

Modified insulin-like growth factor 1 containing collagen-binding domain for nerve regeneration

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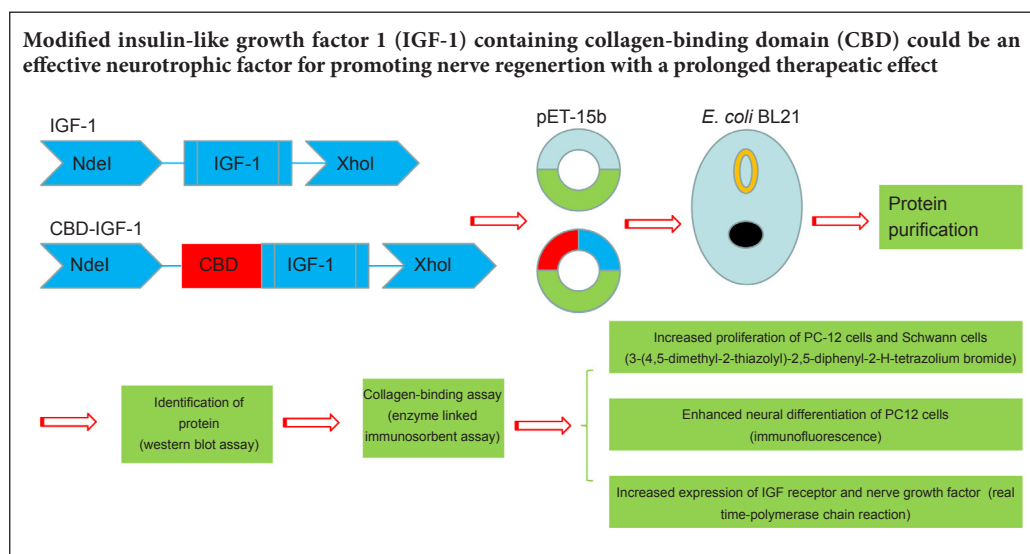
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Graphical Abstract



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Abstract

Insulin-like growth factor 1 (IGF-1) is a potential nutrient for nerve repair. However, it is impractical as a therapy because of its limited half-life, rapid clearance, and limited target specificity. To achieve targeted and long-lasting treatment, we investigated the addition of a binding structure by fusing a collagen-binding domain to IGF-1. After confirming its affinity for collagen, the biological activity of this construct was examined by measuring cell proliferation after transfection into PC12 and Schwann cells using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide assay. Immunofluorescence staining was conducted to detect neurofilament and microtubule-associated protein 2 expression, while real time-polymerase chain reaction was utilized to determine IGF-1 receptor and nerve growth factor mRNA expression. Our results demonstrate a significant increase in collagen-binding activity of the recombinant protein compared with IGF-1. Moreover, the recombinant protein promoted proliferation of PC12 and Schwann cells, and increased the expression of neurofilament and microtubule-associated protein 2. Importantly, the recombinant protein also stimulated sustained expression of IGF-1 receptor and nerve growth factor mRNA for days. These results show that the recombinant protein achieved the goal of targeting and long-lasting treatment, and thus could become a clinically used factor for promoting nerve regeneration with a prolonged therapeutic effect.

Key Words: nerve regeneration; insulin-like growth factor 1; collagen-binding domain; fusion protein; collagenase; targeted therapy; neural regeneration

Introduction

Insulin-like growth factor 1 (IGF-1), a polypeptide growth factor containing 70 amino acids, has a molecular weight of about 7.5 kDa. This factor is an anabolic peptide hormone with structural similarities to proinsulin (Holman and Baxter, 1996; Twigg and Baxter, 1998; Rosen and Pollak, 1999; Clemmons, 2006). While IGF-1 can be synthesized by most organs during fetal development, it is mainly produced by hepatocytes and the pancreas after birth (Fernandez and Torres-Alemán, 2012). The IGF-1 receptor (IGF-1R), which

mediates transmembrane information transfer of IGF-1, is a transmembrane glycoprotein with a tetrameric structure formed by two α and β subunits (Annunziata et al., 2011; Benarroch, 2012).

IGF-1-specific binding to IGF-1R usually triggers two canonical pathways: PI3K/Akt and MEK/ERK (Benarroch, 2012; Hayashi et al., 2013). This factor exerts potent effects on neurogenesis and synaptogenesis, as well as neuroprotective, pro-proliferative, and anti-apoptotic actions. It also has the crucial ability to promote axon regeneration and demy-

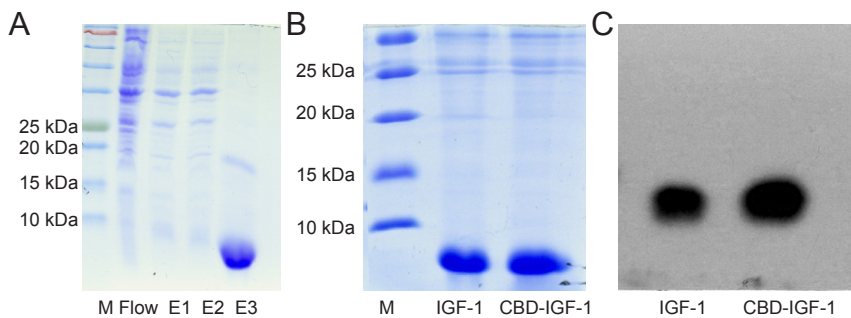


Figure 1 Polyacrylamide gel electrophoresis and western blot assay of recombinant proteins.

