Effects of Tissue Plasminogen Activator and Urokinase on Human Platelet-dependent Thrombin Generation in vitro

Jun Tsubota, Nobuo Aoki, Masashi Homori, Akira MakI, Koichi Kawano, Hideaki Yoshino and Kyozo Ishikawa

The Second Department of Internal Medicine, Kyorin University School of Medicine

Abstract

In patients with acute myocardial infarction, reocclusion after achievement of coronary reperfusion by thrombolytic therapy using urokinase (UK) or tissue plasminogen activator (tPA) has become a problem. The causes of reocclusion may include rethrombosis as well as activation of platelets and coagulation factors by thrombolytic agents. To elucidate the mechanism of reocclusion involved, thrombin generation was measured. Coagulation of platelet-rich plasma was induced by CaCl₂. Each sample was reacted with S-2238 to determine the thrombin generation. UK or tPA was added to this system and the thrombin generation was assessed. The clotting time was shortened by UK and tPA when compared with control. Thrombin generation was increased by UK and tPA. Stimulation of thrombin generation by thrombolytic agents may contribute to reocclusion.

Introduction

In patients with acute myocardial infarction, the use of thrombolytic agents including tissue plasminogen activator (tPA) or urokinase (UK) promotes coronary reperfusion and reduces infarct size, thus decreasing mortality. However, despite coronary artery recanalization by thrombolytic therapy, reocclusion of the coronary artery does occur in some patients. The causes of such reocclusion may include rethrombosis due to regrowth of residual thrombi following platelet activation, and rethrombosis initiated at atherosclerotic plaques in the infarct-related coronary artery through activation of coagulation mediated by tissue factors. Platelet activation or hypercoagulation as indicated by increased fibrinopeptide A (FPA) and thrombin-antithrombin III complex (TAT) during thrombolytic therapy has also been reported. In addition, direct
activation of platelets\(^{28-30}\) and coagulation\(^{25,36}\) by plasmin itself has been suggested as a possible mechanism of reocclusion. However, general agreement on the details has not yet been reached. In the present study, human platelet-dependent thrombin generation was measured in vitro and the effects of UK and tPA were investigated to elucidate the mechanism of reocclusion after thrombolytic therapy.

Methods

(1) Determination of thrombin generation
Thrombin generation was measured according to the method of Aronson et al.\(^{20}\) with slight modifications. Blood was collected into glass tubes containing sodium citrate (3.8% sodium citrate: blood=1:9) from healthy individuals (n=18) in the morning and centrifuged at 800 g at 22°C for 10 minutes. Platelet-rich plasma (PRP) was obtained by collecting the upper 2/3 of the resulting supernatant to avoid contamination by monocytes and other cells. The subjects had taken no drugs that might influence platelet function and coagulation for at least 10 days prior to the blood sampling. The blood remaining after the extraction of PRP was centrifuged at 1,500 g for 15 minutes to obtain platelet-poor plasma (PPP). The platelet count of the PRP was determined with a Coulter counter (S-Pla VI, Coulter Electronics, Healeah, FL, U.S.A.). After confirming the absence of other cells, the platelet count was adjusted to 150,000/\(\mu l\) using PPP, and 500\(\mu l\) of the adjusted sample was placed in a round-bottomed plastic tube (12cm×75mm). Twenty \(\mu l\) of 1 M CaCl\(_2\) was added to initiate coagulation, and 10 \(\mu l\) aliquots of the test sample were added to the wells of a micro titer plate containing 90\(\mu l\) of 3.8% sodium citrate at 10-minute intervals for 60 minutes to terminate the coagulation. Then, 50\(\mu l\) of 0.5 mM S-2238 (Kabi Diagnostica AB, Stockholm, Sweden) dissolved in 1 M Tris (pH=8.1) was added to each well to develop color for 2 minutes. The absorbance was measured at a wavelength of 405 nm on a Vmax microtiter plate reader (EASY READER EAR 304AT, SLT Labinstruments, Vienna, Austria) to determine the thrombin generation.

(2) Assay of UK and tPA
UK (Urokinase, Mochida Pharmaceutical Co. Ltd., Tokyo, Japan) or tPA (Hapase, Zeneca Pharmaceutical Co., Ltd., Irvine, CA, U.S.A.) were used. These drugs were added to the system for estimating the thrombin generation. After 10 minutes of incubation at room temperature, CaCl\(_2\) was added and the thrombin generation was measured. The drugs were added at the following concentrations: UK (n=8), 192, 1,920, and 19,200 U/ml; and tPA (n=10), 120, 1,200, and 12,000 U/ml. Physiological saline was added to PRP as the control.

