Development of a mammalian suspension culture for expression of active recombinant human urokinase-type plasminogen activator

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Abstract

The development of specific catalytic inhibitors for the serine protease urokinase-type plasminogen activator (uPA) has been hindered due to difficulties in producing sufficient amounts of active recombinant uPA that is catalytically equivalent to native uPA. The purpose of this study was to develop an efficient system for the expression of recombinant human uPA that exhibits comparable proteolytic activity to that of the native protein. Since post-translational modifications (e.g. glycosylations) of uPA are necessary for efficient proteolytic activity, we have used a mammalian cell line [Chinese hamster ovary (CHO)-S] to express recombinant human uPA. CHO-S cells were selected to stably express full-length recombinant human uPA containing a hexahistidine tag at its C-terminus to permit purification by nickel-based affinity chromatography. Secretion of recombinant uPA into the culture media was confirmed by immunoblotting and the presence of an *N*-linked glycosylation was confirmed by PNGase sensitivity. Enzymatic activity of purified recombinant uPA was demonstrated using zymography and quantitatively compared to native uPA by kinetic analysis using an uPA-specific substrate. Native uPA and the recombinant uPA demonstrated comparable K_m values (55.7 and 39 μM , respectively). Furthermore, inhibition studies using benzamidine resulted in a K_i of 195 μM for native uPA, while recombinant uPA had a K_i of 112 μM . These data indicate that recombinant human uPA expressed by CHO-S cells is functionally comparable to native uPA.

Abbreviations: CHO-S – Chinese hamster ovary-S cells; PAI-1 – type-1 plasminogen activator inhibitor; PNGase F – peptide-*N*-glycosidase F; SDS-PAGE – sodium dodecylsulfate-polyacrylamide gel electrophoresis; uPA – urokinase-type plasminogen activator

Introduction

The urokinase-type plasminogen activator (uPA) is a serine protease that plays important physiological roles in cell motility that are necessary for basic organism development, tissue remodeling,

wound repair and reproductive cell behavior, such as ovulation and blastocyst implantation (Wang 2001). uPA is responsible for the proteolytic conversion of plasminogen to plasmin. Plasmin has broad substrate specificity and is a primary factor in extracellular matrix (ECM)

dissolution (Pepper 2001), a vital property for cell migration and tissue remodeling. Under normal, homeostatic conditions, uPA synthesis and secretion is tightly regulated. However, tumor cells representing a wide variety of human cancers have been shown to express significantly greater amounts of uPA than their normal counterparts (Sheng 2001; Duffy 2002). It is now established that increased uPA expression directly facilitates tumor matrix dissolution and is a major factor in promoting cancer cell dissemination away from primary tumor sites and colonization at distant secondary sites (Stump et al. 1986; Stephens et al. 1989; Andreasen et al. 1997; Helenius et al. 2001). Such metastatic events typically lead to increased mortality (Ohta et al. 2003). In fact, increased uPA activity is often an independent prognostic indicator of poor patient outcome (Brünner et al. 1994; Stephens et al. 1998; Duffy et al. 1999; Duffy 2001).

Because of the pivotal role played by uPA in tumor cell motility and colonization, a number of inhibitors have been developed that target its proteolytic activity to prevent ECM breakdown and tumor cell metastasis (Rockway and Giranda 2003). The rationale for this approach is sound since the progression of experimental tumors in uPA knock-out animals is delayed when compared to control, wild type animals (Shapiro et al. 1996; Sabapathy et al. 1997; Bugge et al. 1998; Gutierrez et al. 2000; Frandsen et al. 2001). Moreover, because increased uPA activity is common among tumors from a variety of cancers, its inhibition may provide therapeutic strategies with broader scope rather than targeting pathways that are unique to individual tumors. Unfortunately, studies using these inhibitors have met with mixed results and even when positive results were achieved, the effects were not dramatic (Rockway and Giranda 2003). These modest results are largely attributed to poor selectivity and insufficient bioavailability of the inhibitors tested. Small molecule inhibitors of uPA originated with the discovery of aryl guanidines (Yang et al. 1990), aryl amidines (Stürzebecher et al. 1978), or acyl guanidines (Schmitt et al. 1995). These compounds demonstrated poor selectivity because of active-site conservation among the class of serine proteases. Since then, a number of compounds have been developed that provide greater selectivity (reviewed in (Rockway and Giranda 2003)),