(A) SDS-PAGE of soluble mixtures after purification. (B) SDS-PAGE of fusion protein after purification. The molecular weight of fusion protein is between 5 to 10 kDa. (C) Western blot assay of purified proteins. Proteins were confirmed to be CBD-IGF-1 or IGF-1 by western blot assay with an anti-IGF-1 antibody. M: Protein marker; Flow: liquid spilled from gel hole; E1–3: soluble mixtures were purified with Ni-NTA affinity chromatography at pH 6.5, 5.5, or 4.5, respectively. SDS-PAGE: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; IGF-1: insulin-like growth factor 1; CBD: collagen-binding domain.

Table 1 DNA sequences of CBD-IGF-1 and IGF-1

Name of the sequence	Sequence (5'→3')
CBD-IGF-1	<u>CAT ATG ACT AAA AAG ACT CTG AGA ACC GGT GGT GGT GGC GGC GGC CCG GAA ACC CTG TGC GGC GCG GAA CTG GTT GAT GCG CTG CAG TTC GTT TGC GGT GAT CGT GGC TTC TAC TTC AAC AAA CCG ACC GGC TAC GGT AGC AGC AGC CGT CGT GCG CCG CAG ACC GGC ATC GTT GAT GAA TGC TGC TTC CGT AGC TGT GAT CTG CGT CGT CTG GAA ATG TAC TGC GCG CCG CTG AAA CCG GCG AAA AGC GCG TAA CTC GAG</u>
IGF-1	<u>CAT ATG GGT CCG GAA ACC CTG TGC GGT GCT GAA CTG GTT GAT GCT CTG CAG TTC GTT TGT GGT GAT CGT GGT TTC TAT TTT AAC AAA CCG ACC GGT TAT GGT TCT TCT TCT CGT GCT GCT CCG CAG ACC GGC ATT GTT GAT GAA TGT TGC TTC CGT TCT TGT GAT CTG CGT CGT CTG GAA ATG TAT TGT GCT CCG CTG AAA CCG GCT AAA TCT GCT TAA CTC GAG</u>

Underlined zone at the 5' end is an *NdeI* site and underlined zone at the 3' end is an *XhoI* site. The sequence of CBD is marked in red. The gene sequence of the CBD is deduced from human collagenase. The sequence of glycine is labeled in bold. The glycine chains can prevent CBD and IGF from affecting each other. IGF-1: Insulin-like growth factor 1; CBD: collagen binding domain.

elination (Scolnick et al., 2008; Dyer et al., 2016; Rauskolb et al., 2017). Thus, IGF-1 may be a potential therapeutic factor for peripheral nerve repair. However, in its native form, it is an impractical therapy because of its limited half-life of 8–16 hours *in vivo* (Laron, 1999). Thus, short half-life, rapid diffusion, and complications resulting from use of high dosages limit the widespread application of IGF-1. A previous study revealed that many tissues in the body, such as aorta and skin, contain collagens and could therefore be targets of the collagen-binding domain (CBD) (Chen et al., 2007). We hypothesized that CBD could be integrated into IGF-1 to improve its current deficiencies.

In this study, we linked IGF-1 with a CBD deduced from collagenase and expressed the fusion protein using a pET-15b expression system. To verify the biological function of the synthesized protein, we tested its collagen-binding activity and effects on cell proliferation, neural differentiation,

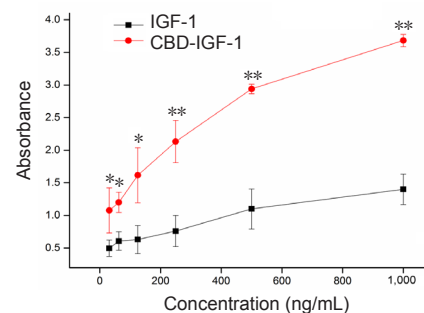


Figure 2 Dose response curve showing binding of IGF-1 and CBD-IGF-1 to rat tail collagen.

The recombinant proteins IGF-1 and CBD-IGF-1 were added to plates containing collagen and incubated overnight. Absorbance values increased with increasing protein concentration. Absorbance at 562 nm for CBD-IGF-1 was significantly higher than that of IGF-1. Data are expressed as the mean \pm SD, as analyzed by two-tailed *t*-test. * $P < 0.05$, ** $P < 0.01$, vs. IGF-1. IGF-1: Insulin-like growth factor 1; CBD: collagen-binding domain.

and levels of IGF-1R-mRNA *in vitro*.