(3) Determination of thrombin-antithrombin III complex (TAT), plasmin \(\alpha\)-plasmin inhibitor complex (PIC), and tPA antigen levels in the PRP supernatant
In PRP combined with UK, tPA, or physiological saline, the levels of TAT, PIC, and tPA antigen as well as the tPA activity were measured in the supernatant at 60 minutes after the addition of CaCl\(_2\). Following the addition of saline, PRP without CaCl\(_2\) was

Abbreviations: FPA, fibrinopeptide A; PIC, plasmin \(\alpha\)-plasmin inhibitor complex; PPP, platelet-poor plasma; PRP, platelet-rich plasma; TAT, thrombin-antithrombin III complex; tPA, tissue plasminogen activator; UK, urokinase.
used as the control. The plasma concentration of TAT, plasmin $\alpha_2$-plasmin inhibitor complex (PIC), and tPA antigen were determined. TAT and PIC levels were measured by enzyme immunoassay using an Enzygnost-TAT kit (Behringerwerke AG Co. Ltd., Marburg, Germany) and a PIC test kit (Teijin Co. Ltd., Tokyo, Japan). TPA-antigen levels were measured by ELIZA-tPA kit (Technoclone, Vienna, Austria).

(4) Statistical analysis
The data were analyzed by repeated measures analysis of variance (ANOVA). When a significant difference was observed, the Bonferroni/Dunn method was used for multiple comparisons. All data are shown as the mean±SEM, and differences were considered significant at a level of $p<0.05$ for each statistical method.

Results

(1) Effects of UK on coagulation and thrombin generation (Fig. 1)
In the saline control (n=10), coagulation was initiated at 40-50 minutes after CaCl$_2$ was added and visible clot formation was observed. In the presence of UK (n=8), coagulation began at 10-20 minutes after the addition of CaCl$_2$ and clot formation also occurred. As the concentration of UK increased, visible clot formation was observed more rapidly. In the saline control, fibrin formed spherical clots during the test. However, in the presence of UK, linear or string-like fibrin clots were common and very few were spherical in shape. In most of the UK-containing samples, the fibrin that formed subsequently dissolved. The thrombin

![Fig. 1](image-url)

Fig. 1 Effects of urokinase (UK) on platelet-dependent thrombin generation. Data are shown as mean±SEM. Thrombin generation increased in proportion to the concentration of UK. ○ indicates control (saline); ▲, UK (192 U/ml); ●, UK (1920U/ml); ■, UK (19200U/ml).
activity was higher in the UK-containing samples than in the saline-containing samples, and as the concentration of UK rose, the thrombin activity increased. The differences among the experimental groups were statistically significant (repeated measures ANOVA, p<0.001). Multigroup comparison of thrombin generation revealed a significant difference between control group and 192 U/ml UK group (p<0.001), control group and 1,920 U/ml UK group (p<0.001), and control group and 19,200 U/ml UK group (p<0.001).

In regard to the data of each measuring time, thrombin activity was significantly greater in the UK groups than in the control group.

(2) Effects of tPA on coagulation and thrombin generation (Fig. 2)

After adding CaCl₂, coagulation was more rapid in the presence of tPA (n=10) than in the saline control samples (n=10). Fibrin often formed linear or string-like clots as in the case of UK, and the fibrin clots that formed usually dissolved subsequently. The thrombin generation was greater in the presence of tPA rather than saline, and increased in proportion to the tPA concentration. The differences among the experimental groups were statistically significant (repeated measures ANOVA, p<0.001). Multigroup comparison of thrombin generation revealed a significant difference between control group and 120 U/ml tPA group (p<0.001), control group and 1,200 U/ml tPA group (p<0.001), control group and 12,000 U/ml tPA group (p<0.001).

