© F. K. Schattauer Verlagsgesellschaft mbH (Stuttgart) 74 (6) 1578–82 (1995)

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).

Correspondence to: Dr. E. Rocha, Hematology Service, University Clinic, P.O. 4209, E-31080 Pamplona, Spain – FAX Number: +34 48 172 294

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 anesthesized 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 disolved 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

	Plasminogen (%)	PA (mU/ml)	PAI (U/ml)	α2-antiplasmin (%)
Control				
- Basal	106.69 ± 7.97	53.89 ± 3.54	6.33 ± 0.40	88.99 ± 5.10
- 2 h	92.22 ± 5.83	45.26 ± 2.86	61.26 ± 2.79	87.20 ± 5.51
- 6 h	90.10 ± 5.70	44.18 ± 2.80	86.72 ± 5.48	73.86 ± 4.67
t-PA (0.2 mg/kg)				
- Basal	100.74 ± 5.62	52.49 ± 6.13	6.96 ± 0.57	90.69 ± 3.19
- 2 h	103.79 ± 5.50	104.96 ± 12.90*	33.76 ± 8.35**	78.16 ± 2.71
- 6 h	110.65 ± 6.30	45.14 ± 8.62	71.68 ± 9.27	71.64 ± 3.85
t-PA (0.7 mg/kg)				
- Basal	100.26 ± 10.97	54.74 ± 5.24	5.65 ± 0.51	94.19 ± 7.27
- 2 h	113.70 ± 15.01	255.96 ± 17.36*	27.51 ± 4.69*	83.82 ± 2.91
- 6 h	115.72 ± 15.62	153.81 ± 14.84*	23.67 ± 5.15*	69.21 ± 3.05
Urokinase				
- Basal	100.94 ± 5.68	62.74 ± 8.96	6.77 ± 0.38	85.87 ± 5.87
- 2 h	96.90 ± 3.94	60.23 ± 8.38	37.23 ± 5.02*	76.24 ± 4.82
- 6 h	99.69 ± 4.41	62.11 ± 6.78	54.97 ± 8.98	66.36 ± 2.40

*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 α_2-antiplasmin concentrations were measured by amidolytic assays (Coatest Plasminogen and Coatest 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.

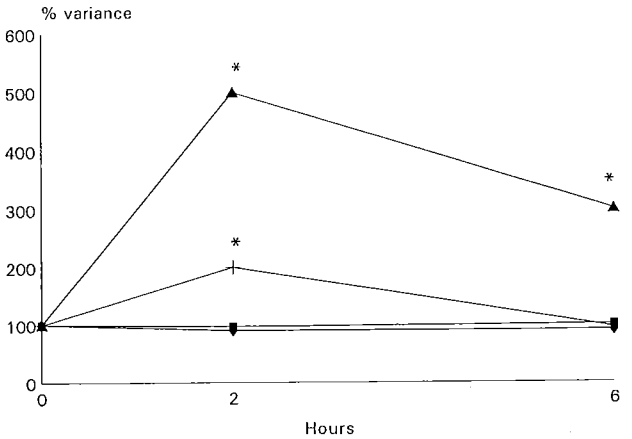

Fig. 1 PA activity levels after endotoxin in control rabbits (◆), and in rabbits receiving low dose of t-PA (+), high dose of t-PA (▲), and urokinase (■). Values are expressed as the mean percent of the initial value before injection of endotoxin. * p <0.0001 as compared to the baseline sample

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 α_2-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

