Characterization of a recombinant chimeric plasminogen activator with enhanced fibrin binding

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Abstract

A recombinant chimeric plasminogen activator (GHRP-scu-PA-32K), consisting of the tetrapeptide Gly-His-Arg-Pro fused to the N-terminus of the low-molecular single-chain urokinase-type plasminogen activator (Leu144^Leu411), was produced by expression in CHO cells. The stable expression cell line was selected for large-scale expression. The product was purified by antibody-Sepharose affinity chromatography with a recovery of 67%. The apparent molecular weight of purified GHRP-scu-PA-32K was 33 kDa according to SDS-PAGE. Its specific activity was 150 000 IU/mg protein according to fibrin plate determination. The conversion of single-chain to two-chain molecules mediated by plasmin was comparable for GHRP-scu-PA-32K (K_m = 4.9 µM, k_2 = 0.35 s⁻¹) and scu-PA-32K. The activation of plasminogen by GHRP-scu-PA-32K (K_m = 1.02 µM, k_2 = 0.0028 s⁻¹) was also similar to that of scu-PA-32K. The fibrin binding of GHRP-scu-PA-32K was 2.5 times higher than that of scu-PA-32K at a fibrin concentration of 3.2 mg/ml. In contrast to scu-PA-32K in vitro ¹²⁵I-fibrin-labeled plasma clot lysis, GHRP-scu-PA had a higher thrombolytic potency, whereas it depleted less fibrinogen in plasma. These results show that GHRP-scu-PA-32K as expected is a potential thrombolytic agent. © 2001 Published by Elsevier Science B.V.

Keywords: Single-chain urokinase-type plasminogen activator of 32 kDa; CHO cell; Fibrin binding

1. Introduction

Urokinase-type plasminogen activator (u-PA), one of the plasminogen activators, is a serine protease which plays an important role in fibrinolysis [1]. Scu-PA is composed of 411 amino acids with M_r 54 000, which could be converted to a two-chain u-PA (tcu-PA) by cleavage of the Lys158^Ile159 peptide bond by plasmin [2]. Single-chain urokinase-type plasminogen activator (scu-PA), also called pro-urokinase (pro-UK), has a higher selectivity for fibrin than tcu-PA [3]. Besides scu-PA, a low-molecular-weight form of scu-PA (scu-PA-32K), lacking the 143 NH2-terminal amino acids, is similar to scu-PA in fibrin specificity and thrombolytic potential [4,5]. But it has a lower molecular weight and a higher stability, which give it advantages in thrombosis [6,7]. Human fibrinogen can be converted to fibrin by thrombin-catalyzed release of small peptides from the amino-terminal segments of the α and β chains that are denoted fibrinopeptides A and B, respectively [8]. As a result of the removal of these peptides, the parent molecule polymerizes spontaneously. The tetrapeptide Bβ15–18 beginning with the sequence glycyl-l-histidyl-l-arginyl-l-proline (Gly-His-Arg-Pro), GHRP, corresponding to the amino-terminal segment of the fibrin β chain, is involved in fibrin lateral associations [9]. The tetrapeptide

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GHRP can interact with a complementary site on the β lobe of fibrin monomers and prevent polymerization [10]. Furthermore, it has been reported that histidine-16 of the Bβ chain plays an important role in the association of fibrin [11]. It is expected that this chimeric plasminogen activator, composed of GHRP and scu-PA-32K, may have a higher fibrin affinity and higher thrombolytic activity.

2. Materials and methods

2.1. Materials

Plasmid pCM-β-neo and CHO/DHFR− cells were kindly provided by Dr. D. Collen (Leuven University, Belgium). Dulbecco’s modified Eagle’s medium, fetal bovine serum, and G418 were purchased from Gibco BRL (USA). Fibrinogen, plasminogen, and thrombin were purchased from Sigma (St. Louis, MO, USA); S2444 (L-pyroGlu-Gly-L-Arg-p-nitroanilide) and S2551 (D-Val-Leu-Lys-p-nitroanilide) were purchased from Kabi Vitrum (Stockholm, Sweden). Mouse monoclonal antibody against the B chain of urokinase (8D2B9) was a gift of Prof. M.H. Hu (Peking University, China). Scu-PA-32K was purified as described previously [12]. GHRP was synthesized by Sbsbio (Beijing, China).