(3) TAT, PIC, and tPA antigen levels and tPA activity in the PRP supernatant (Table 1)
Table 1 TAT, PIC and tPA antigen levels in PRP at 60 minutes after CaCl\textsubscript{2} addition in experimental groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>TAT (ng/mL)</th>
<th>PIC (μg/mL)</th>
<th>tPA-ag (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2±1.5</td>
<td>0.5±0.2</td>
<td>1.5±0.5</td>
</tr>
<tr>
<td>Saline</td>
<td>108.3±11.2***</td>
<td>0.6±0.1</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>tPA</td>
<td>120 U/mL</td>
<td>105.4±6.5***</td>
<td>37.5±7.7*</td>
</tr>
<tr>
<td></td>
<td>1,200 U/mL</td>
<td>102.3±6.4***</td>
<td>46.4±7.7**</td>
</tr>
<tr>
<td></td>
<td>12,000 U/mL</td>
<td>107.8±4.8***</td>
<td>46.9±2.9**</td>
</tr>
<tr>
<td>UK</td>
<td>192 U/mL</td>
<td>98.5±8.4***</td>
<td>9.0±0.8**</td>
</tr>
<tr>
<td></td>
<td>1,920 U/mL</td>
<td>91.6±3.4***</td>
<td>57.8±7.3**</td>
</tr>
<tr>
<td></td>
<td>19,200 U/mL</td>
<td>107.5±3.1***</td>
<td>39.0±8.0*</td>
</tr>
</tbody>
</table>

TAT, thrombin antithrombin-Ⅲ complex; PIC, plasmin \(a_2\)-plasmin inhibitor complex; tPA ag, tissue plasminogen activator; PRP, platelet-rich plasma; Control, saline group without adding CaCl\textsubscript{2}; tPA, tissue plasminogen activator; UK, urokinase Significant difference form control, *p<0.05, **p<0.01, ***p<0.001.

**Discussion**

The pathogenesis of reocclusion after thrombolytic therapy for acute myocardial infarction may include rethrombosis due to residual thrombi\textsuperscript{15,21}, regrowth of thrombi at the sites of injured coronary artery\textsuperscript{13,16}, and activation of platelets and coagulation by the thrombolytic therapy\textsuperscript{15,21}. Recent data have indicated that thrombolytic agents or plasmin generated by these agents can activate platelets and coagulation\textsuperscript{15,20}, providing a possible mechanism for reocclusion. When the effects of tPA or UK on platelet-dependent thrombin generation were examined in the present study, both thrombolytic agents were found to increase the thrombin generation in proportion to the concentration of drug added to the test system.

(1) **Significance of platelet-dependent thrombin generation**

Thrombin production occurs in parallel with the adhesion and aggregation of platelets, and coagulation and platelet activation interact with each other at the sites of fibrin forma-
Platelets activate coagulation factor X, and platelet factor III (phospholipid) promotes the conversion of prothrombin to thrombin\textsuperscript{8,9}. Activation of coagulation factor XI on the platelet surface is considered to trigger the coagulation cascade\textsuperscript{8,9}. In addition, the small amount of thrombin formed during the coagulation process activates platelets to release factor V from their \(\alpha\)-granules. The released factor V then binds to activated factor X, and thrombin generation is further enhanced on the platelet surface. Thus, platelets and thrombin act in a complementary fashion during thrombogenesis, and the coagulation cascade is amplified by several hundred thousand-fold on the platelet membrane. Accordingly, measurements of the thrombin generation in a platelet-rich plasma, as performed in our study, may provide more precise information on coagulation than measurements of the various parameters in the plasma. Aronson et al.\textsuperscript{27} developed a system for the direct determination of thrombin on platelets using S-2238. The thrombin generation measured by this method appears to be a better indicator than the plasma FPA and TAT levels of the regional coagulation process in vivo. Since TAT and antithrombin III are only indirect indicators of thrombin generation, direct measurements of the thrombin activity, as performed in our study, may be useful for understanding the in vivo kinetics of coagulation.

(2) Effects of thrombolytic therapy on platelet function and coagulation

Platelet aggregation has been variously reported to be inhibited\textsuperscript{11,20} or promoted\textsuperscript{12-24} by the addition of thrombolytic agents in vitro. Some studies have also found that these agents have no effect on platelet aggregation\textsuperscript{27}, or that they promote or inhibit aggregation depending on the aggregation stimulant (e.g., ADP or epinephrine) that is used\textsuperscript{13,20}. Thus, no agreement has yet been reached among researchers regarding the effects of thrombolytic agents on platelet aggregation\textsuperscript{29}. However, there is an increasing number of reports demonstrating that thrombolytic agents cause platelets to release the contents of their dense granules\textsuperscript{27} and induce thromboxane A\(_2\), production\textsuperscript{11-16}, suggesting that platelet activation is promoted.

