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High-Level Expression and Characterization of Single Chain Urokinase-type Plasminogen Activator (scu-PA) Produced in Recombinant Chinese Hamster Ovary (CHO) Cells

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Abstract The high-level expression of a human single chain urokinase-type plasminogen activator (scu-PA) was achieved by employing a methotrexate (MTX)-dependent gene amplification system in Chinese hamster ovary (CHO) cells. By cotransfecting and coamplifying a scu-PA expression plasmid and dihydrofolate reductase (DHFR) minigene, several scu-PA expressing CHO cell lines were selected and gene-amplified. These recombinant cell lines, MGpUKs, secreted a completely processed scu-PA of 54 kD and up to 60 mg/L was accumulated in the culture medium when they were adapted to an optimal MTX concentration. Over 95% of the scu-PA expressed was secreted in the culture medium and identified as having the proper function of a plasminogen activator when activated by plasmin. Based on a genomic Southern analysis, a representative subclone, MGpUK-5, exhibited MTX-dependent scu-PA gene amplification, plus the initial single-copy gene of scu-PA eventually turned into about 150 copies of the amplified gene of scu-PA after gradual adaptation to 2.0 μM of MTX. Meanwhile, the transcripts of the scu-PA gene increased, although -early saturation of transcription was identified at 0.1 μM of MTX. The scu-PA production by the MGpUK-5 subclone also increased relative to the gene amplification and increased transcripts, however, the relationship was not linearly proportional. Accordingly, since the MGpUK cell lines expressed elevated levels of enzymatically active scu-PA, these cell lines could be applied to the large-scale production of scu-PA.

Keywords: scu-PA, CHO cells, gene amplification, MTX, copy number, pro-UK

INTRODUCTION

scu-PA was first described in 1973 and later named pro-urokinase (pro-UK) or scu-PA [1]. Since then, it has been investigated for its potential as a better thrombolytic agent. scu-PA is a precursor of urokinase, which is a trypsin-like serine protease and plasminogen activator activating plasminogen to plasmin. This plasminogen activator is mainly made and secreted from several tissues containing human kidney cells in an inactive single-chain structured proenzyme form [2] and has presence in various human fluids including blood. In a number of physiological systems and pathologic conditions, the urokinase-mediated activation of plasminogen to plasmin is critical in the lytic mechanism of fibrinolysis and the maintenance of vascular patency. As such, scu-PA and urokinase have been employed as a potent fibrinolytic agent for patients with thromboembolic diseases, including myocardial infarction, peripheral vascular occlusion, pulmonary embolism, and ischemic stroke [3,4].

Human scu-PA is a glycoprotein with twelve disulfide bonds [5] and has 411 amino acids with a molecular weight of 54 kD. Structurally it consists of three parts, an EGF-like domain, kringle domain, and serine protease domain [6,7]. scu-PA is transformed to an active two-chain molecule, tcu-PA, composed of two polypeptide chains of 20 kD and 34 kD by the cleavage of the Lys158-Ile159 peptide bond following limited digestion with plasmin and other proteases [8-11]. As a result, a conformational change is generated and the affinity of scu-PA for fibrin is diminished [2]. scu-PA exhibits the characteristics of a true proenzyme in terms of its protein structure, its lack of reactivity with inhibitors [12, 13], and its very low activity toward a small synthetic substrate for urokinase [5,12,13]. Also, scu-PA itself displays a weak activity as a plasminogen activator before activation by plasmin.

scu-PA is actually activated and degrades fibrin on the surface of a fibrin clot by a different mechanism from that of urokinase or t-PA (tissue-type plasminogen activator) [14]. Since scu-PA does not degrade many normal plasma proteins, including fibrinogen, in the blood at fibrinolytic concentrations, almost no bleeding is gener-

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ated [14]. Accordingly, it has been postulated that scu-PA has a higher specific thrombolytic activity and better selectivity for fibrin than two-chain urokinase, both in a purified system and in plasma [13,15,16]. Furthermore, the efficacy and specificity of recombinant scu-PA as a thrombolytic agent has already been demonstrated by several studies *in vitro* and in animal models [12]. In a preclinical study, using a canine model of acute myocardial infarction, scu-PA produced a lower incidence of hemorrhagic infarction than t-PA [17]. In contrast to other thrombolytic agents, scu-PA, a rapid-acting, effective, and fibrin-specific thrombolytic agent, has been shown to incur an unusually low degree of reocculusion in animal studies and clinical trials with human patients [18]. scu-PA can bind to platelets and the endothelial membrane, and perhaps, this may protect the artery from reocculusion [19].

Several researchers have already purified scu-PA in urine [8], plasma [20], and mammalian cells [2,5,21,22]. While other research groups have cloned the cDNA of human scu-PA from mammalian cells [22-25] and elucidated the gene structure of scu-PA along with its evolutionary assembly, coded by discrete exons [26,27]. Furthermore, scu-PA has been successfully expressed and purified from cloned human cDNA in a variety of different expression systems, including *Escherichia coli* [21, 25,28], yeast [29], and mammalian cells [30-33]. However, previous studies have shown that a large amount of t-PA (10,000 IU kg^{-1} min^{-1}) and scu-PA (approximately 20 μg kg^{-1} min^{-1}, 60-80 mg for 60-90 min) is needed for treating human patients and animals, such as canines and rabbits [15,17,18,34-37]. Plus, properly processed scu-PA is essential for its activity and resistance in plasma. Accordingly, the current challenge is to identify a recombinant mammalian cell line that can produce high amounts of scu-PA.