however most are still based upon amidine or guanidine templates which demonstrate limited bioavailability due to their charged moieties. Therefore, more studies are needed to improve upon bioavailability while maintaining or improving selectivity.

Although the structure of the active site of uPA has been described both with (Spraggon et al. 1995) and without (Katz et al. 2000; Nienaber et al. 2000) bound inhibitors, more widespread structural studies are lacking due to the poor availability of a cost-effective supply of active human uPA. Expression of recombinant human uPA has been attempted using bacterial (Lijnen et al. 1986; Lenich et al. 1992; Wang et al. 2000), yeast (Agaphonov et al. 2002), and mammalian (Zang et al. 1995) expression systems with mixed results. In many cases, recombinant protein yields are low and/or post-translational modifications are missing or aberrant. This often leads to altered catalytic activity, which likely implies an active-site conformation that is not structurally comparable with the native protein. Using these forms of recombinant uPA for inhibitor development studies would make translation into an *in vivo* setting difficult due to the resulting differences in K_i values between the recombinant uPA and native, endogenous uPA. We report here the development of a mammalian expression system that is capable of producing milligram quantities of recombinant uPA and whose glycosylation profile and activity are comparable to native uPA. As a vehicle for recombinant expression, we chose to use Chinese hamster ovary (CHO)-S cells. CHO-S cells are a clonal variation of normal CHO cells and have been selected for their ability to grow to high densities in suspension cultures (Ciccarone et al. 1999), thus presenting a distinct advantage for the secretion of high concentrations of recombinant protein. In addition, since CHO-S cells do not rely on adhesion for growth, secretion of large amounts of uPA will not affect attachment-dependent cell proliferation. We anticipate that this system will provide a cost-effective source of recombinant human uPA that will generate enough material to aid in the advancement of new inhibitors through structure-based drug design. In addition, this system can also allow for the rapid production of recombinant uPA with active-site, single amino acid substitutions to further define inhibitor binding properties.

Materials and methods

Materials

CHO-S cells, Dulbecco's Modified Eagle Medium (DMEM) and Geneticin were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum, non-essential amino acids, glutamine, streptomycin, and penicillin were from Irvine Scientific (Irvine, CA). Native uPA was purchased from CalBiochem (Catalog no. 672112, San Diego, CA). Goat anti-human uPA polyclonal antibody was raised against low molecular weight uPA and is capable of recognizing both high and low molecular weight forms of uPA (#398, American Diagnostica, Greenwich, CT). Horseradish peroxidase (HRP) conjugated donkey anti-sheep/goat IgG was from Serotec (Raleigh, NC). Ni-chelate affinity matrix, casein and plasminogen were obtained from Sigma-Aldrich (St. Louis, MO). Chromogenic substrate specific for uPA, S-2444 (L-pyroglutamyl-glycyl-L-arginine-*p*-nitroaniline hydrochloride), was purchased from Diapharma (West Chester, OH). Tissue culture plastics were purchased from Corning (Corning, NY) or Greiner Bio-One. All other chemicals were obtained from either Sigma-Aldrich or Calbiochem.

Human uPA expression vector

The cDNA for human uPA (graciously provided by Dr. Jack Henkin, Abbott Laboratories, Abbott Park, IL) was amplified by PCR (1 cycle, 94 °C for 1 min; 30 cycles, 92 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min; 1 cycle, 72 °C for 7 min) and inserted into pcDNA3.1 in frame with a sequence encoding a C-terminal hexahistidine tag. Primer sequences used for PCR were: upstream, GCCACCATGGGAGCCCTGCT; downstream, GAGGGCCAGGCCATTCTCTT. The native uPA signal sequence was included to ensure entrance into the exocytic pathway for secretion.

