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Journal of Chromatography B, 857 (2007) 231–239
JOURNAL OF CHROMATOGRAPHY B
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Purification and characterization of RGD tumor-homing peptide conjugated human tumor necrosis factor α over-expressed in *Escherichia coli*

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Received 17 April 2007; accepted 14 July 2007
Available online 22 July 2007

Abstract

A number of approaches have been investigated to enhance the selective toxicity of tumor necrosis factor α (TNFα) to permit its systemic use in cancer therapy. Because vascular targeting has been proven to be a valid strategy for improving the therapeutic index of TNFα, we prepared RGD-hTNF consisting of human TNF fused with the ACDCRGDCFCG peptide, a ligand of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins. Recombinant RGD-hTNF was produced in *Escherichia coli* as a polyhistidine fusion protein. Between polyhistidine tag and RGD-hTNF, a tobacco etch virus (TEV) protease cleavage site (ENLYFQG) was introduced to ensure the release of intact RGD-hTNF. The purification strategy consisted of the target protein capture step by immobilized metal affinity chromatography (IMAC), TEV protease cleavage of fusion protein, the subtractive depletion of removed His-tag by IMAC and the final gel filtration step. As a result, about 18 mg of intact RGD-hTNF was obtained from 1 l of bacteria culture. The purified RGD-hTNF was characterized by SDS-PAGE, Western blot, mass spectroscopy and gel filtration. Since the RGD-hTNF molecule retained the cytotoxic activity of the TNF moiety and the integrin binding ability of the RGD moiety, the purification method provided material for assessing its anti-tumor activity in animal model.

Keywords: Tumor necrosis factor α; RGD peptide; Fusion protein; Immobilized metal affinity chromatography; Enterokinase; TEV protease

1. Introduction

Human tumor necrosis factor α (TNFα) is a multifunctional cytokine playing a key role in apoptosis and cell survival as well as in inflammation and immunity. Currently it is used in cancer treatment in the isolated limb perfusion (ILP) for soft tissue sarcoma (STS), irresectable tumors of various histological types, and melanoma in-transit metastases confined to the limb [1–4]. Therapeutically effective doses of TNFα cannot be given systemically because of the unacceptably high level of systemic toxicity. The selective targeted delivery of TNFα has been proved to be a valid strategy to improve the therapeutic effects and to reduce the toxic side effects of systemically administered TNFα. Most of targeting strategies are based on antibodies or natural ligands, which can recognize tumor or tumor stroma markers [5,6]. Besides these markers, the ligands of angiogenic markers have been used for targeting TNF to tumor vasculature [7–12]. Targeted delivery of TNF to tumor vessels was achieved by coupling TNF with cyclic CNGRC peptide, an aminopeptidase N (CD13) ligand that targets the tumor neovasculature [9,10]. Further, ACDCRGDCFCG-mouse TNF conjugate (RGD-mTNF) has been proved to have the similar vascular targeting property [11]. These TNF conjugates can increase the local concentration of chemotherapeutic drugs in tumors and their therapeutic index at very low doses [13–15].

Using *in vivo* panning of peptide phage libraries, Ruoslahti et al. identified phage containing ACDCRGDCFC homed to several tumor types (including carcinoma, sarcoma, and melanoma) in a highly selective manner. They have shown the motif can selectively bind to $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin [16–18]. Tumor vasculature is known to over-express $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin. Therefore, the RGD peptide can be very effective in delivering cytotoxic drugs or antibodies to tumor vasculature [11,19].

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1570-0232/$ – see front matter © 2007 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2007.07.022

It is necessary to obtain RGD-hTNF composed of human TNF and the RGD motif to support its further applications in biological and clinical research. In the previous work, Curnis et al. prepared RGD-mTNF using conventional chromatographic separation techniques including hydrophobic interaction chromatography, ion exchange chromatography and gel filtration chromatography [11]. Recently, Wang et al. developed a strategy for purification of RGD4C-rmhTNF composed of human TNF mutant (rmhTNF) and CDCRGDCFC peptide, including ammonium sulfate precipitation and two-step ion exchange chromatography [20]. In our preliminary experiments, we tried to purify RGD-hTNF using these conventional chromatographic techniques, but RGD-hTNF was difficult to recover from crude extracts with the desired purity. Affinity tags are highly efficient tools for the purification of protein without any prior knowledge of its biochemical properties. So, in the present study, recombinant RGD-hTNF was produced in *E. coli* as a polyhistidine fusion protein. The fusion protein was easily purified from crude bacterial extracts by a single immobilized metal affinity chromatography (IMAC).

In the present paper, we report an improved strategy for purification of RGD-hTNF. Fusion proteins were initially purified by the first IMAC capture. After cleavage of tags by recombinant TEV protease, the unwanted byproducts of the digestion were separated from the target protein by the second IMAC capture. A gel filtration step was employed to improve the purity of the target protein. The final product was characterized by SDS-PAGE, Western blot, mass spectroscopy and gel filtration. Furthermore, we evaluated the biology activity of RGD-hTNF by *in vitro* cytotoxicity assay and cell adhesion assay.

2. Experimental

2.1. Construction of expression plasmids

The plasmid pUC18-TNF encoding human TNF-α was a gift from Dr. Xu Xianxiu (Nanjing University, China) and was used as a template for polymerase chain reaction (PCR). The plasmid pET23a hTNF for the expression of human TNF-α was constructed in our laboratory. The clone was further modified to incorporate the RGD sequence at the amino terminus. Sequence encoding ACDCRGDCFCG was inserted, by PCR, upstream of the TNF-coding region, to generate pET23a RGD-hTNF. The upstream primer: 5′-g gaa ttc cat atg gca tgc gac tgc cgt ggt gac tgc ttc tgc ggt gtt cgt tct tct tcc cgt act-3′; the downstream primer: 5′-cg gga tcc tca cag agc gat aat acc gaa-3′. Primer sequences were designed to introduce the Nde I and BamH I restriction sites for the convenience of cloning in pET23a plasmid (Novagen, Madison, WI, USA). A glycine residue between ACDCRGDCFC and the coding region was used as a spacer.