The current study used the CHO cell line [38] along with the widely used DHFR gene amplification system [39], through which inserted genes can be easily amplified for the high-level production of interesting proteins [40]. Many completely processed glycoproteins have already been obtained employing this system [40-43]. In addition, a high-level expression of authentic proteins for therapeutic and prophylactic purposes has also been achieved in recombinant CHO cells [40,43-45]. The amplification of a gene by MTX selection is frequently accompanied by an increased production of the desired gene product [46-48].

scu-PA expression was already studied by the current authors in a recombinant CHO cell line, SVpUK, however, production level of scu-PA was insufficient for scaling-up. In contrast, the present study reports on the high level expression of functional human scu-PA in new recombinant CHO cells, which is sufficient for industrialization and further characterization during gene amplification of the stable cell line expressing scu-PA. The structure of the amplified DNA, its chromosomal location, and stability during cell culture are also addressed.

MATERIALS AND METHODS

Cell Culture and Culture Media

A DHFR-deficient mutant of CHO cell line, DG44 [49], was kindly donated by Professor Chasin (Columbia University, NY, USA). The cell line was sub-cultivated in MEM-α (GibcoBRL, Grand Island, NY, USA) containing nucleosides and deoxynucleosides supplemented with 10% FBS (GibcoBRL) at 37°C in a 5% CO_2 air atmosphere. The recombinant CHO cell lines, MGpUKs, were cultivated in nucleoside- and deoxynucleoside-deficient MEM-α (GibcoBRL) supplemented with 10% dialyzed FBS and 10 KIU/mL aprotinin (Sigma, St. Louis, MO, USA) at 37°C in a 5% CO_2 air atmosphere. During each stage of the gene amplification, an appropriate concentration of MTX (Sigma) ranging from 10 nM to 10 μM was added to the basal medium. The recombinant CHO cell line previously used by the current authors to express scu-PA (SVpUK) was used for comparison [2,50,51]. This cell line was also cultured in the basal medium supplemented with 10 μM MTX.

Preparation of Recombinant Expression Vector and pDCH1P

The scu-PA gene was obtained by a PCR (polymerase chain reaction) of the genomic DNA isolated from SVpUK. The PCR was performed using the sense primer 5′-CCCAAGCTTG CCACCATGAGAGCCCTG-CTGGCGCGCCTG-3′ with a *HindIII* enzyme site and antisense primer 5′-TCCCCCGGGTCAGAGGGCCAG-GCCATT-3′ with a *SmaI* enzyme site. The PCR products of the scu-PA gene, which were about 1.3 kb with a Kozak consensus sequence (GCCACC) before the ATG translation initiation codon, were inserted by cohesive ligation into a pcDNA3.1 mammalian expression plasmid (Invitrogen, Carlsbad, CA, USA). The constructed recombinant scu-PA expression plasmid was named pcPUK (Fig. 1(a)). The DHFR minigene, pDCH1P (Fig. 1(b)), was also a kind gift from Dr. Chasin (Columbia University, NY, USA) [52-54] and used for the selection and gene amplification of those CHO cell lines harboring DHFR minigene and pcPUK.

Transfection of scu-PA Gene, Clonal Selection, and Gene Amplification

The recombinant CHO cell lines, MGpUKs, were obtained from transfectants with the pcPUK plasmid and DHFR minigene. CHO cells in the amount of 2×10^5 were inoculated onto a tissue culture dish (ϕ 60 mm) and incubated for 24 h at 37°C in a 5% CO_2 air atmosphere. pcPUK and pDCH1P were mixed at a ratio of 400:1-100:1 (e.g. 5 μg pcPUK/50 ng pDCH1P) and then transfected into DG44 cells using $CaCl_2$ [55] or DOSPER (Roche, Indianapolis, IN, USA) [56]. The transfected cells were grown for 48 h in a growth medium and then subcultured into a selection medium.

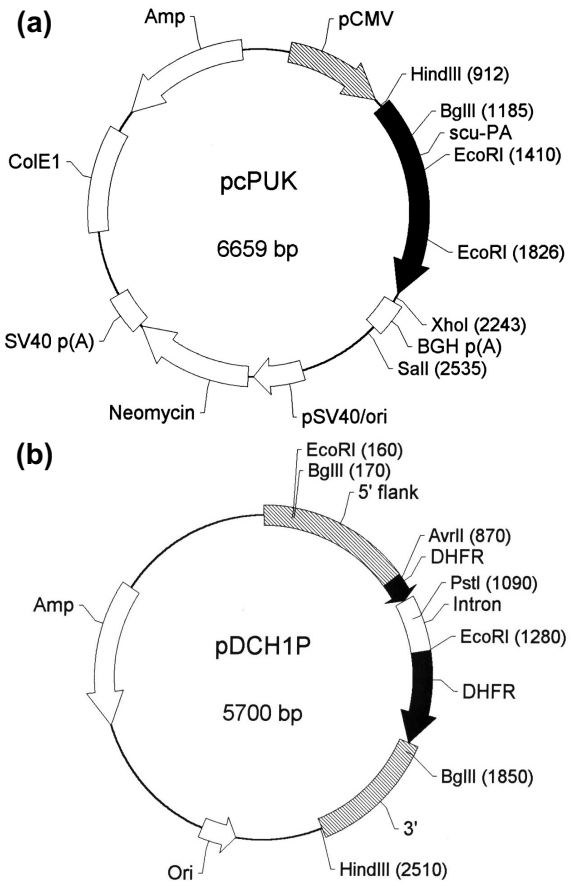

Fig. 1. Plasmids for scu-PA expression using gene amplification in CHO cells. (a) pcPUK expression plasmid containing 1.3 kb cDNA coding scu-PA, (b) pDCH1P plasmid containing 0.7 kb DHFR minigene used for selection and gene amplification.