Cell culture and electroporation

CHO-S cells were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM glutamine, streptomycin (100 $\mu\text{g ml}^{-1}$) and penicillin

(100 units ml^{-1}). Cells were cultured at 37 °C with 5% CO_2 and passaged twice weekly. For electroporation, CHO-S cells were diluted to a concentration of 5×10^6 cells ml^{-1} in serum free DMEM. pcDNA3.1 vector containing uPA cDNA (huPA-pcDNA3.1) or pcDNA3.1 alone (20 μg) was mixed with 500 μl cells in a Gene Pulser cuvette (4 mm, BioRad, Hercules, CA). Cells were then electroporated at 900 μF and 0.5 kV using a Gene Pulser II (BioRad). Immediately following electroporation, cells were diluted with 1.0 ml DMEM containing 10% (v/v) fetal bovine serum and plated. For stable cell selection, cells were grown in complete media supplemented with Geneticin (2 mg ml^{-1}) for a minimum of 10 passages prior to use.

Affinity purification

CHO-S cells, stably expressing recombinant human uPA, were seeded at 2×10^5 cells ml^{-1} in a 500 ml Bellco micro-spinner flask (Vineland, NJ) and incubated at 37 °C/5% CO_2 (60 revolutions per minute). Media was collected after 6–8 days and centrifuged (120 $\times g$, 5 min, 4 °C) to remove cells. High capacity Ni-chelate affinity matrix (5 ml matrix/500 ml culture supernatant) was added to the supernatant and incubated at 4 °C for 16 h with gentle agitation. Ni affinity matrix was then placed in a 1.5 $\times$ 10 cm Flex-column (VWR, West Chester, PA) and washed with >20 column volumes of 20 mM Tris, pH 7.4, 150 mM NaCl (TBS), 10 mM imidazole. Bound protein was eluted with TBS containing 300 mM imidazole and fractions (0.5 ml) were collected. Aliquots of each fraction were mixed with SDS-PAGE sample buffer under non-reducing conditions and separated using precast 10% polyacrylamide gels (Cambrex, Rockland, ME) followed by staining with Coomassie Blue G. Fractions containing protein were pooled and dialyzed against 2 l TBS (3 changes). Culture media from non-transfected CHO-S cells was subjected to the identical Ni-affinity purification protocol and used in subsequent assays as a negative control. Protein concentration was determined by the BCA (bicinchoninic acid) assay according to the manufacturer's protocol (Pierce, Rockford, IL). Results for recombinant uPA were compared with those obtained from a standard curve generated with known concentrations of commercially available

native uPA. Using this purification procedure, we are able to routinely purify 15–50 μg uPA/ 10^9 cells. Densitometric analysis was performed using Scion Image Beta 4.02.

Immunoblotting

Conditioned media or purified uPA was mixed with SDS-PAGE sample buffer supplemented with 2% β -mercaptoethanol, fractionated by 10% polyacrylamide gel electrophoresis and transferred to Immobilon-P (Millipore, Billerica, MA) using a wet tank transfer system (BioRad, Hercules, CA). Membranes were blocked with TBS, 0.1% (v/v) Tween-20, 5% (w/v) non-fat dry milk for 20 min at 23 °C and incubated for 16 h with goat anti-human uPA IgG (4 $\mu\text{g ml}^{-1}$). Membranes were washed three times (10 min each) with TBS, 0.1% (v/v) Tween-20, and bound antibodies were detected with HRP conjugated donkey anti-sheep/goat IgG (1:10,000) followed by chemiluminescence detection according to the manufacturer's instructions (Pierce, Rockford, IL). Images were captured using a Syngene GeneGnome system equipped with a Peltier-cooled 16-bit CCD camera and saturation detection.

