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ORIGINAL ARTICLE

Recombinant human microplasmin: production and potential therapeutic properties

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Summary. The effect of recombinant human microplasmin was studied in ischemic stroke models in mice and in an extracorporeal loop thrombosis model in rabbits. Human microplasminogen (μPlg), which lacks the five 'kringle' domains of plasminogen was expressed with high yield in *Pichia pastoris*. It was purified, converted to microplasmin (μPli) and equilibrated with 5 mmol L^{-1} citrate, pH 3.1, yielding a stable preparation. In mice with middle cerebral artery (MCA) ligation, an intravenous (i.v.) bolus of 5.0 mg kg^{-1} μPli reduced infarct size at 24 h from 27 (26–30) to 25 (21–28) mm^3 (median and range, $n = 16$ each, $P = 0.0001$), whereas 4.0 mg kg^{-1} rt-PA and 40 mg kg^{-1} μPlg had no effect. Infarct reduction was observed with administration at 4 h after occlusion. In mice with MCA, infarct size at 24 h was reduced from 20 (14–30) to 9.1 (3.1–25) mm^3 with 5.0 mg kg^{-1} μPli ($n = 15$ each, $P < 0.002$) and to 11 (5.2–27) mm^3 with 4.0 mg kg^{-1} rt-PA ($n = 6$; $P = 0.02$). Infarct reduction was still observed at 10 h after occlusion with μPli but not with t-PA. In rabbits with radiolabeled clots in an extracorporeal arteriovenous loop, local infusion of 2.5 mg kg^{-1} μPli over 2 h, induced 51 ± 15% lysis (mean ± SD, $n = 11$) vs. a control value of 23 ± 5.5%. μPli did not prolong template bleeding times, whereas equipotent doses of rt-PA were associated with extensive rebleeding. The potency of μPli in both models was similar to that of intact plasmin. These findings indicate that recombinant μPli may be useful for treatment of ischemic stroke and arterial thrombosis.

Keywords: arterial occlusion, ischemic stroke, microplasmin, thrombolysis, thrombosis.

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Intravenous plasmin for thrombolytic therapy has been investigated in humans since the 1950s [1–3]. Administration of a large amount of plasmin, unlike certain other proteolytic enzymes, appeared to be well tolerated, but the development of plasmin for treatment was discontinued in the 1970s.

Nagai *et al*. [4] reported a reduction in infarct size in mice with a permanent ligation of the middle cerebral artery, with a bolus of human plasmin, which was associated with neutralization of plasma α_2-antiplasmin [5]. Marder *et al*. [6] recently demonstrated that human plasmin was comparable to tissue plasminogen activator (t-PA) for local thrombolysis in a rabbit abdominal aorta thrombosis model, but associated with less bleeding. Thus, provided plasmin can be adequately produced and formulated for pharmaceutical use, it could be useful for treatment of ischemic stroke and peripheral arterial thromboembolic disease.

Plasminogen is easily obtained from human plasma or plasma fractions by affinity chromatography on lysine-Sepharose with yields of 0.25 g L^{-1}. Plasminogen cannot readily be expressed in eukaryotic expression systems but has been obtained from the baculovirus/insect cell system [7], which is however, not suitable for large scale production. Plasmin is very unstable in neutral solutions, but can be stabilized with specific amino acids (such as lysine, 6-aminohexanoic acid or tranexamic acid), acid pH (range 2–4) or glycerol (10–50%) [3].

The present study deals with the high yield production, in the yeast Pichia pastoris, of recombinant human microplasminogen (μPlg) and the use of quantitatively activated microplasmin (μPli), stabilized with a dilute citrate buffer at pH 3.1. Microplasmin is a derivative of plasmin which lacks the five kringle domains [8]. Recombinant microplasminogen has previously been expressed in up to 15 mg L^{-1} culture medium [9]. The second order rate constant of the inhibition of microplasmin by α_2-antiplasmin is 2×10^5 mmol L^{-1} s^{-1}, which is about 100 times slower than the inhibition rate of intact plasmin by α_2-antiplasmin ($2–4 \times 10^7$ mmol L^{-1} s^{-1}), due to the absence of the lysine binding site in microplasmin. This lower second order rate constant, corresponds to a half life of microplasmin in circulating blood of 4 s, as compared to a half-life of 0.02 s [10].

