VPA directed against kringle 2 of tPA inhibited plasminogen activation by tPA only when CNBr-fibrinogen fragments were present. This inhibition was apparently competitive and dose-dependent. These data suggest that MPW3VPA interferes with the first phase of fibrin binding to tPA, whereas MPW7VPA interferes with the second phase of fibrin binding to the tPA molecule via kringle 2, resulting in Kcat changes.

Tissue-type plasminogen activator (tPA) released from the vascular endothelium is thought to be a major determinant of fibrinolytic activity in blood. It is believed that tPA function is characterized by the dramatic increase of its plasminogen activating activity in the presence of fibrin. This effect is thought to be brought about by binding of tPA and plasminogen to the fibrin surface, thereby causing a decrease of the Kcat value for plasminogen by about 2 orders of magnitude.

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† To whom correspondence should be addressed.
‡ The abbreviations used are: tPA, tissue-type plasminogen activator; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; pNA, p-nitroanilide.

Fibrin binding to the tPA molecule occurs via the finger and kringle 2 domains of tPA (4). van Zonneveld et al. (5) propose a biphasic model for this mechanism whereby fibrin binds in a first phase to the tPA finger, causing a change in kcat values, and in a second phase to tPA kringle 2 via generated COOH-terminal lysines in the fibrin molecule, resulting in a Kcat change.

It was the aim of this study to obtain monoclonal antibodies which bind to these domains of tPA, to identify the binding sites by use of recombinant deletion mutant proteins, to analyze the effects of such characterized monoclonal antibodies on plasminogen activation by tPA, and to compare these effects with those of CNBr fragments of fibrinogen.

MATERIALS AND METHODS

Protein A-Sepharose CL-4B, Sephacryl S-200, S-300, and S-400, Sephadex G-200 Superfine, and Pharmalyse, pH 3-10, were from Pharmacia (Sweden). Cyrogenic bromide was from Fluka (Switzerland). Freund's adjuvant, complete and incomplete, bovine serum albumin, human serum albumin, goat anti-rabbit IgG and IgM, heavy and light chain peroxidase conjugates, rabbit anti-mouse IgG and IgM, heavy and light chain were from Behringerwerke (Federal Republic of Germany). Microelisa plates were from Dynatec (Great Britain). Tween 20 and Tween 80, trypsin, soybean trypsin inhibitor, elastase from porcine pancreas, ortho-phenylenediamine, and fluorescamine were from Sigma. Rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, and IgM antiserum were from Nordic (Netherlands). Goat anti-mouse IgG and IgM, heavy and light chain peroxidase conjugates, were from Grub (Austria). RPMI 1640, fetal calf serum, and HAT solution (hypoxanthine, 680.5 mg/liter; aminopterin, 8.8 mg/liter; thymidine, 193.8 mg/liter; aminopterin, 8.8 mg/liter; thymidine, 193.8 mg/liter) were from Flow (Great Britain). L-Glutamine, 200 mM, was from Seromed (Federal Republic of Germany). Penicillin-streptomycin was from Gibco (Great Britain). Polyethylene glycol 4000, benzamide chloride, and dithiothreitol were from Merck. Pristane was from EGA-Chemie (Federal Republic of Germany). H-Val-Pro-Arg-pNA (S-2288), H-D-Val-Leu-Lys-pNA (S-300), and human fibrinogen were from Kabi (Sweden). 2-Mercaptoethanol, HRP reagent, gelatin, low and high molecular weight protein standards for SDS gel electrophoresis, acrylamide, bisacrylamide, and sodium dodecyl sulfate were from Bio-Rad. Iodoacetamide, from Calbiochem.