In order to achieve efficient affinity purification of the modified protein, the plasmid pET28a was used to introduce available polyhistidine tag. Additionally, in-frame enterokinase recognition site DDDDK or TEV protease recognition site ENLYFQG was introduced for tag removal. N-terminal amino acid sequences of tagged and cleaved proteins are listed in Table 1.

The cDNA coding for His-EK-RGD-hTNF was obtained by PCR from plasmid pET23a RGD-hTNF. The following primers were used to introduce enterokinase cleavage site DDDDK. The upstream primer: 5′-cg gaa ttc gat gac gat gac aaa gca tgc gac tgc cgt ggt g-3′; the downstream primer: 5′-a ccg ctc gag tta cag agc gat aat acc gaa g-3′. Primer sequences were designed to introduce the EcoR I and Xho I restriction sites for cloning in pET28a plasmid (Novagen, Madison, WI, USA).

The cDNA coding for His-TEV-RGD-hTNF was obtained by PCR from plasmid pET23a RGD-hTNF. The following primers were used to introduce TEV protease cleavage site ENLYFQG. The upstream primer: 5′-cg gaa ttc gaa aac ctg tat ttt cag ggc gca tgc gac tgc cgt ggt g-3′; the downstream primer: 5′-a ccg ctc gag tta cag agc gat aat acc gaa g-3′. Primer sequences were designed to introduce the EcoR I and Xho I restriction sites for cloning in pET28a plasmid (Novagen, Madison, WI, USA).

2.2. Expression and purification of his-tagged fusion protein

The his-tagged fusion protein was expressed in *E. coli* BL21 (DE3) cells (Novagen, Madison, WI, USA). Soluble recombinant protein was recovered from 1-l culture by bacterial sonication in 1 mM PMSF, 20 mM Tris–HCl buffer, pH 8.0, followed by centrifugation (12,000 rpm, 20 min, 4 °C). Solid ammonium sulfate was added to the supernatant to 25% saturation, after which the solution was clarified again by centrifugation as above. The supernatant was then adjusted to 75% saturation with solid ammonium sulfate and incubated on ice for 2 h. After centrifugation, the pellet was dissolved in 150 mM sodium chloride, 50 mM sodium phosphate buffer, pH 8.0. The lysate was loaded onto Ni^{2+} affinity column (1.6 cm × 5 cm) (Sigma, St. Louis, USA) equilibrated with equilibration buffer (150 mM NaCl, 50 mM sodium phosphate, pH 8.0). Non-specific binding proteins were washed with wash buffer (50 mM imidazole, 150 mM NaCl, 50 mM sodium phosphate, pH 8.0). His-tagged fusion protein was eluted with elution

Table 1
N-terminal amino acid sequence of fusion proteins and the cleavage sites

Protein	N-terminal amino acid sequence
His-EK-RGD-hTNF	MGSSHHHHHH. . .GSEFDDDDK*↓**ACDCRGDCFC**GVR↓SSSRTPSDK
His-TEV-RGD-hTNF	MGSSHHHHHH. . .GSEFENLYFQ*↓G**ACDCRGDCFC**GVRSSSRTPSDK

*Asterisk designates the expected cleavage site of the fusion protein. ↓ Arrow designates the actual cleavage site detected by ESI-TOF. The sequence of RGD moiety is marked with bold letters.

buffer (500 mM imidazole, 150 mM NaCl, 50 mM sodium phosphate, pH 8.0). Imidazole in the eluent was removed by Sephadex G-25 gel filtration. Meanwhile, the buffer was changed to 50 mM Tris–HCl buffer, pH 8.0.

2.3. Cleavage of fusion protein by protease

Enterokinase cleavage of fusion protein was performed as described [21]. The fusion protein was incubated with recombinant EK_L-His_6 at 16 °C. Various EK_L-His_6 concentrations and incubation times were tested to optimize the specificity and efficiency of cleavage as described in results. EK_L-His_6 and His-EK-RGD-hTNF (100 μg) were mixed in the mass ratio 1:10,000 and 1:20,000, respectively at 16 °C in 50 mM Tris–HCl buffer, pH 8.0. Samples of the reaction mixture were taken after 0.5, 1, 8, 16 and 30 h for SDS-PAGE analysis.

Recombinant tobacco etch virus (TEV) protease containing a His-tag (His_6-TEV) was used to cleave the affinity-purified His-TEV-RGD-hTNF as described previously [22]. For cleavage specificity and efficiency evaluation, different masses of His_6-TEV were added to 6 μg His-TEV-RGD-TNF in a final volume of 30 μl at 4 °C for 12 h or at 30 °C for 5 h. The mass ratio of TEV enzyme to substrate were 1:5, 1:10, 1:20, 1:50 and 1:100, respectively. Reaction buffer was 50 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT.

For large-scale digestion, after the appropriate adjustment of reaction volume, 20 mg His-TEV-RGD-hTNF was cleaved with 4 mg His_6-TEV in a final volume of 50 ml at 4 °C for 12 h.