2.2. Methods

2.2.1. Construction of expression vector

pCM-β-neo-GHRP-scu-PA-32K and expression in CHO cells

GHRP-scu-PA-32K cDNA was obtained by in vitro site-directed mutagenesis [13] to insert an oligonucleotide sequence (GGTCATAGGCCT) encoding the tetrapeptide Gly-His-Arg-Pro into a DNA fragment encoding scu-PA-32K (144–411). Following this, the mutant cDNA was cloned in pCM-β-neo expression vector and co-transformed CHO-DHFR− cells with pCM-dhfr. One stable expression cell line secreting the highest amount of GHRP-scu-PA-32K was selected with geneticin (G418) and methotrexate (MTX). For large-scale production, the cells were grown in 350 ml of modified Eagle’s medium supplemented with 0.2% NaHCO3, 3 mM glutamine, and 10% fetal calf serum. At confluence, the cells were replaced by serum-free medium supplemented with 10 kallikrein inhibitor units/ml aprotinin. The medium was harvested after 2 days, and centrifuged at 7000 rpm for 15 min.

2.2.2. Purification of GHRP-scu-PA-32K

Purification of the chimeric protein was mainly performed according to Zhao et al. [14]. Briefly, the serum-free culture product was centrifuged at 12000 rpm for 30 min. The centrifuged supernatant was transferred into a monoclonal antibody affinity column against the B chain of pro-UK. The column was washed with 0.1 M phosphate buffer (pH 7.4), followed by 0.1 M NaOAc-HOAc buffer (pH 4.0), and eluted with 2 M KSCN.

U-PA-related antigen was assayed by specific ELISAs. The assay was followed as described [15], utilizing rat antibody against high- or low-molecular urokinase (3C6)-coated polystyrene plates, mouse monoclonal antibody against the B chain of urokinase (8D2B9) and horseradish peroxidase-conjugated goat anti-mouse IgG. The protein concentration was measured as described by Bradford et al. [16], using bovine serum albumin as the standard.

2.2.3. Activity determination

Fibrinolytic activity was determined on bovine fibrin plates after overnight incubation at 37°C by comparison with International Reference Preparation for Urokinase [17]. Amidolytic activity was measured before and after plasmin treatment using the chromogenic substrate S2444 at 37°C [18].

2.2.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to Laemmli [19] with 5% stacking gels and 15% separating gels.

2.2.5. Western blot analysis

Western blot analysis, utilizing a mouse monoclonal antibody against the B chain of urokinase and goat-anti mouse IgG alkaline phosphatase, was performed as described by Towbin et al. [20].

2.2.6. NH2-terminal protein sequence analysis

Protein NH2-terminal analysis was performed by Edman degradation using an Applied Biosystems se-
quencer model 470A connected to a PTH analyzer model, following the standard procedure recommended by the manufacturer.

2.2.7. Treatment with plasmin

The conversion of single-chain to two-chain u-PA moieties was followed as mainly described [21,22]. Scu-PA-32K or GHRP-scu-PA-32K (final concentration, 1–10 µM) was incubated with plasmin (final concentration, 5 nM) in 0.05 mM Tris–HCl, pH 7.4, 38 mM NaCl, and 0.01% Tween 80 at 37°C. At timed intervals (0–3 min), aliquots were removed and the amidolytic activity was measured with S2444 (final concentration, 0.3 mM) after 100-fold dilution of samples. The reaction rate was calculated by the OD increase over time at 405 nm. UK International standards (0.01–5 nM) were used for the standard curve of S2444. The kinetic constants were derived by linear regression analysis from Lineweaver–Burk plots.

