Potential Thrombolysis under Selective Infusion of Autologous Plasmin (AP) Solution

Masahiro Mizutani, M.D., Takeshi Kobayashi, M.D., Masaaki Makita, M.D., Atsushi Miyamoto, M.D., Katsushi Kanamori, M.D., Hiroshi Hasegawa, M.D.,* and Hisakazu Yasuda, M.D.

SUMMARY

Thrombolytic therapy with plasminogen activators, urokinase (UK) and streptokinase (SK), can produce serious complications such as systemic bleeding. We have developed an autologous plasmin (AP) solution as a new potential thrombolytic agent and evaluated its efficacy in animal experiments. The AP solution was prepared by the addition of UK to autologous plasma separated by centrifugation (4°C, 3,000 rpm, 10 min). The plasmin activity in the AP solution was measured by the plasminogen-free fibrin plate method and spectrophotometric assay with the chromogenic substrate S-2251. In animal experiments, the femoral artery of anesthetized mongrel dogs (n=20) was constricted by ligation (1 mm in diameter) and a fibrin clot was embolized into this site. Either AP solution (n=8), UK solution (n=6) or saline (n=6) was selectively infused for 3 min. Prior to the infusion, a temporary flow obstruction was made by inflation of a balloon tip catheter located proximal to the embolized site. The thrombolytic effect was sequentially observed with an ultrasound flow meter for up to 60 min. The total dose of UK was 120,000 IU in both AP and UK solutions. The results showed that the plasmin activity of AP solution was maintained up to 90 min at 22°C with an additional dose of more than 6,000 IU/ml of UK. In animal experiments, a restoration of the flow was observed more frequently with the AP solution than the UK solution within 15 min of the infusion (p<0.01). Thus, a high thrombolytic efficacy was observed with a selective infusion of rich, activated plasmin (AP) solution. This treatment could be applied as a new approach for arterial thrombolysis.

Additional Indexing Words:
Fibrin clot Clot lysis Autologous plasmin

From the Department of Cardiovascular Medicine and the *First Department of Internal Medicine, Hokkaido University School of Medicine, Sapporo, Japan.
Address for reprints: Takeshi Kobayashi, M.D., Department of Cardiovascular Medicine, Hokkaido University School of Medicine, Kita 15 Nishi 7, Kita-ku, Sapporo 060, Japan.
Received for publication May 30, 1988.
Accepted January 6, 1989.

723
RAPID dissolution of intravascular thrombi requires fibrinolysis of the fibrin nets by plasmin, an enzyme that degrades polymeric fibrin to small soluble fragments. Urokinase (UK) and streptokinase (SK) are plasminogen activators that convert plasminogen to plasmin. These activators are presently available as thrombolytic agents. However, they have little specific affinity for fibrin and, therefore, can activate circulating and fibrin-bound plasminogen relatively indiscriminately. Accordingly, when UK or SK is administered, the plasmin in circulating blood will initially be inactivated very rapidly by alpha2-plasmin inhibitor (alpha2-PI), alpha-macroglobulin and antithrombin III, and will lose its thrombolytic efficacy. Once the capacity of these inhibitors becomes exhausted, residual plasmin can degrade several plasma proteins (fibrinogen, coagulant factors V and VIII), which may cause serious bleeding. Moreover, exhaustion of circulating plasminogen results in less effective thrombolysis. This probably explains why thrombolytic therapy with UK or SK has only limited efficacy and may be associated with serious side effects. In an attempt to obtain safer and more effective thrombolytic therapy, we have developed an autologous plasmin (AP) solution and evaluated its physiological efficacy in vivo.

**Methods**

**Preparation of AP solution:**

Peripheral arterial blood from experimental animals was drawn into a syringe containing 3.8% sodium citrate to a final dilution of 9:1. After autologous plasma was separated by 3,000 rpm centrifugation at 4°C for 10 min, the AP solution was quickly prepared at 4°C by the addition of UK to autologous plasma separated by centrifugation at 4°C.

**Measurement of plasmin activity contents in in vitro experiments:**

1) Fibrin plate method
   
   A) UK was added to autologous plasma in a dose range of 93.8 IU/ml to 4,000 IU/ml to prepare AP solutions. The prepared AP solutions were incubated at 37°C for 10 min and the plasmin activities were assayed by measuring the area of digested fibrin on plasminogen-free fibrin plates (pk Kowa, Kowa Pharmaceutical Co., Tokyo, Japan), after incubation at 37°C for 18 hours.

   B) Three preparations were made to compare plasmin activity contents. The first plasmin solution (AP) was prepared by the addition of 2,000 IU/ml UK to autologous plasma separated by centrifugation at 4°C.
Vol. 30
No. 5
POTENTIAL THROMBOLYSIS WITH AP SOLUTION

Fig. 1. Preparation of the AP solution and measurements of plasmin activity in the solution.

(n=6). The second plasmin solution was prepared by the addition of the same dose of UK to autologous plasma separated by centrifugation at 22°C (n=6). The third plasmin solution was prepared by the addition of the same dose of UK to whole blood at 22°C (n=6). The plasmin activity of each solution was measured by the same method as above.

