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Mutation of Angiogenesis Inhibitor TK1-2 to Avoid Antigenicity *In Vivo*

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Abstract – Tissue-type plasminogen activator (t-PA) is a multidomain serine protease containing two kringle domains, TK1-2. Previously, *Pichia*-derived recombinant human TK1-2 has been reported as an angiogenesis inhibitor although t-PA plays an important role in endothelial and tumor cell invasion. In this work, in order to improve *in vivo* efficacy of TK1-2 through elimination of immune reactivity, we mutated wild type TK1-2 into non-glycosylated form (NE-TK1-2) and examined whether it retains anti-angiogenic activity. The plasmid expressing NE-TK1-2 was constructed by replacing Asn^{117} and Asn^{184} with glutamic acid residues. After expression in *Pichia pastoris*, the secreted protein was purified from the culture broth using S-sepharose and UNO S1 - FPLC column. The mass spectrum of NE-TK1-2 showed closely neighboring two peaks, 19631.87 and 19,835.44 Da, and it migrated as one band in SDS-PAGE. The patterns of CD-spectra of these two proteins were almost identical. Functionally, purified NE-TK1-2 was shown to inhibit endothelial cell migration in response to bFGF stimulation at the almost same level as wild type TK1-2. Therefore, the results suggest that non-glycosylated NE-TK1-2 can be developed as an effective anti-angiogenic and anti-tumor agent devoid of immune reactivity.

Key words □ tissue-type plasminogen activator, kringle doamin, angiogenesis, N-glycosylation, site-directed mutagenesis, *Pichia pastoris*

INTRODUCTION

Angiogenesis, the process of new capillary outgrowth from pre-existing vessels, is essential for embryonic development, organ formation, wound healing, female reproduction, tissue regeneration, and remodeling (Folkman and D'Amore, 1996; Risau, 1997). Angiogenesis plays important roles in tumor growth and metastasis (Folkman, 1971, 1995; Hanahan, 1997; Hanahan and Folkman, 1996). Numerous molecules have been found to be involved in this process (Folkman and D'Amore, 1996; Ingber and Folkman, 1989; Risau, 1997). Interestingly, several endogenous angiogenesis inhibitors discovered are protein fragments derived from extracellular matrix or hemostatic system proteins (Browder *et al.*, 2000; Sage, 1997). Tissue-type plasminogen activator (t-PA) is also a fibrinolysis-related protein that consists of a finger domain, an epidermal growth factor-like domain, two kringle domains, and a C-terminal proteolytic domain, and plays an important role in endothelial and tumor cell invasion (Larsen *et al.*, 1988; Stack *et al.*, 1999). In the previous study, the kringle domain of t-PA (TK1-2) with about 29-39% identity to angiostatin kringles has been shown to have the potent inhibitory activity to endothelial cell proliferation and *in vivo* tumor growth, although it doesn't affect fibrinolysis (Kim *et al*, 2003; Shim *et al*, 2005).

When proteins are secreted in eukaryotic cells, they are often glycosylated during transit through the secretary apparatus. Carbohydrate chains can be attached on a serine or threonine (O-linked glycosylation) and an asparagine (N-linked glycosylation). The addition of carbohydrate chains to a protein may have an influence on the folding, structure, secretion, transport, stability, solubility and antigenicity of the protein (Elliott *et al.*, 2004; Helenius and Aebi, 2001; Imperiali and O'Connor, 1999; Khanna *et al.*, 2001; Narhi *et al.*, 1991; Wujek *et al.*, 2004). The mechanism of protein glycosylation is partially understood. N-linked glycosylation is mediated by oligosaccharide transferase and occurs at asparagine residues that are a part of the consensus sequence Asn-X-(Ser/Thr), where X can be any amino acid, except proline (Bause, 1983; Berg and Grinnell, 1993; Imperiali and Shannon, 1991; Roitsch and Lehle, 1989).