Materials and Methods

Construction and expression of plasmids

DNA sequences of CBD-IGF-1 and IGF-1 were designed to generate relative proteins (Kim et al., 2015). The two DNA sequences (Table 1) were designed to include restriction endonuclease sites at both ends (underlined). In addition, the DNA sequence of CBD-IGF-1 was designed to include a CBD sequence (*TKKTLRT*, in red) and glycine sequence (in bold). CBD-IGF-1 DNA sequences and IGF-1 DNA sequences were ligated into a similarly digested pET-15b (+) vector and transformed into *E. coli* BL21 (DE3) host cells, as described previously (Jacobus and Gross, 2015). Subsequently, 100 μ L of host cells carrying recombinant plasmid were plated on solid Luria-Bertani (LB) medium pre-mixed with penicillin (100 μ g/mL). Transfected cells grew overnight at 37°C and then monoclonal colonies were selected and plated in 10 mL of liquid LB medium pre-mixed with penicillin. Afterwards, cells grew overnight in a shaker at 150 r/min and 37°C.

Protein purification

After overnight incubation, 1 mL of transfected cells was added to 1 L of YTA medium containing ampicillin (100 μ g/mL), which was left to recover at 37°C and 150 r/min to an OD_{600}

of 0.8–1.0. Subsequently, it was induced overnight by 1 mL of isopropyl- β -D-thiogalactopyranoside (1 M) at 28°C and 150 r/min. The mixture was pelleted by centrifugation for 30 minutes at 4°C and 4,000 \times g (Heraeus Sepatech, Osterode, Germany). After removal of the supernatant, the remaining pellet was frozen at –80°C for purifying the target protein.

The remaining pellet was resuspended in lysis buffer containing 50 mM phosphate-buffered saline (PBS) and 1 mM phenylmethyl sulfonyl fluoride, pH 6.5. Resuspended cells were sonicated for 20 minutes (300 W, 10 s/10 s). The cell lysate was centrifuged for 30 minutes at 4°C and 10,000 \times g. The supernatant was transferred to a fresh tube for future analysis, while the pellet was washed three times with 30 mL of wash buffer (2 M urea, 50 mM PBS, pH 6.5). Following the last wash, the solution was centrifuged under the same conditions. The insoluble cell pellet was resuspended by rocking in 25 mL of dissolving buffer (8 M urea, 50 mM PBS, 5 M β ME, pH 6.5) overnight at 4°C. Soluble mixtures were centrifuged at 4°C and 10,000 \times g for 30 minutes. For the second round of purification, the supernatant was used for CBD-IGF-1 or IGF-1 purification with Ni-NTA affinity chromatography.

Proteins were eluted by linearly decreasing the urea concentration of the dialysate from 6 M to a final concentration of 0 M (6–0 M). The dialysate was changed every day until there was no urea.

Western blot assay

All samples were analyzed *via* immunoblot with anti-IGF-1 antibody to verify the presence of CBD-IGF-1 or IGF-1. Extracted proteins were transferred to nitrocellulose membranes, blocked, and then incubated with rabbit anti-human IGF-1 monoclonal antibody (1:1,000; Abcam, Cambridge, UK) at 4°C overnight. The following day, membranes were rocked for 1 hour at room temperature with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (1:500; Abcam). After three washes in Tris-buffered saline with Tween, images of bands were acquired using darkroom development techniques for chemiluminescence.

Collagen-binding assay detected by enzyme linked immunosorbent assay (ELISA)

Type I collagen (in 0.5 M acetic acid) was coated onto 96-well plates. After washing with PBS three times, 100 μ L of recombinant IGF-1 or CBD-IGF-1 were added to 96-well plates and incubated at 4°C overnight. After three washes, diluted anti-IGF-1 (1:5,000) was added to the plates and incubated at 37°C for 1 hour. After three washes, 100 μ L of secondary antibody (1:2,000; Earthox, San Francisco, CA, USA) labeled with horseradish peroxidase was added and incubated for 1 hour at 25°C, followed by three washes. The horseradish peroxidase substrate was added and incubated at 37°C for 30 minutes. An Infinite F200 microplate reader (Tecan, Salzburg, Austria) was used to quantify absorbance at 562 nm.

Proliferation effects on PC12 and Schwann cells

As previously described (Zhang et al., 2006), the activity of IGF-1 and CBD-IGF-1 was assayed in both PC12 and

Schwann cells (both from Cell Bioscience, Shanghai, China). Briefly, 5×10^4 cells in 200 μ L of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum were inoculated onto poly-L-lysine-coated 96-well plates for 4 hours. After cells adhered to plates, wells were rinsed with PBS twice and then incubated for 3 days in 200 μ L of DMEM containing 10% fetal bovine serum and 15, 31, 62.5, 125, 250, 500, or 1,000 ng/mL of IGF-1 or CBD-IGF-1. Cell proliferation was evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. Briefly, 20 μ L of MTT solution was added to each well and incubated for 4 hours. After centrifugation, the supernatant was removed and 150 μ L of dimethyl sulfoxide was added to each well. After incubation for 10 minutes, absorbance values were measured at 570 nm with an Infinite F200 microplate reader.