Plasminogen was purified from human plasma by affinity chromatography on lysine-Sepharose (6) followed by gel filtration on Sephacryl S-200 and was obtained in its native form as judged by acid-urea-polyacrylamide gels electrophoresis. CNBr fragments of fibrinogen were prepared according to Blombäck et al. (7). Plasminogen activator inhibitor was purified as reported (8). tPA was purified from cadaver vessel perfusates (9) and supernatants of melanoma cell cultures (9, 10). Human high molecular weight urokinase and low molecular weight urokinase were prepared as described earlier (11, 12). Protein was determined using the dye reagent and standards from Bio-Rad (13). Protein was monitored at 280 nm. Protein in the purified tPA preparations was determined by the fluoroscamine method (14) and its activity compared with the International Refer-
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**Fig. 1. Epitope mapping with a sandwich assay.** To map the epitopes of the tPA molecule for the monoclonal anti-tPA antibodies MPW3VPA (panel A), MPW7VPA (panel B), and MPW6VPA (panel C), the antibodies were coupled to Sepharose and incubated with dilutions of the recombinant tPA deletion mutant proteins containing conditioned media and 125I-labeled monoclonal anti-tPA light chain antibody ESP2. The mutant proteins are designated according to the domains on the molecules: L, light chain; LF, light chain, finger; LFE, light chain, finger, epidermal growth factor; LK1, light chain, kringle 1; LK2, light chain, kringle 2; LK1-2, light chain, kringle 1, kringle 2; LEK1-2, light chain, EGF, kringle 1, kringle 2; rtPA, recombinant tPA; mtPA, melanoma-type tPA.

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**RESULTS**

**Antibody Binding Sites on the tPA Molecule—**To map the epitopes of the tPA molecule for the selected clones MPW3VPA, MPW7VPA, and MPW6VPA the antibodies were coupled to Sepharose and incubated with dilutions of the recombinant tPA deletion mutant proteins containing conditioned media and 125I-labeled monoclonal anti-tPA light chain antibody ESP2. The mutant proteins are designated according to the domains on the molecules: L, light chain; LF, light chain, finger; LFE, light chain, finger, epidermal growth factor; LK1, light chain, kringle 1; LK2, light chain, kringle 2; LK1-2, light chain, kringle 1, kringle 2; LEK1-2, light chain, EGF, kringle 1, kringle 2; rtPA, recombinant tPA; mtPA, melanoma-type tPA.

**Kinetic Studies with the Intact Antibodies—**To characterize the effects of the selected monoclonal antibodies, kinetics of plasminogen activation were studied. The samples were incubated for 17 h while rotating end over end at room temperature. After washing, the samples were counted, and the results were calculated as percent of total input label.

From these data, two monoclonal antibodies of the IgG1 class (MPW3VPA and MPW7VPA) and one monoclonal antibody of the IgM class (MPW6VPA) were selected and further studied. To exclude the possibility that the effects of MPW6VPA might be due to aggregated IgM, 2 mg of MPW6VPA was gel-filtered on a Sephadex S-400 column (100 x 1.6 cm) equilibrated in a buffer containing 0.1 M Tris, pH 8.0. Only one homogeneous protein peak corresponding to M, 900,000 was obtained and used for the kinetic studies.

Furthermore, to exclude the possibility that the MPW6VPA effects were due to the multimeric nature of the antibody, immunologically active fragments of the purified IgM antibody were produced by mild digestion with trypsin as described (21). The fragments were purified by immunoaffinity and tested for their immunological reactivity toward tPA in an ELISA as outlined in this section and under “Results.”

**Kinetic Studies with the Intact Antibodies—**To characterize the effects of the selected monoclonal antibodies, kinetics of plasminogen activation were studied in the presence of the antibodies. Therefore, 100 µl of purified antibody at concentrations of 10, 2, and 1 µg/ml was incubated for 50 min at 37 °C with 100 µl of purified tPA (vascular type) (final concentration, 0.014 nM) as a control, preimmune mouse IgG or IgM, purified as described for the monoclonal antibodies, was used. The mixtures were incubated thereafter at 37 °C with four different concentrations of Glu-plasminogen (from 0.05 to 0.78 µM final concentration) in the absence and presence of CNBr fragments of fibrinogen (55 µg/ml final concentration). Plasmin formation in the incubation mixtures was quantitated by cleavage of H-D-Val-Leu-Lys-pNA (final concentration, 0.6 mM). Increase in absorbance was followed at 405 nm up to 50 min. A ΔA/min of 0.01/min/fliter plasmin was used to calculate the amount of plasmin present in the incubation mixture (22). This value was plotted versus incubation time for each plasminogen concentration used. Plasmin formation/min for the linear part of the reaction (22, 23) was determined and plotted versus the substrate concentration using a Lineweaver-Burk double-reciprocal plot, from which Kₘ and Vₘₐₓ values for plasminogen activation were calculated using the least squares method.