2.4. Purification of recombinant RGD-hTNF

After digestion, the buffer of the reaction mixture was exchanged to 150 mM sodium chloride, 50 mM sodium phosphate buffer, pH 8.0 using a Sephadex G-25 column. In order to remove the polyhistidine tag, un-cleaved protein, His_6-TEV protease and impurities, the cleaved sample was loaded on a Ni^{2+} affinity column (1.6 cm × 5 cm) to remove the remaining un-cleaved protein. The flow-through fraction containing RGD-hTNF was further purified using HiPrep 16/60 Sephacry S-200 HR column on $ÄKTA_{FPLC}$ (Amersham Biosciences, Uppsala, Sweden) system. The final product was stored in multiple aliquots at −80 °C.

Protein concentration was determined by Bradford method. The crude lysates and purified hTNF conjugate were analyzed by 15% SDS-PAGE. Coomassie blue stained gels were scanned using UVP white/ultraviolet transilluminator and quantitative analysis was performed using Grab-it 2.5 and Gelwork software.

2.5. Electrospray ionization mass spectrometry (ESI-MS) analysis

The high resolution MS analysis was performed on an orthogonal time-of-flight mass spectrometer (Agilent Corp., Santa Clara, CA, USA) equipped with an ESI source. All spectra were recorded in positive mode in 100–3000 *m/z* mass range. The conditions of the ESI source were as follows: the capillary temperature, 300 °C; capillary voltage, 3500 V; drying gas (N_2) flow rate, 9 l/min; nebulizer (N_2) pressure, 35 psi.

Prior to mass analysis, the samples were desalted on Centricon PM10 (cut off = 10,000 Da) micro-concentrators (Amicon, Millipore, Bedford MA, USA) in 10 mM ammonium acetate (pH 6.8). At least eight dilution/concentration steps were performed at 4 °C and 4000 rpm. Protein analytes were dissolved in 50% aqueous acetonitrile containing 0.1% formic acid. The molecular weight was estimated by mass analysis in denatured conditions.

All the operations, acquisition and analysis of data were controlled by Agilent LC-MS TOF Software Version A.01.00 (Agilent Technologies, USA) and Agilent TOF Protein Confirmation Version A.01.00 (Agilent Technologies, USA), respectively.

2.6. Western blot analysis

TNF mutants were resolved on 15% SDS-PAGE under reducing condition and transferred to PVDF membranes. After blocking with 5% non-fat milk for 1 h, the PVDF membrane was incubated with anti-human TNFα monoclonal antibody (The Fourth Military Medical University, Xi'an, China). Then the membrane was washed three times with phosphate buffered saline Tween-20 (PBST) and followed by incubation for 1 h with HRP conjugate of anti-mouse antibody. The membrane was washed and then developed with enhanced chemiluminescence (ECL) detection system (Cell Signaling, USA) followed by exposure to X-ray film.

2.7. In vitro cytotoxicity assay

The biologic activity of TNF was determined by using a standard assay, which depends on cytotoxicity in L-929 murine fibroblast cells as previously described [23]. Briefly, 5×10^3 cells were seeded into 96-well microtiter plates in 100 μl of culture medium and incubated 16 h at 37 °C, 5% CO_2. Ten-fold serial of TNF were added in the presence of 1 μg/ml actinomycin D, and after incubation at 37 °C, 5% CO_2 for 24 h, the cytotoxic activity was quantified by standard 3-(4, 5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide assay.

2.8. Cell adhesion assay

Polyvinyl chloride microtiter plates were coated with TNF preparations at various concentrations (100 μl/well in PBS) or 0.2% gelatin as a positive control. After washing with PBS, each well was filled with PBS containing 2% BSA to block non-specific binding sites and washed again. ECV304 cells were harvested, washed with PBS, resuspended in incomplete DMEM and added to wells at a density of 50,000 cells/well. After incubation for 30 min at 37 °C, 5% CO_2, unbound cells were removed by washing with PBS. Adherent cells were stained with 20% methanol containing 0.5% crystal violet. The cells were solubilized in 33% acetic acid and quantified by reading the absorbance of each well at 550 nm using a microplate reader.

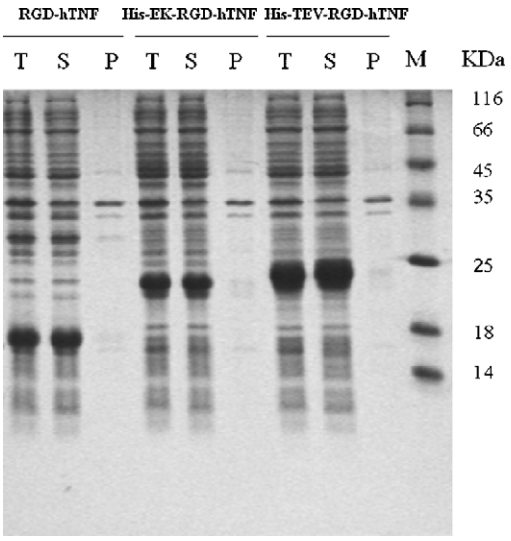

Fig. 1. Analysis of total protein expression, solubility of RGD-hTNF, His-EK-RGD-hTNF and His-TEV-RGD-hTNF using SDS-PAGE. *E. coli* BL21 (DE3) cells harbouring individual expression plasmid were grown in LB medium containing 100 μg/ml ampicillin or 50 μg/ml kanamycin to an optical density of 0.8-1 at 600 nm. The culture was then induced at 37 °C for an additional 3 h by adding isopropyl β-D-thiogalactopyranoside (IPTG) at a final concentration of 0.6 mM. Harvested cells from 1 ml of bacteria culture were re-suspended in 300 μl of 20 mM Tris–HCl buffer, pH 8.0 and lysed by sonication. The total bacterial lysates (T) were divided into soluble (S), and insoluble or pellet (P) fractions. The insoluble fraction was suspended in a volume equal to that of the soluble fraction. Protein size markers (M) are indicated in kDa.