Immunofluorescence staining

Neurofilament (NF200) and microtubule-associated protein 2 (MAP2) are important cytoskeletal proteins in neurons (Chen et al., 2017). Immunofluorescence experiments were performed to characterize NF200 and MAP2 expression. Briefly, after cells were cultured for 7 days, 20 μ L of mouse anti-mouse NF200 or mouse anti-mouse MAP2 (1:200 or 1:100, respectively; Abcam) solution was added to each well and incubated overnight at 4°C. After three washes with PBS, fluorescein conjugated rabbit anti-mouse IgG (1:100; Jackson ImmunoResearch, West Grove, PA, USA) was added to each well and incubated in the dark for 1 hour at 37°C. DAPI was added to counterstain nuclei. Images were observed by confocal laser-scanning microscope (Leica, Germany).

Real time-polymerase chain reaction (RT-PCR) for IGF-1R and nerve growth factor (NGF) mRNA expression

RT-PCR was used to analyze mRNA levels of NGF and IGF-1R in Schwann cells at different experimental periods. Total RNA was extracted from cells using TRIzol (Takara Biotechnology, Dalian, China). RT-PCR reactions were performed using a SYBR Premix Ex Taq II (Tli RNaseH Plus) (Takara Biotechnology). Primer sequences (synthesized by Sangon, Shanghai, China) for NGF, IGF-1R, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: NGF forward, 5'-GTC AGT GTG GGT TGG AGA TA-3', reverse, 3'-GGA GTA TGA GTT CCA GTG CTT G-5'; IGF-1R forward, 5'-AAA CGC TGA CCT CTG TTA CCT CTC-3', reverse, 3'-CAG GTA GTC CGA AGT AGG CG-5'; and GAPDH forward, 5'-ACC ACA GTC CAT GCC ATC AC-3', reverse, 3'-TCC ACC ACC CTG TTG CTG TA-5'. Reactions were carried out as follows: 95°C for 30 seconds, followed by 40 cycles of 95°C for 5 seconds and 56°C for 30 seconds. Amplification was performed using a Stratagene Mx3005p (Agilent, CA, USA). Expression of GAPDH mRNA was determined as an internal control. Final results are expressed as a ratio of the mRNA of interest to that of GAPDH mRNA.

Statistical analysis

Data, expressed as the mean \pm SD, were analyzed using IBM

SPSS statistics 21.0 (IBM, Armonk, NY, USA). The statistical significance of differences was set as $P < 0.05$ and $P < 0.01$ by two-tailed *t*-test.

Results

Construction of CBD-IGF-1 recombinant plasmids and transfection into host cells

Upon harvesting and lysing transfected cells, the soluble mixtures were centrifuged and purified with Ni-NTA affinity chromatography. Coomassie brilliant blue-stained bands of purified proteins were observed at 5–10 kDa on the gel (**Figure 1A, B**). The presence of CBD-IGF-1/IGF-1 was verified by western blot assay with primary anti-human IGF-1 and secondary antibody conjugated to horseradish peroxidase. As observed in the bands of **Figure 1C**, expressed target proteins IGF-1 and CBD-IGF-1 were between 5–10 kDa.

Collagen-binding assay of CBD-IGF-1 and IGF-1

The binding ability of the two recombinant proteins to type I collagen is shown in **Figure 2**, with collagen-binding activity determined by ELISA. As shown in the binding curves, absorbance at 562 nm for CBD-IGF-1 was significantly higher than for IGF-1. This result indicates an increased amount of CBD-IGF-1 bound to type I collagen.

Effect of CBD-IGF-1 on PC12 and Schwann cell proliferation

Effects of the two proteins on cell proliferation were examined by MTT assay. Within the concentration range from 15 to 250 ng/mL, CBD-IGF-1 groups showed similar proliferation rates to the IGF-1 groups (**Figure 3A, B**). However, as the concentration increased, the rate of cell proliferation increased. However, at concentrations of 500 ng/mL and 1,000 ng/mL, the proliferation rate of cells cultured with CBD-IGF-1 was higher than that of IGF-1.

Effect of CBD-IGF-1 on PC12 cell morphology

PC12 cells were cultured in 96-well plates for 3 days. The morphology of PC12 cells was observed by confocal laser-scanning microscope (TCS SP5, Leica). As displayed in **Figure 3C**, both proteins promoted cell proliferation and axon growth, which peaked at 500 ng/mL. The cells observed began to differentiate into neurons and immunofluorescence experiments were performed to further verify cell differentiation. As exhibited in **Additional Figures 1 and 2**, MAP2 and NF200 were observed in cells.