The present study evaluates the effect of recombinant human microplasmin on the reduction of focal cerebral ischemic infarction (ischemic stroke) in two mouse models and on the dissolution of blood clots in an arteriovenous loop thrombosis model in rabbits.

Materials and methods

Vector construction for expression of μPlg in Pichia pastoris

The pPICZαA vector (Invitrogen Corporation, Carlsbad, CA, USA) was used for expression and secretion of recombinant human microplasminogen (μPlg) in *Pichia pastoris*, under a license agreement between Thromb-X NV (Leuven, Belgium) and Research Corporation Technologies Inc (Tucson, AZ, USA). The production was carried out under good laboratory practice (GLP) conditions, at Eurogentech SA (Seraing, Belgium), as described elsewhere [11]. Briefly, the vector contained alcohol oxidase elements to allow methanol inducible expression of transgenes, and a secretion signal for secretion of the heterologous protein in the medium. The μPli cDNA was isolated from the vector Fmyc-μPli [12] by PCR amplification and cloned flush with the secretion signal to obtain expression of μPlg with a native NH_2 terminus. A zeocin resistant plasmid clone pPICZα-MPLG1 (clone #5) containing an insert of the expected size was sequenced to confirm correct insertion of the μPlg coding region (Fig. 1). Compared with the published sequence of human plasminogen [13], the nucleotide sequence differed in 10 positions, but the amino acid sequence was identical.

High level expression and purification of μPlg

Competent *Pichia pastoris* X33 cells (Invitrogen Corporation) were transformed with pPICZα-HPLG1 and a strain with high methanol inducible expression of μPlg (clone X33-MPLG1#5) was selected. Fermentation at a 50 L scale was carried out in four steps and μPlg was purified from the culture broth in a three step process, as described elsewhere [11].

Quantitative activation to and stabilization of μPli

The activation of μPlg to μPli was performed at room temperature, for 30 min, at a molar ratio of 0.5% of staphylokinase (variant SY162 with reduced immunogenicity, comprising 12 amino acid substitutions). The activator was removed (over 99%) from μPli by hydrophobic chromatography on Phenyl Sepharose 6 Fast flow in the presence of 0.1 mol L^{-1} tranexamic acid, which was then removed and μPli equilibrated with 5 mmol L^{-1} citric acid, pH 3.1 by tangential ultrafiltration. The final yield of purified material approached 0.4 g L^{-1} culture broth.

Materials

Human plasmin was prepared from plasma as described elsewhere [4]; rt-PA (Actilyze®) was kindly donated by Boehringer Ingelheim GmbH (Ingelheim, Germany) and the platelet aggregation inhibitor ridogrel was a kind gift from Dr F. Declerck (Janssen Research Foundation, Beerse, Belgium).

Analytical methods

SDS gel electrophoresis was performed using the Mini-Protean II system (Bio-Rad, Nazareth, Belgium). (Micro)plasmin activity was measured with 0.4 mmol L^{-1} of the chromogenic substrate S-2403 (<Glu-Phe-Lys-pNA, Chromogenix, Antwerp, Belgium) using a $\Delta A\ min^{-1}$ at 37 °C, pH 7.4 and I 0.15, of 0.03 per nmol L^{-1} plasmin as determined by the manufacturer. α_2-Antiplasmin levels were measured as described elsewhere [4], and fibrinogen using a routine kinetic turbidimetric assay. Staphylokinase in the final μPli preparation was determined using a specific ELISA [11].

Murine middle cerebral artery ligation model

Focal cerebral ischemia was produced by persistent occlusion of the middle cerebral artery (MCA) according to Welsh *et al.* [14], as described elsewhere [5]. BALB/c mice were preferred because of the large size and low variability in ischemic cerebral infarction [4]. Briefly, mice were anesthetized with 2.5% isoflurane in oxygen and rectal temperature was maintained at 37 °C. The left temporal muscle was transected and the skull exposed. A 1-mm opening was made in the region over the MCA with a hand-held drill under saline superfusion. The meningae were removed, the MCA occluded by ligation with 10–0 nylon thread (Ethylon, Neuilly, France) and transected distally, and the temporal muscle and skin sutured back in place. Study drugs (μPli, μPlg, plasmin, rt-PA or solvent) were given intravenously as a bolus, from 15 min up to 6 h after ligation of the MCA. The surgical operator was blinded with respect to the allocation of animals to control (solvent) or study drug groups.

