·Basic Research·

Cloning and expression of immunotoxin DT_{389} – hbFGF

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Abstract

• AIM: To express the DT389-hbFGF (389 amino acid residues of the N-terminus of diphtheria toxin (human basic fibroblast growth factor) fusion protein for potential targeting therapy towards posterior capsule opacification (PCO) after cataract surgery.

• METHODS: The DNA of inactivated diphtheria bacillus and RNA of 12-week fetal brain cortex were extracted, respectively. The fragments of truncated diphtheria toxin (containing 389 amino acids of N-terminus, DT_{389}) and full-length human basic fibroblast growth factor (hbFGF) sequence (encoding 18kDa protein) were amplified by PCR. The two fragments were inserted into pGEX-4T-1 prokaryotic expression vector to obtain pGEX-DT_{389}-hbFGF prokaryotic expression plasmid. After sequence analysis, the expressing plasmid was transformed into Escherichia Coli BL21 strain and expression was induced under IPTG. The expressed fusion protein was purified and identified.

• RESULTS: The gene fragments encoding DT_{389} and hbFGF were amplified and their gene sequences were confirmed. Hybrid gene expression plasmid pGEX-DT_{389} (hbFGF) was constructed. The fusion protein DT_{389}-hbFGF was expressed and purified.

• CONCLUSION: The successful cloning and expression of DT_{389}-hbFGF immunotoxin provides a foundation for targeting therapy towards posterior capsule opacification.

• KEYWORDS: immunotoxin; DT; hbFGF

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INTRODUCTION

Posterior capsular opacification (PCO), the most frequent complication associated with decreased vision after extra capsular cataract surgery, is a result of lens epithelial cell (LEC) proliferation and migration on the posterior capsular. Therefore, prevention of PCO is an important step towards improving the quality of the surgical outcome. At this time, there is no method of preventing PCO that has proven to be effective, practical and safe for routine clinical procedures. The most convenient method for treating PCO is a neodymium: YAG (Nd:YAG) laser posterior capsulotomy. When an Nd:YAG laser is not available, conventional capsulotomy surgery is performed.

Within the wide range of recent therapeutic targets, cytokines and receptors may play important roles because they are expressed by LECs and can influence postoperative proliferation of LECs in the capsular bag. LECs can express high-affinity fibroblast growth factor (FGFR) and secrete the basic fibroblast growth factor (bFGF) which can induce cell proliferation by binding to FGFR[1]. Diphtheria toxin (DT) is a ribosome-inactivating protein, which consists of the catalytic, transmembrane and cell-binding domains. The 389 amino acids of N-terminus of diphtheria toxin (DT_{389}) include only the catalytic and transmembrane domains. The cell-binding domain of DT was replaced by hbFGF, rendering the fusion protein to specifically bind to human LECs. This report describes how immunotoxin DT389-hbFGF is cloned and expressed.

MATERIALS AND METHODS

The primers used in these studies are given in Table 1.

Cloning DT_{389} Fragment The genomic DNA of inactivated diphtheria bacillus was extracted and amplified to obtain the full-length genomic sequence (1.6kb) by PCR. The DT_{389} fragment was amplified from the full-length genomic sequence. After being separated and purified by 10g/L agarose electrophoresis and QIA quick Gel Extraction Kit (Promega), the DT_{389} fragment was inserted into pGEM-T Easy plasmid by A-T cloning using T-4 ligase. The Escherichia coli (*E. coli*) strain JM109 was transformed by the plasmid and inoculated on an LB plate with ampicillin (Amp 0.1g/L), and incubated overnight at 37℃. The inserted fragment was sequenced by the Chinese National Human Genome Center, Beijing (CHGB).

Cloning hbFGF Fragment The total RNA of embryonic 12-week cortex was extracted and amplified to obtain

Table 1 Primers used in these studies

Sense: 5'-GGC GCT GAT GAT GTT GTT G-3'	Antisense: 5'-CCG GCA CAC GAC CCC ACT A-3'
Sense: 5'-GGCGCTGATGATGTTGTTG-3'	Antisense: 5'-AAATGGTTGCGTTTTATGCC-3'
Sense: 5'-TGAC GGATCC ggcgctgatgatgttgttga-3'	Antisense: 5'- CTGAGAATTCaaatggttgcgttttatgc-3'
Sense: 5'-TCCGCGGAGACAACCAT-3	Antisense: 5'-CGAATAAAGCAAATGCGTGAA-3'
Sense: 5'-CTGA GAATTC atggcagccgggagcatcac-3'	Antisense:5' -TGAC CTCGAG tcagctcttagcagacattg-3'

1,000bp bFGF by one-step RT-PCR Kit (Promega). 500bp fragment encoding 18KD hbFGF was amplified and Eco RI, Xho I restriction sites were inserted by PCR using a 1,000bp bFGF template. The amplified products were separated, harvested and purified as described above.

