J. Microbiol. Biotechnol. (2013), 23(9), 1197–1205
http://dx.doi.org/10.4014/jmb.1305.05004

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Expression, Purification, and Biological Characterization of The Amino-Terminal Fragment of Urokinase in *Pichia pastoris*

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Received: May 3, 2013
Revised: June 3, 2013
Accepted: June 4, 2013

First published online
June 10, 2013

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pISSN 1017-7825, eISSN 1738-8872

Urokinase (uPA) and its receptor (uPAR) play an important role in tumor growth and metastasis. Targeting the excessive activation of this system as well as the proliferation of the tumor vascular endothelial cell would be expected to prevent tumor neovasculature and halt the tumor development. In this regard, the amino-terminal fragment (ATF) of urokinase has been confirmed as effective to inhibit the proliferation, migration, and invasiveness of cancer cells *via* interrupting the interaction of uPA and uPAR. Previous studies indicated that ATF expressed in *Escherichia coli* was mainly contained in inclusion bodies and also lacked posttranslational modifications. In this study, the biologically active and soluble ATF was cloned and expressed in *Pichia pastoris*. The recombinant protein was purified to be homogenous and confirmed to be biologically active. The yield of the active ATF was about 30 mg/l of the *P. pastoris* culture medium. The recombinant ATF (rATF) could efficiently inhibit angiogenesis, endothelial cell migration, and tumor cell invasion *in vitro*. Furthermore, it could inhibit *in vivo* xenograft tumor growth and prolong the survival of tumor-bearing mice significantly by competing with uPA for binding to cell surfaces. Therefore, *P. pastoris* is a highly efficient and cost-effective expression system for large-scale production of biologically active rATFs for potential therapeutic application.

Keywords: Amino-terminal fragment of urokinase, *Pichia pastoris*, recombinant protein production, anti-angiogenesis, tumor therapy

Introduction

Urokinase plasminogen activator (uPA), its receptor (uPAR), and its inhibitors, plasminogen activator inhibitor-1 (PAI-1) and 2 (PAI-2), are important components of the cell surface proteolysis used by tumor cells and capillary endothelial cells, and therefore play crucial roles in the establishment, metastasis, and angiogenesis of most solid tumors [6, 17]. The serine proteinase uPA has the ability to convert plasminogen to active plasmin, and activates matrix metalloproteinases and growth factors, which may play important roles in pericellular proteolysis and angiogenesis [3]. Without the activation by plasmin, single-chain uPA may also have low catalytic activity. Its binding to the cell surface receptor (uPAR) *via* its amino-terminal fragment (ATF) renders it more accessible to plasminogen on the cell surface, thereby facilitating the plasminogen-plasmin transition, which results in an amplified cascade with the activation of uPA by plasmin, and localizes the proteolytic activity of uPA and plasmin on the cell surface [1, 9]. uPAR is a 50-60 kDa extracellular cysteine-rich glycoprotein, congregated in lipid rafts. It is composed of three homologous domains (D1, D2, and D3) belonging to the Ly-6/uPAR/alpha-neurotoxin protein domain family, the last of which attaches to the cell membrane by a glycosylphosphatidylinositol anchor [2, 24]. uPAR is predominantly expressed on inflammatory cells and cancer cells in areas of cancer invasion in several types of cancer,

including gastric, colon, breast, and oral cancers, suggesting that it is involved in both tumor cell invasion and metastasis [8, 11, 14, 27]. In addition, the uPA-uPAR system is implicated in tumor-associated angiogenesis. The binding of uPA to uPAR can not only induce the migration of endothelial cells but also promote the release of several angiogenic factors, such as basic fibroblast growth factor, hepatocyte growth factor, transforming growth factor-β, tumor necrosis factor-α and vascular endothelial growth factor [16, 20, 23].

All these essential roles of the uPA-uPAR system in tumor growth and metastasis make it an ideal candidate for targeted cancer therapy. Therapeutic molecules aimed at interrupting the interaction of uPA and uPAR may inhibit both tumor cell invasiveness and tumor-associated angiogenesis, and thereby might be effective in cancer therapy. For example, the monoclonal antibody against uPA or uPAR has been confirmed as effective to inhibit the proliferation, migration, and invasiveness of cancer cells *in vitro* [15, 25]. Another known antagonist inhibitor of uPA-uPAR is ATF, the amino-terminal fragment of higher-molecular-weight uPA that harbors an epidermal growth factor-like domain and a kringle domain. ATF could efficiently inhibit angiogenesis and tumor invasion *in vitro* and *in vivo* by competing with uPA for binding to both endothelial and tumor cell surfaces [18, 19, 21]. ATF was previously expressed as the inclusion body in *E. coli* [28]. In the present study, we designed and expressed recombinant ATF (rATF) in *P. pastoris*, which is a eukaryotic expression model organism. The purification protocol was simplified and the protein yield was also high. The purified rATF exhibited efficient anti-angiogenesis and tumor growth inhibition function.

Materials and Methods

Strains, Plasmids, and Culturing Conditions

P. pastoris X-33 and the pGAPZαA vector used for *ATF* gene expression were purchased from Invitrogen (Carlsbad, CA, USA). *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA) was used as a host for DNA manipulation and was cultured at 37°C in liquid Luria–Bertani (LLB) medium (10 g/l tryptone, 5 g/l yeast extract, and 5 g/l sodium chloride, pH 7.5) containing 25 μg/ml Zeocin (Invitrogen, Carlsbad, CA, USA). Plasmid pET23a-*ATF* harboring the *ATF* gene was previously constructed and stored in our laboratory [28]. Restriction endonuclease, *Pfu* DNA polymerase, and T4 DNA ligase were bought from TaKaRa Biotechnology Co. (Otsu, Japan). The DNA molecular weight marker was obtained from Fermentas (Burlington, ON, Canada). All chemicals were of reagent grade and were obtained from commercial sources.

