Inhibition of Clot-Bound $\alpha_2$-Antiplasmin Enhances In Vivo Thrombolysis

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Recent experiments in vitro have shown that inhibition of human $\alpha_2$-antiplasmin by a monoclonal antibody (MAb RWR) markedly enhances clot lysis by plasminogen activators. To extend these studies in vivo, we tested whether inhibition of clot or fibrin-bound $\alpha_2$-antiplasmin by MAb RWR could enhance the lysis of a human clot by tissue-type plasminogen activator (t-PA) in a rabbit jugular vein thrombosis model. Compared with a saline placebo or a control antibody, MAb RWR significantly increased thrombolysis by endogenous plasminogen activator in rabbits to which no t-PA was administered ($p<0.05$). In rabbits that received t-PA, the combination of MAb RWR and t-PA caused significantly greater thrombolysis than equivalent doses of t-PA alone ($p<0.05$). However, compared with equipotent doses of t-PA alone, the combination of MAb RWR and t-PA did not increase the nonspecific consumption of fibrinogen. These experiments suggest that the combination of an $\alpha_2$-antiplasmin inhibitor and a plasminogen activator could be a more potent thrombolytic strategy. (Circulation 1990;82:164–168)

Thrombolytic therapy for myocardial infarction has been delayed or underused because of concern about hemorrhagic complications. Indeed, at the doses routinely administered, all plasminogen activators can cause systemic fibrinolysis with degradation of clotting factors such as fibrinogen.1 This complication has prompted a search for novel thrombolytic strategies that can increase clot lysis without causing a systemic lytic state.

One recently reported strategy2,3 is to use a monoclonal antibody (MAb) that inactivates both plasma and clot (fibrin)-bound human $\alpha_2$-antiplasmin, the chief inhibitor of clot lysis.4 When added to clotted plasma in vitro this antibody, RWR, facilitates the "spontaneous" clot lysis induced by endogenous plasminogen activator. When added to clots in combination with tissue-type plasminogen activator (t-PA), MAb RWR interacts synergistically with the t-PA to markedly amplify clot lysis. The combination of RWR and a plasminogen activator increases the potency (for 50% lysis) of streptokinase, urokinase, and t-PA by 20–80-fold yet does not appear to increase nonspecific consumption of fibrinogen in vitro.3

The following in vivo experiments were performed to test whether selective inhibition of fibrin-cross-linked $\alpha_2$-antiplasmin is an effective thrombolytic strategy. We used the rabbit jugular vein thrombolytic model originally described by Collen et al.5 as it has been modified by our laboratory.6 The results suggest that specific inhibition of clot-bound $\alpha_2$-antiplasmin can significantly increase the rate of thrombolysis without increasing the consumption of clotting factors such as fibrinogen.

**Methods**

**Proteins**

t-PA with a specific activity of 580,000 IU/mg was purchased from Genentech, South San Francisco, California. MAb RWR and MAb 40–160 (an antidigoxin, control antibody) were raised as described.3,7 Both MAb's were purified from ascites by ammonium sulfate precipitation followed by ion exchange chromatography on DEAE-Affigel Blue, Bio-Rad, Richmond, California. The concentration of MAb RWR was determined by a competition radioimmunoassay similar to that described by Mariani et al.8 The concentration of antidigoxin MAb 40–160 was measured by spectrophotometry with an extinction coefficient (1% solution, 1 cm path length) of 1.43.
α2-Antiplasmin was purified from human and rabbit plasma by Wiman’s method.5

In Vitro Experiments

Antibody-binding assays were performed to study the binding of MAb RWR to human and rabbit α2-antiplasmin. Wells of a microtiter plate were coated with 25 μL of a 10 μg/mL solution of human or rabbit α2-antiplasmin for 1 hour at room temperature. The wells were then rinsed and treated with 100 μL of a 1% solution of bovine serum albumin (RIA grade) in phosphate-buffered saline containing 0.02% azide (PBSA) to saturate any nonspecific protein-binding sites. The plates were washed again and tapped dry. Dilutions of MAb RWR (from mature hybridoma culture supernatant) were added in triplicate to wells containing human or rabbit α2-antiplasmin or a control (bovine serum albumin alone). After incubation for 1 hour, the wells were washed eight times and tapped dry. Then, 25 μL (about 50,000 cpm) of radioiodinated goat-antimouse F(ab')2 antibody (Cappel Laboratories, West Chester, Pennsylvania) was added to each well and allowed to incubate for 1 hour. Subsequently, the unbound radioactive antibody was aspirated, and the plates were washed and dried. The wells were cut out and gamma-counted to determine the amount of bound antibody.