Construction of Hybrid Gene and Expression Plasmid The DT_{389}-pGEM-T Easy plasmid was extracted using a mini plasmid extracting kit. The Bam HI and Eco RI restriction sites were introduced to the bi-side of DT_{389} by PCR. The DT_{389} containing the restriction sites and pGEX-4T-1 were incubated with Bam HI and Eco RI, respectively, and were linked using T4 ligase to construct pGEX-DT_{389} plasmid. The pGEX-DT_{389} plasmid and hbFGF fragment were incubated with Eco RI and Xho I, respectively, and were linked using T4 ligase to obtain pGEX-DT_{389}-hbFGF expression plasmid. The *E. coli* JM109 was transformed by pGEX- DT_{389}- hbFGF plasmid and cultured in an LB plate with Amp (0.1g/L) at 37℃. The sequence was identified using gene sequencer.

Expression of Fusion Protein Plasmid pGEX-DT_{389}-hbFGF was transformed into the *E. coli* strain BL21 (DE3), which were then inoculated in an LB plate with Amp (0.1g/L) and incubated overnight at 37℃. One clone was transferred into 5mL LB medium with 0.1g/L Amp and cultured at 37℃ until the OD600 of the culture reached 1.0. The bacteria were transferred to 50mL fresh LB medium and continued to be cultured at 37℃. When the OD600 reached 0.6, expression of the hybrid gene was induced by the addition of isopropyl-P-D-thiogalactopyranoside (IPTG, 0.1mmol/L; GIBCO BRL). Bacteria (1mL) were harvested at IPTG induction 0, 1, 2, and 3 hours, and sonicated 5 minutes in an ice-bath, mixed with the same volume of protein electrophoresis loading buffer and boiled 7 minutes for SDS-PAGE. Remaining bacteria were harvested at 3 hours by centrifugation at 5 000g for 10 minutes. The bacterial pellet was resuspended in ice-cold 1mmol/L EDTA/PBS, sonicated 5 minutes, and centrifuged 30 minutes at 15 000g. To determine the localization of expressed protein, the supernatant and pellet, respectively, were loaded for SDS-PAGE. The expression was analyzed on 125g/L SDS-PAGE using a Mini-Protein I1 gel apparatus (Bio-Rad). Proteins were stained with Coomassie brilliant blue.

Purification and Identification of Fusion Protein Several clones were selected and pre-cultured as described above. The bacteria were transferred to 1L LB medium and cultured under IPTG induction 3 hours. The bacteria were harvested and sonicated as described above. The supernatant was collected and added into two Glutathione Sephrose 4B columns. Column I was incubated with GSH (glutamyl cysteinyl glycine) overnight, and the liquid phase was collected (Sample 1). Sample 1 was incubated with thrombin (20U/mL) overnight (Sample 2). Column II was incubated with thrombin overnight and the liquid phase was collected (Sample 3), and then the column II was incubated with GSH overnight, the liquid phase was collected (Sample 4). The four samples were mixed respectively with loading buffer and boiled 7 minutes at 100℃. The expression was analyzed as described above. For immunoblotting, the separated proteins (Sample 3 and bFGF) were transferred to nitrocellulose membranes. Membranes were blocked with 50mL/L milk-containing TBS (20mmol/L Tris, 500mmol/L NaCI, pH7.5) and washed with TTBS (TBS. 0.5g/L Tween-20, pH7.5). Membranes were incubated with rabbit anti-bFGF sera (Santa Cruz), and HRP-conjugated goat anti-rabbit IgG at 1:2 000 dilution was applied with 2 hours incubation. ECL blotting reagents (Amersham Biosciences) was used for protein visualization.

RESULTS

The PCR Products of DT_{389} and hbFGF The full-length sequence of DT was amplified by PCR. Using this sequence as a template, about 1.1kb of the DT_{389} fragment, including the transmembrane and catalytic domains, was obtained. The sequencing data showed that the sequence of the DT_{389} fragment was correct. The 1.0kb bFGF fragment was amplified by RT-PCR from embryonic cortex. The 500bp DNA fragment encoding 18kDa bFGF was amplified and the Eco RI and Xho I restriction sites were introduced.

Construction of Hybrid Gene and Expression Plasmid The DT_{389} containing restriction site and pGEX-4T-1 were incubated with Bam HI and Eco RI, respectively. And were linked using T4 ligase to obtain pGEX-DT_{389} plasmid. The plasmid and hbFGF fragment were incubated with Eco RI and Xho I, respectively, and were linked using T4 ligase to obtain pGEX-DT_{389}-hbFGF expression plasmid (Figure 1). The sequencing data indicated that the construction of expression plasmid was corrected (Figure 2).

