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Matrix-Metalloproteinase-Responsive Brain-Derived Neurotrophic Factor for Spinal Cord Injury Repair

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Abstract: Brain-derived neurotrophic factor (BDNF) plays a vital role in supporting neuronal survival, differentiation, and promoting synaptogenesis, thereby facilitating synaptic plasticity in the central nervous system. Administration of exogenous BDNF is a crucial approach for treating central nervous system injuries. However, the inability of sustained drug release to match disease activity often leads to insufficient drug accumulation in the injured area (ineffectiveness) and severe side effects induced by the drug (toxicity). Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are typically upregulated after tissue damage, and their upregulated expression levels represent the degree of disease activity. In this study, we utilized bioengineering techniques to prepare a BDNF that can specifically bind to collagen and be released in response to MMP substrate cleavage (collagen binding domain tissue inhibitor of matrix metalloproteinases brain-derived neurotrophic factor, CBD-TIMP-BDNF). We verified the ability of CBD-BDNF and CBD-TIMP-BDNF to specifically bind to collagen through collagen binding experiments, examined the characteristics of CBD-TIMP-BDNF in response to MMP-2 to release BDNF, and detected the biological activities of both recombinant proteins. The results demonstrated that the established microenvironment-controlled BDNF release system can respond to MMP-2 to release BDNF. The recombinant proteins CBD-BDNF and CBD-TIMP-BDNF exhibited similar biological activities to the BDNF standard. Targeting the upregulated expression of MMPs after spinal cord injury as a trigger for drug release, it is expected to achieve on-demand release of BDNF in response to the severity of the disease.

Keywords: matrix metalloproteinases (MMPs); brain-derived neurotrophic factor (BDNF); on-demand release; spinal cord injury repair

Citation: He, J.; Cai, H.; Wang, Y.; Yan, J.; Fan, C. Matrix-Metalloproteinase-Responsive Brain-Derived Neurotrophic Factor for Spinal Cord Injury Repair. *Processes* **2024**, *12*, 1510. https://doi.org/10.3390/pr12071510

Academic Editors: Stevo Najman, Sanja Stojanović and Mike Barbeck

Received: 29 May 2024
Revised: 4 July 2024
Accepted: 10 July 2024
Published: 18 July 2024

1. Introduction

Spinal cord injury is a clinically common and severe trauma to the central nervous system, often leading to permanent sensory and motor dysfunction below the level of the injury [1]. When a spinal cord injury occurs, the primary injury can directly cause the death of a large number of nerve cells at the site of injury, followed by secondary inflammatory responses, neuronal necrosis, and apoptosis, resulting in the interruption of neural pathways [2]. Therefore, the key to the treatment of spinal cord injury is to replenish the lost nerve cells and rebuild the neural circuits. However, both exogenous transplanted and endogenous nerve cells face difficulties in long-term survival due to the inhibitory microenvironment at the site of spinal cord injury, often leading to rapid apoptosis or necrosis, which can affect the effectiveness of neural cell therapy for spinal cord injury [1].

Brain-derived neurotrophic factor (BDNF) plays a significant role in the differentiation, survival, and promotion of synaptogenesis of nerve cells. Typically, after ischemia or injury in the central nervous system, nerve cells secrete a certain amount of BDNF autonomously. However, the amount of BDNF secreted by the cells is extremely small, and while it has

an effect on neuronal injury repair, the impact is limited. When BDNF is used for interventional treatment, the degenerative atrophy of nerve cells begins to improve, effectively reducing neuronal apoptosis [3,4]. Numerous research experiments in recent years have confirmed that BDNF can promote the repair and regeneration of axons and nerve fibers, establishing its important position in the field of neurotherapeutics [5]. However, transplanted BDNF is often passively released, making it difficult to maintain effective local therapeutic concentrations during intense injury responses. On the other hand, excessive transient release of the drug during mild injury responses can pose safety risks and fail to achieve the desired repair effects.