After 2 weeks, the formed colonies were pooled and transferred onto microplates by serial dilution. The selection of the scu-PA-producing CHO cell lines was performed by a fibrin plate assay. The fibrin plate assay, based on the fibrinolytic activity of the scu-PA secreted in the culture supernatant, was performed following a previous description [57]. The isolated scu-PA-producing CHO clones were named MGpUKs.

To enhance the expression of scu-PA by amplifying the inserted scu-PA genes, several of the colonies with a high production of scu-PA were gradually adapted using a two- to four-fold increment of MTX from 10 nM to 10 μM.

Assay of scu-PA Production

The scu-PA activity secreted into the culture supernatant was determined based on the fibrinolytic activity [57] and amidolytic activity [58]. Urokinase (Green Cross Corporation (GCC), Korea) purified from plasma was used as the standard, and its specific activity was quantified as being approximately 150,000 IU/mg. The fibrin plate was made by mixing 10 mL of 0.7% human fibrinogen (GCC, Korea) in a 100 mM sodium phosphate buffer (pH 7.8) and 100 μL of thrombin (100 NIH unit/mL in a 50 mM sodium phosphate buffer (pH 7.8) containing 0.25% gelatin). The fibrinolytic activity was determined based on the degree of fibrin lysis after incubating 10 μL of the culture supernatant on a 0.7% fibrin plate for 1 h at 37°C and then staining with a crystal violet solution.

To assay the amidolytic activity of scu-PA, 50 μL of the serial-diluted culture supernatant was added to 96-well tissue culture plates and mixed with 30 μL of an assay buffer (50 mM Tris/HCl (pH 8.8), 80 mM NaCl, 0.02% Tween 80). Subsequently, 10 μL of plasmin (0.5 U/mL) was added and the mixture was incubated for 20 min at 37°C to activate the scu-PA. The reaction was terminated by the addition of 10 μL of aprotinin (100 KIU/ml). Then, 100 μL of a chromogenic substrate solution (6 mM S-2444, pGlu-Gly-Arg-pNitroanilide, in the assay buffer (Sigma)) was added to the reaction solution followed by incubation for 1 h at 37°C. The scu-PA activity was determined by measuring the optical density at 405 nm using an EL311 microplate reader (Bio-TEK Instruments, Winooski, VT, USA), and then subtracting the value of the inactive scu-PA that was not treated with plasmin. Urokinase dissolved in a 50 mM sodium phosphate buffer (pH 7.8) containing 0.25% gelatin was used as the standard.

In most cases, the scu-PA production by the cell lines during gene amplification was determined by measuring the amidolytic activity in the culture supernatant obtained after incubating 1×10^6 cells in 6-well culture plates at 37°C overnight in a 5% CO_2 air atmosphere. The culture supernatant collected after culturing for 4 days, as shown in Fig. 5, was assayed every day using the same method. The mean specific production rate calculated from this scu-PA activity was then used for selecting the high-producing clones for the scale-up study.

Genomic Southern Analysis

Genomic DNA from the recombinant CHO cell lines adapted to different MTX concentrations was isolated using a DNAsol solution (GibcoBRL) and digested with an *HpaI* restriction enzyme. For a Southern analysis, 10 μg of the digested genomic DNA was separated on an 0.8% agarose gel and transferred onto a nylon membrane. To determine the copy number of the amplified scu-PA genes, a pcPUK plasmid digested with an *HpaI* restriction enzyme was serially diluted from 1.0 pg to 1.0 ng, loaded, and separated on the same agarose gel. Thereafter, the blot was hybridized for 15 h at 42°C with a probe specific to the whole scu-PA gene (1.2 kb) in a 50% formamide hybridization solution. The DNA probes were labeled with [^{32}P]-α-dCTP using a random Megaprime system (Amersharm Pharmacia Biotech, Piscataway, NJ, USA). After hybridization, the filter was washed once with washing buffer A (2X SSC, 0.1% SDS) at 42°C and twice with washing buffer B (0.1X

SSC, 0.1% SDS) at 65°C. Then the filter was exposed to a Bio-Imagine Analyzer (BAS1000, Fujifilm, Tokyo, Japan) for quantification of the copy number or to X-ray film (Kodak, Rochester, NY, USA) for autoradiography for 16 h at -70°C.

RNA Isolation and Northern Blot Analysis

The total RNA from 5×10^6 recombinant CHO cells was prepared using mini RNA isolation kits (Qiagen, Hilden, Germany). For a Northern blot analysis, 10 μg of total RNA was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde and transferred onto nylon membranes. Thereafter, the blot was hybridized, washed, and exposed as in the Southern blot analysis except that the second washing was performed at 55°C. The relative amount of transcript was calculated using a phosphorimager.

Western Analysis

To identify and characterize the recombinant scu-PA, a Western analysis was performed using a rabbit polyclonal anti-scu-PA antibody (Technoclone, Vienna, Austria). The cell lysate and culture supernatant of the MGpUK-5 cell line adapted to 2.0 μM MTX was mixed with a protein sample buffer, fractionated in a 4-20% gradient SDS-polyacrylamide gel (Novex, San Diego, CA, USA) and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA). One μg/mL of rabbit polyclonal anti-scu-PA antibody was used as the primary antibody and 0.5 μg/mL of goat anti-rabbit IgG (KPL, Gaithersburg, MD, USA) as the secondary antibody. Also, intracellular and extracellular portions of the total expressed scu-PA were calculated time-dependently to determine the percent of secreted protein.