After 24 h, 3 days or 7 days, the animals were sacrificed with Nembutal (500 mg kg^{-1}, Abbott Laboratories, North Chicago, IL, USA) and decapitated. The brain was placed in a matrix for sagittal sectioning in 1 mm segments. The sections were immersed in 2% 2,3,5-triphenyltetrazolium chloride (TTC) in saline, for 30 min at 37 °C, and placed in 4% formalin in phosphate-buffered saline (PBS), whereby the necrotic infarct area remains unstained (white) and distinguishable from stained (brick red) viable tissue. The sections were photographed and subjected to planimetry, and the focal cerebral ischemic infarct size was determined as the sum of the unstained areas of the sections, multiplied with their thickness.

Murine photochemically induced thrombosis model

Photochemically induced thrombosis was produced with Rose Bengal [15]. Anesthesia was performed with 2.5% isoflurane, rectal temperature was maintained at 37 °C, and a catheter (2FG, SIMS portex Limited, Kent, UK) for the administration of Rose Bengal was placed in the left jugular vein. The temporal muscle was transected and the skull was exposed. A 1.5-mm opening was made over the MCA with saline superperfusion and meningae and dura were removed. Photoillumination of green light (540 nm wave length) was achieved with a xenon lamp (model L-4887, Hamamatsu Photonics, Hamamatsu, Japan) with heat absorbing and green filters, via an optic fiber with a focus of 1 mm, placed on the opening in the skull. Rose Bengal (20 mg kg^{-1}, Sigma, St. Louis, MI, USA) was injected, and photoillumination performed for 10 min. Administration of

