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Recombinant thymosin beta 4 can promote full-thickness cutaneous wound healing

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Abstract

Human thymosin beta 4 (TB4) is a small acidic peptide involved in angiogenesis, wound healing, cancer metastasis and cardiac repair. Currently human TB4 is synthesized chemically for research and this is costly. In order to obtain sufficient biologically active human TB4 economically, we cloned and overexpressed this protein in an *Escherichia coli* system. We also developed a one-step affinity purification method to purify this fusion protein. After the fusion tag was removed from the fusion protein through autohydrolysis by dithiothreitol (DTT), the biological activity and function of this recombinant human TB4 was evaluated by cell proliferation assay using prepared spleen cells and wound assay using a mouse model, respectively. Our data demonstrated that human recombinant TB4 can promote lymphocyte proliferation and differentiation. Further, it can also promote full-thickness cutaneous wound healing in BALB/c mice. To our knowledge, this is the first report of recombinant human TB4 with the ability to promote wound healing.

Keywords: Human thymosin beta 4; Proliferation; Affinity chromatography; Lymphocyte; Wound healing; Cancer metastasis

Thymosin beta 4 is a small amino acid sequence highly conserved water-soluble peptide approximately 5 kDa in size. It has immunomodulatory properties and is a major actin-sequestering peptide in mammalian cells [1–4]. This protein was originally isolated from a thymic extract in 1981 (thymosin fraction 5) [5] and later studies showed that it was present ubiquitously in most tissues and cell lines, in particularly high concentrations in blood platelets and neutrophils [6–8]. Recently, it was reported that thymosin beta 4 also accelerates angiogenesis [9] and wound healing [3,10,11]; promotes cardiac migration, survival, and cardiac repair [12]; and stimulates tumor metastasis by activating cell migration and angiogenesis [13–16]. Therefore, more attention is being paid to TB4 functions and its therapeutic applications [3,17–21]. However, at present human TB4 used for research is synthesized through a prohibitively expensive chemical process. In this study, we explored the other avenue to obtain sufficient human TB4 more economically.

Genetic engineering has been utilized in the production of small peptides for many years [22]. *Escherichia coli* is the most commonly used system in genetic engineering to express recombinant proteins. But there are difficulties in expression of human TB4. We have used pET and pBV220 expression vectors to try to express intact human TB4 and failed (unpublished data). Perhaps this phenomenon is caused by the susceptibility of the small peptides to proteolytic degradation [22]. In 2002 Che YK and his colleagues expressed and purified human TB4 by adding 5 additional amino acids to the N-terminus [23]. This addition of amino acids sequence may have potential to affect the function of the protein. In order to obtain fully functional human TB4, further studies are still necessary. In

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this study, a fusion strategy was used to clone the gene into an expression vector pTXB1and the chitin affinity chromatography was employed to purify human TB4 protein. These strategies allowed us to obtain a relatively large quantity of functional human TB4.

Materials and methods

Materials

Chitin beads and *E. coli* strain ER 2566 were obtained from New England Biolabs (Beverly, MA). Synthetic human TB4 was purchased from GL Biochem (Shanghai, China). The restriction endonucleases NdeI and XhoI and a DNA ligation kit Ver 2.1 were purchased from Takara Biotech (Japan). Taq DNA polymerase, dNTPs and polymerase chain reaction primers were obtained from Sangon (Shanghai, China). A Plasmid Miniprep kit and Gel Extraction kit were purchased from Tiangen (Beijing, China). ConA and reagents for polyacrylamide electrophoresis, such as acrylamide, bis-acrylamide, tricine, ammonium persulfate, TEMED and Coomassie brilliant blue R-250 solution were obtained from Sigma (USA). Reagents for cell culture, including penicillin/streptomycin solution, L-glutamine, and RPMI 1640 medium were purchased from Gibco (NY, USA). Fetal bovine serum was purchased from Hyclone (UT, USA). pGEM-T vector and an MTT assay kit were obtained from Promega (USA). DNA sequencing was performed by Sunbio (Beijing, China). N-terminal sequencing was performed in the College of Life Science, Peking University. Other reagents were analytical grade unless denoted otherwise.