2.2.8. Activation of plasminogen

Activation of Glu-plasminogen (final concentration, 0.1–2 µM) was measured with GHRP-scu-PA-32K or scu-PA-32K (final concentration, 10 nM) in 0.05 mM Tris–HCl, pH 7.4, 38 mM NaCl, and 0.01% Tween 80 at 37°C. Generation of plasmin was monitored for 15 min at 405 nm. The reaction rate was measured as the OD increase over time squared as previously described [22]. The kinetic constants were measured from Lineweaver–Burk plots.

2.2.9. Fibrin binding assay

50 IU/ml scu-PA-32K or GHRP-scu-PA-32K (final concentration, 8 nM) was mixed with different concentrations of fibrinogen (final concentration, 0–3.2 mg/ml) in 50 mM Tris–HCl, pH 7.4, 40 mM NaCl, 0.01% Tween 80. The mixture was clotted by thrombin at a final concentration of 20 NIH/ml, incubated at 37°C for 30 min, and centrifuged at 12,000 rpm for 10 min. The residual activity in the supernatants was measured by fibrin plate determination.

2.2.10. Fibrin clotting analysis

The test was conducted as described by Laudano and Doolittle [23], by adding thrombin to a fibrinogen solution containing GHRP, scu-PA-32K or GHRP-scu-PA-32K at a final concentration of 15 µM. Clotting time was monitored by measuring the scattered light in the spectrophotometer at 350 nm. The final solution contained 1 mg/ml fibrinogen, 0.15 NIH/ml thrombin, 50 mM Tris–HCl, 150 mM NaCl, pH 7.2.

2.2.11. Fibrinolytic and fibrinogenolytic properties in vitro

125I-fibrin-labeled platelet-poor plasma was prepared from normal human plasma. Lysis of 125I-fibrin-labeled plasma clots (60 µl), immersed in citrated normal human plasma (500 µl) with different concentrations of scu-PA-32K or GHRP-scu-PA-32K (final concentration, 0–10 µg/ml), was measured after incubation for 2 h at 37°C as described previously [24]. Lysis was calculated from the release of radioactivity and expressed as a percent of the complete lysis value. In addition, residual fibrinogen levels were measured after incubation for 2 h at 37°C as described by Rampling et al. [25].

3. Result


Scu-PA-32K cDNA was first cloned into M13mp18; next we extracted the M13 single-stranded DNA template. The DNA oligonucleotide (5’-CGACTCCAAAGGCCTTGTATAATTTCAGTGGTGG-3’) was synthesized. M13 single-chain DNA and oligonucleotide were mixed and annealed at 70°C, extended at 16°C. The oligonucleotide (GGTCATAGGCCTTTA- AAATTCAGTGG-3’) encoding the tetrapeptide Gly-His-Arg-Pro was successfully inserted into scu-PA-32K cDNA. The recombinant DNA was checked by restriction analyses and DNA sequencing. Then, the GHRP-scu-PA fragment and expressing vector pCM-βneo were ligated together after digestion with HindIII. The resulting plasmid was pCM-βneo-GHRP-scu-PA-32K, which was used for expression in CHO cells.

3.2. Expression and purification

CHO cells have a relatively high expression effi-
ciency and are stable host cells for expressing eukaryotic protein. Through the expression of the neomycin resistance gene carried by pCM-βneo in this experiment, the transformed cells could be kept alive while growing in the alternative medium including G418. CHO cells were selected with MTX through the expression of dihydrofolate reductase carried by the co-transformed vector pCM-dhfr. Finally the stable expression cell line was selected. The expression level of the cell line cultured in a serum-free medium was 580 IU/10^6 cells/24 h. The large-scale expression medium was harvested and used as the starting material for purification, which has a protein concentration of about 0.9 ± 0.3 mg (mean ± S.D., n = 3) of u-PA-related antigen/l, as determined by ELISA.