In vitro clot lysis assays were performed with fresh-frozen rabbit plasma collected on citrate and pooled from four normal rabbits. The rabbit plasma was mixed with trace amounts of radiiodinated human fibrinogen (Ibrin; Amersham, Arlington Heights, Illinois). Fifty microliters of this mixture (about 15,000 cpm) was combined in a test tube with 50 μL of Tris-buffered saline azide (TBSA) containing 30 mM CaCl₂. The plasma mixture was then incubated at 37°C for 1.5 hours to facilitate clotting. The clots were washed with 1 ml of normal saline, which was removed by aspiration. Then, 50 μL of MAb RWR (37.5 μg) in TBSA or TBSA alone was added to each tube. The tubes were gamma-counted, and 100 μL of TBSA was added to each. The experiment was started by adding 100 μL of t-PA in various amounts (0–10 IU) to the clots. After incubation at 37°C for 1 hour, 250 μL of ice-cold PBSA containing aprotinin (100 kallikrein inhibitor units/ml) was added to each tube. Immediately thereafter, 100 μl of the liquid supernatant was sampled and gamma-counted. The percentage clot lysis was expressed as the ratio of cpm present in the liquid supernatant to cpm remaining in the clot.

Fibrinogen levels were assayed by both the sodium sulfite10 and modified Clauss methods.11 Residual α2-antiplasmin activity was measured by a chromogenic substrate assay12 kit from Stachrom (Asnières, France).

In Vivo Thrombolysis Experiments

Thrombolysis was studied in anesthetized rabbits according to the model originally developed by Collen et al.5 The experiments were performed as previously described by our laboratory,6 with the following modifications. After the [125]I-labeled fibrinogen, human plasma, and red blood cells had clotted in the jugular vein for 30 minutes, t-PA or placebo (saline) was administered by a constant infusion pump through the contralateral, marginal ear vein for 1 hour. In rabbits that received MAb RWR or the control MAb (antidigoxin 40–160), the antibody was given as a single bolus (1.4 mg/kg, 1–2 ml) immediately before the infusion of t-PA or placebo. Two hours after the t-PA or placebo infusion had been terminated, the jugular vein was ligated, excised, and gamma-counted. The percentage lysis was computed from the ratio of the original vein cpm to the final vein cpm as described.5,6 Blood samples (1 ml) were collected before the agents were administered and at the end of the study. The samples were collected on sodium citrate (0.38% final) and P-PACK (10.4 μg/ml final) (Sigma Chemical, St. Louis, Missouri), centrifuged immediately, and frozen at −80°C until assay for fibrinogen and α2-antiplasmin.

All in vivo experiments were performed by one technician who was blinded to the dose of antibody or t-PA. Of the 40 thrombolysis experiments completed according to this protocol, 13 were rejected: eight because of unsatisfactory vein and clot preparation, four because of improper infusion of agent, and one because of excessive movement due to inadequate sedation. Results from the remaining 27 experiments were analyzed and are reported in detail. At the completion of this series, two additional (control) experiments were performed in which the rabbits were given an inert, antidigoxin antibody (MAb 40-160) as a placebo.

Values are given as mean±SEM. A two-sample t test was used to compare the means of different groups.

Results

Because MAb RWR was selected for its ability to bind to human α2-antiplasmin, a radioimmunoassay was performed to test its ability to bind to rabbit α2-antiplasmin. Figure 1 compares the binding of MAb RWR to human and rabbit α2-antiplasmin and a control. The data reveal that RWR bound to human α2-antiplasmin in a concentration-dependent fashion but did not bind significantly to rabbit α2-antiplasmin.