2) Spectrophotometric assay

The changes with passage of time in the plasmin activity contents of the AP solution:

The chromogenic substrate S-2251 (Testzym APL kit, Kabi—Vitrum Diagnostics, Stockholm, Sweden) was used to quantify plasmin activity. Prior to the assay, a standard concentration curve of human plasmin (0.3 CU/ml) was measured from 0% to 120% of active plasmin contents by the absorbance value at 405 nm with a spectrophotometer (Model MPS-2000, Shimadzu Ltd., Kyoto, Japan). The changes in the plasmin activity contents of the AP solutions with the passage of time for a 90 min period at room temperature (22°C), which were prepared with the addition of 2,000–12,000 IU/ml UK, were calculated as a percent from the standard curve.

Thrombolytic effects of the AP solution: In vivo experiments of thrombolytic observation with an ultrasound flow meter:

Twenty mongrel dogs, weighing 10–14 kg, were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg). After endotracheal
intubation, positive pressure respiration (end-expiratory pressure of 2 cm H₂O) was instituted with an animal respirator (Harvard Apparatus Co., Natick, MA, USA). The femoral artery was narrowed to 1 mm in diameter and an autologous fibrin clot (gel prepared by the addition of 0.2 ml, 1/14 mole CaCl₂ to 2 ml autologous plasma) was placed at the site of stenosis via a polyethylene catheter. Thereafter, either AP solution (n=8), UK solution (n=6) or 0.9% saline (n=6) was selectively infused for 3 min via a balloon tip catheter located proximal to the experimental embolus. Two minutes prior to the initiation of the injection, a temporary flow obstruction was made by inflation of the balloon-tip catheter to prevent the compound effects of circulating alpha₂-PI. The balloon was kept inflated for 5 min. To evaluate the thrombolytic effect, the velocity of blood in the femoral artery was measured for 60 min with an extravascular ultrasound flow meter (equipped with 4 MHz pencil probe, Diagnostic Electronics, Inc., USA). The probe was placed and moved for scanning from the 5 cm proximal portion to the site of the stenosis (Fig. 2).

Data analysis:
Values are represented as means±SD. Statistical significance was determined by the Student's t-test and analysis of variance. A multiple range test for paired comparison was performed. A level of p<0.005 was taken as significant.

Results

1) Fibrin plate method
The AP solutions prepared with 93.8-187.5 IU/ml of UK did not pro-
duce measurable fibrin lysis. However, fibrin lysis increased linearly for AP solutions prepared with 250 to 750 IU/ml of UK, reaching a plateau at 1,000 to 4,000 IU/ml (n=6) (Fig. 3). Thus, a UK concentration of 4,000 IU/ml was used for assessing the plasmin activity of AP solutions prepared from the plasma separated at 4°C or 22°C, or from whole blood at 22°C.

The AP solution prepared with plasma separated at 4°C produced a lysed area on the fibrin plate of 86.1±10.3 mm² (Group 1). This was significantly greater than the plate test results from the solutions prepared from either plasma at 22°C, 64.5±5.4 mm² (Group 2), or whole blood, 16.3±3.3 mm² (Group 3). AP solution prepared with plasma separated at 4°C showed the highest plasmin activity contents in the 3 groups. Paired comparison test results were significant [Group 1 (86.1±10.3 mm²) vs. Group 2 (64.5±5.4 mm²), p<0.05, Group 1 (86.1±10.3 mm²) vs. Group 3 (16.3±3.3 mm²), p<0.001].

2) Spectrophotometric assay

The changes with passage of time in the plasmin activity contents of the AP solutions.

A calibration curve with human plasmin was linear from 0% to 120% of active plasmin contents by this assay technique. Self test results obtained by the AP solutions left for a 90 min period at room temperature (22°C) were as follows; The plasmin activity of the 2,000 IU/ml UK solution (Group A, n=7) was 26.3±5.9% of standard active plasmin contents. The 6,000 IU/ml UK solution (Group B, n=7) was 82.1±13.0%, the 12,000 IU/ml solution...
tion (Group C, n=7) was 71.4±13.8%.

The 6,000 IU/ml UK solution (Group B) or the 12,000 IU/ml UK solution (Group C), contained high plasmin activity (range: 70–90% of the standard) for 90 min (Fig. 4). The induced active plasmin contents were significantly high in Groups B and C, compared with Group A (82.1±13.0% vs. 26.3±5.9%, p<0.05, 71.4±13.8% vs. 26.3±5.9%, p<0.01), while there was no significant difference in Groups B and C (82.1±13.0% vs. 71.4±13.8%, NS).