The PA expression product was purified by antibody (monoclonal antibody against the B chain of pro-UK)-coupled Sepharose affinity chromatography. The PA was eluted by KSCN as a single peak, resulting in a nearly 80-fold volume reduction. The whole purification procedure was performed at 4°C. After purification and immunoabsorption of pooled u-PA-containing fractions, approximately 0.6 mg u-PA-related protein was obtained from 1 l of condition medium, with a recovery of 67%. The purity of GHRP-scu-PA-32K was approximately 95%, as determined by comparison of u-PA-related protein with total purified protein. The specific activity of the purified GHRP-scu-PA-32K according to fibrin plate determination was 150 000 IU/mg protein as compared to 110 000 IU/mg for scu-PA-32K, which was similar to the value of scu-PA-32K reported before [5]. It shows that the chimeric protein kept the activity of scu-PA-32K.

3.3. Physicochemical characterization

Purified chimeric protein (about 10 μg) migrated as a single band as shown by reduced SDS-PAGE (Fig. 1).

The apparent molecular weight of purified GHRP-scu-PA-32K was 33 kDa by SDS-PAGE. N-terminal amino acid analysis performed with 150 pmol GHRP-scu-PA-32K yielded the sequence G(97)-H(46)-R(68)-P(88)-L(71)-K(102)-F(87)-Q(53), which was identical to that of the starting GHRP-scu-PA-32K. The chimeric protein had low amidolytic activity (150 IU/mg) which increased dramatically when treated with plasmin. All these data demonstrate that the purified product was in the single-chain form. Western blot with a polyclonal antibody against urokinase indicated that the chimeric protein had a similar antigen binding capacity to urokinase (Fig. 2).

3.4. Analysis of kinetic constants

3.4.1. Treatment with plasmin

The conversion of single-chain to two-chain u-PA moieties followed Michaelis– Menten kinetics as shown by Lineweaver–Burk plots (Fig. 3). The kinetic constants were $K_m = 4.9 \, \mu M$ and $k_2 = 0.35 \, s^{-1}$ for GHRP-scu-PA-32K ($n = 2, \, r = 0.99$) as compared to $K_m = 4.5 \, \mu M$ and $k_2 = 0.27 \, s^{-1}$ for scu-PA-32K ($n = 2, \, r = 0.99$). The catalytic efficiency ($k_2/K_m$) of GHRP-scu-PA-32K (0.07 μM⁻¹ s⁻¹) was similar to that of scu-PA-32K (0.06 μM⁻¹ s⁻¹).

3.4.2. Activation of Glu plasminogen

Upon incubation of single-chain u-PA moieties and plasminogen in the presence of a high concentration of the plasmin-specific chromogenic substrate

Fig. 1. SDS-PAGE analysis on a 10% gel with reduction by dithioerythritol and staining with Coomassie brilliant blue. Lane 1, purified protein GHRP-scu-PA-32K; lane 2, protein calibration mixture. From top to bottom: phosphorylase b (94000), albumin (67000), ovalbumin (43000), carboxic anhydrase (30000), and α-lactalbumin (14400).

Fig. 2. Western blot analysis of GHRP-scu-PA-32K. Lane 1, protein calibration mixture as in Fig. 1; lane 2, GHRP-scu-PA-32K.
S2551, almost all the generated plasmin is shifted into a complex with S2551, preventing the activation of single-chain to two-chain u-PA moieties [21]. Analysis of the kinetic constants revealed that plasminogen activated to plasmin by scu-PA-32K or GHRP-scu-PA-32K also obeyed Michaelis–Menten kinetics. From Lineweaver–Burk plots (Fig. 4), \(K_m = 1.02 \mu M\) and \(k_2 = 0.0028 \text{ s}^{-1}\) for GHRP-scu-PA-32K \((n=2, \ r=0.99)\) and \(K_m = 0.76 \mu M\) and \(k_2 = 0.0018 \text{ s}^{-1}\) for scu-PA-32K \((n=2, \ r=0.99)\) were obtained. The result showed that GHRP-scu-PA-32K had a somewhat lower affinity to plasminogen than scu-PA-32K. But the catalytic efficiency \((k_2/K_m)\) of GHRP-scu-PA-32K \((0.0027 \mu M^{-1} \text{ s}^{-1})\) was somewhat higher than that of GHRP-scu-PA-32K \((0.0024 \mu M^{-1} \text{ s}^{-1})\) as a result of the higher catalytic rate value \((k_2)\).