CBD-IGF-1 promoted IGF-1R and NGF mRNA expression in Schwann cells

Cell proliferation is the result of signal transduction, which is stimulated by IGF-1R (Fernandez and Torres-Alemán, 2012). Levels of IGF-1R were measured by RT-PCR. Expression of IGF-1-induced IGF-1R mRNA increased rapidly and gradually decreased with time. However, IGF-1R mRNA expression induced by CBD-IGF-1 was maintained at a relatively stable level for a longer period of time. Notably, CBD-IGF-1 significantly promoted NGF mRNA expression (**Figure 4**).

Discussion

Neurotrophic factors play an important role in the repair of nerve injury (Zhang et al., 2000; Lee et al., 2003; Yates et al., 2004; Fernandez and Torres-Alemán, 2012; Chaker et al., 2015; He et al., 2016; Sacchetti and Lambiasi, 2017; Wen et al., 2017). Among neurotrophic factors, IGF-1 is a pleiotropic factor that can promote proliferation and differentiation of neurons (Nieto-Estévez et al., 2016). However, like other growth factors, neurotrophic factors are difficult to maintain at the target site for long periods of time because of their diffusion and short half-life. Moreover, a large dose of neurotrophic factors may cause harmful consequences. To overcome these obstacles, many novel drug carriers have been developed to immobilize a drug on target cells, protein, or protein regions. Results of a previous study investigating the four segments of 116-kDa *Clostridium histolyticum* collagenase showed that the S3 segment was the binding domain (Matsushita et al., 1998). In a later study, the same researchers found that the CBD specifically recognized and combined with the triple-helical region of collagen's normal conformation; notably, this binding ability could be enhanced by calcium (Matsushita et al., 2001). The CBD exhibits a broad substrate spectrum because it can combine with any tissue containing type I collagen (Toyoshima et al., 2001). Although, when fusing binding domains to growth factors, it is also important to confirm that the recombinant proteins retain their activities in addition to binding collagen (Yang et al., 2009; Egawa et al., 2011).

We hypothesized that CBD could be integrated into IGF-1 protein to improve its current deficiencies for clinical application. In the present investigation, we constructed fusion proteins containing the CBD deduced from collagenase. As shown in **Figure 1**, the molecular weight of the fusion protein is between 5 to 10 kDa. This is important, as reports suggest that the human immune system recognizes recombinant proteins as endotoxins or antigens when they are larger than 10 kDa (Petsch and Anspach, 2000; Kim et al., 2015). It is also worth noting that function and binding ability can be improved by adding amino acids between growth factors and binding domains (Yang et al., 2009; Egawa et al., 2011). Although CBD binds to the intertwined region of the triple helix, it cannot unwind the helix (Philominathan et al., 2012).

Purified proteins were confirmed to be CBD-IGF-1 or IGF-1 by western blot assay. IGF-1 promotes proliferation of nerve cells by binding to IGF-1R, which can activate two canonical pathways: PI3K/Akt or MAPK (Mairet-Coello et al., 2009; Yuan et al., 2015). To determine if recombinant proteins retained their IGF-1R binding activity and acquired the ability to bind type I collagen, we analyzed IGF-1R mRNA levels and performed binding assays for CBD-IGF-1. The results confirmed the affinity of CBD-IGF-1 towards type I collagen and increased ability to bind collagen. IGF-1R mRNA levels rose rapidly in Schwann cells incubated for 4 hours, and peaked after 1 day. Importantly, levels of IGF-1R mRNA stimulated by CBD-IGF-1 decreased more slowly in the following days. After incubation with the fusion protein, there was also a dramatic increase in amounts of NGF mRNA similar to that