Pichia strains are robust industrial fermentation organisms. However, proteins derived from *P. pastoris* system contain nonhuman N-glycans of the high mannose type, which are

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immunogenic in humans and thus of limited therapeutic value (Ballou, 1990; Hamilton *et al.*, 2003). When expressed in *P. pastoris*, t-PA is glycosylated at three consensus sequences, N117 and N184, and N448 (Bennett *et al.*, 1991). The kringle moiety in t-PA has two glycosylation sites that are N117 and N184, continuously and transiently N-linked glycosylated, respectively (Berg *et al.*, 1993). In the previous work, we confirmed that *Picha*-derived TK1-2 is glycosylated, and this recombinant protein is an effective angiogenesis inhibitor (Kim *et al.*, 2003; Shim *et al.*, 2005). If non-glycosylated TK1-2 is confirmed to retain anti-angiogenic activity, it will be developed as an effective agent to inhibit tumor angiogenesis, devoid of immune reactivity. Therefore, we were interested whether non-glycosylated TK1-2 derived from *Pichia* is able to inhibit angiogenesis. Here, we describe the mutation, expression, purification, and functional test of non-glycosylated TK1-2 protein derived from *Pichia pastoris*.

MATERIALS AND METHODS

Materials

P. pastoris (X-33), Zeocin and yeast nitrogen base (YNB) were purchased from Invitrogen (Carlsbad, CA). Medium 119 (M199), fetal bovine serum (FBS), and trypsin solution used for cell culture were purchased from Invitrogen (Carlsbad, CA). EBM-2 and EGM-2 were purchased from Clonetics (Walkersvillie, MD). Basic fibroblast growth factor (bFGF) was purchased from R&D Systems (Minneapolis, MN). Electrophoresis reagents (electrophoresis grade) were from Bio-Rad (Richmond, CA). *Dpn*I and *E.coli* strain XL10-Gold were purchased from Stratagene (La Jolla, CA). *Sac*I was purchased from Roche Molecular Biochemicals (Mannheim, Germany).

Cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated from fresh cords by an adaptation of the method described by Jaffe *et al.* (1973) and maintained in M199 medium containing 20% FBS, 30 μg/ml endothelial cell growth supplements (Sigma, St. Louis, MO), 90 μg/ml heparin, 25 mM Hepes, 2.2 g/liter sodium bicarbonate, 2 mM L-glutamine, and 1% antibiotics. Cells at passage 2 or 3 were used for the experiments. Cultures were kept at 37°C in a humidified atmosphere of 5% CO_2 in air for HUVECs.

Construction of non-glycosylated TK1-2 by site-directed mutagenesis

In vitro mutagenesis was performed on an pPICZa-C/TK1-2 recombinant plasmid reported previously (Kim *et al.*, 2003). *Dpn*I-mediated site-directed mutagenesis was performed to create the Asn^{117} to Glu^{117} and Asn^{184} to Glu^{184} mutation. Briefly, the oligonucleotide primers (sense strand, containing the desired mutation were extended during thermal cycling by using Native *Pfu* DNA polymerase (Stratagene, La Jolla, CA). Used primers are N117E (5'-GTGCACCAACTGG GAGAG-CAGCGCGTTGG-3'), and N184E (5'-CTG CTACTTTGGG-GAGGGGTCA GCCTACCG-3'). Following thermal cycling, the PCR product and the parental DNA templates were treated with *Dpn*I (target sequence : 5'-G^{m6}ATC-3', Stratagene, La Jolla, CA), which is specific for methylated and hemimethylated DNA, in order to select for mutation containing synthesized DNA. The recombinant plasmid DNA was then transformed into *E. coli* strain XL10-Gold (Stratagene, La Jolla, CA). Positive clones were selected and their DNA sequences were determined to confirm the expected mutation.

Transformation of yeast cell

Plasmid expressing non-glycosylated TK1-2 (NE-TK1-2) was linearized with *Sac*I enzyme (Roche Molecular Biochemicals) and used for homologous recombination into *Pichia pastoris* strain X-33 by electroporation (400 Ω, 25 μF, 1.5 kV) using a Gene Pulser and 0.2 cm cuvettes (Bio-Rad). Immediately after pulsing, 1 ml of cold 1 M sorbitol was added to the cuvette and the mixture was incubated at 30°C for 1 h without shaking. Positive clones were selected in Zeocin resistance (0.5 mg/ml or 1 mg/ml, Invitrogen) YPDS plates (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar). The clones with high production of NE-TK1-2 were selected.