In Situ Hybridization

An *in situ* hybridization was carried out as described elsewhere [59] with some minor modifications. Briefly, the DNA probes were labeled by nick translation with the enzymatic incorporation of biotin-11-dUTP according to the manufacturer's instructions (GibcoBRL). Two hundred ng of labeled DNA probes was precipitated with ethanol and then redissolved in 16 μl of a hybridization mixture (50% deionized formamide/2X SSC/10% dextran sulfate). The metaphase chromosomes were treated with RNase A (100 mg/mL in 2X SSC) for 1 h at 37°C. After rinsing three times with 2X SSC, the slides were dehydrated in a series of ethanol solutions of 70%, 85%, and 100% for 5 min, respectively. The chromosomes were then denatured in a 70% formamide solution for 2 min and dehydrated in another series of ethanol washings. After denaturing at 75°C for 10 min, the DNA probes were then applied to slides, and incubated overnight at 37°C.

After hybridization, the slides were washed in a 50% formamide/2X SSC solution at 42°C three times and then washed three times again at 60°C in an 0.1 X SSC solution for 5 min. Five mg/mL of fluorescein avidin DCS (Vector Laboratories, Burlingame, CA, USA) in a solution of 0.1% Tween 20/4X SSC was placed on each slide, which were then covered with a parafilm and incubated in a humid chamber at 37°C for 30 min. Next, the slides were rinsed three times again in a 0.1% Tween 20/4X SSC solution. For signal amplification, 10 mg/mL of biotinylated anti-avidin D (Vector Laboratories) was applied, and the slides were incubated and rinsed twice repeatedly. The slides were then mounted with an antifade solution containing 1.0 mg/mL of propidium iodide (PI) and diaminophenylindole (DAPI), 1.5 mg/mL of actinomycin D, and 1.0 mg/mL of *p*-phenylenediamine. Actinomycin D was used to enhance the chromosome banding pattern. The slides were examined on an Olympus microscope.

RESULTS AND DISCUSSION

Selection of Recombinant CHO Cell Lines Expressing scu-PA

To gain recombinant CHO cell lines with a higher productivity of scu-PA, a scu-PA expression plasmid, pcPUK, was constructed using a pcDNA3.1 vector which includes a transcription unit consisting of a CMV enhancer, promoter, and BGH poly(A) tail (Fig. 1(a)). The pcPUK plasmid had a Kozak consensus sequence and 1,293 bps of scu-PA cDNA.

Many stable transfectants were obtained by the cotransfection of pcPUK and pDCH1P. These stable cell lines, named MGpUK, were gradually adapted to media containing two-fold increments of an MTX concentration ranging from 10 nM to 10 μM. At an early gene amplification stage, the fibrinolytic activity of the scu-PA secreted into the culture supernatant was assayed on a fibrin plate to examine the *in vitro* activity of the plasminogen activator. As a result, it was found that the secreted scu-PA exhibited a fibrinolytic activity as strong as urokinase (data not shown). To select higher producing clones, the amidolytic activity of scu-PA using a urokinase substrate (S-2444) was analyzed for those cells stably adapted to each stage. At the end of the gene amplification, several high producing clones adapted to 1.0 or 2.0 μM of MTX were selected, which could produce two- to four-fold more scu-PA when compared to SVpUK (Fig. 2(a)). The specific production rate of scu-PA by the MGpUK clones was approximately within a range of 3,000-7,000 IU/10^6 cells/day, whereas that by SVpUK was only 1,750 ± 250 IU/10^6 cells/day. When considering that the specific activity of scu-PA is approximately 150,000 IU/mg, the MGpUK clones had a specific production rate of 20-45 mg/10^6 cells/day. These values were also confirmed using ELISA (Technoclone) (data not shown).

A genomic Southern analysis was also performed to identify the insertion pattern and degree of gene amplification of the scu-PA gene in the genome of the

