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Purification and characterization of a long-acting ciliary neurotrophic factor via genetically fused with an albumin-binding domain

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ABSTRACT

Ciliary neurotrophic factor (CNTF) is a promising candidate for the treatment of neurodegenerative or metabolic diseases, but suffers rapid clearance in body. Herein we constructed a new long-acting recombinant human CNTF (rhCNTF) by genetic fusion with an albumin-binding domain (ABD) through a flexible peptide linker, hoping to endow the new molecule prolonged serum circulation time by binding with endogenous human serum albumin (HSA) and then utilizing the naturally long-half-life property of HSA. This fused protein rhCNTF-ABD was expressed in *Escherichia coli* mainly in the soluble form and purified through a two-step chromatography, with purity of 95% and a high yield of 90–100 mg/L culture. The *in vitro* binding ability of rhCNTF-ABD with HSA was firstly verified by incubation of the two components together followed by HP-SEC analysis. ABD-fused rhCNTF showed similar secondary and tertiary structure as the parent protein. It retained approximately 94.1% of the native bioactivity as demonstrated via CCK-8 cell viability assay analysis. *In vivo* studies in SD rats were performed and the terminal half-life of 483.89 min for rhCNTF-ABD was determined, which is about 14 folds longer than that of rhCNTF (34.28 min) and comparable with 20 k-40 kDa PEGylated rhCNTFs. The new constructed rhCNTF-ABD represents a potential therapeutic modality, and the proposed strategy may also have useful applications for other long-lasting biopharmaceutics' design.

1. Introduction

Ciliary neurotrophic factor (CNTF) is one of the most important neurotrophic factors, which has been functionally demonstrated activity as a survival and differentiation factor for cell of the nervous system. Due to its neuroprotective properties, CNTF is considered as a potential therapeutic for many neurodegenerative diseases such as Parkinson disease, Amyotrophic Lateral Sclerosis, and Alzheimer's disease [1–3]. Moreover, CNTF also exerts metabolic effects resulting in substantial weight loss and therefore is considered potential as metabolic syndrome pathologies treatment such as obesity and type II diabetes [4]. Natural CNTF is a kind of non-glycosylated monomeric cytokine and was initially isolated from chick embryo ciliary neurons. Because of the biological importance and the possible clinical application, approaches for efficiently and large-scalely producing CNTF has been developed through *E. coli* host system, either in soluble form or as inclusion bodies [5,6]. However, short circulatory half-life for recombinant CNTF would still be a limiting factor for its practical applications as an efficacious therapeutic [7].

PEGylation has been used to extend the circulatory life of CNTF in body [8,9]. By chemically conjugated with polyethylene glycol chains to increase its hydrodynamic radius and enhancing physiochemical stability, CNTF was endowed a prolonged half-life of almost 10 h, whereas the half-life for rhCNTF is about half hour [8]. However, recent study reported the unexpected emergence of anti-PEG antibody in patients administered with PEGylated drugs and even in normal persons [10]. PEGylation is now facing the great challenge as a successful strategy for developing long-acting biotherapeutics [11,12].

Binding with endogenous serum albumin as a drug carrier is another increasingly attractive strategy to extend the circulatory

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time for small protein and peptide, utilizing the naturally long-half-life property of human serum albumin (HAS). Several albumin-binding moieties like albumin-binding peptides, small antibodies against albumin, synthetic chemical groups, and albumin-binding domains of bacterial proteins have been extensively described [13]. Albumin-binding domain (ABD), derived from streptococcal protein G [14], stands out because it showed superior affinity (femtomolar to nanomolar) with albumin of human, monkey, mouse, and rat [15,16]. Previous researches revealed that ABD fusion significantly extended the half-lives of a bispecific single chain diabody (scDb) [17], a cancer cell-targeted toxin [18], affibody molecules [19,20], and the glucagon-like peptide 1(GLP-1) [21]. Moreover, ABD is a small peptide with 46 amino acids, which will possibly allow the therapeutic protein to be expressed in *Escherichia. coli* after fusion to ABD at a high level.

These studies triggered our interest in investigating whether ABD fusion is an alternative way to improve the pharmacokinetics of CNTF by taking the advantage of the long half-life of endogenous albumin. In this study, we genetically fused an ABD to the C-terminus CNTF to construct rhCNTF-ABD and, evaluated the impact of the ABD-fusion on albumin binding, *in vitro* activity to support the proliferation of cells, and *in vivo* circulatory half-life in SD rat. All the results suggested that fusion with ABD would be an efficient way to develop long-acting CNTF.

2. Materials and methods

2.1. Materials

Recombinant human CNTF was expressed and prepared in *E. coli* in our lab [8,22], and mPEG20k-CNTF and mPEG40k-CNTF were chemically modified with polyethylene glycol and purified in our lab and, the purity of PEGylated CNTF was verified by SDS-PAGE to be higher than 95% [23]. HSA, Trypan blue, Cell Counting Kit (CCK-8) and Hoechst 33,258 were all purchased from Sigma (USA). All buffers were prepared using deionized Milli-Q water.