3. Results

3.1. Effect of the RGD moiety on protein expression and purification

Human TNF has been successfully over-expressed in *E. coli*, and recovered as soluble homotrimeric protein with high yield. We tested the effect of RGD modification on expression and solubility of RGD-hTNF. To investigate the expression level of soluble recombinant proteins, the cells were harvested and sonicated. The cell lysates were separated by centrifugation into soluble supernatant and insoluble pellet, and each fraction was analyzed with SDS-PAGE. As shown in Fig. 1, the expressed RGD-hTNF was mostly recovered from the soluble fraction, and the expression level was about 27% of the total soluble proteins of *E. coli*. Though the expression level of RGD-hTNF was well, its purification was laborious and inefficient. At the beginning of the study, we tried to purify RGD-hTNF in non-fusion form by ammonium sulfate precipitation, hydrophobic chromatography, ion exchange, and gel filtration chromatography as described. Though hTNF was easily purified by the strategy, RGD-hTNF was difficult to recover from crude extracts with the desired purity. So the recombinant protein RGD-hTNF was expressed as polyhistidine-tagged protein for affinity purification.

When RGD-hTNF was introduced a polyhistidine tag by cloning the target gene directionally into pET28a vector, well-expressed and highly soluble proteins were obtained. The expression levels of His-EK-RGD-hTNF and His-TEV-RGD-hTNF were 26% and 40% of the total soluble proteins, respectively.

3.2. Affinity purification of His-EK-RGD-hTNF and His-TEV-RGD-hTNF

In order to achieve efficient purification of RGD-hTNF, a hexa-histidine tag was introduced to the N-terminus of the molecular. Recombinant His-EK-RGD-hTNF or His-TEV-RGD-hTNF fusion protein was purified by one step Ni^{2+} affinity chromatography. After optimizing imidazole concentration, wash buffer containing 50 mM imidazole was used to reduce non-specific protein binding. The target protein was eluted from column with high purity by elution buffer containing 500 mM imidazole. The two polyhistidine-tagged proteins displayed good chromatographic behavior. All fractions were analyzed by SDS-PAGE and the gels were stained with Coomassie Brilliant Blue as shown in Fig. 2 and Fig. 3. Purified His-EK-RGD-hTNF or His-TEV-RGD-hTNF was exhibited as a homogenous band corresponding to the molecular weight of 22 kDa.

3.3. Cleavage of His-EK-RGD-hTNF and His-TEV-RGD-hTNF

To keep the cytotoxicity of hTNF moiety, the polyhistidine tag must be removed from the fusion protein by a protease.

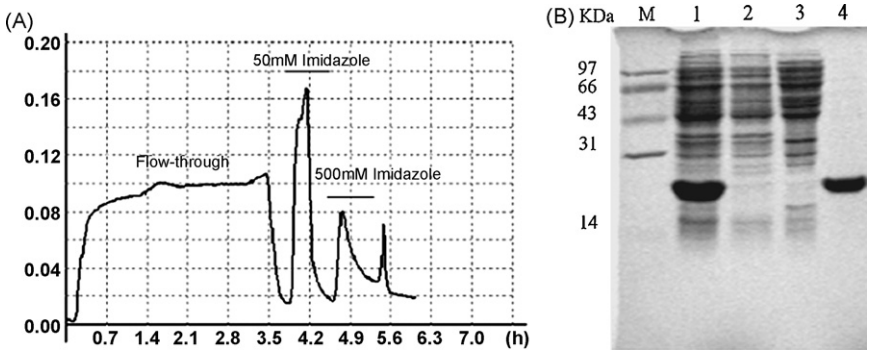

Fig. 2. Affinity chromatogram (A) and SDS-PAGE (B) of His-EK-RGD-hTNF fusion protein from Ni^{2+} affinity column. After ammonium sulfate precipitation, the soluble fraction was loaded onto Ni^{2+} affinity column, and then washed and eluted with different concentrations of imidazole. Lane M, molecular weight markers; lane 1, the crude lysate after ammonium sulfate precipitation; lane 2, the flow-through fraction; lane 3, the wash fraction at 50 mM imidazole; lane 4, the elution fraction at 500 mM imidazole.

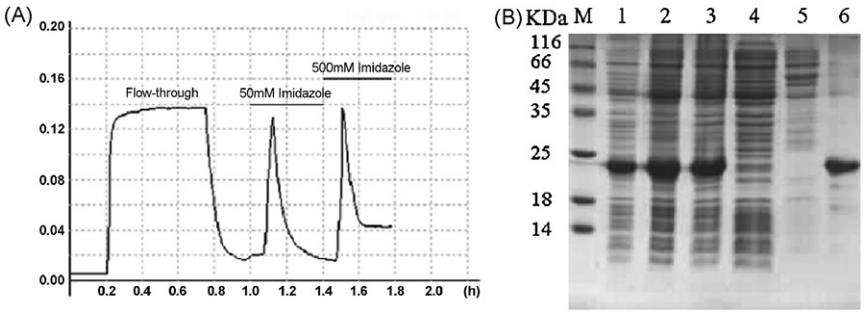

Fig. 3. Affinity chromatogram (A) and SDS-PAGE (B) of His-TEV-RGD-hTNF fusion protein from Ni^{2+} affinity column. After ammonium sulfate precipitation, the soluble fraction was loaded onto Ni^{2+} affinity column and then washed and eluted with different concentrations of imidazole. Lane M, molecular weight markers; lane 1, the total bacterial lysate; lane 2, the soluble fraction; lane 3, the crude lysate after ammonium sulfate precipitation; lane 4, the flow-through fraction; lane 5, the wash fraction at 50 mM imidazole; lane 6, the elution fraction at 500 mM imidazole.

