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2 Cassidy, P., Kerrick, W.G.L., Hoar, P.E., and Malencik, D.A., Pflügers Arch. *392* (1981) 115.
3 Sparrow, M.P., Mrwa, U., Hofmann, F., and Rüegg, J.C., FEBS Lett. *125* (1981) 141.
4 Herzig, J.W., Köhler, G., Pfitzer, G., Rüegg, J.C., and Wölffle, G., Pflügers Arch. *391* (1981) 208.
5 Pfitzer, G., Rüegg, J.C., Flockerzi, V., and Hofmann, F., FEBS Lett. *149* (1982) 171.
6 Lindner, E., Dohadwalla, A.N., and Bhattacharya, B.D., Arzneimittel-Forsch./Drug Res. *28* (1978) 284.
7 Seamon, K.B., and Daly, J.W., J. cyc. Nucleot. Res. *7* (1981) 201.
8 Metzger, H., and Lindner, E., Arzneimittel-Forsch./Drug Res. *31* (1981) 1248.
9 Ross, E.M., and Gilman, A.G., A. Rev. Biochem. *49* (1980) 533.
10 England, P.J., in: Recently discovered systems of enzyme regulation by reversible phosphorylation, pp. 153-174. Ed. Cohen, P., Elsevier Biochemical, Amsterdam/New York 1980.
11 Osterrieder, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., and Hofman, F., Nature *298* (1982) 576.
12 Robertson, S.P., Johnson, J.D., Holroyde, M.J., Kranias, E.G., Potter, J.D., and Solaro, R.J., J. biol. Chem. *257* (1982) 260.
13 Mope, L., McClellan, G.B., and Winegrad, S., J. gen. Physiol. *75* (1980) 271.
14 Ray, K.P., and England, P.J., FEBS Lett. *70* (1976) 11.
15 Portzehl, H., Caldwell, P.C., and Rüegg, J.C., Biochim. biophys. Acta *79* (1964) 581.
16 Laemmli, U.K., Nature *227* (1970) 680.
17 Seamon, K.B., Padgett, W., and Daly, J.W., Proc. natl Acad. Sci. USA *78* (1981) 3363.
18 England, P.J., FEBS Lett. *70* (1975) 11.
19 Jeacocke, S.A., and England, P.J., FEBS Lett. *122* (1980) 129.

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Characterization and antithrombotic action of tissue plasminogen activator[1]

D. Bergqvist[2], I.M. Nilsson and M. Pandolfi

Surgical Department, Coagulation Laboratory and Ophthalmic Department, University Hospital, S-21401 Malmoe (Sweden), 12 July 1982

Summary. An extractive fibrinolytic enzyme has been characterized and found to belong to the class of vascular plasminogen activators. The agent has been found to have an antithrombotic action in the rabbit.

We wish to report the characterization and the antithrombotic effect in the animal of a fibrinolytic agent, D44, which has recently been made available. This agent, extracted from hog ovaries, acts by converting an inert plasma protein, plasminogen, into a serine protease, plasmin, which breaks down fibrin into soluble degradation products (FDP). The body contains 2 types of plasminogen activators[3]: one, urokinaselike, secreted by non-keratinized epithelial cells and whose main action is probably to assure the passage of fluids through narrow ducts; the other, tissue activator (TA) which is produced by the vascular endothelium and has an antithrombotic action. These 2 types of activators have different properties and can be easily differentiated.

We found that D44 clearly belongs to the TA-class of plasminogen activators. The mol.wt of D44 has been determined on polyacrylamide gel electrophoresis[4]. The distance D44 had migrated (determined by testing 1 mm slices of the gel for fibrinolytic activity on fibrin plates) was recorded on a standard curve obtained by plotting the migration of reference substances against their mol.wt. The mol.wt of D44 was found to be about 70,000, (i.e. in the range of mol.wts found for TA[3]).