2.2. Construction, expression and purification of the rhCNTF-ABD

ABD was genetically fused to the C terminus of rhCNTF and a flexible linker $(G_4S)_3$ was inserted between ABD and rhCNTF. The gene encoding rhCNTF-ABD was synthesized based on the amino acid sequences of rhCNTF and ABD035 [15]. A plasmid expressing the ABD fused rhCNTF (denoted as rhCNTF-ABD) was synthesized and constructed in our lab. Briefly, the synthesized gene fragment was digested by the NdeI and XhoI restriction endonucleases and cloned into the pET-28a + vector (Novagen, Madison, WI, USA). The constructed plasmid was transformed into BL21 (DE3) competent cells (Cwbiotech, Beijing, China) and expressed in accordance with the manufacturer's protocol.

The transformed *E. coli* was grown at 30 °C in LB medium supplemented with 100 μg/mL kanamycin and induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) when cell density reached the mid-exponential phase. After fermentation, the bacterial cells were harvested by centrifugation at 4000 rpm for 30 min at 4 °C. The cell pellets were re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.0) followed by sonication on ice. After centrifugation at 10,000 rpm for 30 min at 4 °C, the supernatant was collected and diluted 3 times with dilution buffer (20 mM Tris-HCl, pH 7.0), followed by purification through two chromatographic steps.

Firstly, it was loaded to a Octly Fast Flow column (XK 16 × 100 mm ID, GE Healthcare), equilibrated by 20 mM Tris-HCl, 0.8 M ammonium sulfate, pH 7.0, washed with 10% of elution buffer (20 mM Tris-HCl, pH7.0) and then eluted with 100% of elution buffer (20 mM Tris-HCl, pH 7.0). Secondly, the collected target fraction was further purified by a Ni Fast Flow (XK 26 × 600 mm ID, GE Healthcare), equilibrated with 20 mM Tris-HCl, 0.5 M NaCl, pH 7.0, washed by 1% elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 M imidazole, pH 7.0) and then eluted with 10%,60% and 100% of elution buffer. Each elution peak was collected and its purity was determined by 12% SDS-PAGE. The purified protein was further desalted by a Sephadex G-25 column (XK 26 × 100 mm ID, GE Healthcare) into PBS or 5 mM phosphate buffer and temporarily stored at 4 °C for less than a week before further test.

2.3. Characterization of rhCNTF-ABD

2.3.1. SDS-PAGE

The elution peak of the two-step column chromatography for rhCNTF-ABD and the final product were analyzed by SDS-PAGE according to the methods of Laemmli [24]. The samples were combined with a reducing 5 × sample-loading buffer (Tris-Glycine 5 × SDS sample buffer plus 10% β-mercaptoethanol), heated at 100 °C for 5 min, and applied to 15% Tris-Glycine gels. Gels were stained and analyzed for protein using Coomassie brilliant blue dye.

2.3.2. Reversed-phase high performance liquid chromatography (RP-HPLC)

For the purity analysis of rhCNTF-ABD, 100 μL of final sample was applied to a reversed-phase high performance liquid chromatography (RP-HPLC) column (Shiseido Proteonavi C4, 250 × 4.6mmID, Japan) connected to a HPLC system (Agilent 1100, USA). HPLC solvent A was H_2O (containing 0.1% TFA). Solvent B was acetonitrile (containing 0.1% TFA). A linear gradient from 50% to 90% of solvent B in 30 min was used with a flow rate of 0.5 mL/min. Absorbance was recorded at 280 nm.

2.3.3. MALDI-TOF MS

The molecular weight of rhCNTF-ABD was determined by matrix-assisted laser desorption/ionization time-of-fight mass spectrometry (MALDI-TOF-MS) (SCIEX, USA). 5 μL sample was slowly and directly dispensed onto a MALDI plate followed by a-Cyano-4-hydroxycinnamic acid matrix on it and dried at room temperature. Data was acquired in linear mid-mass positive mode, averaging 2000 laser shots per MALDI-TOF spectrum. Calibration mixtures (Applied Biosystems) were used to calibrate the spectrum to a mass tolerance within 2 Da. The mass range is 15,000–30000 Da.

2.3.4. Circular dichroism (CD)

The far UV spectrum (200–260 nm) CD spectrum was recorded using a J-810 spectrometer (Jasco, Japan) at 25 °C. rhCNTF and rhCNTF-ABD were diluted with 5 mM phosphate buffer to 0.2 mg/mL for analysis. The cuvette path length was 1 mm. Each spectrum was scanned three times, and the average spectrum was plotted.

2.3.5. Intrinsic emission fluorescence

Intrinsic emission fluorescence spectra of rhCNTF and rhCNTF-ABD were analyzed using an F-4500 fluorescence spectrophotometer (Hitachi, Japan). Protein solutions were diluted with 5 mM phosphate buffer to 0.2 mg/mL for immediate analysis. Spectra were recorded on a 1 cm path-length cuvette with an excitation slit width of 5 nm and an emission slit width of 2.5 nm. The excitation wavelength was set at 280 nm for the specific excitation of tryptophan residues, and emission spectra were recorded from 290 to 500 nm at a constant slit of 1 nm.