Deglycosylation of uPA

Native uPA or affinity purified recombinant uPA (1 μg) was incubated with or without 5 units PNGaseF (CalBiochem) at 37 °C for 20 h. Proteins were then mixed with SDS-PAGE sample buffer, supplemented with 2% (v/v) β -mercaptoethanol and fractionated using precast 4–15% polyacrylamide gels. Proteins were then immunoblotted with goat anti-human uPA pAb (4 $\mu\text{g ml}^{-1}$) and bound antibodies were detected with HRP conjugated donkey anti-sheep/goat IgG (1:10,000) followed by chemiluminescence detection.

Zymography

Ten percent polyacrylamide gels containing 1.4 mg ml^{-1} casein and 0.25 $\mu\text{g ml}^{-1}$ plasminogen or containing 1.4 mg ml^{-1} casein (plasminogen deficient, as controls) were prepared. Native uPA

(0.3 ng) or recombinant uPA (0.6 ng) was mixed with sample buffer containing only 0.1% SDS and no reducing agents. Samples were loaded onto the prepared gels and after electrophoretic separation, gels were placed in renaturing buffer (2.5% Triton in ddH₂O) for 30 min, followed by TBS for 30 min at 25 °C. Gels were then transferred into fresh TBS and incubated at 37 °C for 20 h, followed by staining with 0.1% Coomassie G in 7% acetic acid and 5% methanol.

Kinetic analysis

Native or recombinant uPA (0.2 μg) was mixed together with the chromogenic substrate, S-2444 (0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 mM), in a final volume of 500 μl 0.1 M Tris, pH 8.8. Product (*p*-nitroaniline) formation was quantitated by recording absorbance at 405 nm every 10 s for 5 min using a Genesys 10 UV Spectrophotometer (Thermo Spectronic Madison, WI). Moles of product formed were calculated using ϵ_{405} for *p*-nitroaniline (9920 l/mol/cm). Lineweaver–Burk and Eadie–Hofstee analyses were used to determine V_{max} and K_{m} . In all cases, linear regression analysis resulted in r^2 values > 0.96. Benzamidine concentrations of 0.25, 0.5, and 1.0 mM, pH 7.4, were used to determine K_{i} for both native and recombinant human uPA. The protocol used for K_{i} determination was identical to that used to determine K_{m} . Product formation versus substrate concentration was plotted and analyzed by non-linear regression.

Results

Expression of recombinant human uPA by transfected CHO-S cells

uPA is synthesized and secreted by cells as a single-chain polypeptide (Mr, 54 kDa) representing the inactive pro-enzyme form. The pro-enzyme form is subsequently cleaved into its two-chain (Mr, 33 kDa and 25 kDa) proteolytically active form in the extracellular environment by plasmin (Wun et al. 1982). The dimeric structure of the two-chain active form of uPA is preserved by a single disulfide bond that spans the plasmin cleavage site. The 54 kDa form of uPA is

designated as high molecular weight uPA and the 33 kDa polypeptide of the two-chain form, which contains the active site domain, represents low molecular weight uPA. Since the 33 kDa low molecular weight uPA contains the active site, it is proteolytically active alone or together with its native disulfide bonded partner, the 25 kDa sub-unit of two-chain uPA. To demonstrate expression of recombinant human uPA in transfected CHO-S cells, culture media was immunoblotted using an anti-uPA antibody raised against low molecular weight uPA, which is able to recognize both high and low molecular weight forms of uPA. Indeed, both 54 kDa high molecular weight pro-uPA and 33 kDa low molecular weight cleaved form of uPA were found in culture media from uPA-transfected CHO-S cells (Figure 1, lane 2), but not in media from cells transfected with just vector alone (lane 1). A third protein was also detected by the anti-uPA antibody at approximately 95 kDa (lane 2). This size is consistent with recombinant uPA that

is complexed with its natural endogenous inhibitor, type-1 plasminogen activator inhibitor (PAI-1) (Lee et al. 1998). No proteins were recognized by this antibody in culture media from cells transfected with parental pcDNA3.1 alone demonstrating the antibody's specificity. These data clearly show that transfected CHO-S cells are capable of synthesizing and secreting recombinant human uPA and that this uPA is of the expected molecular weight. Immunoblotting also shows that CHO-S cells do not produce any detectable endogenous uPA that is recognized by the anti-human uPA polyclonal antibody.