First, an enterokinase recognition site, DDDDK, was introduced between polyhistidine tag and RGD-hTNF for the release of intact RGD-hTNF without any remaining amino acid residues. The specificity and efficiency of cleavage were tested. Fig. 4 shows the time course for cleavage of His-EK-RGD-hTNF using the mass ratio 1:10,000 or 1:20,000 (EK_L-His_6: substrate) at 16 °C. The cleavage of His-EK-RGD-hTNF was obviously after 8 h incubation. However, the target protein released by EK_L-His_6 was not homogeneous. An extra band smaller than RGD-hTNF appeared, which suggests RGD-hTNF has a non-specific cleavage site recognized by enterokinase. When incubation time increased, the band corresponding to RGD-hTNF decreased in intensity and shift to a lower molecular weight degradation product.

Since RGD-hTNF was non-specifically released by enterokinase, a TEV recognition site was inserted into the fusion protein to substitute for the enterokinase cleavage site. It has been reported that TEV protease is active over a broad temperature range [24]. So we tested His-TEV-RGD-hTNF cleavage by His_6-TEV at 4 °C for 12 h or at 30 °C for 5 h. To optimize TEV protease cleavage, different ratios of His_6-TEV to substrate were tested as shown in Fig. 5. The cleavage of His-TEV-RGD-hTNF was indicated by the appearance of the single band of 18 kDa for RGD-hTNF. However, incubation of His-EK-RGD-hTNF with enterokinase resulted in two fragments. The released large fragment had the same migration as RGD-hTNF protein released by TEV, which confirmed that the small fragment was non-specific cleavage product. His_6-TEV can cleave His-TEV-RGD-hTNF in a dose-dependent manner. Most of His-TEV-RGD-hTNF was cleaved using the ratio 1:5, 1:10 or 1:20 (His_6-TEV to substrate). At the same enzyme/substrate ratio, the cleavage efficiency at 30 °C for 5 h was similar to that at 4 °C for 12 h. But the cleaved RGD-hTNF protein released by TEV at 30 °C displayed a slight diffuse migration band on the gel. These results indicated that the cleavage of His-TEV-RGD-hTNF by TEV was specific and efficient at low temperature. So the digestion temperature was selected at 4 °C and the enzyme/substrate ratio was selected as 1:5 for complete cleavage in large-scale digestion.

3.4. An improved purification of RGD-hTNF

Our protein purification scheme relies on the Nickel binding ability of the polyhistidine tag. His-TEV-RGD-hTNF fusion protein was initially purified by 1st IMAC capture. The tag was

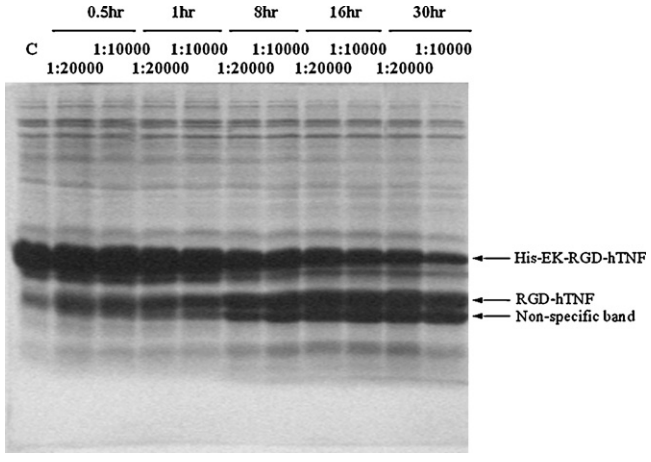

Fig. 4. Cleavage of His-EK-RGD-hTNF fusion protein with enterokinase protease. The incubation time and the mass ratio for EK_L-His_6 to substrate are indicated. Lane C represents the uncut fusion protein control. The arrows indicate the location of His-EK-RGD-hTNF, released RGD-hTNF and non-specific cleavage product, respectively.

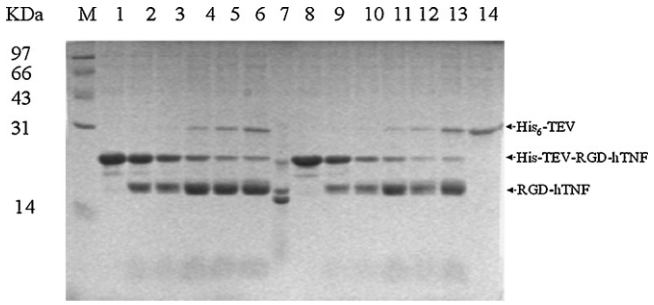

Fig. 5. Cleavage of His-TEV-RGD-hTNF fusion protein with TEV protease. Lane M: molecular weight markers; lane 1, uncut fusion protein incubated at 4 °C for 12 h; lanes 2–6, the digestion reactions were carried out at 4 °C for 12 h, the mass ratios for His_6-TEV to substrate were 1:100, 1:50, 1:20, 1:10 and 1:5, respectively; lane 7, the cleaved protein mixture of His-EK-RGD-hTNF by enterokinase; lane 8, uncut fusion protein incubated at 30 °C for 5 h; lanes 9–13, the digestion reactions were carried out at 30 °C for 5 h, the mass ratios for His_6-TEV to substrate were 1:100, 1:50, 1:20, 1:10 and 1:5, respectively; lane 14, His_6-TEV only. The arrows indicate the location of His_6-TEV, His-TEV-RGD-hTNF and released RGD-hTNF, respectively.