Plasmid construction

The entire cDNA (GenBank Accession No. NM_021109) coding region of thymosin beta 4 was optimized and synthesized by Bioasia (Shanghai, China). The coding region of thymosin beta 4 was amplified by PCR. The PCR conditions were: initial denaturing at 94 °C for 4 min followed by 30 cycles of 30 s at 94 °C, 30 s at 52 °C, and 15 s at 72 °C. The upstream primer corresponding to the TB4 start codon also contained a NdeI restriction site (underlined) (5′-GGT CAT ATG TCT GAC AAA CCG GAC ATG GCT G-3′). The downstream primer included a XhoI restriction site (underlined) (5′-CTC CTC GAG GGA TTC ACC AGC CTG TTT C-3′). The product was cloned into the pGEM-T vector in accordance with the manufacturer's protocol, and then transformed into *E. coli* DH5α. The cloning vector, pGEM-TB4, was extracted from the bacterial culture using a plasmid spin miniprep kit (Tiangen Inc., China) and confirmed by DNA sequencing. Following digestion with the restriction enzymes NdeI and XhoI, the pGEM-TB4 vector was cleaved into two fragments. The smaller fragment encoding human TB4 was recovered using a gel extraction kit (Tiangen Inc., China). The pTXB1 vector was also digested with the restriction enzymes NdeI and XhoI, and the larger fragment was recovered. The expression vector, pTXB–TB4, was constructed through a ligation reaction between the two fragments using the DNA ligation kit Ver2.1 (Takara Biotech, Japan), according to the manufacturer's protocol.

Expression of fusion protein

Escherichia coli ER 2566 (New England BioLabs) was used to express the fusion protein. *E. coli* strain ER 2566 was transformed using recombinant plasmids enabling the expression of the gene under the control of the T7 promoter. In order to express human thymosin beta 4, the *E. coli* strain ER2566 was transformed with pTXB–TB4. The successfully transformed *E. coli* was picked from a single colony and was grown overnight (37 °C, 220 rpm) in the Luria–Bertani (LB) medium (0.5% yeast extract, 1% Bactotryptone and 1% NaCl) supplemented with 100 μg/ml ampicillin. The culture mixture was then inoculated with fresh LB medium (1:100 dilution) containing ampicillin and grown (37 °C, 220 rpm) until the absorbance at 600 nm reached 0.6–0.8. To optimize the culture conditions, human TB4 expression was induced by adding 0.5 mM isopropylthio-β-D-galactoside (IPTG)[1] to the transformed *E. coli* and the bacteria were incubated at 37 °C for a period of 1, 2, 3, 4 or 5 h. The degree of expression was evaluated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Purification of recombinant human TB4 polypeptide

Prior to purification, the induced bacteria were harvested by centrifugation at 8000 rpm for 10 min at 4 °C. The bacterial pellets were then resuspended in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0) and subjected to sonication with 9 s on and 9 s off for 9 min. The lysate was centrifuged (8000 rpm, 20 min, 4 °C) and the supernatant was filtered through a 0.45 μm filter (Millipore) before being mixed with 5 volumes of column wash buffer (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, pH 8.0, 500 mM NaCl and 0.1% Triton X-100). Then the mixture was applied to chromatographic column (diameter: 1 cm; height: 1.5 cm) containing chitin beads. Native proteins from *E. coli* were rinsed from the column with column wash buffer. After immobilization of recombinant protein, the column was filled with cleavage buffer (20 mM Tris–HCl, pH 8.0, 0.1 mM EDTA, pH 8.0, 50 mM NaCl and 30 mM DTT) and the autohydrolysis reaction was continued for 24 h at 4 °C. The human TB4 was eluted with cleavage buffer which did not contain DTT and the eluent was then ultrafiltered through an ultrafiltration device with a molecular weight cutoff of 10 kDa (Pall Corporation, USA). The human TB4 containing solution was

[1] *Abbreviations used:* TB4, human thymosin beta 4; DTT, dithiothreitol; IPTG, isopropylthio-β-D-galactoside; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; CCM, cell culture media.

desalted using a HiTrapTM Desalting column (Pharmacia, USA) and dried at 40 °C using a SPD111V drier (Thermo Electron Corp, USA). The human TB4 powder was stored at −20 °C for later use.