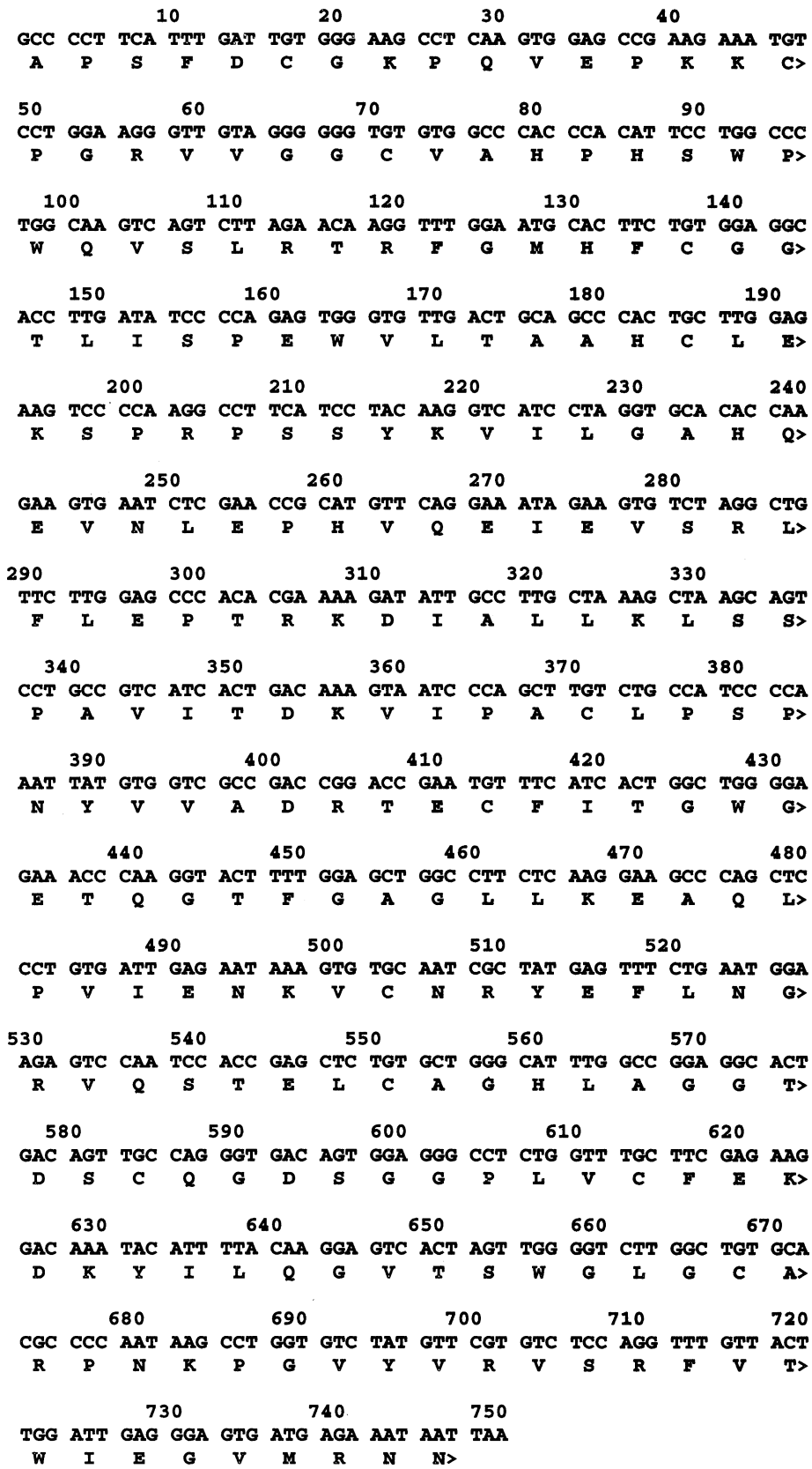

Fig. 1. Nucleotide and deduced amino acid (in single letter code) sequences of human μPlg used in the present study. The first nucleotide in this sequence corresponds to nucleotide 1760 of the human plasminogen cDNA sequence [11]. The first amino acid residue, alanine, corresponds to residue 543 of the human plasminogen amino acid sequence.

study compounds and analysis of cerebral infarct size was carried out as described above.

Rabbit extracorporeal loop thrombolysis model

The thrombolytic effect of μPli, plasmin and rt-PA was studied in an extracorporeal arteriovenous loop thrombus model in rabbits [16]. New Zealand white rabbits weighing 2.9 ± 0.33 kg (mean ± SD) were anesthesized with 1.0 mL of 2% xylazine (Rompun, Bayer, Leverkusen, Germany) and 0.5 mL of ketamine (Ketalin, Apharmo BV, Arnhem, the Netherlands) and additional Nembutal [12 mg (intravenous) i.v. per hour]. A femoral vein catheter was introduced for blood sampling and a femoral artery catheter for blood pressure measurement (PDCR 75, Druck Ltd, Leicester, UK).

A 300-μL thrombus was formed around a woollen thread introduced longitudinally in each of two adapted insulin syringes, from a mixture of ^{125}I-labeled fibrinogen (400 000 cpm), platelet poor rabbit plasma, and 0.07 mL thrombin solution (100 NIHU mL^{-1}). The clot was aged for 30 min at 37 °C and the syringes were inserted in a silicon tubing connecting a femoral artery with a marginal ear vein. Blood flow was regulated via a peristaltic pump (Pump P1, Pharmacia LKB, Piscataway, NJ, USA). Clot extension was prevented by infusion of heparin (300 U kg^{-1} bolus and 200 U kg^{-1} over 2 h) and ridogrel (7.5 mg kg^{-1} bolus, 30 min before the start of the infusion).

Study drugs (6 mL over 2 h) were infused with a pump (Perfuser VI, B. Braun, Penang, Malaysia) via a three way valve, just proximal to the first inserted syringe in the extracorporal loop. Thrombolysis was determined 2.5 h after the start of the infusion, as the difference between the radioactivity originally in the clot and that remaining in the syringes, expressed as percent. Progression of clot lysis was monitored with Geiger counters, linked to a dedicated analysis system (Canberra, Meriden, CT, USA).

Two-milliliter blood samples were drawn into trisodium citrate (0.011 mol L^{-1}), for measurements of fibrinogen, α_2-antiplasmin [3], and activated partial thromboplasmin time. Bleeding times were performed by applying a Symplate II device (Organon Technica, Durham, NC, USA) to a shaved inner thigh surface.

