Endotoxin-Induced Intravascular Coagulation in Rabbits: Effect of Tissue Plasminogen Activator vs Urokinase on PAI Generation, Fibrin Deposits and Mortality

M. J. Paloma, J. A. Páramo, E. Rocha

From the Hematology Service, University Clinic of Navarra, School of Medicine University of Navarra, Pamplona, Spain

Summary

We have evaluated the effect of plasminogen activators (t-PA and urokinase) on an experimental model of disseminated intravascular coagulation (DIC) in rabbits by injection of 20 μg/kg/h of E. coli lipopolysaccharide during 6 h t-PA (0.2 mg/kg and 0.7 mg/kg), urokinase (3000 U/kg/h) and saline (control) were given simultaneously with endotoxin. Results indicated that urokinase and low dose of t-PA significantly reduced the increase of plasminogen activator inhibitor (PAI) activity observed 2 h after endotoxin (p<0.001). High t-PA dose also diminished the PAI levels at 6 h (p<0.0001). A significant reduction of fibrin deposits in kidneys was observed in both t-PA treated groups as compared with findings in the group of rabbits infused with saline solution (p<0.005), whereas urokinase had no significant effect on the extent of fibrin deposition. Finally, the mortality rate in the control group (70%) was reduced to 50% in rabbits receiving high doses of t-PA. In conclusion, treatment with t-PA resulted in reduced PAI generation, fibrin deposits and mortality in endotoxin-treated rabbits.

Introduction

Disseminated intravascular coagulation (DIC) is a pathological process characterized by profound alterations of hemostasis. Its etiology is multifactorial although sepsis is one of the most frequent causes (1, 2).

Several cellular and humoral components are implicated in the endotoxin-induced DIC (3). The generation of thrombin, fibrin deposition in the microcirculation and septic shock are caused by the exposed subendothelium at sites of vascular injury (4) as well as by the liberation of procoagulant substances (5, 6) and endogenous mediators from inflammatory cells (7).

The fibrinolytic system also seems to play an important role in the pathogenesis of DIC. Endotoxin is able to induce a marked increase of plasminogen activator inhibitor (PAI) activity in endothelial cell cultures, animal models and healthy subjects (8-10). High PAI levels have also been found in clinical conditions related to thrombotic processes and in patients with sepsis (11, 12) and preliminary studies indicate that the control of PAI generation may represent a therapeutic approach in experimentally induced DIC (13, 14).

Tissue-type plasminogen activator (t-PA) and urokinase have been found to be useful in different animal models of thrombosis (15-18). In this study we have evaluated the possible therapeutic effect of both plasminogen activators, which have different specificity for fibrin, in a rabbit model of endotoxin-induced DIC.

Materials and Methods

Experimental Studies

Male New Zealand rabbits weighing 2.5-3 kg were anesthetized by intravenous infusion of 10 mg/kg body weight of Nembutal (Abbot Lab) via a marginal ear vein, where a catheter was placed to administer treatment. DIC was induced in 40 rabbits by intravenous infusion, via the contralateral marginal ear vein of 20 μg/kg/h of endotoxin (lipopolysaccharide from E. coli 0128:B8, Sigma Chemical Co., St Louis, MO) during 6 h. Blood samples were taken via a catheter inserted into a femoral vein.

Surviving rabbits were sacrificed 24 h after the start of the experiment by intravenous injection of 60 mg/kg of Nembutal. Kidneys were extracted from all animals (both those which died spontaneously and those which were sacrificed) for subsequent histological studies.

Treatment Schedules

Treatment was started simultaneously with endotoxin infusion. Four different groups (10 rabbits each) were established: (1) Control group. They were infused with saline solution (10 ml/h) during 6 h; (2) Low-dose t-PA group. They received 0.2 mg/kg of t-PA (Boehringer Ingelheim, Germany) diluted in 30 ml of saline during 90 min; (3) High-dose t-PA group. They were treated with 0.7 mg/kg of t-PA (10% administered directly as a bolus and the remaining dissolved in 30 ml of saline and injected over 90 min). Additional saline solution (10 ml/h) was administered in all t-PA-treated rabbits to complete 6 h; (4) Urokinase group. This group received 3000 U/kg/h of two-chain urokinase (Roger Lab S.A, Spain) diluted in 60 ml of saline and injected over 6 h.

Five rabbits not receiving endotoxin were infused with saline solution during 6 h.

Laboratory Methods

Blood samples were taken before endotoxin, at 2 and at 6 h of infusion. Plasma was immediately prepared by centrifugation and kept on ice or frozen at -70°C until tested.