Vector Construction and Transformation

The *ATF* cDNA gene was amplified by polymerase chain reaction (PCR) using plasmid pET23a-*ATF* as a template. The amplification primers containing the restriction sites for *Xho*I and *Xba*I were designed as *ATF*-F, 5′-GGG**CTCGAG**AAAAGAGAGG CTGAAGCTATGAGCAATGAACTTCAT-3′ and *ATF*-R, 5′-GGG **TCTAGA**TTATTTTCCATCTGCGCAGTCATGCACCATGC-3′. The PCR products were double-digested with *Xho*I and *Xba*I, and then ligated into pGAPZαA, forming expression plasmid pGAPZαA-*ATF*. Finally, the recombinant expression vector pGAPZαA-*ATF* was used to transform *E. coli* Top10. Through DNA sequencing, pGAPZαA-*ATF* was confirmed to contain the *ATF* mature cDNA. *P. pastoris* X-33 was transformed with 10 μg of *Bln*I-linearized pGAPZαA-*ATF* vector and parent vector pGAPZαA by electrotransformation, according to Invitrogen's recommendations. The electrotransformation parameters were 1,500 V, 200 Ω, 25 μF, and 5 ms. Transformants were plated on YPDS plates (10 g/l yeast extract, 20 g/l peptone, 20 g/l dextrose, 20 g/l agar, and 1 M sorbitol) containing 100 μg/ml Zeocin to isolate resistant clones. Then, the Zeocin-resistant clones were shifted to YPD-rhodamine B-olive oil medium plates containing 0.08 g/l rhodamine B and 1% (v/v) emulsified olive oil. Transformed colonies were confirmed by both PCR and DNA sequencing.

Expression of rATF in *P. pastoris*

The most efficient rATF-secreting clone was screened by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis for production of the specific protein. A preculture growth step was performed for 24 h in a 250 ml Erlenmeyer flask containing 50 ml YPD medium. This cell culture was further used to inoculate larger yeast cell cultures at an optical density ($OD_{600\ nm}$) of 1, to start the cell growth directly in the exponential growth phase, as well as to establish reproducible cell culture conditions. The yeast was further grown at 30°C with orbital agitation at a rate of 250 rpm. The optimum YPD medium to flask volume ratio for rATF production was found to be 1/5 and the cultures were usually performed in a 1 L Erlenmeyer flask containing 200 ml of YPD medium without any Zeocin. The cultures were stopped after 72 h and the cells were pelleted by centrifugation at 3,000 ×*g* for 20 min. Then 30 μl supernatants were loaded and analyzed on 15% SDS-PAGE and visualized by Coomassie Blue staining.

Purification and Characterization of rATF

The 17 kDa rATF from the culture supernatant was purified after cultivation for 72 h. Culture supernatants from shaker flasks were precipitated with ammonium sulfate (65% saturation). The precipitate was then dissolved in buffer A (20 mM sodium acetate, 1 mM EDTA, 0.5 mM PMSF, pH 4.8), and finally dialyzed against the same buffer at 4°C. Further purification was carried out on a CM Sepharose Fast Flow column (GE Healthcare Life Sciences, Piscataway, NJ, USA). After loading the sample, the column was washed with buffer B (20 mM sodium acetate, 1 mM EDTA, pH 4.8) and stepwise eluted by 0.1, 0.2, and 0.5 M NaCl in buffer

B. The eluted fractions were pooled and the concentration of rATF was determined by the Bio-Rad protein assay method (Bio-Rad, Hercules, CA, USA). The purity was determined on a SDS-PAGE gel stained with Coomassie Blue. The identity of rATF was confirmed by western blotting using polyclonal mouse anti-ATF antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Cell Culture

Human HCT116 colon cancer cell line and human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Philadelphia, PA, USA). HCT116 cells were grown in RPMI 1640 (HyClone, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). HUVECs were grown in Medium 200 (Cascade Biologics, Portland, OR, USA) supplemented with Low Serum Growth Supplement (LSGS). All cells were cultured in a humidified CO_2 incubator at 37°C.

Chick Embryo Chorioallantoic Membrane Assay

The chorioallantoic membrane (CAM) assay was performed as described previously [29]. Fertilized White Leghorn chicken eggs were placed in an incubator under constant humidity at 37°C. Briefly, eggs at the 8th day of chick embryo development were candled using a hand-held egg candler at the blunt end of eggs to identify the air sac and prominent blood vessels. Using a Dremel model drill (Dremel Racine, WI, USA), the CAM was separated from the shell by making a shallow hole at the blunt end of the egg. The rATF at three different doses or buffer control was pipetted onto a cortisone dried filter disk, which had been placed on the chorioallantoic membrane. The window was sealed with sterile Scotch tape and the egg was returned to the incubator. After incubation for 2 days, the possible anti-angiogenic response was evaluated. The tissues directly below the filter disks were fixed with a mixture of methanol and acetone (1:1) for 15 min. CAMs were washed 3 times with phosphate-buffered saline (PBS) and then analyzed using a stereomicroscope. A CAM with an avascular zone larger than 5 mm in diameter was regarded as positive inhibition. The percent of positive inhibition in each group was calculated to quantitatively evaluate the anti-angiogenic activity of rATF.