Statistical analysis

The data are represented as mean ± SD or as median and range of *n* determinations. The significance of differences was determined using analysis of variance followed by Fisher's PLSD test, using the STATVIEW software package or by Student's *t*-test or Mann–Whitney test, as appropriate.

Results

High level expression and purification of μPli

Fermentation at the 50-L scale yielded approximately 0.4 g highly purified recombinant human microplasmin (μPli) per litre fermentation broth. SDS gel electrophoresis revealed a single band with apparent M_r of 30 kDa under non-reducing and a main band of 30 kDa, and two minor low M_r peptides under reducing conditions (Fig. 2). The material was fully active as determined with the chromogenic substrate S2403, using a M_r of 30 000, a of 1.0 for μPli and a $\Delta A\ min^{-1}$ at 405 nm of 0.03 per nmol L^{-1} active plasmin. The residual content of staphylokinase SY162 in the μPli was 0.25% of the original amount added to μPlg (200 parts staphylokinase per million parts μPli). The preparation was stable for at least 1 month at room temperature in solution, with at most a slight increase of low M_r peptide material (Fig. 2).

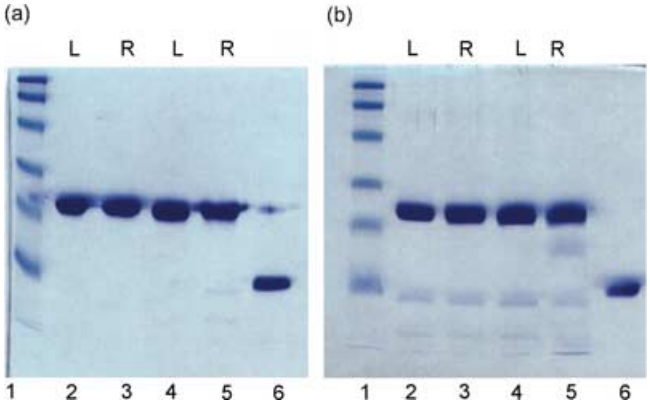

Fig. 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of μPli (10 μg) under non-reducing (a) and reducing (b) conditions. μPli was stored for 1 month as lyophilized powder (L) or as reconstituted material in saline (R). The material was kept at −20 °C (lanes 2 and 3) or at room temperature (lanes 4 and 5); lane 1: molecular weight markers, lane 6: staphylokinase variant SY162 used for activation (2 μg).

Effect of μPli on cerebral infarct size in mice

Bolus injection of μPli decreased plasma α_2-antiplasmin and fibrinogen levels proportionally to the μPli dose and recovered partially within 2 h (Table 1) and fully within 24 h (not shown), suggesting that α_2-antiplasmin depletion was transient during the first hours after the injection of μPli. Bolus injection of 4 mg kg^{-1} t-PA caused minor α_2-antiplasmin reduction without fibrinogen reduction.

Ligation of the MCA induced a cerebral infarct with a volume of 29 mm^3 (range 27–30) ($n = 6$) in inbred BALB/c mice (Table 2). Injection of 2.5 mg kg^{-1} μPli had no significant effect on infarct size, whereas 5.0 mg kg^{-1} μPli, or 5.0 mg kg^{-1} followed 45 min later by another 2.5 mg kg^{-1}, produced a 15% reduction of infarct size. These results are in line with the transient partial reduction of α_2-antiplasmin with the lower dose and the more persistent depletion with 5.0 mg kg^{-1} μPli. Infarct size was reduced with μPli injections up to 4 h after MCA occlusion, and when measured up to 3 days after μPli administration. μPlg at a dose of 40 mg kg^{-1} or rt-PA at a dose of 4.0 mg kg^{-1} did not reduce infarct size (Table 2).

Photoillumination of the MCA produced thrombosis resulting in a cerebral infarct volume of 20 mm^3 (range 16–30)

Table 1 Effect of μPli on plasma, α_2-antiplasmin and fibrinogen levels in mice

		α_2-Antiplasmin (%)				Fibrinogen (%)			
Agent	Dose (mg kg^{-1})	0 min	15 min	1 h	2 h	0 min	15 min	1 h	2 h
	2.5	94 ± 2.1	73 ± 16	75 ± 3.6	100 ± 12	100 ± 12	92 ± 32	78 ± 2.4	75 ± 4.2
μPli	5.0	–	30 ± 22	25 ± 10	34 ± 6.7	–	32 ± 17	15 ± 3.4	64 ± 17
t-PA	4.0	–	85 ± 4.1	–	–	–	100 ± 9.2	–	–

The data represent the mean ± SD of groups of at least three animals.