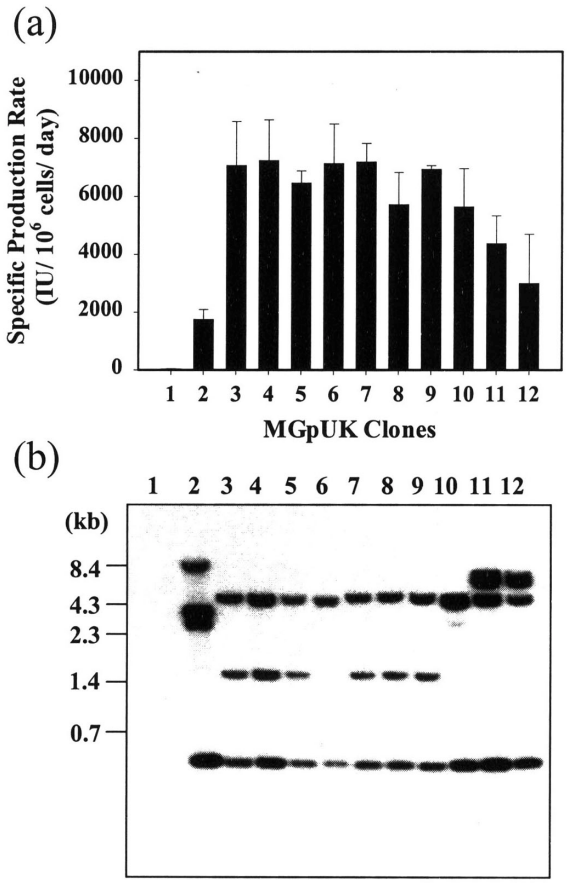

Fig. 2. Determination of scu-PA activity and genetic pattern of MGpUK cell lines. (a) scu-PA activity secreted into culture supernatant, prepared after cultivating for 17 h with 1×10^6 cells/2 mL and (b) genomic Southern analysis performed on 2.0 μM MTX-adapted MGpUK cell lines. CHO cells (Lane 1) and SVpUK clone (Lane 2) were also included as a mock and control, respectively. Lanes 3 - 12 correspond to MGpUK-4, -5, -12, -13, -16, -18, -19, -34, -40, and -58 cell lines adapted to 2.0 μM MTX, respectively. The error bars indicate standard errors obtained by independent experiments (n = 3 to 5).

MGpUK cell lines. The genomic Southern analysis for many MGpUK stable clones adapted to 1.0 or 2.0 μM of MTX showed three different patterns based on *EcoRI* digestion distinct from those of SVpUK (Fig. 2(b)) and an MTX-dependent gene amplification on a chromosome. In addition, it also showed that the copy number of amplified genes was not higher than that of the control cell line, SVpUK.

Based on these results, MGpUK-5 from among the MGpUK clones, which exhibited a high production of scu-PA and stable growth rate and cell morphology, was selected as a candidate industrial clone and analyzed further.

Identification of scu-PA

To identify the expressed scu-PA and calculate the ratio of scu-PA expressed in the cell lysate to that secreted into the culture supernatant, a Western analysis was performed using the scu-PA expressed from the MGpUK-5 clone at two time points (Fig. 3). Fig. 3 shows that uniform scu-PA of 54 kD was secreted into and accumulated in the culture medium. After 24 h of culture, over 95% of the produced scu-PA had been secreted into the culture supernatant and all of it was in a single-chain form. After 48 h of culture, more than 90% of the scu-PA in the culture broth was present as a single-chain urokinase type, plus a trace amount of two-chain forms of 34 kD and 20 kD also appeared. This conversion or degradation of scu-PA would appear to be attributable to several proteases secreted into the culture supernatant by the CHO cells [31,32,60].

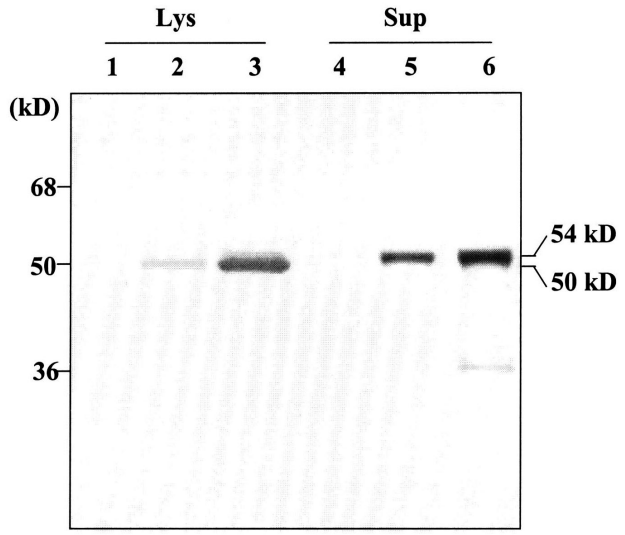

Fig. 3. Western analysis for scu-PA expressed in cell lysate (Lys) and secreted into culture supernatant (Sup) from 2.0 μM MTX-adapted MGpUK-5 cell line. The scu-PA was separated on a 4-20% SDS-PAGE and analyzed as described in the Material and Methods section. Lanes 1 and 4 depict a sample of CHO cells cultured for 24 h as a mock. MGpUK-5 cells were cultivated for 24 h (Lanes 2 and 5) and 48 h (Lanes 3 and 6), respectively.

Furthermore, Fig. 3 indicates that the secreted scu-PA of 54 kD was properly glycosylated in contrast to the scu-PA of 50 kD expressed in the cell lysate, which was a mixture of a non-glycosylated and glycosylated form. These results are consistent with previous reports where biologically active glycoproteins expressed in CHO cells have properly-processed *N*-glycosylation similar to native *N*-linked carbohydrate patterns [30,40,42,43,61]. Therefore, the recombinant scu-PA of this study would appear to be a useful therapeutic agent that can be produced economically. The presence of a proper carbohydrate moiety is known to be critical for the activity of scu-PA *in vivo,* although there are reports that the *in vitro* fibrinolytic properties of non-glycosylated scu-PA are essentially equivalent to those of a natural single-chain u-PA [29]. It has also been noted by many other

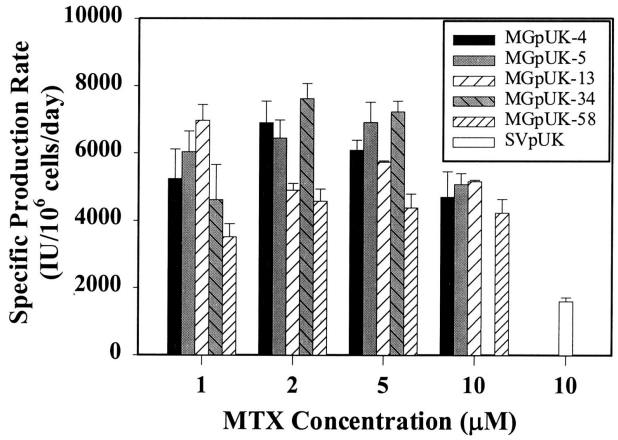

Fig. 4. Determination of optimum MTX concentration for scu-PA expression from MGpUK clones. The error bars indicate standard errors obtained by independent experiments (n = 3 to 5).

investigators that proper glycosylation increases the circulation time *in vivo*, catalytic potential, and resistance to proteolytic degradation [18,62,63].