**Thrombolytic effect with ultrasound flow meter**:

The AP solution (autologous plasma 10 ml±12×10⁴ IU UK, n=8) rapidly induced complete reperfusion in 5 of 8 cases (5/8) within 15 min. It induced partial reperfusion in 1 of 8 cases (1/8) and no reperfusion in 2 of 8 cases (2/8) over the 60 min observation period. By contrast, UK solution (saline 10 ml±12×10⁴ IU UK, n=6) induced complete reperfusion in 1/6 cases, partial reperfusion in 1/6 cases, and no reperfusion in 4/6 cases. Saline alone (10 ml) was ineffective in all 6 animals (Fig. 5). The restoration of femoral blood flow after infusion was more frequently observed by AP solution than by the other two solutions. In particular, a significant dif-
Fig. 5. Sequential changes in femoral blood flow before and after selective infusion of the AP solution (top), UK solution (middle) or saline (bottom). C=control; S=stenosis; E=embolization.

A difference (p<0.01) in the thrombolytic effect between the AP solution and UK solution was obtained from 15 min after infusion (Fig. 6), although the complete or partial reperfusion rates between the AP solution and UK solution were not significantly different.
Intracoronary or intravenous infusion with UK or SK in evolving myocardial infarction has recently been undertaken to limit myocardial damage, thereby reducing immediate and long-term mortality and morbidity. Since this therapy sometimes induces fatal bleeding complications, it has not yet come into general use. Rentrop\(^9\) reported that the incidence of bleeding associated with high-dose, short-term intravenous infusion of SK varied between 0\% and 20\%.

The occurrence of bleeding has usually been associated with plasma proteolysis and a resulting fibrinolytic state. This state is characterized by a decrease in plasminogen, shortening of the whole blood euglobulin lysis time, a decrease in circulating fibrinogen, the appearance of fibrinogen and fibrin degradation products (FDP-fdp), and prolongation of the prothrombin and thrombin times.

Basic and essential thrombolysis must be performed by the adsorption of activated plasmin onto the fibrin surface. Several plasminogen activators have no direct fibrinolytic action. Only plasmin\(^{10}\) has direct action for fibrinolysis, but it has less specific affinity for fibrin than plasminogen activators. Accordingly, the selective infusion of an AP solution is an alternative strategy for thrombolysis. This study investigated whether it could be used safely and efficaciously for dissolving experimental thrombi.

*In vitro* experiments have indicated that a greater amount of activated plasmin was induced in AP solution prepared by centrifugation at 4\(^\circ\)C cooling than in other plasmin solutions which were prepared by centrifugation at 22\(^\circ\)C non-cooling, or prepared by the addition of UK to whole blood. In
addition, the plasmin activity in this type of preparation was stable for 90 min at room temperature. The stability of the solutions prepared with at least 6,000 IU/ml UK indicates that plasma plasminogen, the plasminogen-UK reaction and released plasmin are not appreciably inhibited in the preparation.

In vivo experiments have indicated that the UK solution had less thrombolytic efficacy than the AP solution. However, the experimental conditions may not be representative of clinical conditions. For example, the homogeneous fibrin clot that was used in the experiments lacked blood cell components of endogenous thrombi such as platelets, red or white blood cells. Moreover, the access of circulating plasminogen to the clot was impeded by balloon inflation or ligation. Thus, it is possible that the thrombolytic efficacy of the AP solution can be attributed to experimental conditions that maximized the adsorption of activated plasmin onto the fibrin clot.

Several attempts have been made to increase the affinity of UK and SK for fibrin. One approach has relied upon prior administration of Lys-plasminogen, which had higher affinity than Glu-plasminogen. A second approach is based upon the removal of alpha2-PI by prior infusion of plasmin, dextran or orthothymotic acid. However, none of these methods have gained general acceptance. Moreover, the three most promising approaches towards fibrin—specific thrombolysis have been the development of stable acyl derivatives of SK—plasminogen complex (UPSAC), the isolation of human tissue-plasminogen activator (T-PA), and the isolation of a single-chain form of UK (pro UK). Some of these agents have been administered in practice. For the patients with acute myocardial infarction within 6 hours from the onset of symptoms, the recanalization rates associated with intravenous application of these drugs are considerably higher than rates achieved by intravenous infusion of SK. However, significant systemic bleeding (estimated as blood loss of 250 ml) involving the gastro-intestinal tract (5%), genito-urinal tract (4%), retroperitoneal (1%) or intracranial sites (1.3%), has been reported after intravenous application of T-PA (100–150 mg). Accordingly, additional trials will be necessary to assess these thrombolytic agents for side effects.

Intravenous plasmin therapy was also reported by Clifton and Ruegsegger in the 1950's; however, it could not be widely applied because the plasmin itself had antigenicity and was immediately inhibited by alpha2-PI.

In this study, we found that the AP solution prepared from autologous plasma had no antigenicity and maintained high plasmin activity for 90 min after preparation. Consequently, this plasmin solution was safer and had more thrombolytic efficacy than UK when directly infused near the fibrin
clot. For clinical practice, the total time for preparation of the AP solution was 20 min [drawing blood (20–40 ml), centrifugation, addition of UK to the plasma, and final warming up to 37°C before injection]. All of these procedures are feasible during initial evaluation procedures such as coronary angiography. Thus, this treatment is a practical, new approach for arterial thrombolysis.

REFERENCES