In Vitro Wound-Healing Assay

Cell motility was assessed using a modified wound-healing assay [29]. Briefly, HUVECs plated onto fibronectin-coated (10 mg/ml) 24-well plates were serum-starved overnight, and then wounded with a 200 µl pipette tip, washed with PBS, and incubated in the medium containing 10% FBS with rATF (PBS used as buffer control) at a concentration of 3 µg/ml or 6 µg/ml for 24 h. The migration of the wounded cells was visualized and quantified under a microscope with a magnification of ×100. All groups of experiments were conducted in triplicates, and the cell number was counted by Image-Pro Plus 6.0 software.

Boyden Chamber Matrigel Invasion Assay

The effect of rATF on the inhibition of invasion of HCT116 cells was determined in a 2-compartment Boyden chamber (Transwell; Costar Corp., Cambridge, MA, USA), according to previously reported methods [13, 30]. The invasion ability of tumor cells was determined by the mean number of cells migrating through the membrane. Each treatment was done in triplicates.

In Vivo Animal Tumor Model Experiment

Athymic nude mice (6-8 weeks of age) were obtained from Shanghai Laboratory Animal Center (Shanghai, China) and housed in a temperature-controlled sterile room where the humidity and light were carefully monitored. Animal welfare and experimental procedures were performed strictly in accordance with high standard animal welfare and other related ethical regulations approved by the Nanjing University Animal Care and Use Committee. Tumors were generated by subcutaneous injection of HCT116 cells (2×10^6 cells in 50 µl) into the dorsal flanks of each mouse. Tumor measurements were converted to tumor volume (V) using the formula ($L \times W^2 \times 0.52$), where L and W are the length and width, respectively. Measurements were made with a vernier caliper. All tumor-bearing mice were divided randomly into three groups, and treatment was initiated on the 7th day when the volume of tumor reached a size of approximately 50 mm^3. The mice were injected intraperitoneally (i.p.) with rATF (2 mg/kg) or cyclophosphamide (CTX; 30 mg/kg) every 2 days for a total of 19 days. Control mice received i.p. injections of PBS. The antitumor activity of treatments was evaluated by tumor growth inhibition, tumor doubling time (TDT), and animal survival prolongation. The TDT was calculated using GraphPad Prism v 5.0. TDT values were generated from exponential growth curves, which had been fitted to % change in tumor volume data ($r^2 > 0.70$).

In a parallel animal assay (totally 3 groups, and 3 mice per group), the tumor establishment and drug treatment were the same as described earlier. On the 19th day, the mice were euthanized. Tumors were collected, fixed with 4% formaldehyde, embedded in paraffin, and sectioned for hematoxylin and eosin (H&E) staining according to standard histological procedures.

Data Analysis and Statistics

Statistical analysis was carried out using the SPSS software (v. 11.0, SPSS, Chicago, IL, USA). Data were expressed as the mean ± SD and analyzed using one-way ANOVA and the least-significant difference tests. A value of $P < 0.05$ was considered statistically significant.

Results

rATF Expression and Purification

The *Pichia* expression system was used to prepare rATF in soluble form. Twenty-five colonies obtained after the transformation of *P. pastoris* were randomly picked to test

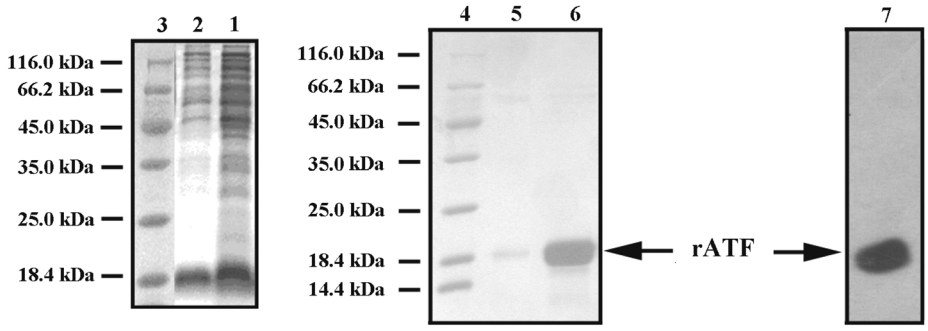

Fig. 1. Production and validation of rATF.
Purified rATF was analyzed by 15% SDS-PAGE. Lane 1, culture supernatant. Lane 2, ammonium sulfate precipitation. Lane 3 and Lane 4, protein Marker. Lane 5, 0.1 M NaCl elution. Lane 6, 0.2 M NaCl elution. Lane 7, Western blotting using poly-antibody against ATF. rATF in the 0.2 M NaCl elution migrated at around 17 kDa.

the protein expression on a small scale. The clone with the highest expression level of rATF was selected. For purification purposes, after ammonium sulfate precipitation, the target protein was concentrated in a small buffer volume and significant removal of some contaminants was achieved. In the ion-exchange purification step, rATF was eluted as a single homogenous peak at 0.2 M NaCl. After the final step, the desired level of product purity (>98%) was achieved. On 15% SDS-PAGE, the mobility of the purified protein was found to correspond to a molecular mass of about 17 kDa (Fig. 1, lane 6). The summary of purification is listed in Table 1 and the yield was about 28.4 mg per liter of cultures. The purified protein was further examined by western blot assay using polyclonal anti-ATF antibody. As shown in Fig. 1, lane 7, the rATF migrated homogenously at 17 kDa as expected, and no degradation was observed.