Given that MAb RWR did not appreciably bind to purified rabbit α2-antiplasmin alone, we tested whether MAb RWR could bind to and inhibit fibrin-crosslinked or clot-bound rabbit α2-antiplasmin in an in vitro, rabbit plasma clot lysis assay. In a previous report, we demonstrated that RWR, because of its ability to bind to and inhibit human α2-antiplasmin, markedly accelerates the lysis (by 20–30-fold) of human plasma clots by t-PA. In a similar assay in rabbit plasma (Figure 2), although present in molar excess of rabbit α2-antiplasmin, RWR did not accelerate the lysis of rabbit plasma clots by t-PA. Thus, by radioimmunoassay and clot lysis experiments, RWR
FIGURE 1. Plot of binding of monoclonal antibody RWR to human and rabbit \( \alpha_t \)-antiplasmin. A solid-phase radioimmunoassay was used to compare the binding of monoclonal antibody RWR with that of human \( \alpha_t \)-antiplasmin (●), rabbit \( \alpha_t \)-antiplasmin (○), and control (bovine albumin, □). Compared with the control monoclonal antibody, RWR binds significantly to human \( \alpha_t \)-antiplasmin but not to the rabbit \( \alpha_t \)-antiplasmin. Each point represents the mean (±SEM) of triplicate observations.

shows no significant binding or inhibition of rabbit \( \alpha_t \)-antiplasmin.

Because MAb RWR binds to and inhibits human but not rabbit \( \alpha_t \)-antiplasmin, we were able to construct an experimental model to test the hypothesis that inhibition of clot-bound \( \alpha_t \)-antiplasmin would accelerate thrombolysis in vivo. Radiolabeled human clots were formed in rabbit jugular veins to compare the thrombolytic effects of placebo (saline or an inert, control antibody), t-PA, RWR, or the combination of t-PA and RWR. Because MAb RWR only inhibits human \( \alpha_t \)-antiplasmin, any thrombolytic effect of the antibody must be due to inhibition of clot-bound (i.e., human) \( \alpha_t \)-antiplasmin. Figure 3 shows the percent clot lysis in rabbits that received t-PA alone or the combination of t-PA and MAb RWR. Rabbits receiving RWR and t-PA showed significantly greater lysis than those receiving an equivalent dose of t-PA alone (p<0.05). Compared with the dose-response curve for t-PA alone, the dose-response curve for RWR and t-PA appears shifted to the left and upward, with RWR increasing the thrombolytic potency of t-PA by twofold to threefold.

Figure 4 compares the amount of lysis in rabbits receiving saline placebo, a control (antidigoxin) antibody, or RWR alone. In rabbits that received saline placebo, mean clot lysis was 16.1±3.1%. In those that received control MAb, mean lysis was 10.3±3.8%. However, in rabbits that received RWR without t-PA, mean lysis was 25.9±2.8%, which is greater than and significantly different from that obtained in the saline or antibody placebo experiments (p<0.05). Thus, in addition to enhancing the thrombolytic potency of exogenous (administered) t-PA, RWR increased

FIGURE 2. Plot of effect of monoclonal antibody RWR on rabbit clot lysis by tissue-type plasminogen activator (t-PA) in vitro. Pooled fresh-frozen rabbit plasma was labeled with \(^{125}\text{I}-\text{fibrinogen} \) and clotted. Various amounts of t-PA (0.123–10 IU) were added to the clots and incubated at 37°C. The percentage clot lysis was compared for clots incubated with monoclonal antibody RWR (●) and without (○). Each point represents the mean±SEM of triplicate observations.

FIGURE 3. Plot of thrombolysis by tissue-type plasminogen activator (t-PA) and monoclonal antibody RWR in vivo. The dose-response curves for t-PA alone (○) and for t-PA and monoclonal antibody RWR in combination (●) are shown. The number of rabbits that received each agent are in parentheses. Compared with rabbits receiving equal doses of t-PA alone, the rabbits that received the combination of t-PA and antibody RWR had significantly greater clot lysis (p<0.05). Each point represents the mean lysis±SEM.

FIGURE 4. Bar graph of thrombolysis by monoclonal antibody RWR in vivo. The amount (mean±SEM) of clot lysis is shown for rabbits receiving saline placebo alone (n=4), an inert control monoclonal antibody alone (n=2), or monoclonal antibody RWR alone (n=3). Compared with saline placebo or the control antibody, antibody RWR caused significantly more clot lysis (p<0.05). There was no significant difference (p=0.32) in the amount of lysis produced by the control monoclonal antibody compared with the saline placebo.
thrombolysis by the rabbit's endogenous plasminogen activator.