Expression and purification of recombinant uPA using CHO-S cells

We chose to use CHO-S cells as a vehicle for uPA expression. These cells are a clonal variation of normal CHO cells and have been selected for their ability to grow in suspension cultures (Ciccarone et al. 1999). Suspension cultures typically provide a distinct advantage for the production of large amounts of recombinant protein since their growth is not limited to two dimensional surfaces, thus enabling them to achieve very high densities. CHO-S cells also demonstrate high transfection efficiencies which allows for rapid selection of cells that are stably expressing the recombinant protein of interest. In addition, cell cultures that grow in suspension are less likely to lose uPA expression because of selection pressure due to repetitive passaging of cells. We reasoned that overexpression of recombinant uPA in attachment-dependent cells would be selected out of the cultured population of cells with increased passages since uPA is responsible for extracellular matrix degradation and is likely to adversely effect the function of cell adhesion molecules which are responsible for the attachment-dependent phenotype. In order to simplify the purification of recombinant uPA, we engineered it to contain a hexahistidine tag at its C-terminus to permit affinity purification using nickel-based chromatography. CHO-S cells transfected with huPA-pcDNA3.1 were cultured in media containing geneticin to select for cells stably expressing recombinant uPA. Following a one-step purification procedure using conditioned media from stably transfected cells, the recombinant uPA was compared to commercially

Figure 1. Expression of recombinant human uPA by CHO-S cells. CHO-S cells were transfected by electroporation with pcDNA3.1 alone (lane 1) or pcDNA3.1 containing the cDNA for human uPA (lane 2, huPA-pcDNA3.1). Cells were cultured in serum-free media for 24 h post-transfection. Secreted proteins in the conditioned media (4 μ l) were fractionated by 10% SDS-PAGE under reducing conditions and immunoblotted with anti-human uPA polyclonal antibody (4 μ g ml⁻¹).

available native uPA by SDS-PAGE analysis under non-reducing conditions. Recombinant uPA demonstrated identical molecular mass as high molecular weight native uPA (Figure 2a). By densitometric analysis, we estimate purity of our recombinant uPA to be $\sim 80\%$. Protein quantitation indicates that we are able to achieve yields of $15\text{--}50\text{ }\mu\text{g uPA}/10^9\text{ cells}$. A 95-kDa protein was also observed to co-purify with recombinant uPA during the affinity chromatography step. Since this protein was not seen when non-transfected CHO-S cell conditioned media was subjected to Ni-chelate chromatography (data not shown), we believe that its presence is dependent on recombinant uPA expression and likely represents recombinant uPA coupled to its endogenous inhibitor, PAI-1 (Hekman and Loskutoff 1988).

Glycosylation analysis

Human uPA contains a single *N*-linked carbohydrate addition found on asparagine residue 302 within its 33 kDa subunit (Steffens et al. 1982; Bergwerff et al. 1995). This carbohydrate moiety is known to contribute to the active site conformation as previous studies have shown that its absence affects uPA's catalytic properties (Sarubbi et al. 1989; Lenich et al. 1992; Li et al. 1992).

