Bacterial expression and purification of biologically active human TFF2

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Abstract: Human trefoil factor 2 (hTFF2) is considered as one of the most important initiators of mucosal healing in the gastrointestinal tract by promoting cell migration and suppressing apoptosis. However, it is hard to obtain hTFF2 from human tissue and many recombinant hTFF2 produced in vitro exist as fusion proteins. The purpose of the present study was to produce native hTFF2 while maintaining its biological activities. The open reading frame of hTFF2 was inserted into a pET-32a(+) expression vector, and hTFF2-TRX fusion protein was successfully expressed in Escherichia coli and purified by Nickel-nitrilotriacetic acid affinity chromatography and reverse-phase HPLC steps. The recombinant fusion protein (purity>95%) was cleaved by Factor Xa at 23 °C to release hTFF2. After removal of Factor Xa and undigested fusion proteins, hTFF2 was purified and identified by SDS-PAGE and Western blotting. The yield of recombinant hTFF2 was about 5 mg/L. The recombinant hTFF2 could promote IEC-6 cells migration and in vitro wound healing via the activation of ERK1/2. Recombinant hTFF2 could also inhibit apoptosis of HCT-116 cells induced by 50 μmol/L ceramide.

In summary, our results showed that the recombinant hTFF2 was expressed in E. coli and successfully purified after cleavage with the fusion partner with high yield while maintaining its biological activities. Recombinant hTFF2 might be useful for investigating the molecular mechanism of hTFF2 and development of hTFF2-related drugs.

Key words: TFF2; Expression; Cell migration; Anti-apoptosis; Wound healing

Abbreviations: TFF, trefoil factor; SP, spasmolytic polypeptide; TRX, thioredoxin; NSAIDs, non-steroidal anti-inflammatory drugs; ERK, extracellular signal-regulated kinase; pERK1/2, phospho-ERK1/2; PARP, poly ADP-ribose polymerase; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; FCS, fetal calf serum; BSA, bovine serum albumin; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; Ni–NTA, Nickel-nitrilotriacetic acid

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Trefoil factors (TFFs) are a family of peptides that share three-looped TFF-domains, each of which is composed of 38 or 39 amino acid residues in which six cysteine residues are linked in the configuration of l-5, 2-4, and 3-6, thus forming a characteristic three-leaved structure (Thim, 1989). Human TFFs, including hTFF1 (pS2), hTFF2 (spasmolytic peptide, SP), and hTFF3 (intestinal trefoil factor, ITF), are synthesized and secreted mainly by mucin-secreting epithelial cells in the gastrointestinal tract (Suemori et al, 1991). Both hTFF1 and hTFF3 only have a single TFF-domain, whereas hTFF2 has two TFF-domains (Sands & Podolsky, 1996). The TFF peptides are believed to contribute to mucosal healing and restitution by promoting cell migration and suppressing apoptosis (Dignass et al, 1994; Taupin et al, 2000).

The hTFF2 is mainly expressed in the mucous neck cells of the gastric mucosa where it boosts epithelial restitution and prevents infection (Sands & Podolsky, 1996; Taupin et al, 2000). Recent reports showed that the alteration of hTFF2 expression was closely associated with carcinogenesis and progression of gastric cancer by affecting cell proliferation, migration, infiltration, and apoptosis (Cook et al, 1999; Dignass et al, 1994; Fox et al, 2007; Hanby et al, 1993; Oertel et al, 2001). In TFF2-deficient mice, gastric epithelial cell proliferation decreased, and acid secretion and susceptibility to non-steroidal anti-inflammatory drugs (NSAIDs) increased (Farrell et al, 2002). However, the injury induced by NSAIDs in stomachs could be prevented or cured after TFF2 administration (Poulsen et al, 1999). Although diverse roles of hTFF2 have been investigated, the detailed molecular mechanism of hTFF2-mediated signaling transduction is largely unknown, partly because there is an insufficient amount of hTFF2. Thus, the expression of hTFF2 in vitro would be beneficial for the mechanism study of hTFF2.