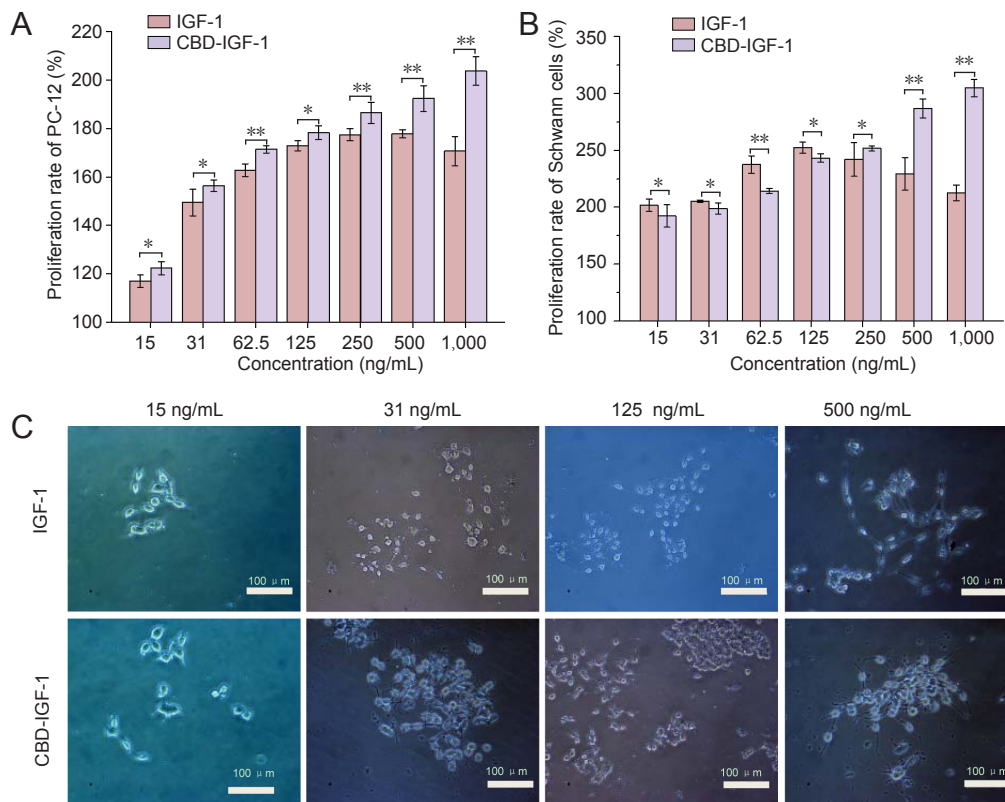


Figure 3 Effect of CBD-IGF-1 on PC12 and Schwann cell proliferation.

(A) Proliferative effects on PC12 cells. As concentration increased, the cell proliferation rate increased. Compared with IGF-1, CBD-IGF-1 had similar or better biological activity for promoting PC12 cell proliferation. (B) Proliferative effects on Schwann cells. Similar to PC12 cells, the cell proliferation rate of Schwann cells induced by CBD-IGF-1 increased with increasing concentration. However, the proliferation rate of Schwann cells induced by IGF-1 reached its peak at 125 ng/mL. (C) Morphology of PC12 cells as observed by confocal laser-scanning microscope. Numbers of cells and lengths of axons increased with increasing concentrations of the two proteins. Data are expressed as the mean \pm SD, as analyzed by two-tailed *t*-test. **P* < 0.05, ***P* < 0.01. IGF-1: Insulin-like growth factor 1; CBD: collagen-binding domain.

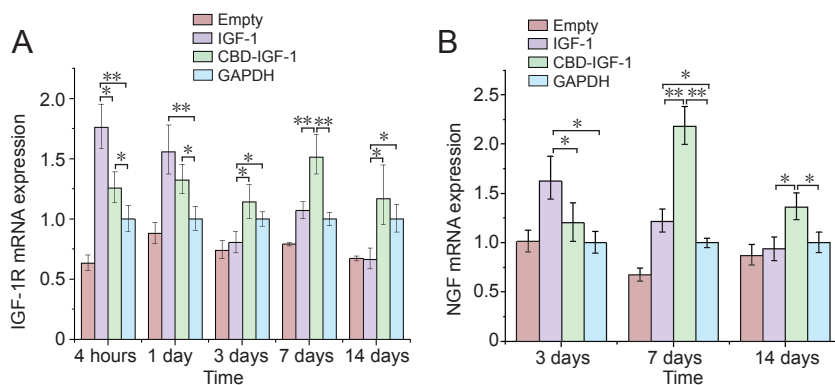


Figure 4 Effect of CBD-IGF-1 on IGF-1R and NGF mRNA levels in Schwann cells detected by RT-PCR.

After treatment with IGF-1 or CBD-IGF-1, IGF-1R and NGF mRNA were expressed in Schwann cells in both groups. IGF-1 led to a rapid increase in mRNA levels, while CBD-IGF-1 led to more sustained elevation. Empty: Tube contained all reagents except template RNA. (A) IGF-1R mRNA levels in Schwann cells. After 4-hour treatment with IGF-1, mRNA rapidly increased, but then declined rapidly over the following days. In contrast, IGF-1R mRNA expression induced by CBD-IGF-1 increased slowly and decreased slowly. (B) NGF mRNA levels in Schwann cells. Change in mRNA levels of NGF was similar to that of IGF-1R. Data are expressed as the mean \pm SD, as analyzed by two-tailed *t*-test. **P* < 0.05, ***P* < 0.01. IGF-1: Insulin-like growth factor 1; CBD: collagen binding domain; IGF-1R: insulin-like growth factor 1 receptor; NGF: nerve growth factor; RT-PCR: real time-polymerase chain reaction.

observed for IGF-1R mRNA. These results suggest that the half-life of CBD-IGF-1 is longer than that of IGF-1.