Matrix metalloproteinases (MMPs), especially MMP-2 and MMP-9, are overactivated after spinal cord injury, leading to an imbalance between MMPs and tissue inhibitors of metalloproteinases (TIMPs) in spinal cord tissue. This imbalance rapidly degrades a large amount of proteins, thereby aggravating secondary spinal cord injury [6,7]. Studies have reported that the peak expression of MMP-9 occurs between 12 and 24 h after spinal cord injury, and the degree of elevation is directly proportional to the severity of the injury, with expression persisting for up to 7 days. In contrast, MMP-2 levels begin to rise 5 days after injury [8]. Currently, one of the hot topics in research is the design of drug delivery systems triggered by MMPs in the tumor microenvironment to achieve tumor imaging and treatment. The high selectivity of enzymes confers significant advantages to MMP-responsive nanoformulations in both imaging and antitumor applications. This suggests that we can target the upregulated expression of MMPs after spinal cord injury to design and construct environmentally responsive and highly functional biomaterials that regulate the microenvironment of spinal cord injury. The realization of microenvironment-controlled release hinges on the combination of growth factors with biomaterials. Biomaterial delivery factors can be achieved through physical encapsulation or physical binding, which belong to passive diffusion and are less regulated. Chemical crosslinking is another approach, but direct crosslinking may affect the activity of growth factors. The fusion expression of collagen-binding domains (CBD) does not alter the biological activity of growth factors while enhancing their binding affinity to collagen materials. Therefore, the use of CBD fusion to express growth factors can achieve specific binding between growth factors and collagen materials.

In summary, promoting neural cell differentiation and survival is the key to successfully repairing spinal cord injuries. The use of scaffold materials loaded with growth factors to improve the injured microenvironment is an important method for regulating neural cell fate. BDNF can both promote neural cell differentiation and survival, as well as neurite outgrowth. To enable BDNF to be released as needed to better exert its effects, controlling the active release of BDNF in response to the spinal cord injury microenvironment can achieve neural cell differentiation, survival, and neurite outgrowth, thereby improving the repair effect of spinal cord injuries. To achieve microenvironment-responsive release, this study introduces MMPs substrate peptides (TIMP) by modifying growth factors and prepares CBD-TIMP-BDNF using CBD fusion expression to improve binding capacity with collagen materials. It can respond to the upregulation of MMPs expression in the spinal cord injury microenvironment to release BDNF. Collagen materials loaded with CBD-TIMP-BDNF are expected to achieve on-demand release of BDNF in response to changes in the injured microenvironment, laying the foundation for the development of environmentally responsive drugs for the efficient treatment of spinal cord injuries.

2. Materials and Methods

2.1. Prokaryotic Expression and Purification of Recombinant Proteins

The prokaryotic expression vectors pET28-CBD-BDNF encoding the CBD and the mature peptide sequence of BDNF with a histidine affinity tag, as well as pET28-CBD-TIMP-BDNF containing the encoding sequence for the short peptide substrate of MMP-2/9, were transformed into Escherichia coli receptor cells DH5α. The transformed cells were cultured on LB plates at 37 °C, and then the selected cells were transferred to LB liquid medium for

amplification and cultivation for 12 to 24 h at 37 °C. PCR and sequencing were performed to verify the correctness of the encoding sequence for the MMP-2/9 substrate short peptide and the BDNF sequence. Subsequently, the well-constructed vectors were transformed into E. coli BL21 cells. After IPTG-induced expression, the expression of recombinant proteins was analyzed via sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Concurrently, the protein concentration was determined using the Bradford method.

2.2. Analysis of Binding Abilities of CBD-BDNF and CBD-TIMP-BDNF to Collagen

A 96-well plate was coated with 100 μL/well of collagen gel (100 μg/mL) and incubated overnight at 4 °C. The unbound collagen gel was removed the next day, and the plate was air-dried and washed three times with PBS. BDNF fusion proteins (BDNF, CBD-BDNF, and CBD-TIMP-BDNF) were directly added to the coated wells and incubated for 1 h at room temperature. After incubation, the supernatant was aspirated and stored at −20 °C. The unbound factor quantity in the supernatant was measured on the second day using an ELISA kit for double insurance. The wells were washed twice with PBS to remove unbound proteins. Blocking was performed with 2.5% BSA containing 0.1% Tween 20 for at least 2 h at 37 °C. The plate was washed once with PBS, and the primary antibody (anti-BDNF, 1:500; Abcam, Hangzhou, China) was added for incubation at 37 °C for 1 h. After three washes with PBS, the secondary antibody (anti-mouse IgG: AP-conjugated, A5153, 1:10,000; Merck, Beijing, China) was added for incubation at 37 °C for 1 h. The plate was washed five times with PBS, and AP substrate P-NPP disodium (2 mg/mL, P7998; Merck, China) was added (PNPP should be dissolved in AP buffer, which has a formula of 0.1 M glycine-NaOH, 1 mM $MgCl_2$, 1 mM $ZnCl_2$, pH 9.6). After 10 min, the reaction was terminated with an equal volume of 0.2 M NaOH if individual wells were sampled separately. If a multi-channel pipette was used for sampling, termination was generally not required to eliminate time differences in sampling. Termination had the advantage of allowing for periodic observation of color changes and multiple readings with a machine. Optical density values were measured at 405 nm every 10 min for six readings, followed by an additional reading on the next day.