To determine whether clot-specific inhibition of α2-antiplasmin affected the fibrin selectivity of t-PA, we measured the residual fibrinogen levels for RWR and t-PA in combination and those for t-PA alone (Figure 5A and B). Fibrinogen levels remained substantially unchanged as a function of clot lysis for both combination and single-agent regimens. In addition, we measured antiplasmin levels for the two regimens (Figure 6). Although the antiplasmin level for rabbits receiving t-PA without MAb RWR appeared slightly less at the 26% lysis point, this difference was the result of one outlying value. Thus, there was no apparent decrease in α2-antiplasmin levels in rabbits that received the α2-antiplasmin inhibitor, confirming our previous results (Figure 2) that MAb RWR has no measurable inhibitory effect on rabbit α2-antiplasmin.

Discussion

We have exploited the species selectivity of MAb RWR to test whether inhibition of clot-bound α2-

antiplasmin enhances clot lysis by t-PA. Compared with saline placebo or an inert MAb, MAb RWR significantly augmented clot lysis by the rabbit's endogenous plasminogen activator. When administered in combination with t-PA, MAb RWR also significantly increased thrombolysis compared with the lysis achieved by equivalent doses of t-PA alone. This increased lysis by the combination of t-PA and MAb RWR occurred without decreasing circulating fibrinogen or α2-antiplasmin levels. Previous in vitro experiments suggest that this enhanced clot lysis is due to the fact that both agents increase the concentration of plasmin by independent mechanisms: t-PA catalyzes the activation of plasminogen, and MAb RWR inhibits the inactivation of plasmin by α2-antiplasmin. The net result is a synergistic interaction between the two agents.3

In vivo, α2-antiplasmin is present in two forms: circulating free in the plasma and covalently cross-linked to the α chain of fibrin. Both forms inhibit fibrinolysis,13 although it has been hypothesized that the role of free α2-antiplasmin is largely to inhibit circulating plasmin and prevent a systemic lytic state.14,15 Sakata and Aoki4 have shown that fibrin-bound α2-antiplasmin is a critical inhibitor of clot lysis. Their experiments demonstrate that clots from α2-antiplasmin–deficient patients undergo spontaneous lysis even when those clots are suspended in plasma containing normal levels of free α2-antiplasmin. We have extended their findings by demonstrating that an inhibitor of clot-bound antiplasmin can augment thrombolysis even when it is administered after clot formation to an animal whose circulating levels of free α2-antiplasmin are normal.

In rabbits, t-PA is the most potent and clot-specific thrombolytic agent.5 Thus, the improved lytic potency of t-PA by MAb RWR was a rigorous test of the feasibility of a clot-specific inhibitor of α2-antiplasmin. Furthermore, these experiments demonstrated that the combination of t-PA and MAb RWR did not increase fibrinogen consumption over that obtained with equipotent doses of t-PA alone. As such, clot-specific inhibition of α2-antiplasmin enhances the potency of t-PA and preserves its fibrin selectivity. In the case of an agent such as urokinase, which is not selective for fibrin, it is likely that a clot-specific inhibitor of α2-antiplasmin would cause a similar, or more pronounced, enhancement in potency and lead to less fibrinogen consumption as well.

Like all in vivo models, these experiments are imperfect approximations to a human thrombolytic situation. Yet this model has permitted the initial test of a new thrombolytic strategy. We have previously shown in vitro that inhibition of both plasma and clot-bound α2-antiplasmin increases clot lysis without increasing fibrinogen consumption.3 We have extended these studies in vivo and demonstrated that selective inhibition of clot-bound α2-antiplasmin alone improves the potency of t-PA and, as we would expect, preserves its fibrin selectivity. These results suggest that the combination of a clot-specific inhibitor of

Figure 5. Plot of fibrinogen consumption by tissue-type plasminogen activator (t-PA) and monoclonal antibody RWR. Residual fibrinogen levels were measured by the sulfite precipitation assay (A, n=18) or by the modified Clauss assay (B, n=20). Each graph shows the mean values±SEM for t-PA alone (○) and the combination of monoclonal antibody RWR and t-PA (●).

Figure 6. Plot of effect of tissue-type plasminogen activator (t-PA) and monoclonal antibody RWR on rabbit α2-antiplasmin consumption. The mean residual α2-antiplasmin levels (±SEM, n=22) are shown for rabbits given t-PA alone (○) and the combination of t-PA and monoclonal antibody RWR (●).
\( \alpha_2 \)-antiplasmin and a plasminogen activator could be a more effective thrombolytic strategy.

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**KEY WORDS** • plasminogen activator • monoclonal antibodies • fibrinolysis • antiplasmin
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