Inhibition of Angiogenesis on CAM by rATF

The chick embryo CAM model was used to evaluate the anti-angiogenic activity of rATF. Dried filter disks, adsorbed with rATF at 0.5, 1.0, and 2.0 μg/embryo doses, were implanted on the top of growing CAMs. Two days later, rATF induced a strong anti-angiogenic response in the CAM tissues in a dose-dependent manner (Figs. 2A and 2B), as shown by the decreased number of branching vessels in the center of the filter disk.

Table 1. Purification of recombinant ATF from *P. pastoris*.

Purification step	Total rATF (mg/l)	Recovery (%)
Culture supernatant	39.3	100
Ammonium sulfate precipitation	37.1	94
CM Sepharose Fast Flow chromatography	28.4	76

Inhibition of HUVECs Migration by rATF

Wound-healing scratch assays were performed to examine whether rATF could inhibit the migration of

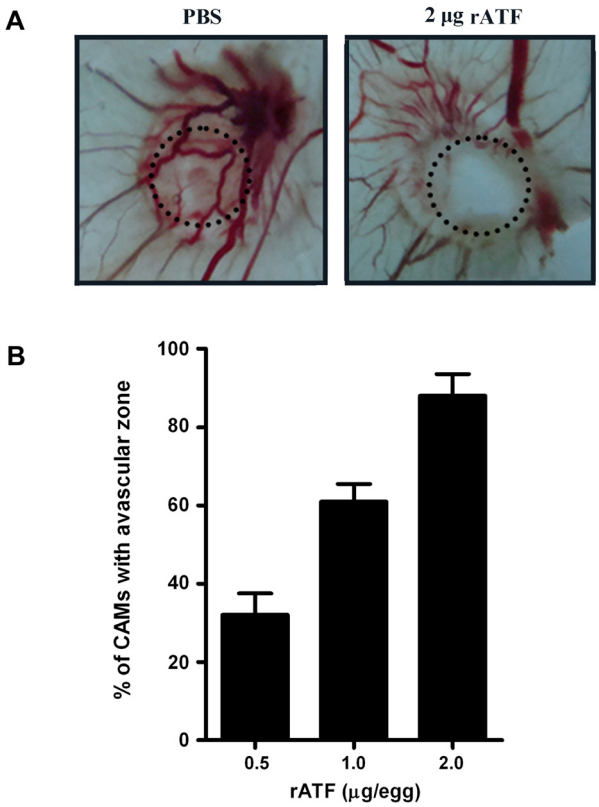

Fig. 2. Effect of rATF on angiogenesis by chick CAM assay.
(**A**) Representative CAMs from 8-day-old chick embryos, which were treated with different doses of rATF (0.5, 1.0, 2.0 μg/egg) for 48 h. (**B**) The data were calculated as the percentage of the positive eggs (formation of avascular zones ≥ 5mm in diameter) relative to the total eggs tested. N = 15. PBS was used as the buffer control. Experiments were conducted in triplicates.

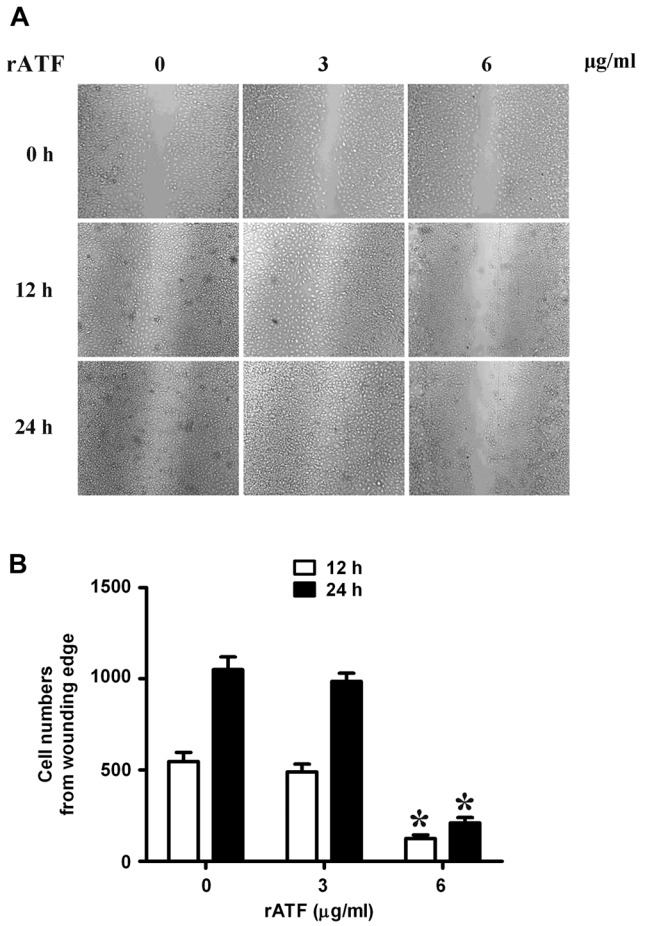

Fig. 3. Effect of rATF on endothelial cell migration by wound-healing assay.

HUVECs were treated with different doses of rATF (0, 3, 6 μg/ml). After a 24 h healing period, cells migrating into the wound area were visualized (**A**) and quantified with a magnification of ×100 (**B**). $N = 3$. Experiments were conducted in triplicates. $^*p < 0.05$ as compared with the control.