Experiments were made to visualize the action of D44 on plasminogen. Human plasminogen was incubated for various lengths of time together with D44, urokinase (UK) and TA (a purified tissue activator obtained from hog ovaries with another extractive method and kindly provided by P. Kok, Umeå). After incubation and reduction with β-mercapto-ethanol the mixtures were run on SDS polyacrylamide gel disc electrophoresis or on polyacrylamide gradient gel slabs (Pharmacia, Fine Chemicals, Uppsala). While UK caused the well-known pattern of limited proteolysis of plasminogen with the fading out of the plasminogen-band and the appearance of the heavy and light chains of plasmin, neiter D44 nor TA produced any significant effect on plasminogen (fig. 1). This was due to the absence of fibrin in the system. It is known that, fibrin is necessary for the activation of plasminogen by TA, but not by UK[3].

The role of fibrin in plasminogen activation by D44 was confirmed in a series of experiments with cross-immunoelectrophoresis and autoradiography. D44, UK, TA were labeled with I^{125}. Plasma incubated with D44 and run against anti-α_2-AP (α_2-antiplasmin, the main plasmin inhibitor of plasma) did not show any complex, nor did we find any mobility change of plasminogen. These results

Table 1. Mean fibrinolytic activity and range (mm^2 lysis) of resuspended euglobulin precipitate

	Before injection	After injection
Controls (10)	30 (0-50)	32 (0-60)
D44 50,000 U (10)	61 (35-150)	41 (33-48)
D44 100,000 U (5)	42 (25-64)	96 (30-169)

Table 2

	Frequency of thrombosis (%)	Frequency of occl. thrombi (%)
Controls (10)	72	50
D44 50,000 U (10)	73	28
D44 100,000 U (5)	45*	33

*$p < 0.05$ for 100,000 U compared with both 50,000 U and controls.

Table 3

	Weight (mg) of: Femoral thrombi	Jugular thrombi	Total mean
Controls (10)	1.0 (0.1-2.2)	34.4 (0.6-122.7)	24.9
D44 50,000 U (10)	1.2 (0.1-5.2)	27.6 (0.1-82.8)	18.5
D44 100,000 U (5)	1.6 (0.6-4.3)	10.6* (0.9-23)	6.6

*$p < 0.05$ for 100,000 U compared with both 50,000 U and controls.

showed that no plasmin was formed. Complex formation between α_2-AP and plasmin was observed with plasma + UK. Complex formation between α_2-AP and plasmin was observed when TA and D44 were incubated with plasminogen in presence of fibrin.

A further similarity between D44 and TA (and a further difference from UK and UK-like activators) was the effect of increasing concentrations of EACA on lysis of standard fibrin clots according to Thorsen and Astrup[5]: while increasing concentrations of EACA had a progressively inhibiting effect on the lysis of the clot by TA and D44 a biphasic (inhibition - potentiation) effect was observed in clots lysed by UK or KK (keratokinase, a corneal UK-like activator[6]).

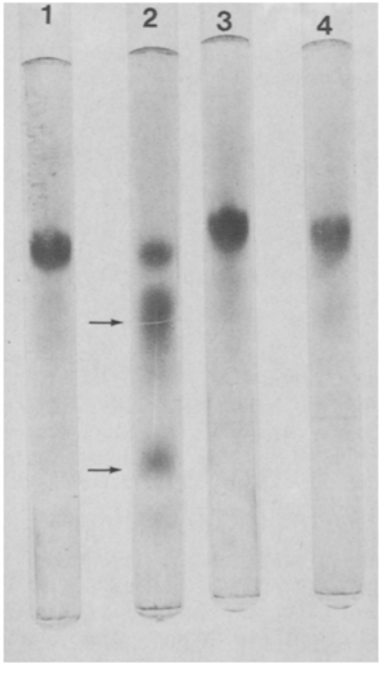

Figure 1. Pattern of limited proteolysis induced in the molecule of plasminogen by UK (2), TA (3), D44 (4). Only in (2) the band of plasminogen decreases and bands corresponding to plasmin's heavy and light chain appear (arrows). (1): buffer + plasminogen. SDS PAA electrophoresis.