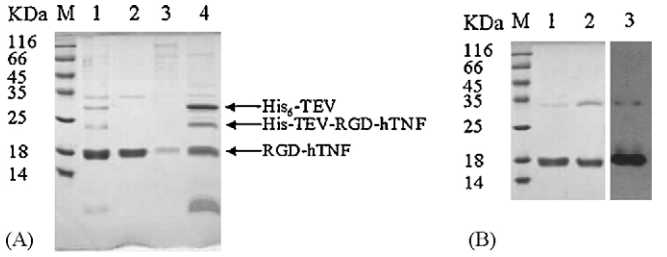

Fig. 6. (A) Purification of recombinant RGD-hTNF during the second IMAC step for removal of polyhistidine tag and His_6-TEV protease. The flow-through fraction contained the majority of the target proteins, while the elution fraction at 500 mM imidazole contained His_6-TEV protease and the uncleaved proteins. Lane M, molecular weight markers; lane 1, the reaction mixture after TEV treatment; lane 2, the flow-through fraction; lane 3, the wash fraction at 50 mM imidazole; lane 4, the elution fraction at 500 mM imidazole. (B) SDS-PAGE and Western blot analysis of the purified RGD-hTNF. Lane M, molecular weight markers. Lane 1 represents RGD-hTNF under reducing conditions. Lane 2 represents RGD-hTNF under non-reducing condition. Lane 3 represents Western blot of RGD-hTNF under reducing conditions.

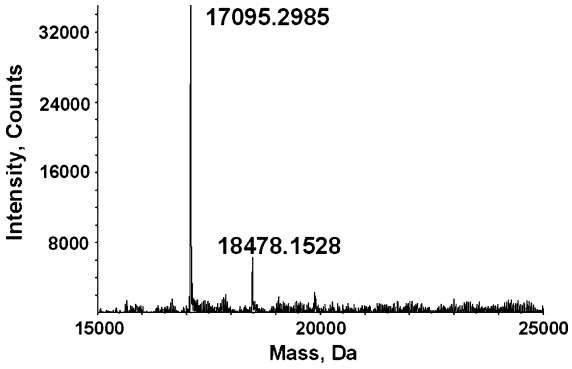

Fig. 7. Deconvoluted ESI-TOF mass spectrum of the reaction mixture after digestion of His-EK-RGD-hTNF by enterokinase. The 18,478 Da peak represents the signal of the cleavage product at the engineered DDDDK site. The 17,095 Da peak represents the non-specific product corresponding to cleavage after Arg2 of hTNF motif.

cleaved from the purified fusion protein using a polyhistidine-tagged version of TEV protease (His_6-TEV). The polyhistidine tag, the recombinant TEV and the un-cleaved protein were separated from the target protein through subtractive IMAC purification as shown in Fig. 6A. After the subtractive purification, the homotrimers were further purified by gel filtration. Because hTNF has been proved to be a compact homotrimeric protein [25–27], only fractions corresponding to trimeric species were collected during the final gel filtration chromatography. The final product was analyzed by gel filtration chromatography on the same column. The retention volume of RGD-hTNF was consistent with the retention time of hTNF, which corresponds to trimers (data no shown). The purity of the preparation and the molecular mass of the RGD-hTNF protein were checked by ESI-MS.

Reducing SDS-PAGE of RGD-hTNF showed a major band with molecular weight of 18 kDa for RGD-hTNF monomers and a minor band with molecular weight of 36 kDa for non-reducible dimmers. The fact that these bands were immunoreactive by anti-human TNF-α monoclonal antibody suggested that they correspond to RGD-hTNF monomers and dimers. The formed non-reducible dimmers for hTNF were also observed elsewhere [9,28,29]. Under non-reducing condition, the level of dimers was about 17% of the total protein, suggesting the presence of subunits with intra- and inter-chain disulfide bridges. The 18 kDa subunits with intra-chain disulfide bridges accounted for about 82% of the total material. Table 2 shows the yield of each step of RGD-hTNF purification. As a result, about 18 mg of pure RGD-hTNF was obtained from 1 l of bacteria culture.

3.5. Characterization of the protein of interest after cleavage by ESI-MS

In order to reveal the non-specific cleavage site in His-EK-RGD-hTNF fusion protein, the reaction mixture after digestion was subjected to ESI-MS. The mass spectrum after deconvolution of the raw data shows a minor peak of 18,478 Da and a major peak of 17,095 Da (Fig. 7). Analysis of the amino acid sequence indicated that the former value corresponded to the cleavage product at the expected DDDDK site (M_w = 18,484 Da) and the latter value corresponded to the mass of $hTNF_{3\text{-}157}$ (M_w = 17,097 Da). This suggested that enterokinase had recognized a secondary cleavage site within the target protein. The truncated form was produced by cleavage after Arg2 of hTNF motif.

It is necessary to characterize the protein of interest after cleavage from the affinity label to assure that there are no changes in covalent structure of the protein of interest [30]. The purified protein was subjected to ESI mass spectrometry to confirm identity and integrity. The major signals observed in the mass spectrum were due to multiply charged ions of RGD-hTNF monomer with ions ranging from *m/z* 900 to *m/z* 2800. The expected molecular mass of RGD-hTNF subunit is 18,541 Da (Fig. 8). The deconvoluted mass spectrum of

Table 2
Purification of RGD-hTNF from *E. coli* extract (1 l of fermentation)[a]

Fractions	Total protein[b] (mg)	Purity[c] (%)	RGD-hTNF (mg)	Step recovery (%)
Crude extract	235.3	34.1	80.2	100
Ammonium sulfate precipitation	159.0	35.0	55.7	69.5
The capture step by IMAC-1	61.7	76.4	47.1	84.6
The subtractive step by IMAC-2	35.3	89.0	31.4	66.7
Gel filtration chromatography	17.8	95.9	17.1	54.5

[a] Data are normalized to quantities obtained per liter growth medium that approximately 3.6 g wet weight of cells.
[b] Determined by Bradford method.
[c] Determined by densitometric scanning of the SDS-PAGE gel.