In the present work, we used RT-PCR techniques to amplify hTFF2 cDNA and inserted it into an N-terminal histidine tag containing bacterial expression vector. After the N-terminal histidine tag was cleaved by Factor Xa, the tagless recombinant hTFF2 was purified with high yield. The recombinant hTFF2 was able to induce rat intestinal epithelial cell IEC-6 migration, promote in vitro wound healing, and inhibit human colorectal cancer cell HCT-116 apoptosis.

1 Materials and Methods

1.1 Construction of hTFF2 expression plasmid

Total RNA was extracted from surgical resection specimens of human gastric mucosa using a TRIzol Reagent kit (Tiangen Biotech, China) according to the manufacturer’s instructions. The cDNA of hTFF2 was amplified by a reverse transcriptional reagent kit (TaKaRa, China). The open reading frame (ORF) of hTFF2 was inserted into a pMD-19T simple vector (TaKaRa, China) by conventional methods. Briefly, a polymerase chain reaction (PCR) was conducted with the sense primer used was: 5′-ggtaccatgggaagggagagacccctcctcctg-3′, which containing a Kpn I site (italicized), the coding sequence for a Factor Xa recognition site (boldface), and N-terminal residues of hTFF2 (underline). The antisense primer used was: 5′-gaattcattagcgcggagacccctcctcctcctcctg-3′, which containing an EcoR I site (italicized), the stop codon (boldface), and C-terminal residues of hTFF2 (underline). The PCR was performed with an initial denaturation at 94 °C for 4 min, followed by 35 cycles of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 45 s, and a final extension at 72 °C for 7 min. The PCR products
were first subcloned into the pMD-19T simple vector. After digestion with *Kpn I* and *EcoR I* on the pMD-19T-hTFF2 plasmid, the recovered hTFF2 ORF was ligated into the pET-32a(+) vector (Novage, Germany) at *Kpn I* and *EcoR I* sites to construct the expression plasmid, designated as pET-32a(+-)hTFF2. Clones with a correct sized insert were verified by DNA sequencing.

### 1.2 Expression, purification, and identification of recombinant hTFF2

The *E. coli* expression strain BL21 (DE3) (Novage, Germany) was transformed with pET-32a(+-)hTFF2 plasmid. Transformed cells were grown at 37 °C up to an absorbance of about 0.6–0.8 at 600 nm and then induced with 0.5 mmol/L isopropyl-1-thio-β-D-galactopyranoside (IPTG) and cultivated for an additional period of 3.5 h at 37 °C. The cells were centrifuged at 8 000 r/min for 10 min and resuspended in 100 mL equilibration buffer (50 mmol/L sodium phosphate, pH 8.0, with 0.3 mol/L NaCl and 10 mmol/L imidazole). The cells were then lysed by sonication (Ultrasonic Cell Crusher JY92-2D) at 350 W for 80 cycles (6 s working, 10 s free) in an ice-water bath. The supernatant recovered by centrifugation at 16 000 r/min for 30 min was applied to a Ni-Sepharose column packed with 3 mL nickel-nitrilotriacetic acid (Ni–NTA) resin (Sigma, American). Elution of the fusion protein was carried out with elution buffer (50 mmol/L sodium phosphate, pH 8.0, with 0.3 mol/L NaCl and 250 mmol/L imidazole). The fractions containing thioredoxin (TRX)-hTFF2 were pooled and applied on a reverse-phase HPLC (RP-HPLC) Zorbax 300 SB C4 column (Elite, China) equilibrated with 0.1% (v/v) trifluoroacetic acid/water. The elution was performed at a flow rate of 0.7 mL/min. At the acetonitrile concentration of 60%, a peptide peak was collected and verified to TRX-hTFF2 by Western blotting. The TRX-hTFF2 concentration was determined by a protein assay kit (Bio-Rad, USA), then 50 μg TRX-hTFF2 fusion protein was incubated with 1 μg Factor Xa (Biolabs, New England) for 16 h at 23 °C in cleavage buffer (20 mmol/L Tris-HCl, 100 mmol/L NaCl, and 2 mmol/L CaCl2, pH 8.0). After digestion, the products were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and hTFF2 was further purified by Factor Xa removal resin column (Qiagen, USA) and a Ni–NTA column to remove the TRX-tag and undigested fusion protein according to the manufacturer’s instructions. The flow-through fractions were collected and dialyzed against 10 mmol/L sodium phosphate (pH 7.5) and lyophilized. The recombinant hTFF2 was analyzed by SDS-PAGE and Western blotting with anti-hTFF2 antibody (Santa Cruz, USA).