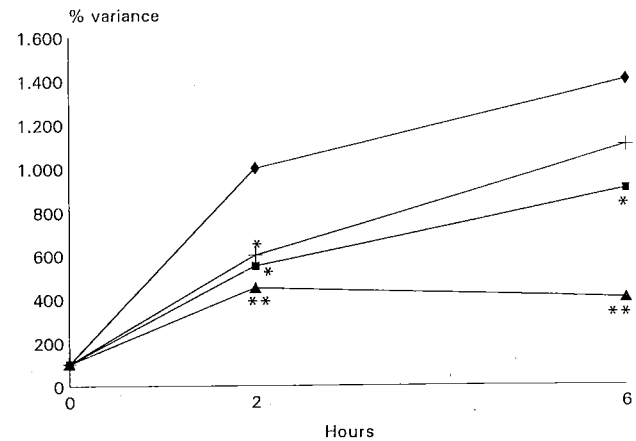

Fig. 2 PAI activity levels after endotoxin in control rabbits (◆), and in rabbits receiving low dose of t-PA (+), high dose of t-PA (▲), and urokinase (■). * p <0.001 and ** p <0.0001 as compared to the control group

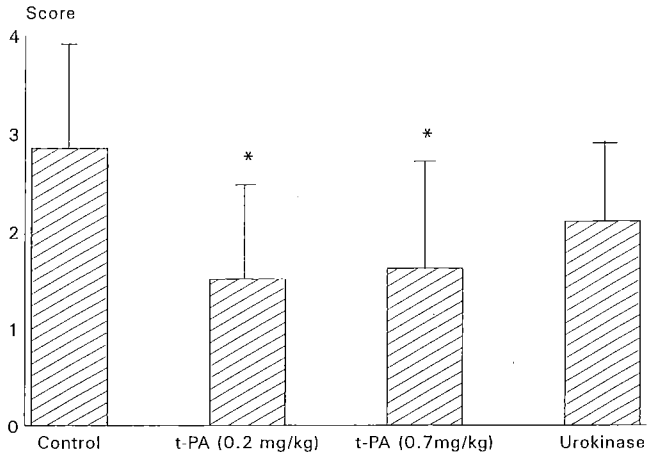

Fig. 3 Score of fibrin deposition in kidneys induced by endotoxin in control rabbits, and and in rabbits receiving t-PA (low and high dose) and urokinase. * $p < 0.005$ as compared to control

decrease to control values. High t-PA doses induced a significant increase in PA activity after 2 h which was maintained in the sample obtained 6 h after endotoxin ($p < 0.0001$). Urokinase did not substantially change the PA activity level which remained similar to baseline values throughout the experiment.

As shown in Fig. 2, whereas a low dose of t-PA reduced the increase of PAI activity in the sample taken at 2 h ($p < 0.001$), the high t-PA dose had an effect 2 and 6 h after endotoxin injection ($p < 0.0001$). Similarly, urokinase significantly reduced the increase in PAI activity, mainly 2 h after injection of endotoxin, as compared with values obtained in the control group ($p < 0.001$). As reported in Table 1 none of the treatments used significantly changed the levels of α_2-antiplasmin with respect to those found in the control. In the group of rabbits without endotoxin, no changes in the fibrinolysis parameters analyzed were observed (not shown).

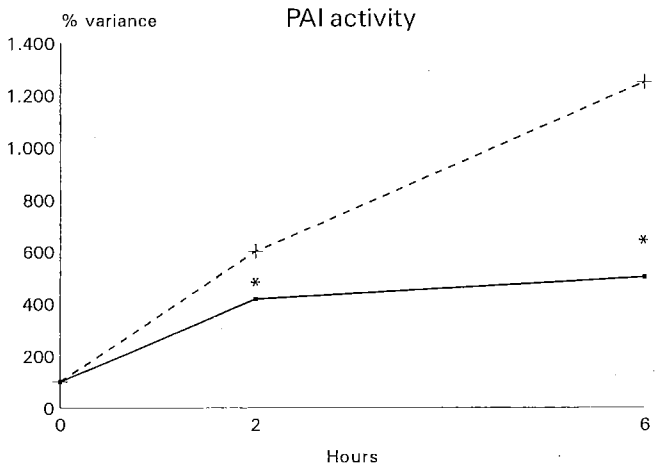

Fig. 4 PAI levels as related to mortality in endotoxin-treated rabbits. The increase in plasma PAI activity in animals that died (n = 26) following endotoxin (---) was significantly higher than in survivors (n = 14) (—). * $p < 0.01$