2.3. MMP Responsiveness and BDNF Release

To evaluate the specific regulatory role of MMPs on the release of BDNF from CBD-TIMP-BDNF, we conducted in vitro release experiments in the presence of MMP-2. The specific steps are as follows: (1) coat a 96-well plate with 100 μL/well of collagen gel (100 μg/mL) and leave it overnight at 4 °C; (2) the next day, remove the unbound collagen gel, air-dry the plate, and wash it three times with PBS; (3) add 3 μM CBD-TIMP-BDNF directly to the coated wells and incubate at room temperature for 1 h; (4) divide the samples into four groups: 3 μM CBD-BDNF+MMP2, 3 μM CBD-TIMP-BDNF+PBS, 3 μM CBD-TIMP-BDNF+MMP2, and 3 μM CBD-TIMP-BDNF+MMP2+Marimastat; (5) prepare MMP2 (62 kDa) with a concentration of 20 nM (theoretically, 3 mL is needed each time); ese 10 μg per tube and add 8.0645 mL of PBS to achieve a working concentration of 20 nM; (6) wash the wells three times with PBS to remove unbound proteins; (7) add 200 μL of PBS buffer containing 0.02% sodium azide (including 20 nM MMP2) to each well; (8) place the plate in a shaker at 37 °C and 80 rpm; (9) collect the supernatants at time points of 1 day, 5 days, 7 days, and 10 days, and replace the buffer; (10) finally, use a BDNF ELISA kit to detect the amount of BDNF in all collected supernatant samples simultaneously. By following these steps, we aim to assess the specific effects of MMPs, particularly MMP-2, on the release of BDNF from the CBD-TIMP-BDNF construct. The experiment allows us to gain insights into the potential role of MMPs in modulating the release profile of BDNF, which is crucial for understanding its therapeutic effects and optimizing its delivery systems.

2.4. Detection of Activity of CBD-BDNF and CBD-TIMP-BDNF

First, dorsal root ganglia (DRG) of neonatal rats were isolated and stripped under a dissecting microscope. A 48-well plate was coated with 1 mg/mL mouse tail collagen (200 uL/well).

Unbound collagen was aspirated, the plate was air-dried using a hairdryer, followed by three washes with PBS. The DRG were seeded onto the plate and incubated in DMEM/F12 medium containing 10% FBS. After the DRG adhered to the plate, 0.01 μmol/L of CBD-BDNF or CBD-TIMP-BDNF was added to each well, while the control group received an equal volume of PBS. The plate was then placed in a cell culture incubator for 48 h. Immunofluorescence staining of nerve fibers was performed, and statistical analysis was carried out using Image-pro plus software 5.0. The biological activity of CBD-BDNF and CBD-TIMP-BDNF was evaluated based on their ability to stimulate the growth of axons in the DRG.

2.5. Statistical Analysis

The statistical software GraphPad Prism 8 was utilized to process all experimental data, which were expressed as means ± SD. The significance between two groups was analyzed using Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) with Tukey's test was performed. A statistically significant difference was considered at $p < 0.05$.

3. Results

3.1. Preparation of Recombinant Protein CBD-TIMP-BDNF

The coding sequences of CBD, the MMP-2/9 substrate short peptide, and the mature peptide of BDNF were linked together using gene synthesis methods (Figure 1 and Table 1). PCR analysis was performed using the above synthetic sequence as a template. The primer sequences for CBD-TIMP-BDNF were TGGTGCCGCGCGGCAGCCATATG-CACTCTGCCCGCCGAGGGGAGC (forward primer; 5′–3′), GTACCTACCACCACCGC-CGCCGCCAGGCCCAGCGGGCCTCTTCCCCTTTTAATGGTCAA (reverse primer 1, 5′–3′), TTAAGTACGCAGGGTTTTCTTAGTACTACCACCACCGCCGCCC (reverse primer 2, 5′–3′), and CAAGCTTGTCGACGGAGCTCGAATTCTTAAGTACGCAGGGTTTTCTTA (reverse primer, 5′–3′). The coding sequence of CBD-TIMP-BDNF was inserted into PET28b, and the successful ligation of the MMP-2/9 substrate short peptide coding sequence with the BDNF sequence was verified through PCR and sequencing. The constructed vector was then transformed into Escherichia coli BL21 cells. After IPTG-induced expression, protein polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed that CBD-TIMP-BDNF was preferentially expressed in the supernatant under optimized experimental conditions. Furthermore, the supernatant was purified using a His purification column, and the purity of the protein was detected by SDS-PAGE. As shown in Figure 2 below, the two recombinant protein bands were single and without obvious impurities, indicating that they can be directly used for subsequent functional detection.