FPA, an in vivo marker of fibrin formation, has been reported to be elevated during thrombolytic therapy for acute myocardial infarction\textsuperscript{17-20}, suggesting that hypercoagulability may develop during thrombolytic therapy. TAT has also been shown to increase during thrombolytic therapy\textsuperscript{18,20}, indicating the production of thrombin. However, the TAT level does not reflect the total thrombin generation, since thrombin binds not only to AT-III but also to heparin cofactor II, \(\alpha\)-macroglobulin and thrombomodulin. In addition, TAT is only an indirect index of thrombin generation and not a direct measure of the thrombin activity.

(3) Clinical significance

When the effects of UK or tPA on human platelet-dependent thrombin generation were investigated in vitro, it was found that the thrombin generation was increased in a concentration-dependent manner. Coagulation occurred at all concentrations tested. In the presence of physiological saline alone, the formation of spherical fibrin clots was noted from 40-60 minutes after CaCl\(_2\) addition. However, in the presence of UK or tPA, there were several cases in which fibrin formation did not lead to clear clot formation or in which the thrombi formed were subsequently lysed. Despite this, the thrombin generation
was significantly higher in the presence of UK and tPA than in the control. It appears therefore that thrombolytic agents such as UK and tPA not only possess a clear thrombolytic effect but also promote thrombin generation. In acute myocardial infarction, even if reperfusion is successfully achieved by thrombolytic therapy, thrombus regrowth may be initiated at residual thrombi or at the sites of injured coronary artery by the effects of the above drugs on thrombin generation, leading to reocclusion. Because the nidus of coronary thrombus usually is not removed, rethrombosis easily occur. And plasminogen activation with exogenous compounds has the paradoxical effect of activating both platelets and thrombin in the acute phase\(^{25}\). Also, incomplete lysis of thrombus and plaque swelling after thrombolysis may be stimuli for rethrombosis\(^{26,30}\).

(4) Thrombin generation and molecular markers of coagulation

In the presence of physiological saline, the TAT value was markedly increased at 60 minutes after adding CaCl\(_2\) as compared to without CaCl\(_2\), clearly indicating the generation of thrombin in the PRP supernatant and supporting the validity of the method of Aronson et al.\(^{27}\) In contrast, there was no significant change in TAT level in the presence of saline, UK, and tPA at 60 minutes after adding CaCl\(_2\). Since no significant difference was observed among the three groups, no significant changes in TAT levels were elicited by the AT-III that is neutralized with thrombin in the PRP. In the presence of saline, there was no significant change in PIC level both before and after addition of CaCl\(_2\). Since no lysis occurred when clots formed in the presence of saline, plasmin generation in the PRP was presumed to be limited. After adding CaCl\(_2\), the PIC value was higher with both UK and tPA than with saline. These findings indicate that production of plasmin was occurred after addition of UK or tPA.

(5) Limitations of the present study

Increased platelet-dependent thrombin generation by UK or tPA were demonstrated in the present study, although the mechanism remains unknown. UK and tPA are serine proteases, so that they may have acted on the coagulation cascade in PRP, degraded various coagulation factors, and stimulated thrombin production from prothrombin. As shown by Aronson et al.\(^{27}\) thrombin generation apparently decreases in PPP when compared with PRP, and there is almost no stimulatory effect of UK and tPA on thrombin generation in PPP. UK and tPA do not therefore appear to be very potent serine proteases in vitro. In vivo animal studies are needed to clarify this point.

Prevention or inhibition of the stimulatory effects of UK and tPA on thrombin generation could not be investigated in the present study. Further research is required to develop drugs that can inhibit the stimulation of thrombin generation by thrombolytic agents, which is thought to be one underlying mechanism of reocclusion, and to establish experimental systems for detecting such drugs.

Conclusion

Human platelet-dependent thrombin generation was measured and the effects of UK and tPA on thrombin generation were investigated in vitro. Both UK and tPA increased the thrombin generation in proportion to the concentration of drugs added. The stimulation of thrombin generation by thrombolytic agents including UK and tPA may be one cause of reocclusion following thrombolytic
References