Expression and purification of recombinant TK1-2 and NE-TK1-2 in *P. pastoris*

Large scale expressions of TK1-2 and NE-TK1-2 protein were performed in 2 liter flasks. The *P. pastoris* transformant was cultured in 500 ml YPD (1% yeast extract, 2% peptone, 2% dextrose) at 30°C with shaking (220 rpm) until the culture reached OD_{600} = 3~6, approximately 1 day. Cells were collected by centrifugation at 1,500g for 10 min and resuspended in 100 ml BMM (buffered minimal methanol medium: 100 mM potassium phosphate buffer, pH 6.0, 1.34% Yeast nitrogen base, 4×10^{-5}% biotin, 1% methanol). Pure methanol was added every 24 h to a final concentration of 1%. After 96 h, cells were centrifuged at 5,000g for 15 min and culture broth was stored at 4°C until use. Crude culture broth containing

TK1-2 or NE-TK1-2 protein was clarified by centrifugation at 14,000g for 20 min, concentrated, and diafiltated against buffer 1 (20 mM potassium phosphate buffer and 24 mM citric acid, pH 5.0) by ultrafiltration using a YM3 membrane (Millipore, Billerica, MA).

S-sepharose (Pharmacia Biotech, Uppsala, Sweden) was used for an ion exchange chromatography. S-sepharose column was packed and equilibrated with buffer 1. Samples were applied to columns, which were then washed with 5 resin volumes of buffer 1, and then protein was eluted with buffer 2 (20 mM potassium phosphate buffer, pH 7.2 or 5.0 with NaCl in a range from 20 mM to 1000 mM by linear gradient manner). Fractions were dialyzed for 16 h against 50 mM sodium acetate, pH 5.0. Then, FPLC was performed using UNO S1 column (Bio-rad) to increase the protein purity. Purified protein samples were dialyzed against 50 mM sodium acetate (pH 5.0) or PBS (pH 7.2) and concentrated by centricon YM-10 (Millipore). We removed endotoxin from the sample for cell assays by extraction with Triton X-114 followed by treatment with SM-2 beads. The endotoxin level of the purified protein was less than 0.01 unit/mg protein.

MALDI-TOF analysis

Mass spectrometric analysis of protein using MALDI-TOF (matrix-assisted laser desorption ionization time of flight) mass spectrometer, Voyager-DE STR Biospectrometry Workstation (Applied Biosystems Inc. Foster City, CA) was performed at the National Center for Inter-university Research Facilities at Seoul National University, Korea.

SDS-PAGE

SDS-PAGE was performed using a precast 14% polyacrylamide gel according to the manufacturer's instructions (BioRad). Samples under reducing (containing β-mercaptoethanol) and non-reducing conditions were dissolved in the SDS sample buffer.

Protein analysis by CD-spectrum

The CD spectra of wild type TK1-2 and NE-TK1-2 were measured on a JASCO J-715 spectrophotometer (Jasco International, Tokyo, Japan) in the wavelength range 200-250 nm at 25°C. The measurement was performed at Korea Basic Science Institute, Dae-jeon, Korea. Both of the proteins were dissolved in PBS (pH 7.2), and filtered through a 0.22 mm-pore size filter unit (Millipore). Determined protein concentration of the final solution using the Bradford assay was 430 μg/ml. Spectra were obtained over an average of 5 scans with a scan speed of 50 nm/min and detection limit of 50 mdeg.

Wound migration assay

HUVECs were cultured in 48 well plates containing EGM-2 (Clonetics) and grown to confluence. Then, the cells were washed with PBS and starved with serum free EBM-2 for 4 h. The endothelial cells were scraped with a 200 μl pipette tip and washed with PBS to remove cellular debris. Endothelial cells were pretreated with fresh EBM-2 in the presence or absence of TK1-2 or NE-TK1-2 for 30 min, and then added with 1% FBS containing bFGF (3 ng/ml). The cells were incubated for 8 h at 37°C, 5% CO_2 and 95% humidity condition. Wound regions were photographed under an inverted light microscopy at 100 X magnification using an Olympus C-5050 digital camera at 0 and 8 h.