PA activity was determined by spectrophotometric assay as described by Verheijen et al. (19). Briefly, diluted euglobulin fraction was mixed in a microtiter plate to a final volume of 200 μl with 0.02M Tris,HCl, pH 7.5, 0.1% Tween 80, 0.3 mM S-2251 (Kabi Diagnostica, Sweden), 0.13 μM human plasminogen (Biopool, Sweden) and 0.13 mg/ml CNBr fibrinogen fragments (Chromogenix, Sweden). The plate was incubated at 37°C and the change in
Table 1  Hemostatic parameters before and after endotoxin infusion in rabbits in the four different groups (n = 10). Results are expressed as mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Plasminogen (%)</th>
<th>PA (mU/ml)</th>
<th>PAI (U/ml)</th>
<th>α2-antiplasmin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Basal</td>
<td>106.69 ± 7.97</td>
<td>53.89 ± 3.54</td>
<td>6.33 ± 0.40</td>
<td>88.99 ± 5.10</td>
</tr>
<tr>
<td>- 2 h</td>
<td>90.32 ± 5.81</td>
<td>45.26 ± 2.86</td>
<td>61.26 ± 2.79</td>
<td>87.20 ± 5.51</td>
</tr>
<tr>
<td>- 6 h</td>
<td>90.10 ± 5.70</td>
<td>44.18 ± 2.80</td>
<td>64.72 ± 5.48</td>
<td>73.86 ± 4.67</td>
</tr>
<tr>
<td><strong>t-PA (0.2 mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Basal</td>
<td>100.74 ± 5.62</td>
<td>52.49 ± 6.13</td>
<td>6.96 ± 0.57</td>
<td>90.69 ± 3.19</td>
</tr>
<tr>
<td>- 2 h</td>
<td>103.79 ± 5.50</td>
<td>104.96 ± 12.90*</td>
<td>33.76 ± 8.35**</td>
<td>78.16 ± 2.71</td>
</tr>
<tr>
<td>- 6 h</td>
<td>110.65 ± 6.30</td>
<td>45.14 ± 6.82</td>
<td>71.68 ± 9.27</td>
<td>71.64 ± 3.85</td>
</tr>
<tr>
<td><strong>t-PA (0.7 mg/kg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Basal</td>
<td>101.26 ± 10.97</td>
<td>54.74 ± 5.24</td>
<td>5.65 ± 0.51</td>
<td>94.19 ± 7.27</td>
</tr>
<tr>
<td>- 2 h</td>
<td>113.70 ± 15.01</td>
<td>235.96 ± 17.36*</td>
<td>27.51 ± 4.69*</td>
<td>83.82 ± 2.91</td>
</tr>
<tr>
<td>- 6 h</td>
<td>115.72 ± 15.62</td>
<td>153.81 ± 14.84*</td>
<td>23.67 ± 5.15*</td>
<td>69.21 ± 3.05</td>
</tr>
<tr>
<td><strong>Urokinase</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Basal</td>
<td>100.94 ± 5.68</td>
<td>62.74 ± 8.96</td>
<td>6.77 ± 0.38</td>
<td>85.87 ± 5.87</td>
</tr>
<tr>
<td>- 2 h</td>
<td>96.99 ± 3.94</td>
<td>60.23 ± 8.38</td>
<td>57.23 ± 5.02*</td>
<td>76.24 ± 4.82</td>
</tr>
<tr>
<td>- 6 h</td>
<td>99.29 ± 4.41</td>
<td>62.11 ± 6.78</td>
<td>54.97 ± 8.98</td>
<td>66.36 ± 2.40</td>
</tr>
</tbody>
</table>

*p < 0.0001, **p < 0.001 as compared with the control group.

absorbance at 405 nm was measured. Results were expressed in mU/ml. t-PA inhibitor capacity of rabbit plasma (referred to as PAI activity) was measured by an amidolytic assay as previously described (20). PAI activity was expressed in units of t-PA inhibited per ml. Plasminogen and α2-antiplasmin concentrations were measured by amidolytic assays (Coastet Plasminogen and Coastet Antiplasmin, Chromogenix, Sweden).

**Histological Examination**

Sections of kidneys were fixed in formalin, stained with hematoxylin-eosin and Masson’s trichrome and examined for the presence of fibrin microthrombi by a pathologist who was unaware of the experimental protocol for the individual animals. Tissue sections were scored on a scale from 0 to 4 as previously described (13).

**Data Analysis**

Results are expressed as mean ± SEM. Two-way analysis of variance followed by Tukey’s multiple comparison test for all pairs was applied for purposes of group comparison. For differences in mortality between control and treated groups the Fisher’s exact test was used.