Protein concentration determination and SDS–PAGE analysis

Protein concentration was determined by using the Coomassie protein assay kit (Beyotime Biotech. Co. Ltd., China) according to the manufacturer's instructions. The assay is based on the method of Bradford [24] and bovine serum albumin was used as a reference standard. The expressed fusion proteins were analyzed by 12% SDS–PAGE [25], and the purified human TB4 was analyzed by 16.5% Tricine–SDS–PAGE as described by Hermann [26].

N-terminal sequencing

For N-terminal sequencing, the polypeptide (5–10 μg), which was separated on a Tricine–SDS–PAGE gel, was electro-transferred to a polyvinylidene difluoride (PVDF) membrane (Pall, USA) for 25 min at 55 V using CAPS transfer buffer (10 mM CAPS and 10% methanol). The PVDF membrane was then stained with Coomassie blue R-250, followed by destaining with 50% methanol. The stained protein bands were excised with a clean blade, placed in a 1.5 ml conical centrifuge tube and subjected to N-terminal sequencing. The N-terminal sequencing was performed by automated Edman degradation method on an Applied Biosystems 491 protein sequencer.

MALDI-TOF-mass spectroscopy

The purified protein sample of recombinant human TB4 was analyzed by MALDI-TOF-MS using Applied Biosystems (ABI) 4800 MALDI-TOF mass spectrometry.

Biological activity

The biological activity of recombinant and synthetic human TB4 was evaluated by its promotion of T lymphocyte proliferation and differentiation as described previously [1,27,28] with some modification. The lymphocyte proliferation assay was performed as previously described [29]. Briefly, the spleen was aseptically removed from a BALB/c mouse. A suspension of single spleen cells was prepared by lysing the red blood cells and collecting into cell culture media (CCM) that contained RPMI1640 (Gibco), 2 mM glutamine, 10% fetal bovine serum (Hyclone), and 100 μg/ml of penicillin and streptomycin. Triplicate cultures were grown in 96-well flat-bottomed tissue culture plates at 5 × 105 cells/well. For the activity assay, recombinant or synthetic human TB4 was added to the cell suspension at a final concentration of 2–10 μg/ml and afterwards the splenocytes were incubated for 6 h in the presence of ConA (4 μg/ml) at 37 °C in a humidified incubator with 5% CO_2 and 95% air. Splenocytes were cultured for 72 h. Then activity assay was performed using CellTiter aqueous one solution cell proliferation assay (MTS) kit (Promega, USA) according to the manufacturer's instructions.

Assay of promoting wound repair

Wound assay

Four full-thickness 3 mm punch wounds were made on the dorsal surface of each mouse as previously described [11,30]. Recombinant human TB4 was applied topically to two of the wounds on each mouse (5 μg in 50 μl) at the time of wounding and again after 48 h. Controls for the TB4-topical treatment (the two remaining wounds on each mouse) received identical amounts of saline at the time of wounding and at 48 h. Mice were not bandaged. The experiment was terminated on day 7 post wounding and analysis of wounds was made as detailed below.

```
N  TCT GAC AAA CCC GAT ATG GCT GAG ATC GAG AAA TTC GAT AAG TCG AAA
S  TCT GAC AAA CCG GAC ATG GCT GAA ATC GAG AAA TTC GAC AAG TCC AAA
      S  D  K  P  D  M  A  E  I  E  K  F  D  K  S  K

N  CTG AAG AAG ACA GAG ACG CAA GAG AAA AAT CCA CTG CCT TCC AAA GAA
S  CTG AAG AAG ACC GAA ACC CAG GAG AAA AACCCGCTG CCG TCC AAA GAA
      L  K  K  T  E  T  Q  E  K  N  P  L  P  S  K  E

N  ACG ATT GAA CAG GAG AAG CAA GCA GGC GAA TCG
S  ACC ATC GAA CAG GAG AAA CAG GCT GGT GAA TCC
      T  I  E  Q  E  K  Q  A  G  E  S
```

Fig. 1. The amino acid sequence of human TB4 protein and comparison of the nucleotide sequences of the native human TB4 gene (N) and the optimized synthetic human TB4 gene (S) used in the pTXB–TB4. The altered nucleotides are shown in bold in the "S" sequence.