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 plasminogen activators. The average value according to our scale was 2.85 ± 1.04. High and low doses of t-PA significantly reduced ($p < 0.005$) fibrin deposits in kidneys (score 1.62 ± 1.17 and 1.51 ± 1.31 respectively), while urokinase had no significant effect on the extent of fibrin deposition (score 2.10 ± 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 ± 8.40 U/ml vs 25.20 ± 6.21 U/ml) and at 6 h (75.68 ± 9.31 U/ml vs 29.89 ± 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, plasminogen and α_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 thombotic 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 Educación y Ciencia, Spain.

References

1. Colman RW, Robboy SJ, Minna JD. Disseminated intravascular coagulation: a reappraisal. Ann Rev Med 1987; 30: 359-74.
2. Bick RL. Disseminated intravascular coagulation. Hemat Oncol Clin North America 1992; 6: 1259-85.
3. Morrison DC, Ulevitch RJ. The effects of bacterial endotoxins on host mediation systems. Am J Pathol 1978; 93: 527-618.
4. Gaynor E, Bouvier CA, Spaet TH. Vascular lesions: possible pathogenetic basis of the generalized Schwartzman reaction. Science 1970; 170: 986-88.
5. Rickles FR, Rick PD. Structural features of Samonella typhimurium lipopolysaccharide required for activation of tissue factor in human mononuclear cells. J Clin Invest 1977; 59: 1188-95.
6. Bone RC. Modulators of coagulation. A critical appraisal of their role in sepsis. Arch Intern Med. 1992; 152: 1381-9.
7. Morrison DC, Ryan JL. Endotoxins and disease mechanisms. Ann Rev Med 1987; 38: 417-32.
8. Colucci M, Páramo JA, Collen D. Generation in plasma of a fast-acting inhibitor of plasminogen activator in response to endotoxin stimulation. J Clin Invest 1985; 75: 818-24.
9. Crutchley DJ, Conanan LB. Endotoxin induction of an inhibitor of plasminogen activator in bovine pulmonary artery endothelial cells. J Biol Chem 1985; 261: 154-9.
10. Suffredini AF, Harpel PC, Parrillo JE. Promotion and subsequent inhibition of plasminogen activation after administration of intravenous endotoxin to normal subjects. N Engl J Med 1989; 320: 1165-72.
11. Rocha E, Páramo JA, Fernández FJ, Cuesta B, Hernández M, Paloma MJ. Clotting activation and impairment of fibrinolysis in malignancy. Thromb Res 1989; 54: 699-707.
12. Páramo JA, Fernandez FJ, Rocha E. Plasminogen activator inhibitor activity in bacterial infection. Thromb Haemost 1988; 59: 451-4.
13. Gómez C, Páramo JA, Colucci M, Rocha E. Effect of heparin and/or antithrombin III on the generation of endotoxin-induced plasminogen activator inhibitor. Thromb Haemost 1989; 62: 694-8.
14. Paloma MJ, Páramo JA, Rocha E. Effect of DDAVP on endotoxin-induced intravascular coagulation in rabbits. Thromb Haemost 1992; 68: 306-9.
15. Matsuo O, Rijken DC, Collen D. Thrombolysis by human tissue plasminogen activator and urokinase in rabbits with experimental pulmonary embolus. Nature 1981; 291: 590-1.
16. Korninger C, Matsuo O, Suy R, Stassen JM, Collen D. Thrombolysis with human extrinsic (tissue-type) plasminogen activator in dogs with femoral vein thrombosis. J Clin Invest 1982; 69: 573-80.
17. Collen D. Stassen JM, Verstraete M. Thrombolysis with human extrinsic (tissue-type) plasminogen activator in rabbits with experimental jugular vein thrombosis. Effect of molecular form and dose of activator, age of the thrombus, and route of administration. J Clin Invest 1983; 71: 368-76.
18. Agnelli G, Buchanan MR, Fernández F, Boneu B, Hirsh J, Collen D. Hemorrhage-free thrombolysis by tissue plasminogen activator in rabbits: a comparison with streptokinase. Circulation 1985; 72: 178-84.
19. Verheijen JH, Mullaart E, Chang GTG, Kluft C, Wingaards G. A simple, sensitive spectrophotometric assay for extrinsic (tissue type) plasminogen activator applicable to measurements in plasma. Thromb Haemost 1982; 48: 266-9.
20. Páramo JA, Alfaro MJ, Rocha E. Postoperative changes in the plasmatic levels of tissue-type plasminogen activator and its fast-acting inhibitor. Relationship to deep venous thrombosis and influence of prophylaxis. Thromb Haemost 1985; 54: 713-6.
21 Bone RC. Sepsis and coagulation. An important link. Chest 1992; 101: 594-5.
22. Hajjar KA, Hamel NM, Harpel PC, Nachman RL. Binding of tissue plasminogen activator to cultured human endothelial cells. J Clin Invest 1987; 80: 1712-9.