Optimization of Gene Amplification and Production of scu-PA

In the current study, gene amplification was performed by adding MTX from 10 nM to 10 μM in a medium based on two- to four-fold increments of concentration, and each amplification cycle took 2-4 weeks depending on the clone. To obtain the optimal degree of amplification for a better production of scu-PA, the specific production rate of scu-PA was measured for each clone after the culture stabilized.

The higher producing clones, MGpUK-4, -5, -13, -34, and -58, showed a similar specific production profile of scu-PA relative to the concentration of MTX (Fig. 4). Clones MGpUK-4, -34, and -58, exhibited their maximum specific production rate of scu-PA at an amplification stage treated with 2.0 μM of MTX, whereas clones MGpUK-13 and -5 showed their maximum with 1.0 μM and 5.0 μM of MTX, respectively. Most clones showed their maximum value in the stages treated with 1.0 to 5.0 μM of MTX. The specific production rate of scu-PA for each clone was within a range of 4,000-7,500 IU/10^6 cells/day, and no significant difference was found between the values of each clone. Most of the clones showed a two- to four-times higher expression level than that with SVpUK, the previously constructed clone [2,50,51].

Accordingly, clones MGpUK-4, -5, -13, and -34, adapted to 2.0 μM of MTX, were selected for further examination of their cell growth profile and scu-PA production during 4 days of culture. As illustrated in Fig. 5(a), the cells propagated to 1 × 10^6 cells/mL after 4 days of culture, whereas 3 days of culture was sufficient for the SVpUK control cells to reach the same number. The cell growth rate for all the new clones was slower

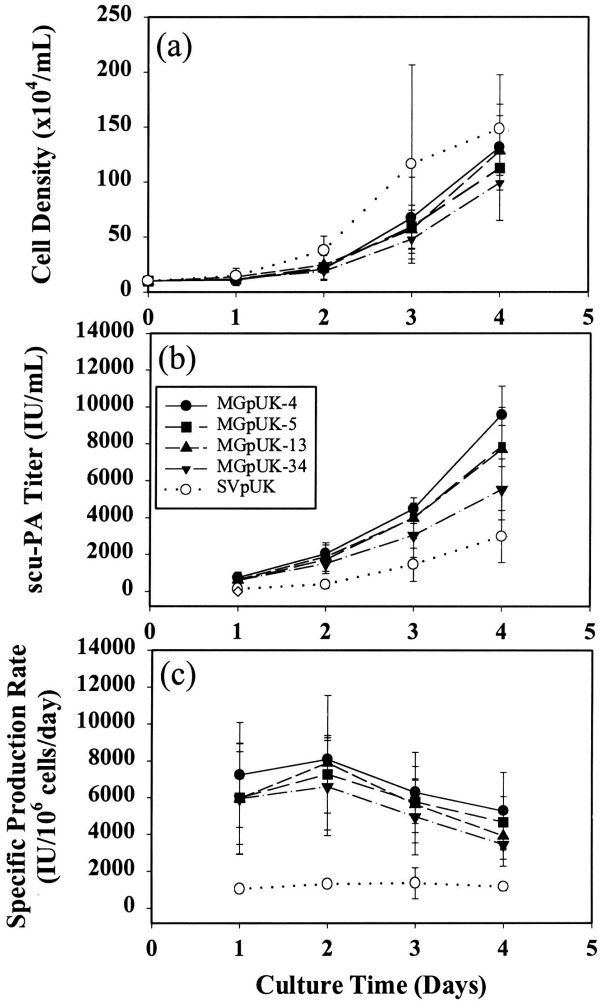

Fig. 5. Cell growth of MGpUK clones and their scu-PA production. The error bars indicate standard errors obtained by independent experiments (n = 3).

than the control cell line, SVpUK. Nonetheless, the scu-PA production by the new clones was much higher than that by SVpUK and ranged on average from 5,000 to 9,000 IU/mL, whereas that by SVpUK was about 2,300 IU/mL after 4 days of culture (Fig. 5(b)). Therefore, the scu-PA production by the new clones was two- to four-times higher than that by the SVpUK cell line. In terms of the specific production rate, the production by all the new clones was maximal after 2 days of culture and ranged from 6,500-8,000 IU/10^6 cells/day (Fig. 5(c)). However, it was hard to determine the best clone due to a minimal difference in the scu-PA production levels between the new clones. Therefore, the MGpUK-5 clone was picked as the representative clone due to its stable growth rate and cell morphology.