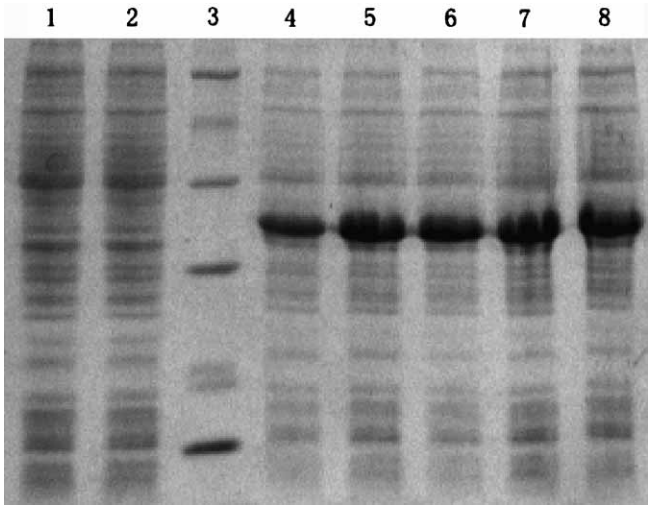

Fig. 2. SDS–PAGE analysis of the recombinant human TB4 fusion protein expressed by *E. coli*. The expression was induced by 0.5 mM IPTG at 37 °C for different periods. Lane 1, 5 μl total protein of *E. coli* ER2566; lane 2, 5 μl total protein of *E. coli* ER2566/pTXB–TB4 before induction; lane 3, protein molecular weight marker (97.4, 66.2, 43, 31, 20.1 and 14.4 kDa); lanes 4–8, 5 μl total protein of *E. coli* ER2566/ pTXB–TB4 after induction with 0.5 mM IPTG for 1, 2, 3, 4 and 5 h, respectively.

Histological analysis

As described previously [11,30], wound tissue was collected at the time of euthanasia and fixed in 10% buffered formalin. The samples were sectioned from the middle of the wounds and were stained with hematoxylin and eosin or with Masson's Trichrome (Department of Pathology, People's Hospital, Peking University, Beijing). All subsequent analyses were performed by observers blinded to treatment. Histological sections were used to measure the re-epithelialization, collagen content and vessel counts. Keratinocyte migration was determined by measuring the lengths of the epidermal tongues from both wound edges in a microscope with a ruler, and data are expressed as percentage wound closure (distance of migrated keratinocytes from the wound edge/total wound width × 100). Collagen content was estimated from the Masson's trichrome staining in the center of the wound versus the unwounded areas on a scale of 1–5, with 5 being the stain intensity observed in the unwounded area, i.e., maximal collagen deposition. Vessel counts were performed by first identifying vascular spaces by their endothelial lining. All such vessels in the wound bed were counted, including those at the junction of the dermis and the subcutis, since angiogenesis into the wounds occurs to a great extent from these vessels. The numbers were averaged into vessel counts per 10 high-powered fields (40×). Data were analyzed by Student's *t*-test, with a P value of <0.05 considered statistically significant.