HUVECs. Equal numbers of growing cells (1×10^5) were plated onto fibronectin-coated 24-well plates, and after 8 h, cells were cleared within a defined area by scratching with a pipette tip, washed with PBS, and allowed to migrate into the cleared area in the presence of serum. At 24 h after the scratch, whereas control untreated cells had migrated into and largely covered the original wound area, those cells treated with rATF at the dose of 6 μg/ml failed to cover a substantial portion of the wound. The cells migrating into the wound area were counted. rATF treated at 6 μg/ml decreased the migrating cells by 79.32% compared with the control group (Fig. 3).

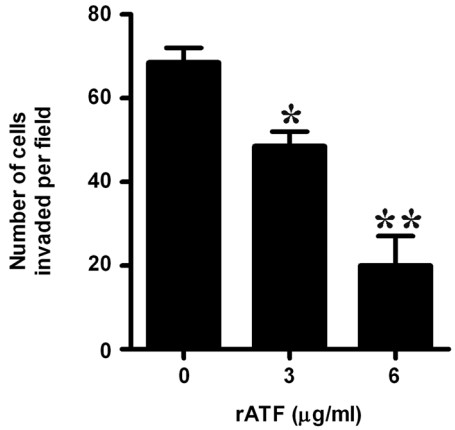

Fig. 4. Effect of rATF on tumor cell invasion.

In the *in vitro* Matrigel invasion assay, HCT116 cells (5×10^4 cells in 0.1 ml of medium) were seeded in the upper compartment of transwell chambers containing filters coated with Matrigel. After a 24 h incubation, under serum-free conditions (RPMI 1640), cells that invaded through the 8 μm pores were stained and counted. The data are the mean ± SD of 3 separate experiments. The invaded cells in the treatments of rATF were dose-dependently decreased compared with that of control treatment. $^*p < 0.05$, $^{**}p < 0.01$ as compared with the control.

Inhibition of HCT116 Cells Invasion by rATF

The effect of rATF on the invasiveness of tumor cells was studied using the Matrigel model. We found that rATF could reduce the invaded cell number in a dose-dependent manner (Fig. 4), suggesting an obvious inhibitory effect on the invasion of HCT116 cells by rATF.

Inhibition of Tumor Growth by rATF

The antitumor effect of rATF was analyzed in a xenograft tumor model by transplanting HCT116 cancer cells into athymic nude mice. On the 7th day post-implantation, mice were randomly divided into three groups before the tumor was palpated, with at least eight tumor-bearing mice in each group. Mice with tumors were injected i.p. every other day for a total of 19 days with rATF at 2 mg/kg or CTX at 30 mg/kg. Both CTX and rATF significantly inhibited the growth of xenograft tumors (on day 9-19, $p < 0.05$, compared with control). On the 19th day, the mean volume of tumor was 425.5 ± 94.65 mm^3, with 76.16% inhibition when using rATF at 2 mg/kg, whereas treatment with CTX resulted in 42.63% inhibition of tumor growth (Fig. 5A). The tumor doubling time was prolonged from 3.44 days in mice receiving PBS, to 7.19 days in mice receiving rATF ($p < 0.05$; Fig. 5B). In addition, rATF induced