### 1.3 Cell culture

Rat intestinal epithelial cell line IEC-6 and human colorectal cancer cell line HCT-116 were obtained from the American Type Culture Collection (Manassas, USA). The IEC-6 cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) containing 5% fetal calf serum (FCS), 100 U/mL penicillin, and 100 mg/mL streptomycin. The HCT-116 cells were cultured in DMEM containing 10% FCS, 100 U/mL penicillin, and 100 mg/mL streptomycin. The cells were grown in a humidified atmosphere with 5% CO2 at 37 °C.

### 1.4 Cell migration assay

Cell migration activity was tested with a modified Boyden chamber assay as described previously (Liu et al., 2008). The bottom sides of the Millicells (8 μm pore, Millipore, USA) were coated with collagen type 1 (10 μg/mL) from rat tail (Sigma). After starvation overnight, the IEC-6 cells were detached, then 1×10^5 cells were planted into the top of each chamber. The recombinant hTFF2 was added into the bottom of the chambers. After 16 h, the non-migratory cells on the upper membrane surface were removed with a cotton swab, and the migrated cells were dyed with freshly prepared 0.025% crystal violet in 0.1 mol/L borate buffer (pH 9.0) containing 2% ethanol for 30 min at room temperature. The bound crystal violet was eluted with 1 mL 10% acetic acid and the migration activity was expressed as the value monitored at 586 nm of extraction.

### 1.5 in vitro wound healing assay

Wound healing assay was performed as reported previously (Hoying & Williams, 1996), with minor modification. The IEC-6 cells cultured to confluent in 6-well plates were starved overnight, and the cell monolayer was scratched by a standard 200-μL pipette tip across the diameter of the wells. The medium and non-adherent cells were removed, and the plates were rinsed twice with PBS. The fresh medium containing recombinant hTFF2 was added into the plates and changed every 24 h. Time-lapse photography of the wounding edges was performed under an inverted-phase microscope within 72 h.

### 1.6 The phosphorylation level of ERK1/2

Confluent monolayers of IEC-6 cells were treated with recombinant hTFF2 for 0 min, 15 min, 30 min, and 60 min. The collected cells were then washed and immediately lysed on ice with the lysis buffer (50
mmol/L HEPES, pH 7.4, containing 5 mmol/L EDTA, 50 mmol/L NaCl, 1% Triton X-100, 50 mmol/L NaF, 5 mg/mL aprotinin, 5 mg/mL leupeptin, 1 mmol/L Na3VO4, and 1 mmol/L PMSF). After centrifugation, the lysate (20 μg) was loaded on a SDS-PAGE gel and transferred onto a PVDF membrane. The membrane was subsequently blocked with 3% BSA and incubated with anti-ERK2 and anti-pERK1/2 primary antibodies and secondary antibodies (Santa Cruz, USA). Protein bands were visualized with super signal reagents (Pierce, USA) as described previously (Taupin & Podolsky, 1999).

1.7 Anti-apoptosis activity assay

Anti-apoptosis effect was assessed by measuring the cleavage of caspase substrate p116-[poly ADP-ribose polymerase (PARP)] as described in previous research (Kinoshita et al, 2000). Confluent monolayers of HCT-116 cells in 6-well plates were starved and then treated with recombinant hTFF2 for 24 h. Ceramide (50 μmol/L) was added to the treated HCT-116 cells, and the cells were incubated for a further 24 h. Apoptosis activity was assessed by measuring the cleavage of caspase substrate p116-PARP as analyzed by Western blotting with anti-PARP antibody (Santa Cruz, USA) (Kinoshita et al, 2000).

1.8 Statistical analysis

The data were expressed as mean±SD. Comparisons were performed by Student’s t-test. P<0.05 was considered statistically significant.