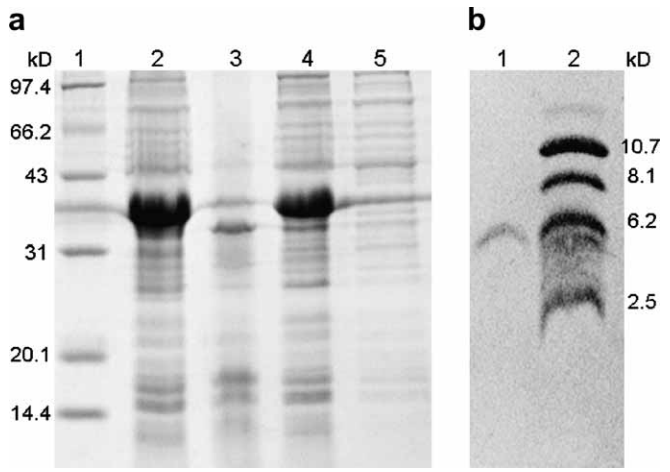

Fig. 3. Human TB4 fusion protein was purified from a crude soluble fraction using a chitin-affinity column. (a) 12% SDS–PAGE analysis. Lane 1, protein molecular weight marker; lane 2, induced bacteria lysate; lane 3, pellet of induced bacteria; lane 4, supernatant of induced bacteria lysate; lane 5, run through. (b) 16.5% Tricine–SDS–PAGE analysis. Lane 1, purified human TB4; lane 2, low molecular weight markers.

Results and discussion

Human TB4 gene synthesis and construction of the prokaryotic expression plasmid pTXB–TB4

DNA sequence analysis of genes encoding either high abundance or low abundance proteins in *E. coli* has revealed a pattern of favored codon usage; highly expressed genes show the greatest degree of conformity to the preferred codons, which correspond to the most abundant tRNAs in the cell [31]. Therefore we synthesized human TB4 cDNA using *E. coli* preferred codons without changing the amino acid sequence (Fig. 1). The human TB4 cDNA fragment was amplified by PCR and successfully inserted into the pTXB1 vector, which was confirmed by

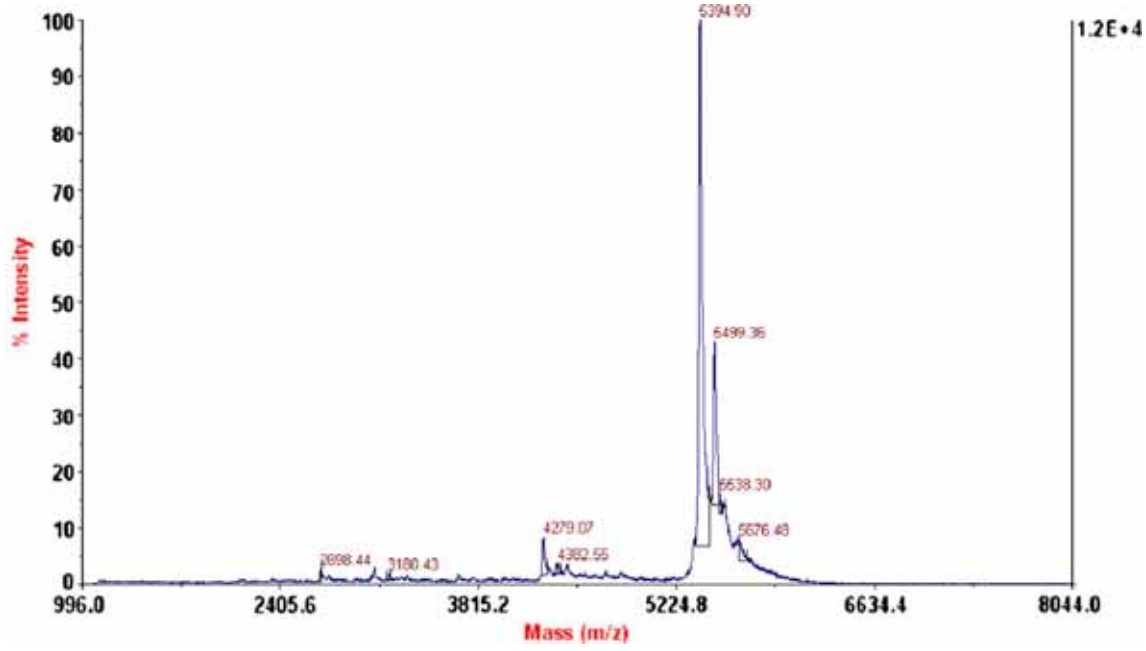

Fig. 4. Mass spectroscopy of purified recombinant human TB4. The large peak denotes the molecular mass for recombinant TB4, 5394.90. Mass per charge is indicated by use of m/z.

DNA sequencing. The final pTXB–TB4 construct was able to express the recombinant human TB4 as a fusion protein with a fusion tag, intein-CBD, at the C-terminal. With the fusion tag the recombinant human TB4 can be purified by chitin affinity chromatography and the intein-CBD tag can be removed through autocatalytic cleavage by DTT. This plasmid was used to transform the *E. coli* strain ER2566.