Amplification of scu-PA Gene associated with Transcription and Translation

In order to evaluate the colinearity between the am-

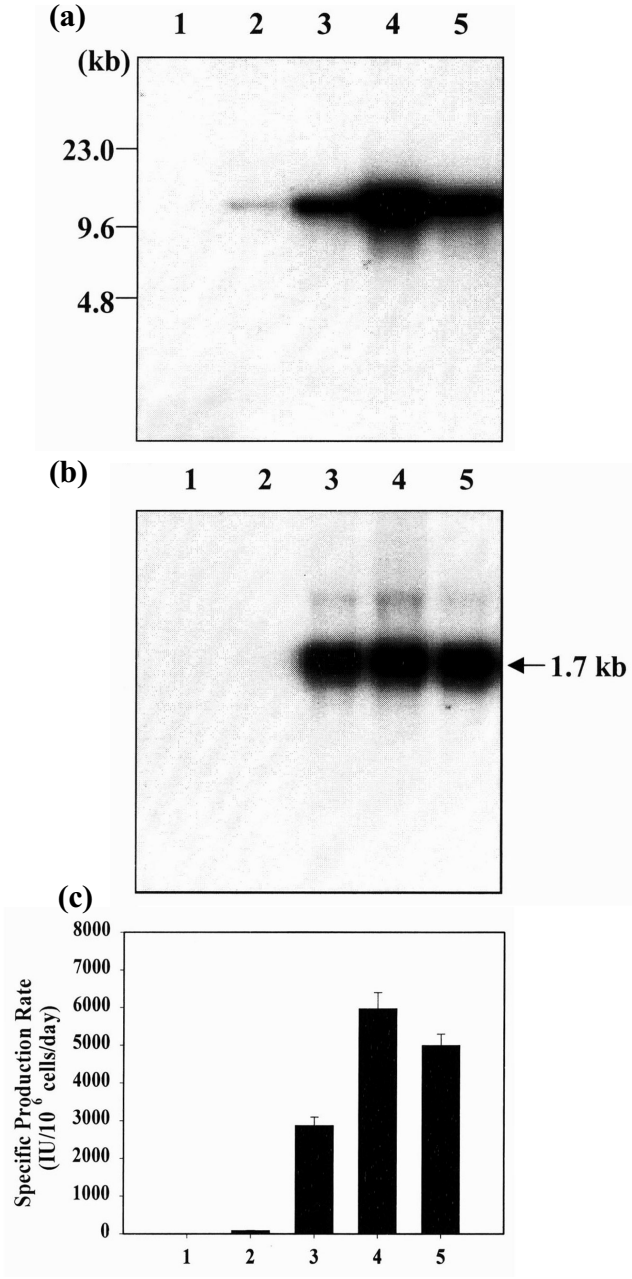

Fig. 6. MTX-dependent gene amplification, increment of transcripts, and scu-PA production. (a) Genomic Southern and (b) Northern analyses were carried out on MGpUK-5 cell line adapted to selection media containing 0, 0.1, 2.0, and 10 μM of MTX (c) The scu-PA activity expressed by the cell line at different stages of gene amplification was determined and compared. In panels (a), (b), and (c), Lane 1 represents CHO (DG44) cells as the control cell line and Lanes 2 - 5 correspond to the MGpUK-5 cell line adapted to selection media containing 0, 0.1, 2.0, and 10 μM of MTX , respectively. In panel (c), the error bars denote standard deviations where n = 3.

plified gene copy number and the scu-PA expression, the DNA, RNA, and protein quantity in the scu-PA was examined. First, to characterize the genetic structure and copy number of the scu-PA gene inserted into and amplified in the genome of the MGpUK cell lines, a genomic Southern analysis was performed on the finally selected MGpUK-5 cell line (Fig. 6(a)). The genomic Southern analysis resulted in one fragment after digestion with *HpaI*, whose active site is lacking in the pcPUK expression plasmid. This result implies that the inserted expression plasmid was in the same unit of gene amplification and had not changed during the MTX selection and gene amplification. Also this blot presents that the initial scu-PA gene insert was within one site as a single copy, and, when compared with the standard plasmid loaded with quantified gene copies, the amplified genes of scu-PA were approximately 150 copies, assuming that a diploid animal cell has 6 pg of chromosome DNA. In addition, the gene amplification of the scu-PA gene was MTX-dependent and highest at the amplification stage with an MTX concentration of 2.0 μM.

A Northern analysis showed a specific transcript of 1.7 kb for the scu-PA gene and its increment was associated with the MTX concentration (Fig. 6(b)). The saturation level for the transcript began close to 0.1 μM of MTX and a plateau was formed with 2 μM of MTX. The maximal amount of transcript was about 45 times more than that in the unamplified MGpUK-5 cell line. This result suggests that the enhancement of the mRNA formation was not proportionally dependent on the MTX concentration, as such, the transcriptional machinery may have certain limitation in transcribing all the amplified DNAs. The production of scu-PA also exhibited some correlation with the degree of amplification of the scu-PA gene in an MTX-dependent manner in terms of the specific production rate (Fig. 6(c)). This value was highest at the amplification stage with 2.0 μM of MTX at 6,000 ± 500 IU/10^6 cells/day.

The influence of the gene amplification on transcription and translation was analyzed by comparing the relationship between the amplified gene copy numbers, the transcript amount, and the secreted protein of the MGpUK-5 cell line (Table 1). With gene amplification up to the stage of 2.0 μM of MTX, the scu-PA gene was amplified approximately 150-fold, its transcripts 45-fold, and the scu-PA protein 70-fold, based on two independent experiments. The gene amplification was three and two times higher than the RNA and protein increment, respectively. In particular, transcription was the least efficient or inactive in the same gene copies, notwithstanding sufficient amplification of gene copies. The inefficient transcription and translation may have occurred from a deficiency and limitation of related factors and enzymes in the transcription, protein synthesis, and post-translational processing system. Previous reports have postulated that tandem repeats of amplified genes resulting from an over-amplification of a gene induce gene silencing and transcriptional inactivation [64,65]. Also, it is conceivable that the position of the inserted and amplified genes in the chromosome may have a critical influence on the transcriptional activity [66,67]. Generally, mammalian cells, including the CHO

Table 1. Comparison of amplification ratios of DNA, transcript, and production of recombinant scu-PA in MGpUK-5 cell line

MTX (μM)	DNA (pg)		Transcript (ng)		Scu-PA (IU/10^6 cells/day)	
0.0	2.2[a]	1.0[b]	10.3[a]	1.0[b]	87[a]	1.0[b]
0.1	42.8	19.5	364.0	35.3	2875	33.0
2.0	337.8	153.5	457.0	44.3	5975	68.9
10.0	131.0	59.5	433.0	42.0	5025	57.8

[a] Average of two independent experiments; inter-experimental deviation was no more than 20%.