To further specify the stimulating effect of IgM antibody MPW6VPA, kinetic studies as described above were performed, but intact IgMs were replaced by immunologically active fragments (1, 0.2, and 0.1 µg/ml final concentration). In addition, kinetic studies were performed with fragments of MPW6VPA (1 µg/ml final concentration), as described above, after tPA was preincubated for 50 min at 37 °C without or with three different concentrations of MPW3VPA (1, 0.2, and 0.1 µg/ml final concentration).

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**As can be seen from Fig. 1, MPW3VPA reacted only with the deletion mutant protein containing finger and EGF do-**
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![Graphs showing Lineweaver-Burk plots](image)

**Fig. 2.** Lineweaver-Burk plots of $1/v$ versus $1/[\text{plasminogen}]$ for the activation of plasminogen by tPA in the absence (○) and presence of three different concentrations of the monoclonal anti-tPA antibody MPW3VPA (1 μg/ml (○), 0.2 μg/ml (Δ), and 0.1 μg/ml (□)). Vascular tPA was used in a final concentration of 0.014 nM. Plasmin formation was quantified by the cleavage of H-D-Val-Leu-Lys-pNA in a final concentration of 0.6 mM. Panel A shows kinetic analysis in the absence of, and panel B in the presence of, CNBr fragments of fibrinogen (55 μg/ml final concentration). Pn, plasmin; Pgn, plasminogen.

Km remains, indicating that its antigenic binding site is located close to these domains. MPW6VPA reacted with the deletion mutant containing the EGF and the finger domains and slightly with a mutant containing only the EGF, but not the finger domain. MPW7VPA reacted only with mutants containing kringle 2.

Kinetic analysis of the effect of the antibodies on plasminogen activation in the absence and presence of CNBr fragments of fibrinogen are shown in Figs. 2-4. Fig. 2 shows the effect of MPW3VPA on plasminogen activation by tPA in the absence and presence of CNBr fragments of fibrinogen. No effect was seen in the absence of fibrinogen fragments (Fig. 2A), whereas in the presence of fibrinogen fragments MPW3VPA inhibited plasminogen activation in a non-competitive way by changing $k_{cat}$ values from 0.15 to 0.12 s$^{-1}$ at 0.1 μg/ml, to 0.08 s$^{-1}$ at 0.2 μg/ml, and to 0.07 s$^{-1}$ at 1 μg/ml antibody. The $K_m$ value remained unchanged and was 0.05 μM. $K_i$ was calculated to be 3.14 nM.

MPW7VPA, in contrast, inhibited plasminogen activation by tPA in the presence of fibrinogen fragments in a competitive way by increasing $K_m$ from 0.05 to 0.12 μM at 0.1 μg/ml, to 0.3 μM at 0.2 μg/ml, and to 1.0 μM at 1.0 μg/ml antibody. $V_{max}$ values remained unchanged, and a $K_i$ of 0.57 nM was calculated (Fig. 3B). No effect was seen in the absence of CNBr fragments of fibrinogen (Fig. 3A).

Fig. 4A shows the results obtained with MPW6VPA in the absence of fibrinogen fragments. As can be seen, MPW6VPA stimulated plasminogen activation by increasing $V_{max}$ values in a dose-dependent fashion, resulting in a $k_{cat}$...
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FIG. 4. Lineweaver-Burk plots of $\frac{1}{v}$ versus $\frac{1}{[\text{plasminogen}]}$ for the activation of plasminogen by tPA in the absence (○) and presence of three different concentrations of the monoclonal antibody MPW6VPA (1 μg/ml (□), 0.2 μg/ml (△), and 0.1 μg/ml (◇)). Vascular tPA was used in a final concentration of 0.014 nM. Plasmin formation was quantified by the cleavage of H-D-Val-Leu-Lys-pNA in a final concentration of 0.6 mM. Panel A shows kinetic analysis in the absence of, and Panel B shows kinetic analysis in the presence of, CNBr fragments of fibrinogen (55 μg/ml final concentration).

change. $K_m$ decreased from 6.6 to 0.38 μM at all three antibody concentrations used. The $k_{cat}$ changed from 0.06 to 0.07, 0.12, and 0.23 s$^{-1}$ when 0.1, 0.2, and 1.0 μg/ml MPW6VPA, was used. MPW6VPA did not influence plasminogen activation in the presence of fibrinogen fragments (Fig. 4B).