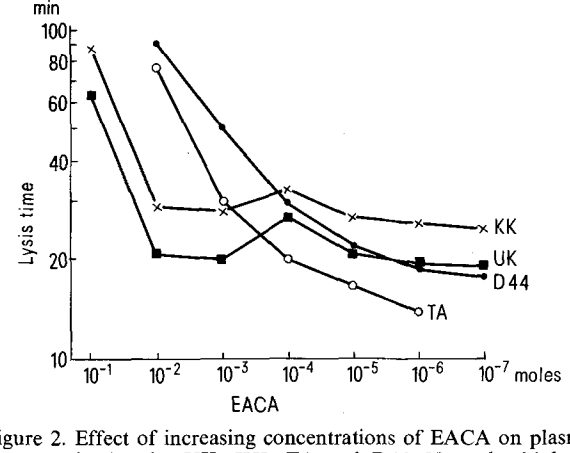

Figure 2. Effect of increasing concentrations of EACA on plasminogen activation by UK, KK, TA and D44. Note the biphasic effect with paradoxycal enhancement of the activation of plasminogen by UK and KK at EACA concentration of 10^{-4} M in contrast to the progressive inhibition of both TA and D44.

Experiments performed according to the method of Thorsen et al.[7] showed that both D44 and TA had a tendency to become bound to fibrin which was significantly stronger than that of UK. These properties confirmed that D44 belongs to the class of TA (endothelial) plasminogen activators.

The antithrombotic activity of D44 was demonstrated as follows. 25 rabbits of both sexes (1.9-4.0 kg) were used. After pentobarbital anesthesia both jugular and femoral veins were isolated. 1-cm-long segments were isolated for thrombosis induction mainly according to a model previously described[8]. A modification was introduced, i.e. ethoxysclerol was used instead of sodium morrhuate for the denudation of the endothelium. After contact between ethoxysclerol and endothelium for 5 min ethoxysclerol was removed with saline and the puncture hole sealed with cyanolite adhesive. A stasis ligature corresponding to a 23 gauge needle was placed proximal to the denuded area allowing blood to pass by for 45 min. Thereafter the veins were inspected. We noted: 1. the occurrence of thrombosis, 2. whether the thrombi were occlusive or not, 3. the thrombus wet weight.

Three groups of rabbits were studied: 1. 10 rabbits were given 50,000 units/kg D44 in saline i.v. 10 min before thrombus induction. 2. 5 rabbits were given 100,000 units/kg D44 in saline i.v. 10 min before thrombus induction. 3. 10 rabbits were given the corresponding volume of saline injected as above. This group served as a control group.

Blood samples were obtained from the central ear artery. Two ml of blood in 0.5 ml 3.8 trisodium citrate was aspirated before injection and after removal of the thrombi. The fibrinolytic activity of citrated plasma and resuspended euglobulin precipitate was measured on unheated fibrin plates[9]. The activity was expressed in mm^2 of lysed area[9].

The fibrinolytic activity is shown in table 1. Only 100,000 units/kg induced fibrinolysis. An effect was only seen in the resuspended euglobulin precipitate, all the citrate plasma samples being negative. Table 2 shows the frequency of thrombosis, which is significantly less when the higher D44 dose was used vs both the control group and the group given D44 50,000 units/kg. The weight of femoral thrombi did not significantly differ between the groups, whereas jugular thrombi were significantly decreased in weight with the highest D44 dose (table 3). One reason of the difference may be that the jugular vein is larger than the femoral vein, thus allowing blood flow during a longer period before occlusion and thereby a longer time for fibrinolytic activity to work.

The results show that D44 has properties similar to those of the endothelial plasminogen activator and suggest that it may be of clinical value in the prevention and treatment of thrombosis.

1 Provided by Dr L. Dussourd d'Hinterland, Laboratoires Pierre Fabre, Castres, France. 1000 units have a fibrinolytic activity of about 15 PU urokinase.
2 To whom reprint requests should be addressed.
3 Pandolfi, M., Thromb. Res. (1983) in press.
4 Weber, K., and Osborn, M., J. biol. Chem. *244* (1969) 4406.
5 Thorsen, S., and Astrup, T., Thromb. Diath. Haemorrh. *32* (1974) 306.
6 Pandolfi, M., and Lantz, E., Exp. Eye Res. *29* (1979) 563.
7 Thorsen, S., Glas, P., and Astrup, T., Thromb. Diath. Haemorrh. *28* (1972) 65.
8 Arfors, K.-E., Bergqvist, D., and Tangen, O., Acta chir. scand. *141* (1975) 40.
9 Nilsson, I.M., and Olow, B., Acta chir. scand. *123* (1962) 247.

0014-4754/84/010067-02$1.50 + 0.20/0