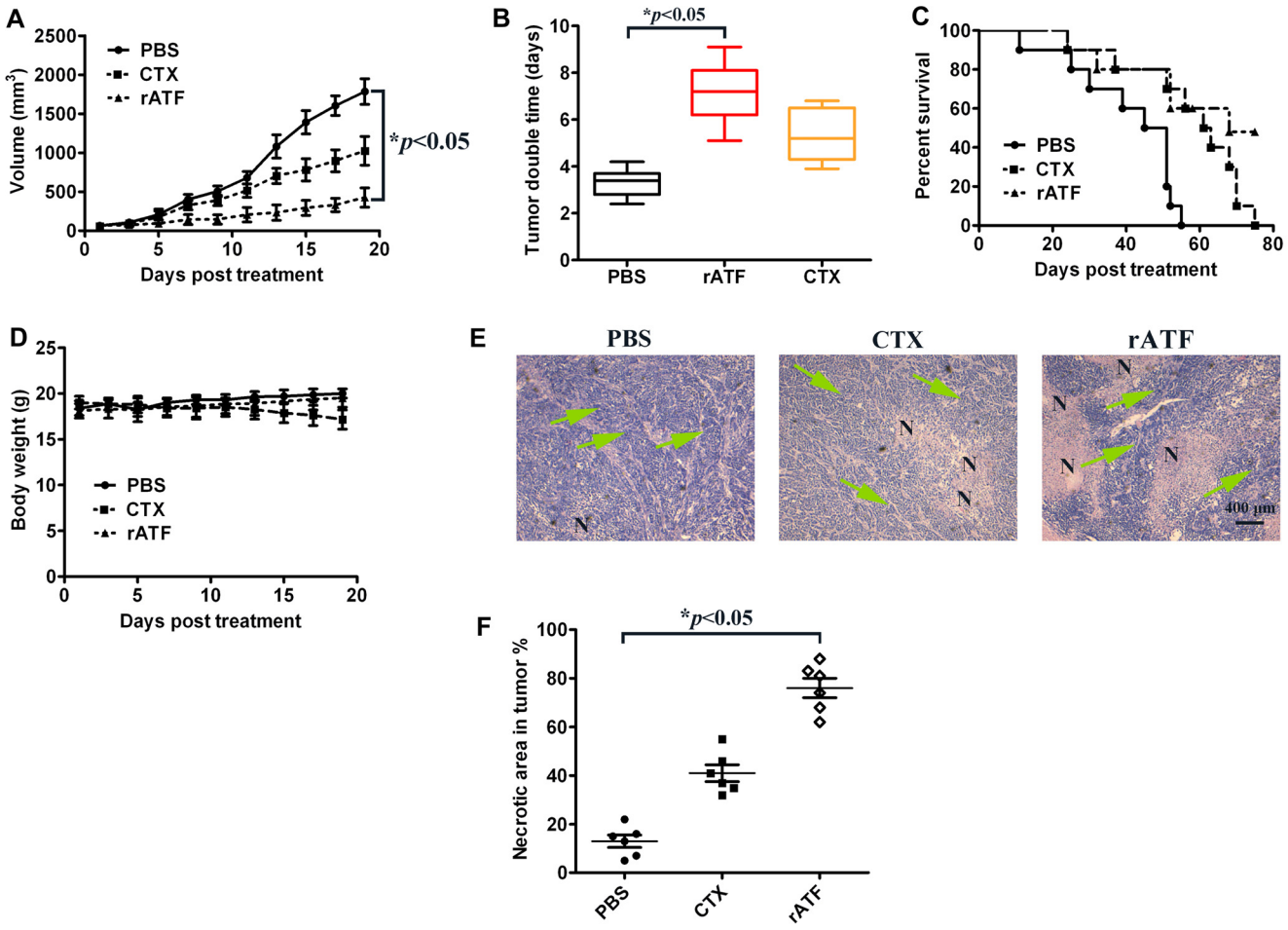

Fig. 5. Effect of rATF on the xenograft of colon cancer.

HCT116 cells were injected subcutaneously into the dorsal flanks of athymic nude mice. When tumors reached a size of approximately 50 mm^3, mice were injected i.p. with rATF or CTX every 2 days for a total of 19 days. (**A**) The tumor growth inhibitory effects of different treatments were compared. Values are expressed as the mean ± SD from eight animals. *$p < 0.05$ as compared with the control. (**B**) Tumor doubling time of each group. Data are shown as box-and-whiskers plots. *$p < 0.05$ as compared with the control. (**C**) rATF significantly improved the survival of mouse bearing colon tumor xenografts. Mouse survival kinetic estimate was carried out during and post treatment. **$p < 0.01$ compared with PBS, *$p < 0.05$ compared with CTX. (**D**) The fluctuation of mouse body weight in the course of treatment was recorded and represented as the mean of each group. (**E**) Determination of tumor necrosis after treatment with rATF or CTX. Tumor necrosis areas were shown by H&E staining and observed under a light microscope (×100). The viable tumor cells are indicated by a green arrow. (**F**) Quota of tumor necrosis. Tumor necrosis was determined by Image J software (NIH, USA). Two sections/mouse and three mice were prepared (mean ± SD, *$p < 0.05$).

significant survival benefits as compared with control and CTX therapy (Fig. 5C). As expected, the rATF had no obvious side-effect on animals, compared with CTX, as evidenced by the continuous increase of body weight and normal structure of spleens (Fig. 5D and data not shown). Light microscopy further revealed that tumor tissues in mice receiving rATF displayed more severe necrosis than control or CTX treatment. CTX treatment or untreated controls displayed tissue necrosis interspersed with viable tumor cells, whereas rATF induced large areas of continuous necrosis within tumors (Figs. 5E and 5F).

Discussion

Cancer is one of the most common causes of death, taking nearly 7 million lives each year worldwide. To date, cancer is still one of the most life-threatening diseases. However, in spite of the advances in surgical techniques

and radiotherapy, most cancer patients will eventually progress due to micrometastases and die of their disease. Therefore, current treatments to cure cancer have been focusing on drugs, biological molecules, and immune-mediated therapies. In this regard, targeted cancer therapy may point to a more promising modality for treating cancer. New cancer targeted therapies using therapeutic small molecules or antibodies have made treatment more tumor-specific and less toxic. Antibodies against specific tumor antigens are now becoming increasingly recognized as the most important biological agents for targeted cancer therapy. Several antibodies that target specific molecules, such as Herceptin (anti-Her2), Rituxan (anti-CD20), and Avastin (anti-VEGF), have been successfully applied to treat human tumors derived from diverse tissues [12, 22, 26]. Although the number of therapeutic antibodies is increasing quickly, the ideal targets are limited. Generally, an ideal target needs to follow these standards: (1) the molecule is predominantly expressed on tumor cells or tumor-related tissues; (2) the targeting molecule locates on the cell surface; (3) the molecule plays key roles in the regulation of proliferation and metastasis of tumors; and (4) blocking the molecule has little or no effect on normal tissues. In this regard, uPAR was identified to be an ideal targeting molecule. Previous research has shown that interference with the uPA/uPAR system is an efficient therapy for cancer [15, 25]. Unfortunately, as far as we are aware, there is still no ideal therapeutic antibody against uPA or uPAR that has been successfully applied to clinical treatment. ATF, the enzymatically inactive uPA fragment, has high affinity to uPAR. This characteristic makes it widely applicable to interference with uPA-uPAR interaction, and therefore tumor invasion and metastasis can be inhibited efficiently [19, 21].