Table 2 Effect of systemic administration of μPli, μPlg and rt-PA on focal cerebral ischemic injury in mice with permanent middle cerebral artery (MCA) ligation

				Cerebral infarct size		
Compound	Dose (mg kg^{-1})	Injection time (h after MCA-O)*	Sacrifice (days after MCA-O)	Control	Compound	P vs. control
	2.5	0.25	1	29 (27–30) (6)	29 (27–30) (6)	0.74
μPli	5.0**	0.25	1	id	26 (21–28) (6)	0.04
	5.0 + 2.5	0.25 and 1	1	id	26 (20–28) (6)	0.02
	5.0**	0.25	1	27 (26–29) (6)	24 (22–27) (6)	0.01
	5.0	1	1	id	25 (20–27) (6)	0.04
	5.0	2	1	id	24 (22–28) (6)	0.02
	5.0	4	1	id	25 (21–28) (6)	0.05
	5.0	6	1	id	26 (24–32) (6)	0.65
	5.0**	0.25	1	27 (26–29) (6)	23 (22–27) (6)	0.01
	5.0	0.25	3	24 (22–28) (6)	22 (21–24) (6)	0.01
	5.0	0.25	7	12 (9.0–18) (6)	11 (5.2–13) (6)	0.15
Plasmin	7.5	0.25	1	28 (25–32) (6)	24 (20–26) (6)	0.01
μPlg	40	0.25	1	26 (25–28) (6)	26 (24–27) (6)	0.87
rt-PA	4.0	0.25	1	28 (25–32) (6)	26 (22–31) (6)	0.11

Cerebral infarct size is expressed as median and range of the number of experiments given between brackets; *MCA-O: middle cerebral artery occlusion; **pooled data of 5.0 mg kg^{-1} μPli vs. control 27 (26–30) vs. 25 (21–28) mm^3 (median and range, $n = 16$, $P < 0.0001$).

($n = 6$) in the control group (Table 3), which was reduced to 4.8 mm^3 (3.1–22) ($n = 6$) with 5.0 mg kg^{-1} μPli ($P < 0.01$) and to 11 mm^3 (2.1–22) ($n = 6$) with 5.0 plus 2.5 mg kg^{-1} ($P = 0.04$). Infarct size was reduced with μPli up to 10 h after occlusion and when measured up to 3 days after its administration. Plasmin at a dose of 7.5 mg kg^{-1} also did, but μPlg at a dose of 40 mg kg^{-1} did not reduce infarct size significantly. rt-PA reduced infarct size significantly when administered 15 min after illumination but not after 4 h (Table 3).

Effect of μPli on extracorporeal loop clot lysis in rabbits

In control animals, the spontaneous decrease of radioactivity in the inserted syringes 23 ± 5.5% ($n = 6$) (Table 4). A dose-dependent degree of clot lysis was observed both with μPli and plasmin. With a blood flow of 0.5 mL min^{-1}, μPli, at doses of 0.63–5.0 mg kg^{-1}, induced clot lysis ranging from 43 ± 4.5% ($n = 6$) to 64 ± 13% ($n = 6$) whereas plasmin, at doses of 2.5 and 5.0 mg kg^{-1}, induced 44 ± 2.5% ($n = 2$) and 53 ± 3.5% ($n = 2$) clot lysis, respectively (Table 3). Administration of 5.0 mg kg^{-1} μPli produced minor α_2-antiplasmin reduction (67 ± 11% residual plasma level) and fibrinogen breakdown (67 ± 5.8% residual level) and did not significantly prolong template bleeding time. Infusion of 2.5 or 5.0 mg kg^{-1} plasmin reduced α_2-antiplasmin and fibrinogen to 80% with minor effect on the bleeding time. With a blood flow of 0.1 mL min^{-1}, 2.5 mg kg^{-1} μPli induced clot lysis of 73 ± 11% ($n = 8$), whereas 2.5 mg kg^{-1} plasmin induced 57 ± 4.6% ($n = 2$) lysis with marginal effects on α_2-antiplasmin, fibrinogen and bleeding time (Table 4). Thus, the extent of clot lysis by μPli and plasmin is mainly determined by the dose and delivery in the vicinity of the thrombus, with marginal changes in fibrinogen, and α_2-antiplasmin levels and in bleeding time.