Thus, non-glycosylated recombinant uPA obtained from bacterial sources is not likely to be a suitable source for the development of synthetic inhibitors since the active site conformation of uPA produced in prokaryotes is not equivalent to native mammalian uPA. For this reason, we determined if our recombinant uPA possesses an *N*-linked carbohydrate addition. Toward this end, we incubated native uPA and purified recombinant uPA with PNGaseF, which cleaves *N*-linked carbohydrate chains from core proteins, and compared their relative molecular masses by polyacrylamide gel electrophoresis. Without PNGaseF treatment, we found that both native and recombinant uPA demonstrated identical molecular masses (Figure 2b). Following enzymatic release of *N*-linked carbohydrates with PNGaseF, the 33 kDa subunit of native and recombinant uPA were both found to have reduced ($\sim 2\text{ kDa}$) molecular mass due to the loss of sugar residues. These results indicate that recombinant uPA does indeed contain an *N*-linked glycosylation on its 33 kDa subunit.

Qualitative examination of recombinant uPA activity

We used zymography to initially determine if our purified recombinant uPA is proteolytically active.

Figure 2. SDS-PAGE analysis of purified recombinant uPA and glycosylation profile analysis. (a) Native uPA or affinity purified recombinant uPA ($2\text{ }\mu\text{g}$) was fractionated by 4–15% SDS-PAGE under non-reducing conditions, followed by staining with Coomassie G. (b) Native uPA or purified recombinant uPA ($1\text{ }\mu\text{g}$) was incubated with (+) or without (–) 5 units PNGaseF at $37\text{ }^{\circ}\text{C}$ for 20 h. Proteins were fractionated by 4–15% SDS-PAGE under reducing conditions and immunoblotted with anti-human uPA pAb ($4\text{ }\mu\text{g ml}^{-1}$). Reducing conditions were chosen because the single *N*-linked carbohydrate addition to uPA is found on its 33 kDa low molecular weight form (Bergwerff et al. 1995).

Polyacrylamide gels were prepared with incorporation of casein to serve as a proteolytic substrate. Plasminogen was included in one gel and the presence of uPA activity is expected to generate localized plasminogen-to-plasmin conversion and hydrolysis of casein due to plasmin's broad substrate recognition (Roche et al. 1983; Alexander et al. 1998; Davis et al. 2003; Pejler et al. 2003; Hall et al. 2004). Plasminogen was absent from the control gel to demonstrate that the hydrolysis of casein is solely due to the presence of uPA and not other proteases that are secreted by CHO-S cells. As shown in Figure 3, both native (lane 1) and recombinant uPA (lane 3) demonstrate proteolytic activity at 54 kDa, which corresponds to the molecular mass of high molecular weight two-chain uPA. In the absence of plasminogen, no hydrolysis of casein was evident (lanes 2 and 4).

Figure 3. Recombinant human uPA activity as assessed by zymography. Native uPA (0.3 ng) and purified recombinant uPA (0.6 ng) were separated under non-reducing conditions using 10% polyacrylamide gels containing casein (1.4 mg ml^{-1}) and plasminogen ($0.25 \text{ } \mu\text{g ml}^{-1}$) (+) or casein alone (–). Gels were incubated in renaturation buffer (2.5% Triton X-100), followed by Tris-buffered saline and developed for 20 h at 37°C . Gels were then stained with Coomassie G and photographed. Clear zones represent areas of proteolysis.

Inhibition of recombinant uPA activity by PAI-1

An important property in assessing the specificity of uPA activity is whether it is able to serve as a substrate for PAI-1, the natural physiologic inhibitor of uPA. PAI-1 is a suicide inhibitor of uPA as defined by its ability to form a covalent acyl linkage with uPA's critical active site serine residue. Thus, the association of PAI-1 with uPA is highly dependent on uPA's unique catalytic structure and forms a 1:1 molecular complex. To determine if our recombinant uPA is inhibited by PAI-1 in a similar manner as native uPA, we incubated both forms of uPA with varying concentrations of human PAI-1 and quantitated proteolytic activity using an uPA-specific substrate, S-2444 (Paar and Maruhn 1980). We found that PAI-1 inhibited both recombinant and native uPA in a dose-dependent manner and with similar titration profiles (Figure 4). Moreover, when PAI-1 was present in excess (4:1 molar ratio, PAI-1:uPA), little or no enzymatic activity remained in the recombinant uPA sample indicating that all proteolytic activity in our recombinant fraction is due specifically to uPA.