In the present study, we designed and produced a recombinant ATF in *P. pastoris*, providing an efficient way to produce this protein. The *P. pastoris* system has been successfully used to produce a wide variety of heterologous proteins, originating from viruses, bacteria, fungi, animals, plants, and human beings [5]. As a eukaryotic organism, *P. pastoris* has many of the advantages of higher eukaryotic expression systems, especially post–translational modifications such as glycosylation, carboxylation, hydroxylation, sulfation, amidation, and protein secretion into the medium, among which the latter facilitates easy purification while being manipulated as easily as *E. coli* or *S. cerevisiae* [7]. It is faster, easier, and less expensive to use when compared with other expression systems such as baculovirus or mammalian systems. Additionally, compared with other expression systems such as S2-cells from *Drosophila melanogaster* or Chinese hamster ovary cells, *P. pastoris* usually gives a much higher yield [10]. The major advantage of *P. pastoris* over *E. coli* is that *P. pastoris* is capable of producing correctly folded glycosylations and carboxylations in proteins [7]. This means that in cases where glycosylations are necessary, *E. coli* might produce an inactive or insoluble protein [4]. These features along with convenient maintenance, easy scale-up, and inexpensive growth requirements make *P. pastoris* a very useful protein expression system. In our study, using *P. pastoris* as the expression organism, a high yield (about 30 mg/l culture) was obtained, while the one-step purification protocol was very simple as compared with previous research [31]. The *P. pastoris*-derived rATF was isolated to homogeneity with a purity as high as 98% by using one-step CM ion-exchange chromatography with a gradient of sodium chloride elution. Our laboratory previously reported a system for the efficient expression and purification of intact and biologically active rATF in *E. coli* [28]. In that system, rATF was expressed as an inclusion body. After refolding and purification, the final yield was about 12 mg/l. Thus, compared with previous studies, this study described a simplified expression and purification method for the high yield of rATF in *P. pastoris*. In addition, *in vitro* and *in vivo* studies showed that rATF produced from *P. pastoris* in this study could efficiently inhibit angiogenesis, endothelial cell migration, tumor cell invasion, and xenograft tumor growth, by competing with uPA for binding to cell surfaces. In addition, rATF induced significant tumor growth inhibition and survival benefits as compared with cyclophosphamide, a chemotherapy drug conventionally used for the treatment of several types of cancers. Moreover, at a relatively lower dosage (2 mg/kg/2 days) as compared with a previous study using *E. coli*-derived ATF [28], *P. pastoris*-derived rATF in this study induced more significant xenograft tumor growth inhibition, indicating that high protein activity was achieved when using a eukaryotic expression system. We assumed that the antitumor function of rATF was achieved by its suppressive capacity against angiogenesis, which owes to its competitive interaction with uPAR towards uPA. These results suggest that rATF produced in *P. pastoris* can efficiently block uPAR signaling and kill tumor cells, and therefore is an ideal uPAR-targeted therapeutic molecule.

In conclusion, we have expressed and purified rATF in *P. pastoris,* using a simplified purification protocol, at a high yield. We also show that the *P. pastoris*-produced rATF has an efficient effect in anti-angiogenesis and tumor therapy.

Acknowledgments

The authors are grateful for the grants from the Ministry of Science and Technology (2012CB967004, 2012AA020304, 2008BAI51B01, and 2012ZX09401012), the Chinese National Natural Sciences Foundation (81121062, 31200572, and 31071196), the Jiangsu Provincial Nature Science Foundation (BK2011228, BK2011573, BK2011048, and BZ2012050), and the Bureau of Science and Technology of Changzhou, Jiangsu, China (CZ20110028, CZ20120004, and CM20122003).