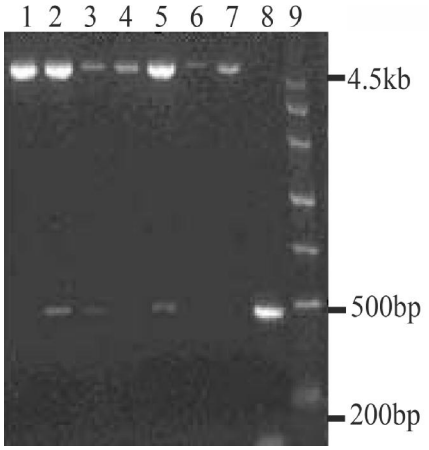

Figure 1 Agarose electrophoresis Line 1: pGEX- DT_{389} plasmid; Line 2-7: the products of pGEX-DT_{389}-hbFGF cut with Eco RI, Xho I; Line 8: the hbFGF fragment; Line 9: Molecular markers (200bp, 500bp, 800bp, 1.2kb, 2 kb, 3 kb, 4.5kb)

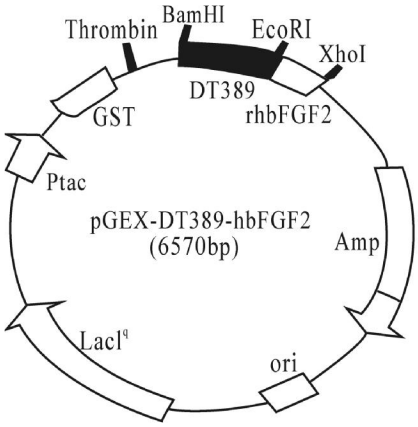

Figure 2 Construction of pGEX –DT_{389} –hbFGF expression plasmid

Expression of Fusion Proteins Coomassie brilliant blue staining indicated there was a very bulky band about 86KD after IPTG induction. The molecular weight coincided with GST-DT_{389}-hbFGF fusion protein. Location analysis showed that the fusion protein was expressed in cytosol mainly, but there was also a small quantity of protein in inclusion (Figure 3).

Purification and Identification of Fusion Protein Because the labeling protein GST can bind with Glutathione Sephrose 4B, the fusion protein was absorbed by the Glutathione Sephrose 4B column. GSH can elute the GST from Glutathione Sephrose 4B column, and the thrombin can cut off GST from the fusion protein. So, Sample 1 is GST-DT_{389}-hbFGF, and its molecular weight is about 86kDa; Sample 2 includes GST (26kDa) and DT_{389}-hbFGF (60kDa); Sample 3 is purified fusion protein (60kDa); Sample 4 is GST (26kDa) (Figure 4). Western blot indicated that the fusion protein is able to bind to the hbFGF antibody and the molecular weight is about 60kDa, implying that the fusion protein is DT_{389}-hbFGF (Figure 5).

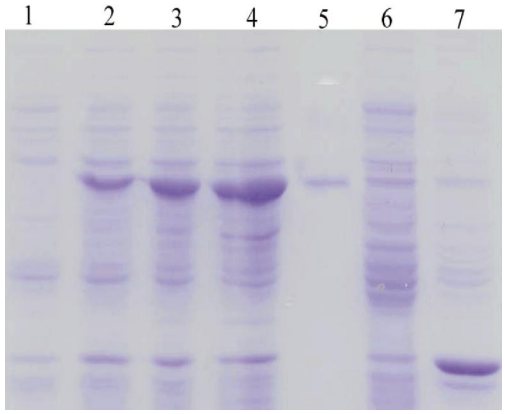

Figure 3 Expression products after IPTG induction Line 1: Before IPTG induction; Line 2: 3, 4: 1, 2, and 3 hours, respectively, after IPTG induction; Line 5: The supernatants of 3 hours after IPTG induction; Line 6: The sonicated product of 3 hours after IPTG induction; Line 7: The Pellet of 3 hours after IPTG induction

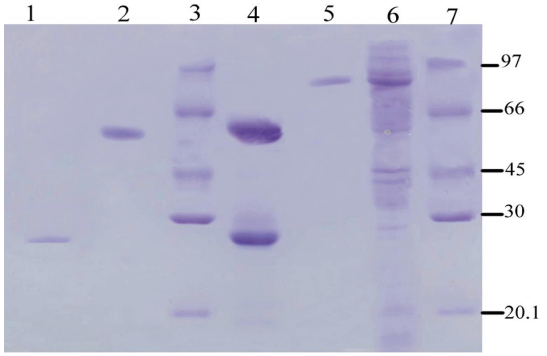

Figure 4 Expression and purification of recombinant toxin DT_{389}–hFGF2 Line 1: Sample 4 (GST); Line 2: Sample 3 (fusion protein); Lines 3 and 7: Molecular Weight Markers; Line 4: Sample 2 (GST fusion protein); Line 5: Sample 1 (fusion protein); Line 6: Sonicated products before purification