Expression and purification of the human TB4 polypeptide

After induction with IPTG, *E. coli* ER2566 transformed with pTXB–TB4 produced a fusion protein of approximately 32 kDa, which contained an intein-CBD tag of 27 kDa as shown in Fig. 2. To determine the optimal induction period, the bacteria were incubated with IPTG at 37 °C for 1, 2, 3, 4 and 5 h, respectively, and the cell lysates were subjected to analysis of protein production. The results showed that when the incubation time exceeded 2 h, the yield of the product was not significantly raised (Fig. 2). SDS–PAGE analysis also demonstrated that recombinant human TB4–intein-CBD was highly and inducibly expressed in a soluble form, accounting for about 50% of the total soluble protein as assessed by densitometric scanning (Fig. 3a).

The fused human TB4 in the soluble fraction was separated using the chitin-affinity column according to the protocol provided by the manufacturer. The recombinant

Table 1
Biological activity of recombinant human TB4

Final concentration (μg/ml)	OD_{490nm}	
	rTB4	sTB4
0 (control)	0.654±0.011	0.654±0.011
2	0.716±0.013*	0.697±0.021*
5	0.786±0.011*	0.787±0.015*
10	0.717±0.007*	0.71±0.016*

rTB4, recombinant human TB4; sTB4, synthetic human TB4.
* $p < 0.05$ vs. control (one-tailed Student's *t*-test).

Table 2
Wound healing parameters after 7 days in normal mouse

Measurement	Control	rhTB4	Significance
Epidermal closure[a]	47 ± 5.0	88.7 ± 5.8	$p < 0.01$
Collagen[b]	2.1 ± 0.2	3.5 ± 0.6	$p < 0.01$
Vessels/10 HPF[c]	1028 ± 109	1834 ± 113	$p < 0.05$

Data were analyzed by Student's *t*-test ($n = 6$).
[a] Epidermal closure is expressed as percentage of wound closure.
[b] Collagen content is expressed on a scale of 1–5 based on Masson's stain with 5 showing the most deposition of collagen. Scoring was performed by observer blinded to treatment.
[c] HPF, high power field.