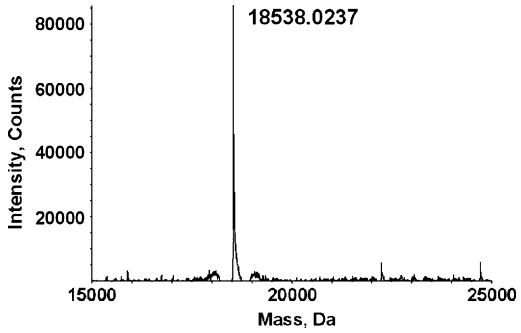

Fig. 8. Deconvoluted ESI-TOF mass spectrum of RGD-hTNF obtained under denaturing conditions. The 18,538 Da peak represents the intact RGD-hTNF released by TEV protease at the engineered ENLYFQG site.

RGD-hTNF showed an intense peak corresponding to a mass of 18,538 Da, in good agreement with the value expected for RGD-hTNF monomer. Thus the 18,538 Da peak represents the full-length target protein.

3.6. Biological activity of RGD-hTNF

The *in vitro* cytolytic activities of hTNF and RGD-hTNF were measured by standard cytolytic assay with L929 mouse fibroblasts. The *in vitro* cytolytic activities of hTNF and RGD-hTNF were 2.45×10^7 and 4.15×10^7 units/mg, respectively. These results indicate that the RGD domain of RGD-hTNF does not interfere with TNF receptor binding and RGD-hTNF maintains the biological activity of the TNF moiety.

RGD-peptide, as a ligand of $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin, is well known for its binding to integrins on the surface of endothelial cells. To determine whether the addition of RGD-motif to hTNF could enhance its binding capability to endothelial cells, the binding specificity of RGD-hTNF was tested against ECV304 cells. At the beginning, the expression of integrins on the membrane of ECV304 cells was detected using flow cytometry. Integrin flow cytometry analysis with specific antibodies showed that ECV304 cells express both $\alpha_v\beta_3$ integrin and $\alpha_v\beta_5$ integrin as expected (data no shown). So ECV304 cells were used to evaluate the binding activity of RGD-hTNF. BSA-blocked wells were used as a negative control, and 0.2% gelatin-coated wells were used as a positive control. We observed adhesion and spreading of ECV304 cells on plates coated with RGD-hTNF but not with hTNF (Fig. 9A and B). RGD-hTNF could increase cell attachment in a dose-dependent manner. Cell adhesion was completely abrogated by depletion of Ca^{2+} and Mg^{2+} with EDTA (data not shown), as expected for divalent ion-dependent integrin binding. These results suggest that the RGD domain of RGD-hTNF fold properly and is able to interact with adhesion receptors on the surface of ECV304 cells.

4. Discussion

Curnis et al. firstly reported that targeted delivery of TNF to tumor vessels by coupling TNF with cyclic CNGRC peptide could enhance the anti-tumor effect of TNF. About 17.25 mg of murine NGR-TNF and 16 mg human NGR-TNF could be purified from 11 culture using conventional chromatographic techniques [9]. However, RGD modification resulted in a low production and about 2 mg of purified RGD-mTNF was recovered from 11 of *E. coli* culture using the similar scheme [11]. These results suggest that RGD modification and NGR modification have different effects on expression and purification. In this paper, we expressed and purified intact RGD-hTNF by his-tag fusion strategy. We unambiguously demonstrated that recombinant His-EK-RGD-hTNF or His-TEV-RGD-hTNF could be eluted from Ni^{2+} affinity column with high purity by imidazole as high as 500 mM with good chromatographic behavior. In previous reports, various histidine-tags were fused to the N-terminus of full-length TNF and to the truncated form (dN6) of TNF. Because of very strong binding for affinity resin, the fusion proteins had to be recovered by elution buffer containing EDTA [31,32]. To avoid the difficulty that the previous reports had met with, our purification procedure was designed different from the reported methodology in both the polyhistidine tag length and position.

Enterokinase and recombinant enterokinase light chain (rEK_L) have been used widely to cleave fusion proteins with the target sequence of $(Asp)_4$-Lys. Enterokinase is able to

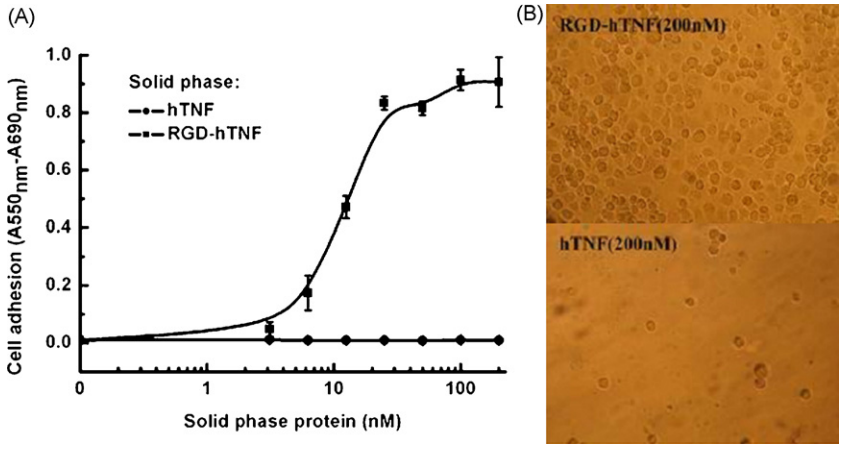

Fig. 9. Adhesion of ECV304 cells to solid phases coated with RGD-hTNF or hTNF. Cell adhesion assay was performed as described in Section 2. Dose-dependent cell adhesion kinetic analysis of RGD-hTNF or hTNF (A). Microphotograph of well coated with 100 nM RGD-hTNF or hTNF, at 200× magnifications (B).