The biological activities of fusion proteins were examined using an MTT assay. With increasing concentrations, rates of cell proliferation increased. Thus, CBD-IGF-1 exhibited similar biological activities to IGF-1 *in vitro*. Interestingly, at concentrations of 500 ng/mL and 1,000 ng/mL, the proliferation rate of cells cultured with CBD-IGF-1 was higher than that of IGF-1. One possible reason for this phenomenon was that cells secreting type I collagen and CBD-IGF-1 bound to type I collagen, leading to a high local concentration. Morphological observation of PC12 cells showed that CBD-IGF-1 promoted cell proliferation and axon growth. Immunofluorescence experiments conducted to verify neuronal differentiation showed increased MAP2 and NF200 in cells, indicating

that the fusion protein can promote neural differentiation of PC12 cells. This result, which is similar to previous research showing potent actions of IGF-1 on axon regeneration (Fernandez and Torres-Alemán, 2012), demonstrated that the recombinant proteins retain their biological activity. Moreover, addition of the CBD promoted maintenance of a relatively high concentration of IGF-1 to promote cell proliferation.

The primary limitation of this research is the need to test the immunogenicity and biological activity of CBD-IGF-1 *in vivo*. As such, further *in vivo* studies are currently underway to determine the clinical efficacy of CBD-IGF-1.

In summary, we synthesized a fusion protein containing IGF-1 and CBD, and provided a preliminary evaluation of its effectiveness. Our results achieved the goal of a targeted and long-lived recombinant CBD-IGF-1 protein, which may

be an effective neurotrophic factor suitable for clinical use.

Author contributions: JAL, Chang-fu Zhao (CFZ), SJL, Chun-fang Zan (CFZ) and XYY searched literatures and wrote the paper. XYY and JAL designed the study, obtained funding, and provided technical support. Chang-fu Zhao (CFZ), SJZ, JZ, and Chun-fang Zan (CFZ) searched literatures and participated in follow-up experiments. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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Open peer review report:

Reviewer: Aldo Calliari, Universidad de la República, Uruguay.

Comments to authors: The manuscript is an interesting work intended to develop a collagen-targeted neurotrophic factor as a starting point to develop a pharmacological tool for nerve repair. The manuscript intended to develop a pharmacological tool (a recombinant molecule resulting from the fusion of IGF and a collagen binding domain from a bacterial collagenase), designed to efficiently target the nerve tissue. Overall, the research is well conducted and the results are clearly described.

Additional file:

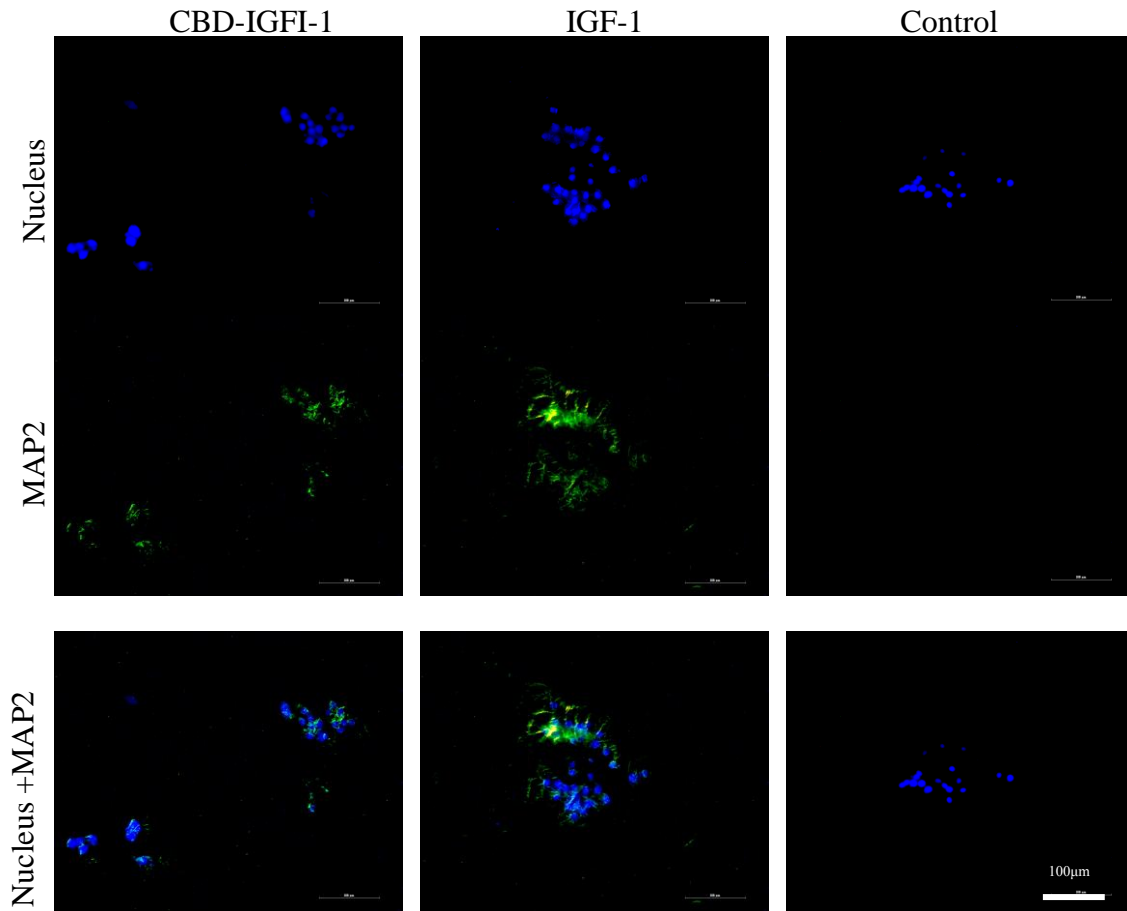
Additional figure 1: Immunofluorescence staining of MAP2.