23. Colucci M, Páramo JA, Collen D. Inhibition of one-chain and two-chain forms of human tissue-type plasminogen activator by the fast-acting inhibitor of plasminogen activator in vitro and in vivo. J Lab Clin Med 1986; 108: 53-9.
24. Gelerther TD, Sznycer-Laszuk R. Thrombin induction of plasminogen activator-inhibitor in cultured human endothelial cells. J Clin Invest 1986; 77: 165-9.
25. Bevilacqua MP, Schleef RR, Gimbrone MA Jr, Loskutoff DJ. Regulation of the fibrinolytic system of cultured vascular endothelium by interleukin 1. J Clin Invest 1986; 78: 587-91.
26. Van Hinsberg VWM, Kooistra T, Van den Berg EA, Princen HMG, Fiers W, Emeis JJ. Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells in vitro and in rats in vivo. Blood 1988; 72: 1467-73.
27. Krishnamurti C, Barr CF, Hasset MA, Young GD, Alving BM. Plasminogen activator inhibitor: a regulator of ancrod-induced fibrin deposition in rabbits. Blood 1987; 69: 798-803.
28. Krishnamurti C, Bolan C, Colleton CA, Reilly TM, Alving BM. Role of plasminogen activator inhibitor-1 in promoting fibrin deposition in rabbits infused with ancrod or thrombin. Blood 1993; 82: 3631-6.
29. Sawa H, Fujii S, Sobel B. Augmented arterial wall expression of type-1 plasminogen activator inhibitor induced by thrombosis. Arterioscler Thromb 1992; 12: 1507-15.
30. Mason JW, Colman RW. The role of Hageman factor in disseminated intravascular coagulation induced by septicemia, neoplasia or liver disease. Thromb Diath Haemorrh 1971; 26: 235-331.
31. Colman RW. The role of plasma proteases in septic shock. N Engl J Med 1989; 320: 1207-9.
32. Collen D, Lijnen HR. Basic and clinical aspects of fibrinolysis and thrombolysis. Blood 1991; 78: 3114-24.
33. Agnelli G, Buchanan MR, Fernández F, Hirsh J. The thrombolytic and hemorrhagic effects of tissue type plasminogen activator: influence of dosage regimens in rabbits. Thromb Res 1985; 40: 769-77.
34. Bergstein JM. Tissue plasminogen activator therapy of glomerular thrombi in the Shwartzman reaction. Kidney Int 1989; 35: 14-8.
35. Kliman A, Mckay DG. Prevention of the generalized Shwartzman phenomenon by fibrinolysis. Fed Proc 1958; 17: 443-6.
36. Matsuo O, Mihara H. Threshold phenomena in blood fibrinolysis. Thromb Res 1977; 10: 753-8.
37. Gurewich V, Pannell R, Lovie S, Kelley P, Suddith R, Greenlee R. Effective and fibrin specific clot lysis by a zymogen precursor form of urokinase (pro-urokinase). A study in vitro and in two animal species. J Clin Invest 1984; 73: 1731-9.

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