Preimmune mouse IgM used as a control did not alter the pattern of plasminogen activation in either the absence or presence of CNBr fragments of fibrinogen.

When purified MPW6VPA fragments were applied to SDS gel electrophoresis under nonreducing conditions, two major protein bands at $M$, 110,000 and 85,000 were detected, whereas under reducing conditions only one stainable protein band at $M$, 85,000 was visible (data not shown). These fragments showed approximately the same 50% binding efficiency when compared with the intact antibody in an ELISA system using tPA coated to the plate. The values were 0.29 μg/ml for MPW6VPA fragments and 0.25 μg/ml for intact MPW6VPA. When these immunologically characterized active fragments of MPW6VPA were used for kinetic studies, fragments of MPW6VPA showed a similar pattern of stimulation in the absence of fibrinogen fragments. The $k_{cat}$ values were 0.24 s$^{-1}$ at 1 μg/ml, 0.12 s$^{-1}$ at 0.2 μg/ml, and 0.08 s$^{-1}$ at 0.1 μg/ml MPW6VPA fragments, as compared with a $k_{cat}$ of 0.06 s$^{-1}$ without antibody fragments.

Fig. 5 shows that the stimulating effect of MPW6VPA fragments could be reversed by preincubation of tPA with different concentrations of MPW3VPA. $k_{cat}$ changed from 0.24 s$^{-1}$ without preincubation with MPW3VPA to 0.15 s$^{-1}$ at 0.1 μg/ml, to 0.08 s$^{-1}$ at 0.2 μg/ml, and to 0.06 s$^{-1}$ at 1 μg/ml MPW3VPA.

**DISCUSSION**

Three monoclonal antibodies against fibrin binding domains in the tPA molecule were obtained. Two IgG antibodies, MPW3VPA and MPW7VPA, were directed against an epitope close to the finger and EGF domains and to the kringle 2 domain, respectively, and one IgM antibody, MPW6VPA, reacted with an epitope similar but not identical to the binding site of MPW3VPA.

MPW3VPA inhibited plasminogen activation by tPA in the presence of fibrinogen fragments in a noncompetitive way by decreasing $k_{cat}$ values. Since van Zonneveld et al. (5) proposed a biphasic model of fibrin binding to the tPA mole-
cule in which fibrin binds in a first phase to tPA via the finger domain, thereby causing changes in $k_{cat}$ values, it can be concluded from the kinetic analyses that MPW3VPA interferes with that type of fibrin binding to the finger domain. In contrast, MPW7VPA, which binds to kringle 2, inhibits plasminogen activation by tPA in the presence of fibrinogen fragments in a competitive way by increasing $K_m$ values. According to van Zonneveld et al. (5), fibrin binds to tPA in a second phase via kringle 2, thereby causing a decrease in $K_m$. Our data obtained by kinetic analysis suggest that MPW7VPA interferes with the binding of fibrin to kringle 2.

MPW6VPA, however, stimulates plasminogen activation by tPA in the absence of fibrinogen fragments by increasing $k_{cat}$ values. MPW6VPA binds to an epitope close to the finger and EGF domains. The stimulating effect of this antibody was not restricted to the intact molecule but was also brought about by immunologically active fragments. This finding suggests that the stimulating effect is not caused by steric effects due to the $M_r$ 900,000 IgM molecule. Furthermore, the stimulating effect was reversed by preincubation of tPA with MPW3VPA, which inhibits fibrin effects in a noncompetitive way by binding to an epitope close to the EGF and finger domains, as described above. From the binding data and the kinetic pattern of the stimulating effect caused by MPW6VPA we conclude that this antibody mimics the initial binding of fibrin to the tPA molecule via the finger domain, which results in $k_{cat}$ changes as described above.

In summary, our data support the finding of others that fibrin binding to tPA occurs via two different domains, both located on the heavy chain (4). Furthermore, the data obtained by kinetic analysis using these antibodies support the model proposed by others (5, 24) in which fibrin binding to tPA takes place in a biphasic reaction whereby the initial binding of fibrin via the finger domain results in $k_{cat}$ changes, whereas fibrin binding in the second phase via kringle 2 causes a decrease in $K_m$ values of tPA-mediated plasminogen activation.

**REFERENCES**