RESULTS AND DISCUSSION

Previously, TK1-2 was produced as insoluble and soluble forms in *E. coli* and *P. pastoris*, respectively. Two types of TK1-2 showed the potent inhibitory effect on *in vitro* endothelial cell proliferation and *in vivo* tumor growth (Kim *et al.*, 2003; Shim *et al.*, 2005). However, two types of TK1-2 have some problems. Insoluble TK1-2 expressed in *E. coli* has to be denatured and refolded in vitro to produce active protein, and TK1-2 derived from *P. pastoris* has N-glycan that might have antigenicity in human body (Ballou, 1990; Hamilton *et al.*, 2003). To obtain functionally effective and more useful TK1-2, we made non-glycosylated TK1-2 by site-directed mutagenesis and *P. pastoris* – expression system. Mutation was performed by site-directed mutagenesis to change two asparagine residues to glutamic acid residues (N117E and N184E). Mutant TK1-2 was expressed in *P. pastoris* and purified from total culture broth by S-sepharose and UNO-S1. In this purification system, NE-TK1-2 was produced in a soluble protein with 37.6% purification yield from culture broth (Table I). When purified NE-TK1-2 was checked by band shifting compared with wild type

Table I. Purification of NE-TK1-2

Expression and purification step	Protein concentration (mg/ml)	Volume (ml)	Total protein (mg)	Yield (%)
Pichia expression	0.0676	400	27.03633	100
Ultrafiltration	0.3789	66	25.00882	92.5008
S-sepharose	1.0572	13	13.74298	50.8315
UNO-S1	4.0679	2.5	10.16981	37.6153

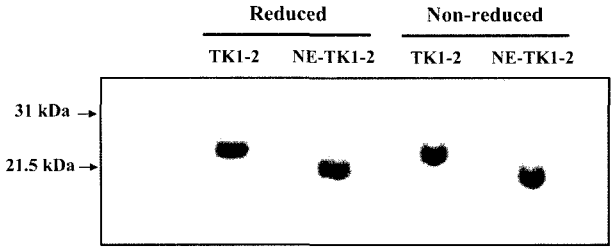

Fig. 1. Purified proteins, wild type TK1-2 and NE-TK1-2 were analyzed by SDS-PAGE under reducing and non-reducing conditions, and Coomassie staining.

TK1-2 in SDS-PAGE (Fig. 1), the size of NE-TK1-2 protein was smaller than wild type TK1-2, and corresponded with the expected size of non-glycosylated TK1-2 (19,969 Da).

In mass spectrum analysis, purified NE-TK1-2 showed closely neighboring two peaks, 19,631.87 and 19,835.44 Da. The difference in molecular weight between two peaks is about 203.57 Da. In an independent experiment, two peaks obtained are 19,884.24 Da and 19,715.48 Da, 168.76 Da apart from each other. As shown in fig. 1, it migrated as one band in SDS-PAGE. Thus, the small mass difference of two peaks doesn't seem to be due to glycosylation modification. In the previous report, the mass spectrum of wild type TK1-2 also showed

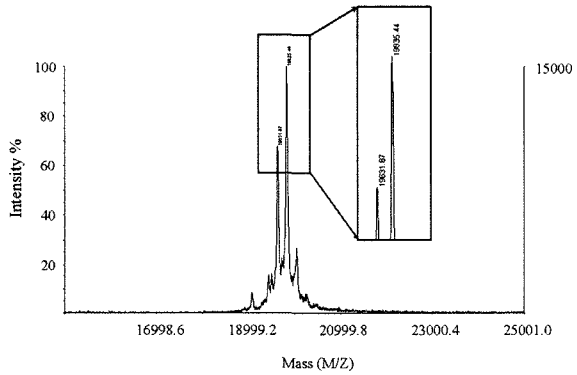

Fig. 2. The mass spectrum of purified NE-TK1-2 measured by MALDI-TOF. Mass of NE-TK1-2 was determined to be 19,631.87 and 19,835.44 Da, which are comparable to that of its calculated mass (19,969 Da).

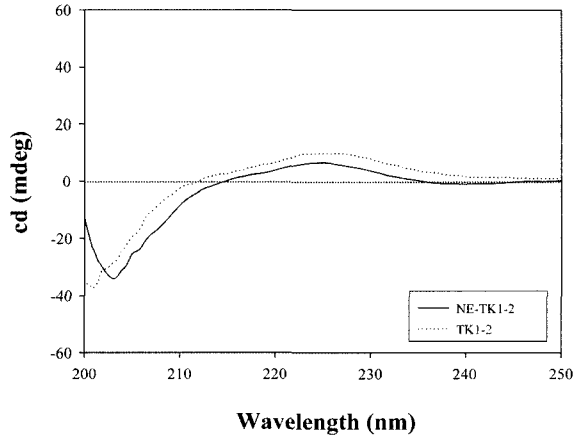

Fig. 3. Spectroscopic analysis of wild type TK1-2 and NE-TK1-2. Far UV CD spectra were recorded at 25°C by using 0.1 cm path-length cuvette and protein solutions containing PBS. The concentration of wild type TK1-2 or NE-TK1-2 was 430 μg/ml.