Kinetic comparison of recombinant and native human uPA

In order to determine if our recombinant uPA is comparable in enzymatic activity to native uPA, we investigated their kinetic properties using the uPA-specific chromogenic substrate, S-2444. As expected, both enzyme preparations approached maximal hydrolysis with increasing concentrations of substrate (Figure 5, upper panels). We interpreted these data by both Lineweaver–Burk (middle panels) and Eadie–Hofstee (lower panels) analyses and found that recombinant and native uPA demonstrate comparable V_{max} and K_{m} values (Table 1). These values are also comparable to previous measurements of human uPA using the S-2444 substrate (uPA Standard established by Chromogenix Instrumentation Laboratory, Milano, Italy).

To determine if the inhibition properties of recombinant and native uPA are also equivalent, we measured their catalytic properties in the presence of varying concentrations of the well-characterized aryl amidine inhibitor, benzamidine

Figure 4. Inhibition of recombinant uPA and native uPA by human PAI-1. 0.2 μ g native uPA (filled circles) or recombinant uPA (open circles) was incubated with varying concentrations of human PAI-1 at 37 °C for 60 min. uPA enzymatic activity was quantitated by recording absorbance (405 nm) after a 10 min incubation with the chromogenic substrate, S-2444 (1.0 mM).

(Stürzebecher and Markwardt 1978). We found that benzamidine inhibited enzymatic activity of both native and recombinant uPA in a dose-dependent manner (Figure 6, upper panels). Lineweaver–Burk (middle panels) and Eadie–Hofstee (lower panels) transformations of these data clearly indicate the competitive nature of benzamidine inhibition. Comparable K_i values were determined; 195 μ M for native uPA and 112 μ M for recombinant uPA (Table 1). These values are similar to those reported previously for benzamidine inhibition of uPA (Renatus et al. 1998; Katz et al. 2000; Zeslowska et al. 2000).

Discussion

Continued progress toward the development of more effective and more specific inhibitors of uPA has been hampered by the lack of sufficient quantities of the protease for structural studies. Structural studies typically require milligram quantities of protein for analysis and acquiring such quantities of uPA from commercial sources is prohibitively expensive for the average laboratory. To overcome this limitation we have developed a cost-effective, mammalian expression system for the production of large quantities of active

recombinant human uPA. As a vehicle, we chose to use CHO-S cells. These cells are a clonal variation of normal CHO cells and have been selected for their ability to grow to high densities in suspension cultures (Ciccarone et al. 1999) and thus present a distinct advantage for the secretion of high concentrations of recombinant protein. These cells also demonstrate high transfection efficiencies. We engineered the recombinant uPA to contain a hexahistidine tag at its C-terminus to allow for affinity purification using nickel-based chromatography. Following a one-step purification procedure using conditioned media from stably transfected cells, we obtained a recombinant uPA preparation that demonstrated ~80% purity with yields of 15–50 μ g uPA/ 10^9 cells. Since individual cells express recombinant proteins at widely varying levels, we anticipate being able to obtain even greater yields after using clonal selection to isolate cells expressing recombinant uPA at the highest levels. In addition, deglycosylation analysis using PNGase F showed that both native and recombinant uPA are sensitive to this enzyme as they demonstrate identical changes in molecular mass by SDS-PAGE analysis. These data indicate that recombinant uPA, like native uPA, contains an *N*-linked carbohydrate addition and that this modification is present on the 33 kDa subunit of

Figure 5. Kinetic analysis of recombinant uPA versus native uPA. 0.2 μg native uPA (filled circles, left panels) and recombinant uPA (open circles, right panels) were incubated with varying concentrations of chromogenic substrate, S-2444 (0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.6, 0.8 mM). Hydrolysis was measured by recording absorbance (405 nm) at 10 s intervals for 5 min. Lineweaver-Burk (middle panels) and Eadie-Hofstee (lower panels) analyses were used to determine $V_{\max}$ and K_m values for comparison.

the enzyme. We also found the purified recombinant uPA to be highly active as visualized by zymographic analysis. Moreover, kinetic analysis

using the uPA-specific substrate, S-2444 (Paar and Maruhn 1980), showed that the native and recombinant uPA demonstrate comparable $V_{\max}$

Table 1. Comparison of Kinetic constants for native and recombinant uPA.