2 Results

2.1 Construction of the pET-32a(+)–hTFF2 expression plasmid

To construct an effective expression system for hTFF2, the plasmid pET-32a(+) was selected as the expression vector. As shown in Fig. 1A, the protein sequence of cloned hTFF2 was identical to that of GenBank (Accession No.7032), and Kpn I (at N-terminus) and EcoR I (at C-terminus) sites were introduced to construct pET-32a(+)–hTFF2 plasmid. The Factor Xa sequence provided a cleavage site (Fig. 1A) to obtain the recombinant hTFF2 without additional amino acid at its N-terminus. The hTFF2 ORF (310 bp) was ligated into pET-32a(+) vector between the Kpn I and EcoR I sites. The recombinant plasmid was confirmed by releasing a 310 bp DNA fragment after restriction enzyme digestion (Fig. 1B), and by DNA sequencing (data not shown).

2.2 Expression and purification of recombinant hTFF2

The pET-32a(+)–hTFF2 expression plasmid was transformed into BL21 (DE3) cells. As shown in Fig. 2A, after induction by IPTG for 3.5 h, the cells were collected and lysed by sonication, and the TRX-hTFF2 fusion protein mainly existed in the ultrasonic supernatant. His-tag in fusion protein provided an effective purification step by His-select Ni–NTA column.
The fusion protein was finally eluted with elution buffer containing 250 mmol/L imidazole. The elution was then subjected to RP-HPLC, and the highest peak was the recombinant TRX-hTFF2 protein (Fig. 2A). The TRX-hTFF2 fusion protein was then cleaved with Factor Xa and further purified by Factor Xa removal column and the Ni–NTA resin. The flow-through fractions were the recombinant hTFF2 without any extension amino acids at its N-terminus, and SDS-PAGE showed a single band with the theoretical molecular weight of 1.3×10^4. In addition, the single band was verified to human hTFF2 by Western blotting analysis with the special anti-hTFF2 antibody (Fig. 2B).

2.3 hTFF2 promoted cell migration and in vitro wound healing

One of the most important roles of TFFs is to promote cell migration and wound healing (Taupin & Podolsky, 2003). The recombinant hTFF2 showed strong activity in promoting cell migration and wound healing in vitro. After treatment with hTFF2 for 16h, the motility of IEC-6 cells was increased in a dose-dependent manner and the activity was about 2-fold greater in 200 nmol/L hTFF2 compared with the negative control (BSA) (Fig. 3A). In the in vitro wound healing assay, the IEC-6 cell monolayer was “wounded” by 200-μl standard tips. Obvious increases in the rate of wound closure were found at each time point in the presence of 200 nmol/L hTFF2, and the wound achieved almost 95% closure at 72 h (Fig. 3B).

2.4 Activation of ERK1/2 in IEC-6 cells stimulated by hTFF2

Phosphorylation of ERK1/2 is important for signal transduction of TFFs, especially for cell migration (Taupin & Podolsky, 2003). The phosphorylation levels of ERK1/2 in IEC-6 cells stimulated with recombinant hTFF2 were analyzed. As shown in Fig. 3C, 200 nmol/L hTFF2 significantly enhanced the phosphorylation level of ERK1/2.

2.5 The anti-apoptotic activity of hTFF2

Suppressing apoptosis is an important procedure at the early stages of wound healing (Bossenmeyer-Pourié et al, 2002). In the present study, apoptosis of HCT-116 cells was assessed by analyzing the cleavage of p116-PARP. Results showed that 50 μmol/L ceramide induced obvious apoptosis as observed by the disappearance of the 1.16×10^5 band of PARP in HCT-116 cells. However, after pre-incubation with hTFF2 protein for 24 h, the HCT-116 cells were further treated with C2-ceramide for another 24 h. The cleavage of 1.16×10^5 band was inhibited, suggesting that hTFF2 suppressed the apoptosis of HCT-116 cells induced by ceramide (Fig. 4).