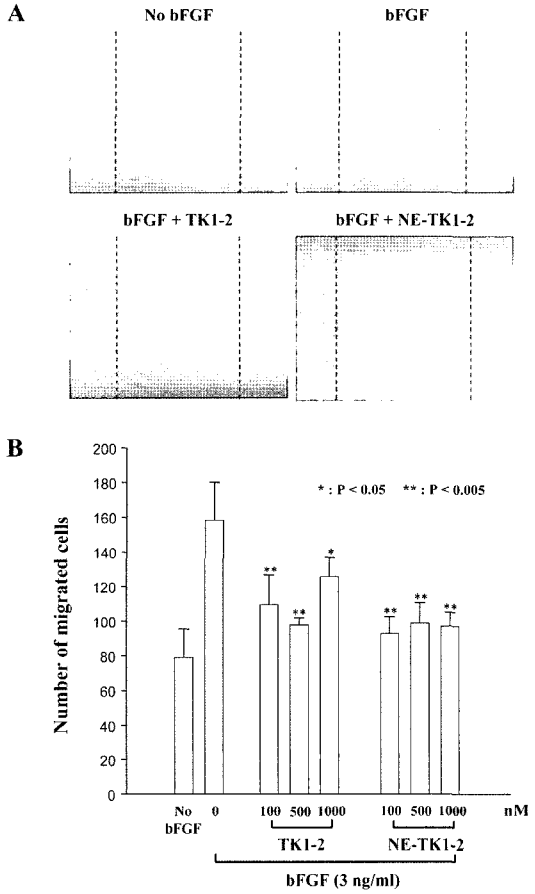

Fig. 4. Inhibition of HUVEC migration by NE-TK1-2 and wild type TK1-2. HUVECs were plated, scraped, and incubated for 8 h in EBM-2 supplemented with 3 ng/ml bFGF and 1% FBS in the presence or absence of various concentrations (100-1,000 nM) of NE-TK1-2 and wild type TK1-2. (A) Representative photographs of non-stimulated cells, bFGF-stimulated control cells, the cells treated with bFGF and wild type TK1-2 (1,000 nM), or treated with bFGF and NE-TK1-2 (1,000 nM) were shown. Lines indicate the initial scraping region. (B) The cell migration upon TK1-2 or NE-TK1-2 treatment was quantitatively measured. Data are expressed as the number of cells that migrated in the bFGF-stimulated cultures (mean ± SD). *p< 0.05; **p<0.005, protein treated groups versus bFGF-stimulated control.

close two peaks or one broad peak (21,804.23 and 21,990.54 or 23,940.81 Da, respectively). Overall, the mass of NE-TK1-2 was smaller than that of wild type TK1-2, approximately 1.8~4 kDa. Such large difference in mass between NE-TK1-2 and wild type TK1-2 might be due to high-mannose carbohydrate on proteins. In addition, NE-TK1-2 showed a typical and similar secondary structure by CD-spectra (Fig. 3). These data demonstrated that N-glycan on TK1-2 was successfully removed by mutagenesis and removal of sugar moiety in TK1-2 by mutation did not affect secondary structure of NE-TK1-2.

Next, we tested the activity of NE-TK1-2 by employing a wound migration assay. Purified NE-TK1-2 was able to inhibit endothelial cell migration in response to bFGF stimulation in a dose-dependent manner at the same level as wild type activity *in vitro* (Fig. 4). In case of t-PA, N-glycan is related to binding affinity and specificity to plasmin, plasminogen, or fibrinogen (Aoki *et al.*, 2001; Asselbergs *et al.*, 1993; Berg *et al.*, 1993; Wilhelm *et al.*, 1990). However, in regard to TK1-2, the results suggest that N-glycan on TK1-2 may not affect the anti-angiogenic activity of TK1-2 and NE-TK1-2 can be used effectively as an anti-angiogenic agent.

Therefore, we report a new efficient way of producing non-glycosylated functional TK1-2. Since most of proteins attached N-glycan derived from eukaryotic system is accompanied by antigenicity in human body, our study should provide non-immunogenic recombinant protein NE-TK1-2 for clinical application.

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