Kinetic constant	Native uPA	Recombinant uPA	uPA standard ^a
$V_{\max}$ (nmol min ⁻¹ μg^{-1} protein)	28.0	19.5	13.0
K_m (μM)	55.7	39.0	60.0
K_i (μM) benzamidine	195.0	112.0	N.D.

^aAs determined by Chromogenix Instrumentation Laboratory using S-2444 substrate; N.D.: not determined.

and K_m values. We further compared inhibitor binding properties between the two forms of uPA using the aryl amidine, benzamidine (Renatus et al. 1998; Sturzebecher and Markwardt 1978), and found them to also demonstrate similar K_i values.

While designing this system, an important goal that we have focused upon is to generate recombinant uPA that is functionally comparable to native uPA. We plan to use our recombinant uPA for structure-based inhibitor design studies which involve the analysis of ligand/inhibitor interactions within the active site. The usefulness of recombinant uPA for such studies depends on the

protein being properly folded and maintaining an active site conformation that is identical to that of native uPA. With this identity, it will then be possible to extrapolate inhibitor measurements made with recombinant uPA to native uPA *in vivo*. Since we demonstrate comparable K_m and K_i values for our recombinant uPA as compared to native uPA, we are confident that the active site conformations of recombinant and native uPA are equivalent.

Other investigators have attempted to generate recombinant sources of human uPA with mixed results. Bacterial expression systems have been used to produce uPA; however, the recombinant uPA

Figure 6. K_i determination for recombinant uPA and native uPA using benzamidine. Kinetic analysis of native uPA (left panels) and recombinant uPA (right panels) was performed as described in Figure 5. K_i determination was carried out by performing kinetic analyses in the absence (filled circles) or presence of 0.25 mM (open circles), 0.5 mM (filled triangles), or 1.0 mM (open triangles) benzamidine. Lineweaver-Burk (middle panels) and Eadie-Hofstee (lower panels) analyses were used to determine K_i values for comparison.

demonstrated altered activity when compared to the activity of the native protein (Lijnen et al. 1986; Lenich et al. 1992; Wang et al. 2000). The altered activity was attributed to the lack of proper post-translational modifications; specifically the inability of bacteria to properly glycosylate recombinant mammalian proteins. Yeast expression systems for uPA have also been attempted because yeast are capable of glycosylating proteins during exocytic transport. However, secretion of uPA by yeast was hampered due to the formation of large aggregates in the endoplasmic reticulum (Agaphonov et al. 2002). Zang and colleagues have previously explored the use of a mammalian expression system to avoid complications observed with prokaryotic or yeast systems (Zang et al. 1995). Using normal, wild type CHO cells, they were able to generate reasonable quantities of recombinant uPA, however it was not reported in this study if the recombinant uPA was proteolytically active or structurally comparable to native uPA.

The primary objective of the present study was to develop an expression system that produces recombinant human uPA that is structurally and catalytically equivalent to native uPA. Because of uPA's prominent role in the metastatic phenotype of many cancers and since it serves as an important target for the prevention of tumor cell dissemination, we believe that this system will aid in the search for more effective and more specific catalytic inhibitors that will block uPA activity *in vivo*. We anticipate that this system will provide sufficient quantities of recombinant protein to overcome current limitations to drug development and provide enough material for crystallographic analysis to further refine our understanding of inhibitor/active site interactions.

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