**Results**

**Effect of t-PA and Urokinase on Endotoxin-induced Changes in Fibrinolysis**

Table 1 shows the plasma levels of the different fibrinolysis parameters analyzed throughout the experiment in the control and treated groups. No differences in any of these parameters were observed among groups in the baseline samples.

In control rabbits there was a significant decrease of plasminogen and PA activity 2 and 6 h after endotoxin infusion (p < 0.05) as well as a reduction of α2-antiplasmin in the sample taken at 6 h (p < 0.001). PAI levels showed a marked increase 2 h after endotoxin infusion (p < 0.0007) to maximum values at 6 h (p < 0.0001).

We observed that injection of low and high t-PA doses, as well as of urokinase, were sufficient to ameliorate the moderate plasminogen decrease observed in the control group after endotoxin. The changes in the PA activity in the control and treated groups are shown in Fig. 1. Treatment with low doses of t-PA produced a rapid and significant increase of PA in the sample taken at 2 h (p < 0.0001) followed by a

**Fig. 1**  PA activity levels after endotoxin in control rabbits (○), and in rabbits receiving low dose of t-PA (+), high dose of t-PA (▲), and urokinase (■). Values are expressed as the mean percent of the initial value before injection of endotoxin. * p < 0.0001 as compared to the baseline sample.

**Fig. 2**  PAI activity levels after endotoxin in control rabbits (○), and in rabbits receiving low dose of t-PA (+), high dose of t-PA (▲), and urokinase (■). * p < 0.001 and ** p < 0.0001 as compared to the control group.
Histological Findings

Histological analysis of kidney sections was performed in all surviving rabbits as well as in animals which died during the experiment. Intense deposits of fibrin within glomerular capillaries were observed in the majority of endotoxin-treated rabbits not receiving plasinogen activators. The average value according to our scale was $2.85 \pm 1.04$. High and low doses of t-PA significantly reduced ($p < 0.005$) fibrin deposits in kidneys (score $1.62 \pm 1.17$ and $1.51 \pm 1.31$ respectively), while urokinase had no significant effect on the extent of fibrin deposition (score $2.10 \pm 1.33$) (Fig. 3).

No hemorrhagic areas were observed in any of the treated groups.

Mortality Rate

In the control group, 7 out of 10 rabbits (70%) died shortly after the endotoxin infusion. The same mortality rate was observed in rabbits treated with low dose of t-PA and with urokinase, whereas administration of high doses of t-PA reduced the mortality rate to 50% (5 out 10 rabbits), although such difference was not significant. No deaths were observed within the first 6 h of the beginning of the experiment in any of the groups.

An interesting finding was that the increase in plasma PAI activity was significantly higher ($p < 0.01$) at 2 h ($36.23 \pm 8.40$ U/ml vs $25.20 \pm 6.21$ U/ml) and at 6 h ($75.68 \pm 9.31$ U/ml vs $29.89 \pm 7.84$ U/ml) in the 26 rabbits that died following endotoxin infusion than in survivors (Fig. 4).

Discussion

Administration of endotoxin to rabbits resulted in DIC as demonstrated by the intense presence of fibrin deposition in kidneys and the impairment of fibrinolysis observed in the control group. Endotoxin induced a progressive decrease in PA, plasinogen and $\alpha_2$-antiplasmin and a significant increase in PAI activity levels, which agrees with previous clinical and experimental studies (8, 13, 14, 21).

The decrease of PA has already been described in endothelial cell cultures stimulated by endotoxin, in experimental animals after injection of lipopolysaccharide and after endotoxin administration to healthy volunteers (8-10). Such decrease could be related to a direct vascular injury, binding of t-PA to endothelial cells or as a result of an increase in PAI which would result in the formation of enzyme-inhibitor complexes (22, 23).

We found high PAI activity in control rabbits 2 and 6 h after endotoxin as previously reported (13, 14). Thrombin, key enzyme in DIC, as well as other humoral factors generated in response to endotoxin, may stimulate the release of this inhibitor from the endothelium (24-26). High PAI inhibitor activity, by reducing fibrinolysis, could contribute to the intense fibrin deposition observed in endotoxin-treated rabbits (13, 27, 28) Additional support for the role of PAI in thrombosis comes from in vivo experiments showing increased inhibitor gene expression in endothelial cells induced by thrombosis (29).

The mortality rate was also greatly elevated in control animals, which could be related to the intense fibrin deposits associated with DIC. Other events could also contribute to the high mortality rate after endotoxin infusion, such as the generation of anaphylatoxins as a result of the activation of the complement system, the liberation of vasoactive substances from platelets and the bradykinin production (30, 31).