Infusion of rt-PA at doses between 0.31 and 2.5 mg kg^{-1} produced 65–70% clot lysis, without a clear dose–response, but with a dose-related prolongation of the template bleeding time. A remarkable difference between rt-PA and μPli was that rebleeding and oozing from template skin wounds was consistently observed, with rt-PA at all doses tested, but not with μPli.

Discussion

In the present study a preparation of human plasmin, suitable for pharmaceutical use, was developed. This was achieved by expression of human microplasminogen (μPlg) lacking the five

Table 3 Effect of systemic administration of μPli, plasmin, μPlg or rt-PA on focal cerebral ischemic injury in mice with photochemically induced MCA thrombosis

Compound	Dose (mg/kg)	Injection time (h after MCA-O)	Sacrifice (days after MCA-O)	Cerebral infarct size (mm^3) Median and rage		
				Control	Compound	P vs. control
μPli	2.5	0.25	1	20 (16–30) (6)	17 (7.6–29) (6)	0.32
	5.0	0.25	1	id	4.8 (3.1–22) (6)	<0.01
	5.0 + 2.5	0.25 and 1	1	id	11 (2.1–22) (6)	0.04
	5.0	0.25	1	26 (15–35) (17)	14 (4.3–24) (12)	<0.01
	5.0	1	1	id	14 (2.4–23) (12)	<0.01
	5.0	2	1	id	9.4 (2.7–31) (12)	<0.01
	5.0	4	1	id	16 (2.7–25) (12)	<0.01
	5.0	6	1	id	16 (4.4–28) (12)	<0.01
	5.0	8	1	id	21 (5.7–33) (12)	<0.01
	5.0	10	1	id	21 (9.0–27) (12)	0.03
	5.0	0.25	1	20 (15–27) (6)	10 (5.1–20) (6)	0.01
	5.0	0.25	3	26 (9.7–30) (6)	11 (3.9–21) (6)	0.03
	5.0	0.25	7	7.7 (3.1–12) (6)	6.7 (3.1–9.0) (6)	0.3
μPlg	40	0.25	1	21 (17–25) (6)	19 (9.1–33) (6)	0.6
Plasmin	7.5	0.25	1	28 (25–33) (6)	14 (4.3–30) (6)	0.01
	4.0	0.25	1	28 (25–33) (6)	11 (5.2–27) (6)	0.02
rt-PA	4.0	4	1	22 (17–25) (6)	25 (20–30) (6)	0.04

Cerebral infarct size is expressed as median and range of the number of experiments given between brackets. MCA-O: middle cerebral artery occlusion.

Table 4 Effect of local infusion of μPli, plasmin and rt-PA on clot lysis and hemostasis parameters in rabbits with extracorporeal loop thrombosis model

Substance	Blood flow ($mL\,min^{-1}$)	Dose ($mg\,kg^{-1}$)	*n*	Clot lysis (%)	Residual fibrinogen (%)*	Residual α_2-AP (%)*	Bleeding time(s)*
Solvent		–	7	23 ± 5.5	110 ± 6.2	100 ± 2.0	110 ± 25
μPli	0.5	0.6	6	43 ± 4.5	93 ± 8.0	97 ± 10	100 ± 25
	0.5	1.3	7	52 ± 20	93 ± 5.0	96 ± 12	140 ± 58
	0.5	2.5	11	51 ± 15	79 ± 16	71 ± 15	120 ± 64
	0.5	3.8	6	64 ± 13	75 ± 4.0	71 ± 14	150 ± 53
	0.5	5.0	6	61 ± 14	67 ± 5.8	67 ± 11	90 ± 19
	0.1	2.5	8	73 ± 11	84 ± 10	88 ± 9.0	100 ± 35
Plasmin	0.5	2.5	2	44 ± 2.5	82 ± 6.8	80 ± 22	120 ± 42
	0.5	5.0	2	53 ± 3.5	80 ± 3.3	86 ± 3.0	150 ± 85
	0.1	2.5	2	57 ± 4.6	94 ± 10	93 ± 6.0	120 ± 85
rt-PA	0.5	0.31	6	77 ± 8.9	95 ± 1.0	98 ± 7.0	130 ± 24
	0.5	0.63	6	65 ± 12	94 ± 8.0	88 ± 3.0	150 ± 56
	0.5	1.3	6	68 ± 9.6	87 ± 5.0	74 ± 5.0	170 ± 53
	0.5	2.5	6	68 ± 11	75 ± 12	41 ± 12	180 ± 55