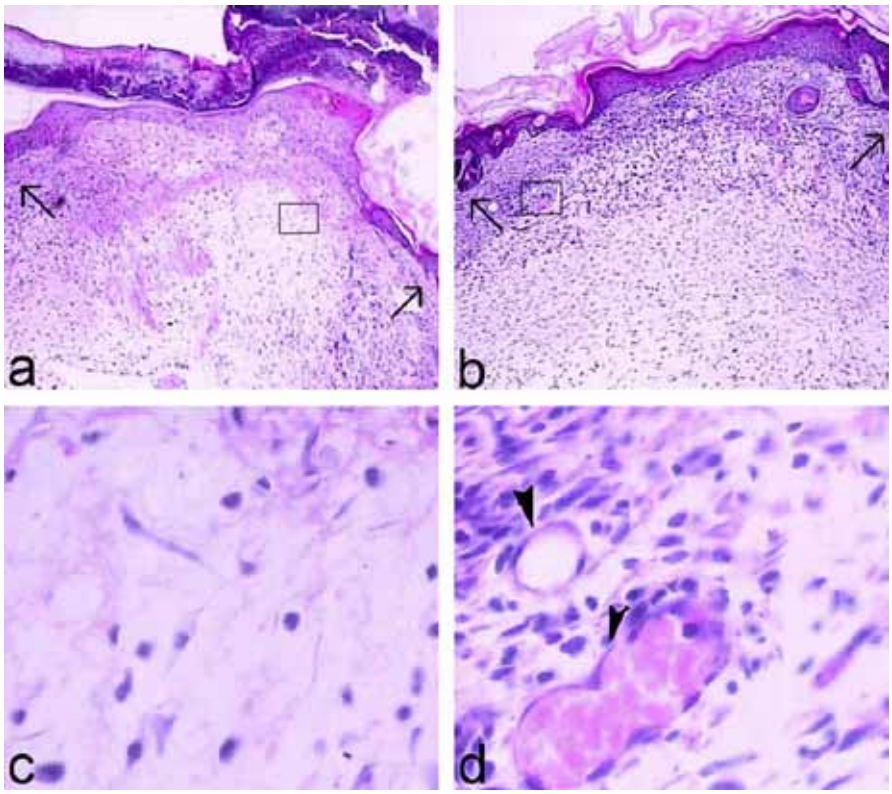

Fig. 5. Histological sections of 7 d wounds showing re-epithelialization and angiogenesis. Arrows indicate the edge of the original wound. (a) Control wound treated with saline. Migration of the epithelium is visible at the wound edge. (b) Topical treatment (5 μg per 50 μl of recombinant human TB4) resulted in increased re-epithelialization of the wound epidermis. Boxed areas are the location of the higher magnification fields (c and d). (c and d) Dermis near dermal and epidermal junction. (c) Control showing few cells near the dermis and little neovascularization. (d) Dermis showing granulation tissue infiltrated with fibroblasts and extensive neovascularization (arrowheads). Topical application resulted in significant new capillaries.

human TB4 was cleaved from the fusion protein at the cleavage site (LEGSS↓C) to release it from the intein-CBD tag, and eluted, leavine the tag on the column. The human TB4 was ultrafiltered and the filtrate was analyzed by Tricine–SDS–PAGE. The result appeared as a single band (Fig. 3b). N-terminal sequencing of the purified recombinant human TB4 revealed that the first five residues were identical to the counterpart of the human TB4 derived from its DNA sequence (SDKPD). The yield of the purified recombinant human TB4 was approximately 4 mg per liter culture.

MALDI-TOF-mass spectroscopy

To determine the exact molecular weight and further confirm the identity, recombinant human TB4 was subject to MS analysis. The molecular mass determined for recombinant human TB4 is 5394.90 (Fig. 4), which is compatible with the calculated value of 5394.95. Because there are five additional amino acids (LEGSS) left at the C-terminal after the DTT-induced autohydrolysis, recombinant human TB4 is about 0.5 kDa larger than native human TB4 in size.

Biological activities of the recombinant human TB4

In Table 1, the value of OD_{490nm} is used to indicate the cell numbers in the culture; its increase means the increase of cell numbers in the culture. From this table, we can see that purified recombinant human TB4 is able to promote lymphocyte differentiation and proliferation as effectively as synthetic one. This finding was consistent with previous work [1,15,27,28], and this indicates that our cloning strat-