[b] Relative ratio of each value when values of unamplified MGpUK-5 were considered as 1.0.

cell line, often suppress the expression of a transfected foreign gene by many different mechanisms. As such, since amplified multi-copy genes can be easily deleted or silenced in mammalian cells, the expression of foreign genes transfected into them is not copy number-dependent. Therefore, it is necessary to develop mammalian expression vectors and establish stable CHO cell lines that can help overcome this position effect so as to accomplish copy number-dependent expression.

Production Stability

To investigate the effect of MTX on scu-PA production by MGpUKs during a long-term culture, MGpUK clones adapted to 2.0 μM MTX were continuously subcultured in MEM-α media with or without MTX, respectively. After culturing for 60 days, the secreted scu-PA was quantified (Fig. 7). A reduction of 30-50% in the scu-PA production was observed without the selection pressure of MTX. However, the nucleotide sequence of the scu-PA from the MGpUK cells was identical based on sequencing with scu-PA cDNA subcloned from cells grown without selection pressure (data not shown). Therefore, MTX would appear to be necessary for the clones to express scu-PA properly, even though the integrity of the expressed scu-PA was unaltered. It is possible that the reduced scu-PA production may have been attributable to a loss in the gene copy number due to deletion or the transcriptional inactivity of the scu-PA genes.

To detect the location of the amplified scu-PA genes in the chromosome, *in situ* hybridization was performed on the genomic DNA of the MGpUK-5 cell line using a biotin-labeled specific scu-PA gene probe (Fig. 8). Most of the amplified scu-PA genes were located within one chromosome, yet 5-10% of them were also found in other chromosomes including the previous one. When considering the results of the Southern analysis and *in situ* hybridization, it would appear that the transcription unit and remaining expression vector acted as a gene amplification unit and were translocated from the inserted genome site to other sites.

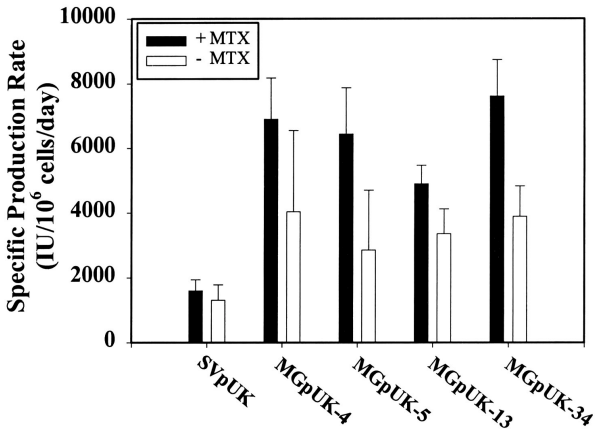

Fig. 7. Stability of scu-PA production by MGpUK cell lines in long-term culture without MTX. The error bars denote standard errors obtained by independent experiments (n = 3).

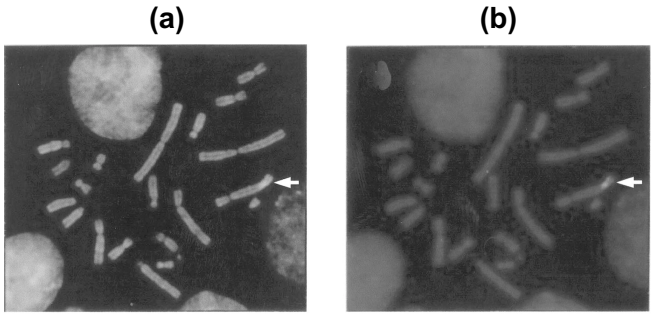

Fig. 8. *In situ* hybridization of MGpUK-5 cell line. In order to examine the location of the scu-PA gene, the metaphase chromosome of the MGpUK-5 cell line was hybridized with a biotin-labeled scu-PA specific probe and treated with fluorescein avidin. The slides were mounted with PI and DAPI, and then analyzed and photographed. The figure shows the photographs stained with (a) DAPI and (b) fluorescein avidin, respectively. The arrow indicates the labeled scu-PA gene in the chromosome.

In conclusion, stable recombinant CHO cell lines, MGpUKs, with a high scu-PA expression ability were obtained by gene amplification using MTX. Most of the scu-PA secreted from these cell lines exhibited a proper *in vitro* physiological activity and the specific production rate was about 45 mg/10^6 cells/day, and up to 60 mg/L was accumulated during batch cultures. The gene amplification, transcription, and production of scu-PA was MTX-dependent and optimal with 1.0 - 2.0 μM of MTX. In particular, most of the transfected scu-PA gene was stable at the initially inserted site during gene amplification. Therefore, MGpUK cell lines and especially subclone MGpUK-5 show great potential for the large-scale production of scu-PA.

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