### 3.5. Fibrin binding and fibrin clotting analysis

Fig. 5 shows that the chimeric protein had a higher affinity for the fibrin clots than did scu-PA-32K. At 3.2 mg/ml of fibrin clots, the affinity for fibrin of GHRP-scu-PA-32K was 2.5 times higher than that of scu-PA-32K. Besides, fibrin clotting times were prolonged when the polymerization system was incubated with GHRP or GHRP-scu-PA-32K (Fig. 6). This result indicates that GHRP interfered with fibrin polymerization after binding to the complementary location of fibrin.

### 3.6. Fibrinolytic and fibrinogenolytic properties in vitro

Dose-dependent lysis of platelet-poor plasma submerged in human plasma was obtained with single-chain plasminogen activators. Fig. 7 presents dose-response curves of 125I-fibrin-labeled plasma clot lysis, showing GHRP-scu-PA-32K had a higher fibrinolytic potency than scu-PA-32K. Fifty percent clot lysis in 2 h was obtained at a concentration of 3.9 \(\mu g/ml\) for scu-PA-32K, whereas 2.1 \(\mu g/ml\) was required with GHRP-scu-PA-32K as shown in Fig. 7A.
After incubation for 2 h at 37°C, residual fibrinogen levels were measured. At a concentration yielding 50% clot lysis in 2 h, the residual fibrinogen level was 49% for scu-PA-32K, while it was 76% for GHRP-scu-PA-32K as shown in Fig. 7B.

4. Discussion

Scu-PA is one of the most effective thrombolytic agents. Its thrombolytic activity is higher than that of tissue plasminogen activator (t-PA), but its affinity for fibrin is lower than t-PA. In therapeutic application scu-PA may overactivate plasminogen and overconsume $\alpha$-2 antiplasmin, leading to systemic bleeding. A low-molecular-weight form of scu-PA (scu-PA-32K) lacking the epidermal growth factor domain and the Kringle domain, has a similar characterization as scu-PA in thrombosis [6,7]. Though scu-PA-32K has several advantages as a thrombolytic agent, it has the limitation of low direct fibrin selection [26]. However, the use of a plasminogen activator with higher fibrin affinity may reduce fibrinogen breakdown and systemic activation of the fibrinolytic system. GHRP is a tetrapeptide of the fibrin $\beta$ chain, which plays an important role in the process of fibrin polymerization. Furthermore, GHRP can bind to fragments D of all sizes, preventing the polymerization of fibrin.

In this study, the chimeric plasminogen activator GHRP-scu-PA-32K, combining scu-PA-32K (Leu144–Leu411) with GHRP (fibrin peptides), was successfully constructed. The chimeric protein was expressed in CHO cells and purified from conditioned medium. The specific fibrinolytic activity of GHRP-scu-PA-32K was comparable to that of intact scu-PA-32K. The analysis of the kinetic parameters revealed that GHRP-scu-PA-32K had very similar characteristics to scu-PA-32K in the conversion by plasmin and the activation of plasminogen. The activation of plasminogen by GHRP-scu-PA-32K also confirmed that the scu-PA moiety had an appreciable intrinsic plasminogen activity with high affinity ($K_m$) and a low catalytic rate constant ($k_2$). Fibrin binding experiments demonstrated that GHRP-scu-PA-32K had a higher fibrin binding capacity than scu-PA-32K, suggesting that GHRP-scu-PA-32K could be targeted to fibrin clots with GHRP. It was concluded that insertion in scu-PA-32K of GHRP, which could bind to fibrin, conferred a higher affinity to fibrin. At high concentrations of GHRP-scu-PA-32K, the fibrin polymerization time could be prolonged, indicating that GHRP might act as a potent inhibitor. In fibrin clot lysis in human plasma, the chimeric protein showed a higher fibrinolytic potency, whereas it consumed less fibrinogen.

GHRP-scu-PA-32K, retaining thrombolytic and fibrin-specific properties, may be useful as a thrombolytic agent. The efficacy for the chimeric protein in appropriate animal models needs to be further investigated.

References