References

1. Andreasen PA, Kjoller L, Christensen L, Duffy MJ. 1997. The urokinase-type plasminogen activator system in cancer metastasis: a review. *Int. J. Cancer* **72:** 1-22.
2. Behrendt N, Ronne E, Ploug M, Petri T, Lober D, Nielsen LS, *et al*. 1990. The human receptor for urokinase plasminogen activator. NH_2-terminal amino acid sequence and glycosylation variants. *J. Biol. Chem.* **265:** 6453-6460.
3. Binder BR, Mihaly J, Prager GW. 2007. uPAR-uPA-PAI-1 interactions and signaling: a vascular biologist's view. *Thromb. Haemost.* **97:** 336-342.
4. Brondyk WH. 2009. Selecting an appropriate method for expressing a recombinant protein. *Methods Enzymol.* **463:** 131-147.
5. Cereghino JL, Cregg JM. 2000. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol. Rev.* **24:** 45-66.
6. Choong PF, Nadesapillai AP. 2003. Urokinase plasminogen activator system: a multifunctional role in tumor progression and metastasis. *Clin. Orthop. Relat. Res.* S46-S58.
7. Cregg JM, Tolstorukov I, Kusari A, Sunga J, Madden K, Chappell T. 2009. Expression in the yeast *Pichia pastoris*. *Methods Enzymol.* **463:** 169-189.
8. Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, Skriver L. 1985. Plasminogen activators, tissue degradation, and cancer. *Adv. Cancer Res.* **44:** 139-266.
9. Dass K, Ahmad A, Azmi AS, Sarkar SH, Sarkar FH. 2008. Evolving role of uPA/uPAR system in human cancers. *Cancer Treat. Rev.* **34:** 122-136.
10. Demain AL, Vaishnav P. 2009. Production of recombinant proteins by microbes and higher organisms. *Biotechnol. Adv.* **27:** 297-306.
11. Foekens JA, Peters HA, Look MP, Portengen H, Schmitt M, Kramer MD, *et al*. 2000. The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients. *Cancer Res.* **60:** 636-643.
12. Goldstein D, Gofrit O, Nyska A, Benita S. 2007. Anti-HER2 cationic immunoemulsion as a potential targeted drug delivery system for the treatment of prostate cancer. *Cancer Res.* **67:** 269-275.
13. Guo Y, Higazi AA, Arakelian A, Sachais BS, Cines D, Goldfarb RH, *et al*. 2000. A peptide derived from the nonreceptor binding region of urokinase plasminogen activator (uPA) inhibits tumor progression and angiogenesis and induces tumor cell death *in vivo*. *FASEB J.* **14:** 1400-1410.
14. Kim TD, Song KS, Li G, Choi H, Park HD, Lim K, *et al*. 2006. Activity and expression of urokinase-type plasminogen activator and matrix metalloproteinases in human colorectal cancer. *BMC Cancer* **6:** 211.
15. Kobayashi H, Gotoh J, Shinohara H, Moniwa N, Terao T. 1994. Inhibition of the metastasis of Lewis lung carcinoma by antibody against urokinase-type plasminogen activator in the experimental and spontaneous metastasis model. *Thromb. Haemost.* **71:** 474-480.
16. Koolwijk P, van Erck MG, de Vree WJ, Vermeer MA, Weich HA, Hanemaaijer R, *et al*. 1996. Cooperative effect of TNFalpha, bFGF, and VEGF on the formation of tubular structures of human microvascular endothelial cells in a fibrin matrix. Role of urokinase activity. *J. Cell Biol.* **132:** 1177-1188.
17. Laufs S, Schumacher J, Allgayer H. 2006. Urokinase-receptor (u-PAR): an essential player in multiple games of cancer: a review on its role in tumor progression, invasion, metastasis, proliferation/dormancy, clinical outcome and minimal residual disease. *Cell Cycle* **5:** 1760-1771.
18. Li H, Griscelli F, Lindenmeyer F, Opolon P, Sun LQ, Connault E, *et al*. 1999. Systemic delivery of antiangiogenic adenovirus AdmATF induces liver resistance to metastasis and prolongs survival of mice. *Hum. Gene Ther.* **10:** 3045-3053.
19. Li H, Soria C, Griscelli F, Opolon P, Soria J, Yeh P, *et al*. 2005. Amino-terminal fragment of urokinase inhibits tumor cell invasion *in vitro* and *in vivo*: respective contribution of the urokinase plasminogen activator receptor-dependent or -independent pathway. *Hum. Gene Ther.* **16:** 1157-1167.
20. Mars WM, Jo M, Gonias SL. 2005. Activation of hepatocyte growth factor by urokinase-type plasminogen activator is ionic strength-dependent. *Biochem. J.* **390:** 311-315.
21. Mohanam S, Chandrasekar N, Yanamandra N, Khawar S, Mirza F, Dinh DH, *et al*. 2002. Modulation of invasive properties of human glioblastoma cells stably expressing amino-terminal fragment of urokinase-type plasminogen activator. *Oncogene* **21:** 7824-7830.
22. Moreau P, Voillat L, Benboukher L, Mathiot C, Dumontet C, Robillard N, *et al*. 2007. Rituximab in CD20 positive multiple myeloma. *Leukemia* **21:** 835-836.
23. Odekon LE, Sato Y, Rifkin DB. 1992. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *J. Cell Physiol* **150:** 258-263.
24. Pollanen J, Stephens RW, Vaheri A. 1991. Directed plasminogen activation at the surface of normal and malignant cells. *Adv. Cancer Res.* **57:** 273-328.

25. Rabbani SA, Gladu J. 2002. Urokinase receptor antibody can reduce tumor volume and detect the presence of occult tumor metastases *in vivo*. *Cancer Res.* **62:** 2390-2397.
26. Shih T, Lindley C. 2006. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. *Clin. Ther.* **28:** 1779-1802.
27. Sidenius N, Blasi F. 2003. The urokinase plasminogen activator system in cancer: recent advances and implication for prognosis and therapy. *Cancer Metastasis Rev.* **22:** 205-222.
28. Sun Q, Xu Q, Dong X, Cao L, Huang X, Hu Q, *et al*. 2008. A hybrid protein comprising ATF domain of pro-UK and VAS, an angiogenesis inhibitor, is a potent candidate for targeted cancer therapy. *Int. J. Cancer* **123:** 942-950.
29. Sun QM, Cao L, Fang L, Chen C, Dai J, Chen LL, *et al*. 2005. Expression, purification of human vasostatin120-180 in *Escherichia coli*, and its anti-angiogenic characterization. *Protein Expr. Purif.* **39:** 288-295.
30. Wang X, Hou M, Tan L, Sun X, Zhang Y, Li P, *et al*. 2005. A hybrid protein of the amino-terminal fragment of urokinase and mutant plasminogen activator inhibitor-2 efficiently inhibits tumor cell invasion and metastasis. *J. Cancer Res. Clin. Oncol.* **131:** 129-136.
31. Zhao G, Yuan C, Bian C, Hou X, Shi X, Ye X, *et al*. 2006. Protein expression and preliminary crystallographic analysis of amino-terminal fragment of urokinase-type plasminogen activator. *Protein Expr. Purif.* **49:** 71-77.