3 Discussion

Trefoil factors are small secretory stable peptides.
Due to the compact TFF-domain formed by three intrachain disulfide bonds among six conserved cysteine residues, TFFs are resistant to protease (Kinoshita et al, 2000). Additionally, TFFs play important roles in mucosal repair, cytoprotection, and tumor suppression in the gastrointestinal tract (Perry et al, 2008; Wong et al, 1999). The roles of TFFs in vivo and in vitro indicate that TFFs may have potential value in drug development for curing gastrointestinal tract mucosa injury. In a rat model, porcine TFF2 accelerated the healing of gastric ulceration (Poulsen et al, 1999), recombinant human hTFF2 and TFF3 also protected against both ethanol and indomethacin induced gastric injury markedly (Babyatsky et al, 1996). Because hTFF2 has very important biological functions but very limited amounts can be prepared from human tissue extracts, much effort has been made to express hTFF2 peptide in yeast in vitro (Thim et al, 1993) or in Escherichia coli with an additional tag linked to hTFF2 (Sun et al, 2010). Improved quality and quantity of hTFF2 is urgently needed to help explore its functions.

In this study, the pET-32a(+) system with TRX-tag was used to express hTFF2. Using the TRX-tag as the fusion partner significantly enhanced the solubility of human hTFF2 and facilitated the formation of disulfide bonds, which is important for hTFF2 to maintain its conformation and exert biological activities. Moreover, His-tag could be used to purify fusion protein by Ni-NTA resin (LaVallie et al, 1993; Stewart et al, 1998). As shown in Fig. 2A, most recombinant TRX-hTFF2 produced in E. coli was soluble. After purification by Ni-NTA resin and RP-HPLC, the purity of the fusion protein was above 95%. To exclude the influence of TRX, the TRX-tag was removed from the fusion protein by Factor Xa, Factor Xa removal resin column, and Ni-NTA column. The recombinant native hTFF2 without additional amino acids was finally produced. The yield of recombinant hTFF2 was approximately 5 mg/L. The SDS-PAGE showed a single band with the theoretical molecular weight of about 1.3×10^4 (Fig. 2A), and the 1.3×10^4 band was verified to human hTFF2 by Western blotting with anti-hTFF2 antibody (Fig. 2B).

Together with epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), TFFs participated in the re-epithelization of wounded tissue by promoting mucosal repair and wound healing (Wong et al, 1999). As an acute-phase protein, hTFF2 was speculated to facilitate mucosal healing by promoting cell migration in the early phase (Alison et al, 1995). In this study, 50–200 nmol/L hTFF2 significantly promoted IEC-6 cell migration in a dose-dependent manner. Similar results were also obtained in the wound healing model. The 200 nmol/L hTFF2 significantly increased wound closure, with the wound achieving almost 95% closure at 72 h (Fig. 3B). The results showed that recombinant hTFF2 had similar activities of cell migration and wound healing as Bm-TFF2, a two-domain TFF from frog species Bombina maxima expressed by the pET-32a(+) system (Yu et al, 2010). It is well known that cell migration-promoting activity is dependent on the activation of ERK1/2 (Chwieralski et al, 2004; Klemke et al, 1997; Storensund et al, 2008). In our study, 200 nmol/L recombinant hTFF2 significantly enhanced the phosphorylation level of ERK1/2, suggesting that the promotion of cell migration activity of hTFF2 was ERK1/2-dependent. Additionally, hTFF2 can act as a morphogen in the presence of TFF1 and promote cell survival via inhibition of cell apoptosis (Lalani et al, 1999). Consistent with hTFF2 inhibited apoptosis of colon cancer cell lines and breast cancer cell lines (Siu et al, 2004), native hTFF2 can also inhibit HCT-116 cell apoptosis induced by C2-ceramid. In contrast with the results, the recombinant TFF3 protein increased cartilage-degrading and promote chondrocyte apoptosis, which was a previously unrecognized pro-apoptotic function of TFFs (Rösler et al, 2010). In addition, the recombinant hTFF2 in the present research also resisted trypsin degradation (data not shown).

In summary, native human hTFF2 was successfully expressed by a pET-32a(+) expression system. The recombinant hTFF2 without additional amino acid tags promoted cell migration and wound healing and inhibited cell apoptosis. The expression of hTFF2 in E. coli may be useful for studying molecular mechanisms of TFF2-mediated signaling and the development of TFF2-related drugs.

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