Additional figure 2: Immunofluorescence staining of NF200.

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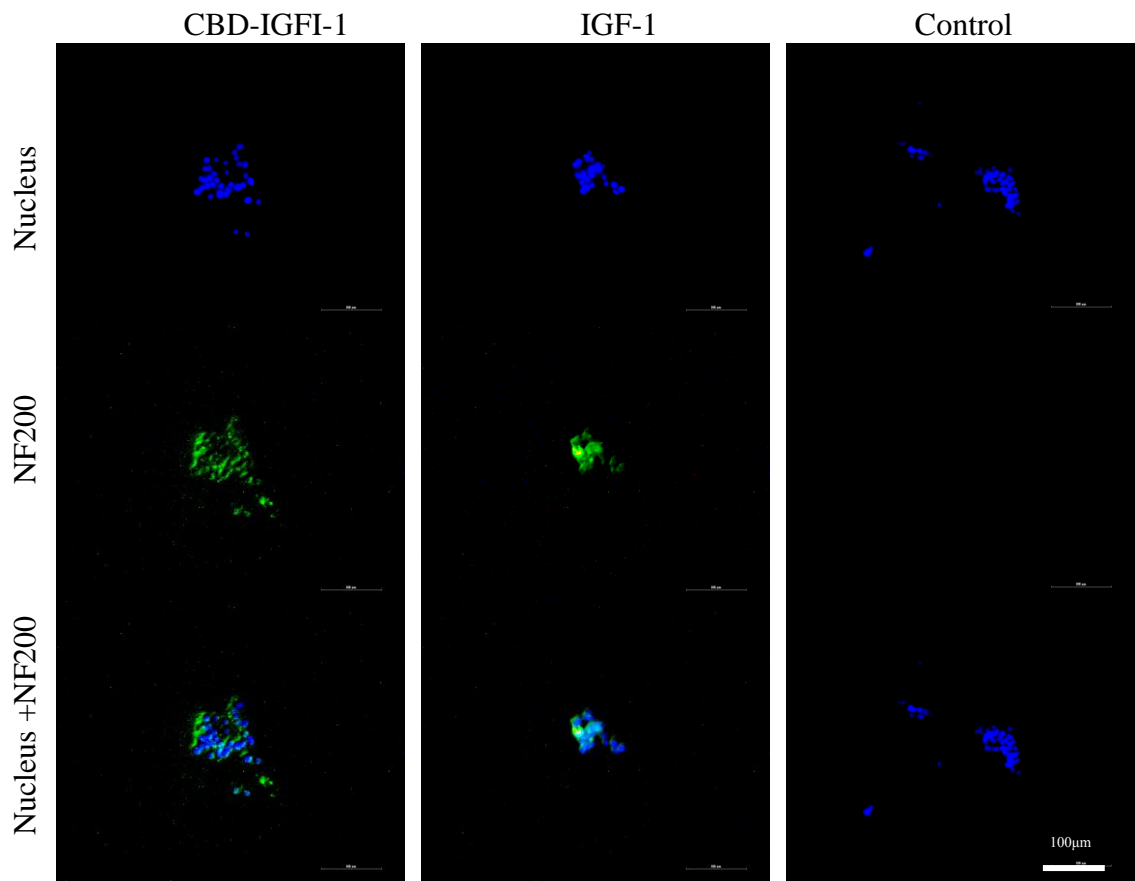
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Additional Figure 1 The immunofluorescence staining of MAP2.

The concentration of IGF-1/CBD-IGF-1 was 250 ng/mL. The culture medium in the control group was consistent with IGF-1/CBD-IGF-1 group except IGF-1/CBD-IGF-1. There were MAP2 in the cells.



Additional Figure 2. The immunofluorescence staining of NF200.

The concentration of IGF-1/CBD-IGF-1 was 250 ng/mL. The culture medium in the control group was consistent with IGF-1/CBD-IGF-1 group except IGF-1/CBD-IGF-1. There were NF200 in the cells.