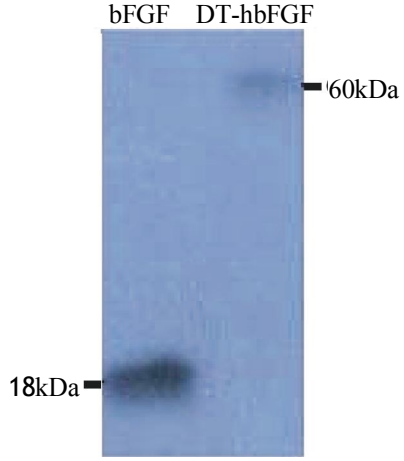

Figure 5 Western blot

DISCUSSION

Only partial sequences of bFGF can be obtained in many kinds of tissues such as kidney, placenta, fetal liver, and fetal heart by RT-PCR because bFGF mRNA is unstable. We

made use of the fact that human brain glial cells can highly express bFGF [2] and therefore could easily obtain the full-length sequences of bFGF from 12-week fetal brain, which provide a simple way to detect bFGF cDNA.

Not only can they secrete bFGF, but LECs can also highly express high-affinity FGFR. bFGF has an important role in the formation of PCO because it can induce LEC proliferation, migration, and production of extracellular matrix. So, some studies regarding the use of the bFGF antibody, which is the inhibitor of the bFGF receptor or antisense nucleic acid of FGF, for inhibiting LEC proliferation. Francine[3,4] added bFGF (SAP or rbFGF-SAP, respectively, into culture medium of bovine LECs or rabbit capsules after cataract surgery and proved that this immunotoxin can inhibit the proliferating of LECs.

Using gene recombinant technique, the receptor binding domain of natural toxin can be replaced by antibodies or cytokines. The recombinant immunotoxin can specifically recognize and kill target cells, but causes little injury to other cells. DT, which has strong cytotoxicity, consists of three independent structure regions: the catalytic domain, transmembrane domain and the receptor binding domain. It is suitable for gene recombination and maintaining the toxin [5]. ONTAK (DAB_{389}-IL2) has been approved by the US FDA for treatment of cutaneous T-cell and B-cell lymphomas (CTCLs)[6].

Cloning and expression of the DT-bFGF fusion protein has not been reported before. In the present study, we have successfully cloned and expressed a fusion protein-DT_{389}-hbFGF, in which DT_{389} represents the N-terminal 389 amino acids of DT, including the catalytic domain and transmembrane domain, while the cell-binding domain of DT was replaced by hbFGF. In the next step, we will determine the efficacy and safety of the DT_{389}-hbFGF, and investigate its potential application as an immunotoxin for prevention of PCO.

REFERENCES

1 Seghezzi G, Patel S, Ren CJ, Gualandris A, Pintucci G, Robbins ES, Shapiro RL, Galloway AC, Rifkin DB, Mignatti P. Fibroblast growth factor2 (FGF2) induces vascular endothelial growth factor (VEGF) expression in the endothelial cells of forming capillaries: an autocrine mechanism contributing to angiogenesis. *J Cell Biol*1998;141(7):1659-1673

2 Gomez-Pinilla F, Lee JW, Cotmam CW. Distribution of basic fibroblast growth factor in the developing rat brain. *Neuroscience*1994;61(4):911-923

3 Behar-Cohen FF, David T, Buechler Y, Nova MP, Houston LL, Pouliquen YM, Courtois Y. Cytotoxic effects of FGF2-saporin on bovine epithelial lens cells *in vitro*. *Invest Ophthalmol Vis Sci*1995;36(12):2425-2433

4 Behar-Cohen FF, David T, D'Hermies F, Pouliquen YM, Buechler Y, Nova MP, Houston LL, Courtois Y. *In vivo* inhibition of lens re-growth by fibroblast growth factor 2-saporin. *Invest Ophthalmol Vis Sci*1995;36(12):2434-2448

5 Choe S, Bennett MJ, Fujii G, Curmi PM, Kantardjieff KA, Collier RJ, Eisenberg D. The crystal structure of diphtheria toxin. *Nature*1992;357(6375):216-222

6 Frankel AE, Powell BL, Hall PD, Case LD, Kreitman RJ. Phase I trial of a novel diphtheria toxin/granulocyte macrophage colony-stimulating factor fusion protein (DT388GMCSF) for refractory or relapsed acute myeloid leukemia. *Clin Cancer Res*2002;8(5):1004-1013