In this study we report the effect of two fibrinolytic agents, t-PA and urokinase, on endotoxin-induced DIC. Both agents are broadly used in
different human clinical conditions related to thrombotic phenomena, although they differ with respect to the mechanism of activation of the fibrinolytic system in the presence of fibrin, since t-PA has specific affinity for fibrin while urokinase induces systemic fibrinolysis (32).

The plasminogen levels tended to normalize after administration of plasminogen activators independently of the type of activator as compared to the values observed in the control group, probably reflecting a lower plasminogen consumption in the treated groups. Low doses of t-PA reduced the PAI activity at 2 h of endotoxin infusion whereas both high doses of t-PA and urokinase were able to diminish the PAI increase observed 2 and 6 h after endotoxin. The PAI reduction might be explained by the formation of enzyme-inhibitor complexes, although the possibility of an effect of the activator treatment or the fibrin lysis on the inhibitor synthesis cannot be ruled out (29).

In our study low doses of t-PA were as effective as high t-PA doses at causing a decrease in fibrin deposits, indicating that the lysis of fibrin is not necessarily related to the levels of activator as reported by Agnelli et al. (33). Our results would also agree with findings of Bergstein et al. who reported that 0.5 mg/kg of t-PA significantly decreased the presence of fibrin deposits within glomeruli in an experimental model of Shwartzman reaction (34). The increased PA activity still observed 6 h after the start of t-PA infusion might have been due to a reduced clearance of this activator during sepsis.

Urokinase did not reduce the fibrin deposits in our experimental model. It is possible that the dose used in this study was insufficient for a reduction of fibrin deposits, although a similar scheme (3000 U/kg/h) is given to patients with pulmonary embolism. In this study rabbits were infused with a dose of urokinase 10 times lower than that used by Collen et al. in the rabbit jugular vein thrombosis model to get 40% reduction of fresh clots (17). Furthermore, other authors have experimentally demonstrated that renal bilateral necrosis, which is a typical finding of the Schwartzman reaction, can be prevented by using a dose of urokinase much higher than that used in this work (35).

Treatment with high doses of t-PA achieved a moderate reduction in the mortality rate to 50% as compared to 70% in the control group. When used at doses of 0.2 mg/kg, t-PA had no effect on mortality despite the fact that this dose reduced the fibrin deposits. The reason for that is not completely understood, although it is possible that low t-PA doses could not overcome the generalized microthrombosis present in endotoxin-induced DIC. Thus, the effect of high t-PA doses on the mortality rate would be related to the generalized fibrinolysis and not so much to the local lysis of fibrin deposits in kidneys. On the other hand since PAI activity was significantly higher in animals that died than in survivors it could be speculated that the control of endotoxin-induced PAI generation could have a beneficial effect.

The dose of urokinase used had no effect in this model of DIC, but it is important to point out that rabbits are particularly resistant to this pharmacological agent (36, 37).

In the light of our results we conclude that the treatment with high doses of t-PA in rabbits infused with endotoxin reduces mortality in our model of endotoxin-induced DIC. The fact that low doses of t-PA also diminished the amount of renal fibrin deposits indicates that lysis is not necessarily related to the dose of activator.

Acknowledgements

This work was supported by grant PM92-0167 from the DGICYT of the Ministerio de Educacion y Ciencia, Spain.

References


Received November 14, 1994 Accepted after resubmission August 18, 1995

This new edition of Antibiotic Therapy gives a clear, systematic and up-to-date presentation of antimicrobials together with practical guidance in their use.

Without any doubt, this text will be of great value to clinical specialists as well as to general practitioners, doctors in training, pharmacologists, pharmacists, and to all concerned with the diagnosis and management of infection. A wide range of substances recently developed such as new penicillins, new cephalosporins, new macrolides as well as the new quinolones are dealt with. Modern approaches to the treatment of tuberculosis, fungal and viral infections (particularly AIDS and its associated opportunistic infections) are described.

The main sections contain chapters on the properties of the single antimicrobial agents, the treatment of infections and infectious disease along with general rules for antibiotic therapy. Special attention has been paid to recent scientific publications which have been included in the extensive bibliography to each chapter together with more general guidance for further reading.

Therefore, this text provides latest therapeutic strategies as well as basic information about pharmacologic properties, side effects, indications and contraindications of antimicrobial agents. Moreover, this book is working into a truly European text having achieved seven German editions as well as numerous editions in other European countries, where expert co-authors have closely cooperated in this project.