The data represent mean ± SD of *n* experiments; *measured at the end of the experiment.

kringles (comprising amino acids Ala543–Asn791) in high yield ($>0.5\,g\,L^{-1}$ fermentation broth) in *Pichia pastoris*, purification to homogeneity, quantitative conversion to microplasmin (μPli), stabilization of the enzyme in a dilute citrate buffer at pH 3.1 and lyophylization. The potential therapeutic efficacy of the homogeneous, fully active material is illustrated in animal models of ischemic stroke (using systemic intravenous administration) and of arterial thrombosis (using local administration).

Ischemic stroke due to thrombotic occlusion of a cerebral artery is amenable to therapy with antithrombotic and thrombolytic agents. The use of rt-PA within 3 h of symptom onset improves neurologic outcome, but may cause acute hemorrhage in the brain. In previous studies we observed that α_2-antiplasmin gene deficient mice had smaller cerebral infarct size after middle cerebral artery (MCA) ligation [5] and that reduction of α_2-antiplasmin with a single bolus of human plasmin, or of an anti-α_2-AP Fab fragment reduced infarct size [4]. This suggested that reduction of circulating α_2-antiplasmin may constitute a new approach to reduce cerebral infarct size in ischemic stroke in the absence of reperfusion, possibly via a neuroprotective mechanism of action.

In the present study we have explored the effect of μPli in two mouse models consisting of permanent ligation or photochemically induced thrombosis of the MCA. In the first model, μPli reduced cerebral infarct size by approximately 15%, comparable with that of full length plasmin [4]. In the photoactivation induced thrombosis model, the extent of cerebral infarct size reduction was much larger (approximately 50%), possibly due to a combined effect of reperfusion-dependent and reperfusion-independent mechanisms. Microplasmin lacks fibrin affinity and has short half-life in blood, suggesting that its thrombolytic potency might primarily result from enhanced endogenous thrombolytic after reduction of α_2-AP [17,18].

The beneficial effect of μPli was observed when administered for 4–10 h after occlusion whereas the effect of rt-PA had disappeared after 4 h, when infarct expansion was observed. This may be explained by the recent observation that, in the absence of reperfusion, rt-PA causes a dose-related infarct expansion [19], which may be due to its neurotoxic effect via plasminogen activator mediated proteolysis and activation of the NMDA receptor [20]. Consequently, provided these observations can be extrapolated to patients with ischemic stroke, microplasmin might constitute a safer alternative to rt-PA.

In patients with peripheral artery occlusion disease, intra-arterial thrombolysis may prevent complex surgical procedures and allow simpler elective procedures to correct unmasked lesions. The most feared complication of intra-arterial thrombolysis with plasminogen activators is, however, intracranial bleeding which occurs in 1–2% of treated patients. Local infusion of plasmin obtained from plasma plasminogen has recently been shown in a rabbit model to dissolve arterial clots with less bleeding time prolongation than rt-PA [6]. The present study confirms and extends these observations to recombinant human microplasmin.

In conclusion, a preparation of μPli was developed, which appears to be suitable for pharmaceutical use. Being a truncated derivative of plasmin lacking the lysine binding sites, it has no affinity for fibrin and it is inhibited more slowly by α_2-antiplasmin than intact plasmin. In *in vivo* models of ischemic stroke and peripheral arterial occlusion, μPli appeared to be comparable in potency to full length plasmin. In line with its lack of fibrin affinity and its short plasma half life, μPli did not cause a bleeding tendency nor hemostatic plug dissolution at distant sites. The present results warrant further investigation of μPli both for the treatment of ischemic stroke by systemic administration and of arterial thromboembolic disease by local catheter delivery.

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