cleave fusion protein without leaving any remaining amino acid residues at the N-terminus of the target protein, which makes it more ideal for tag removal. In our previous studies, the purified EK_L-His_6 demonstrated excellent cleavage activity towards fusion proteins containing the recognition sequence [33,34]. In the case of His-EK-RGD-hTNF, degradation pattern was observed and a secondary cleavage site was identified after Arg2 of hTNF moiety using ESI-MS. Kenig et al. had observed non-target cleavage of hTNF moiety when His_{10}-TNF was digested with recombinant enterokinase previously. N-terminal sequencing showed that 80% of cleavage occurred after the first arginine (Arg2) and 20% after the second (Arg6) [32]. These data show that enterokinase is not appropriate for digesting the fusion protein containing hTNF moiety. Since RGD-hTNF was non-specifically released by enterokinase, a TEV recognition site was created in the fusion protein. Highly specific and efficient cleavage of His-TEV-RGD-hTNF at 4 °C was achieved as expected, and there was no non-specific degradation occurred on the final product RGD-hTNF.

The use of affinity tags provides a generic strategy for purification. The strategy might make it possible to purify proteins that would be difficult or even impossible to obtain by traditional techniques. Affinity-based purification methods have been developed in several areas of research, particularly in high-throughput protein production for structure genomics. Most of the studies employ a two-step affinity chromatography procedure, with an optional polishing step to improve the purity of the target protein [35,36]. Our purification process included the first IMAC capture, the cleavage of the fusion protein with polyhistidine-tagged TEV protease, the second IMAC to capture the unwanted byproducts of the digestion (polyhistidine tag, His_6-TEV, and any undigested fusion protein). Though the cleavage efficiency was good (about 93% of the fusion protein), the final yield was compromised by the interaction between undigested His-TEV-RGD-hTNF and final product RGD-hTNF. So as we observed in Fig. 6A, the band of RGD-hTNF appeared in both flow-through fraction and the elution fraction. In a previous study, Kenig et al. detailed the effect of the cleavage efficiency and the degree of oligomerization of the protein on the final yield using hTNF fusion protein bearing different histidine tags as a model protein. Despite high cleavage efficiency (95%), the yield from the second IMAC was rather low, less than 65%. In the flow-through fraction, there was only cleaved protein. In the elution fraction, there were both uncleaved and cleaved proteins with a ratio of 1:1.76. Since one tag is sufficient for binding to IMAC resin, each oligomeric molecule bearing even single histidine tag is lost during the second IMAC [32]. In our research for the purification of RGD-hTNF, the ratio for uncleaved protein to cleaved protein in the elution fraction was 1:2.5, giving that the most of trimers have one histidine-tagged subunits. The yield from the second IMAC step was about 66.7%. These results suggest that the Ni affinity adsorbents exhibit strong binding capacities for trimers containing one histidine tag, which is consisted with the previous results. However, the proposed method still allowed us to obtain RGD-hTNF with an overall yield of 21%, which is due to high efficiency of affinity purification and TEV protease cleavage. In previous reports, about 2 mg of RGD-mTNF was purified from 1 l of *E. coli* culture, and 3.9 mg of RGD4C-rmhTNF was recovered from 1 g wet weight of cells [11,20]. Our purification procedure produced a higher yield than ever reported.

ESI-MS is a widely recognized technique for mass measurements of individual proteins with high precision. Quality assurance of proteins by mass spectrometry is necessary. In this study, we used the benefits of ESI-MS to unambiguously determine the integrity of RGD-hTNF, which further confirmed the correct cleavage of His-TEV-RGD-hTNF fusion protein using TEV protease. Moreover, ESI-MS can distinguish individual ingredient in protein mixture, which has been used to reveal the non-specific cleavage sites in a thioredoxin-fused protein [37]. Using the same method, we revealed the non-specific site in His-EK-RGD-hTNF, which might help us to design point mutation to eliminate non-specific cleavage.

5. Conclusion

In this study, we have shown that the fusion protein of RGD-hTNF with polyhistidine tag can be produced in soluble form in the cytoplasm of *E. coli*. Separation of RGD-hTNF from the fusion protein, however, was not straightforward as we experienced difficulties with non-specific cleavage of RGD-hTNF by enterokinase. We have constructed a novel expression plasmid where the enterokinase recognition site was replaced by the TEV protease recognition site. Finally, we have isolated a soluble intact recombinant RGD-hTNF that was characterized by SDS-PAGE, Western blot, mass spectroscopy and gel filtration. We demonstrated that the *in vitro* cytotoxic activity of RGD-TNF was similar to that of hTNF and the RGD domain of RGD-hTNF was able to interact with adhesion receptors on the surface of ECV304 cells.

Acknowledgements

The project was supported by grants from the Chinese National Nature Sciences Foundation (30330530, 30425009) and Jiangsu Provincial Nature Sciences Foundation (BK2007715) to Zi-chun Hua.

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