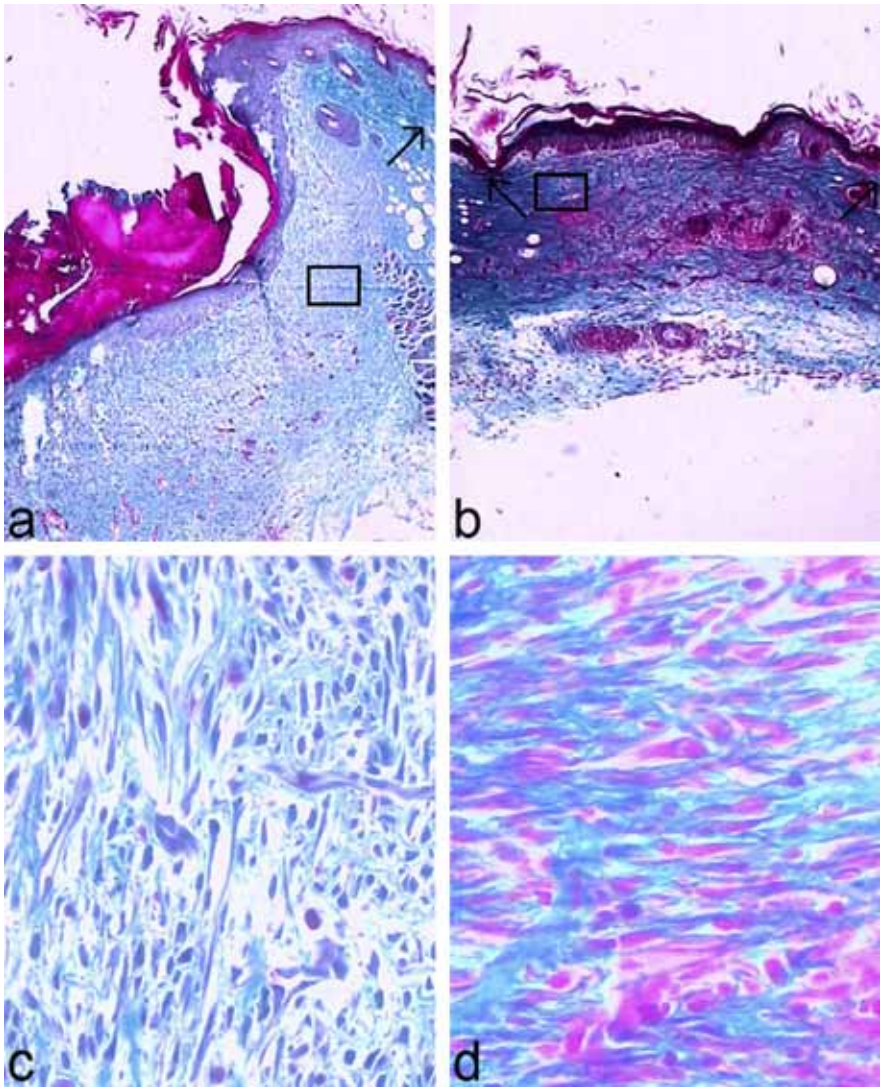

Fig. 6. Histological sections of 7 days wounds showing collagen deposition/accumulation. Masson's trichrome staining shows collagen in blue and endothelial cells in red. (a) Low magnification view of a control wound treated with saline. (b) Low magnification view of wounds where recombinant human TB4 was applied topically. Boxed areas are the location of the higher magnification fields (c and d). (c) Control wound at higher magnification showing baseline collagen accumulation. (d) Treatment topically resulted in enhanced collagen production/accumulation compared with wounds treated with saline. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

egy of the human TB4 gene is appropriate and the methods for expression and purification of the functional recombinant TB4 protein are effective.

Promotion of wound repair

The effect of recombinant human TB4 on wound healing was studied in a full thickness cutaneous mouse wound model. Fig. 5 shows a comparison of a typical control (a) and TB4-treated (b) sections of 7 day wounds. Treatment with TB4 resulted in increased re-epithelialization (Table 2) and considerable capillary (Fig. 5d) in growth. Vessel counts showed a significant increase in the number of vessels in TB4-treated wounds (Table 2). We also observed an increase in the deposition/accumulation of collagen in TB4-treated wounds (Fig. 6 and Table 2). Thus, our recombinant human TB4 exhibits the ability to promote wound healing.

Human thymosin beta 4 is an important peptide in the body [7]. It participates in angiogenesis, cell migration, tumor metastasis, wound healing and cell differentiation [9,11–13,21,30,32]. Now more and more groups focus on its therapeutic application in wound healing and some commercial companies (such as RegeneRX, www.regenerx.com) are attempting to use TB4 for treatment of epidermolysis bullosa and corneal wound healing. Thus, searching other ways to produce active human TB4 at low cost is critical important. In our study, we have expressed, purified and characterized a functional human TB4 protein using a simple method. The recombinant human TB4 promoted lymphocyte proliferation and differentiation. Its biological activity is as strong as that of the synthetic TB4. As expected, the purified recombinant human TB4 could promote full thickness cutaneous wound healing. In the future, further work should be done on the molecular mechanisms of TB4 function. To date, some groups have shown that TB4 can increase the expression of metalloproteinase [33,34] and laminin-5 [35], two important factors for wound healing, and decrease inflammation-mediated tissue injuries [34]; These preliminary reports may have indicated the future direction for further study.

In conclusion, our expression system is found to be a good approach for production of a biologically active and difficultly expressed peptide such as human thymosin beta 4. Our results clearly demonstrate that recombinant human TB4 is functional and has the ability to promote wound healing. Therefore, recombinant human TB4 can be used for future research to better understand the molecular mechanisms of TB4 function.

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