Table 1. Gene and amino acid sequence of CBD-TIMP-BDNF.

Coding Gene:
>*BDNF-TIMP-Linker-CBD-486bp*
ATGGGCAGCAGCCATCATCATCATCATCACAG-CAGCGGCCTGGTGCCGCGCGGCAGCCATATGCAC- TCTGACCCTGCCCGCCGAGGGGAGCTGAGCGTGTGTGACAGTATTAGTGAG- TGGGTAACGGCGGCAGACAAAAAGACTGCAGTGGACATGTCGGGCGGGAC- GGTCACAGTCCTTGAAAAGGTCCCTGTATCAAAAGGCCAACTGAAGCAA- TACTTCTACGAGACCAAGTGCAATCCCATGGGTTACACAAAAGAAGGCTG- CAGGGGCATAGACAAAAGGCATTGGAACTCCCAGTGCCGAACTACCCAGTCG- TACGTGCGGGCCCTTACCATGGATAGCAAAAAGAGAATTGGCTGGCGAT- TCATAAGGATAGACACTTCTTGTGTATGTACATTGACCATTAAAAGGGGAA- GAGGCCCGCTGGGCCTGGCGGGCGGCGGTGGTGGTAGTACTAA-GAAAACCCTGCGTACTTAAGAATTC
Expression Vector:
The cDNA sequence was cloned into the PET28b expression vector.
>*BDNF-TIMP-Linker-CBD* (159AAs; 17.51KDa; pI9.90)
MGSSHHHHHHSSGLVPRGSHMHSDPARRGELSVCDSISEWVTAADKKTAVDMSGGTVTVLEKVPVSKGQLKQYFYETKCNPMGY TKEGCRGIDKRHWNSQCRTTQSYVRALTMDSKKRIGWRFIRIDTSCVCTLTIKRGRGPLGLAGGGGGSTKKTLRT

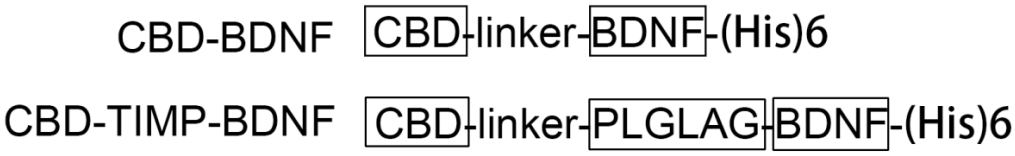

Figure 1. Schematic diagram of the structure of CBD-BDNF and CBD-TIMP-BDNF.

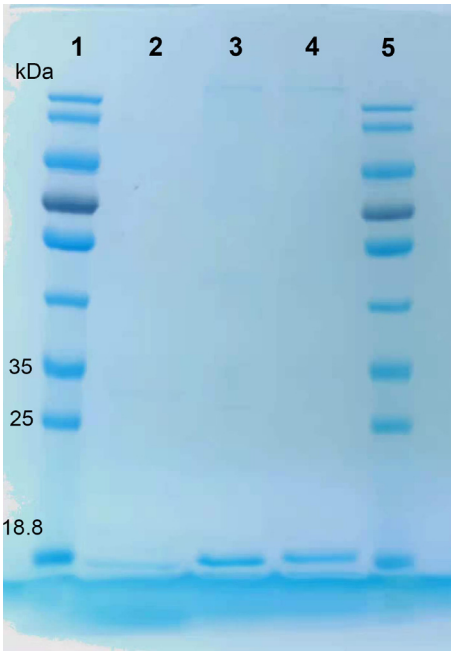

Figure 2. SDS-PAGE analysis of CBD-BDNF and CBD-TIMP-BDNF expression: Lane 1 and Lane 5, molecular weight markers; Lane 2, BDNF standard; Lane 3, CBD-BDNF; Lane 4, CBD-TIMP-BDNF.

3.2. The Recombinant Protein CBD-TIMP-BDNF Can Specifically Bind to Collagen

To verify the binding ability of the recombinant protein to collagen, the amount of BDNF bound to collagen under different concentrations was analyzed. As shown in Figure 3, the optical density values of CBD-BDNF and CBD-TIMP-BDNF binding to collagen at 405 nm were higher than that of BDNF at different concentrations, and significant differences were observed at high concentrations. The collagen binding assay verified the ability of CBD-BDNF and CBD-TIMP-BDNF to specifically bind to collagen and detected the characteristic of CBD-TIMP-BDNF responding to MMP-2 to release BDNF and the biological activities of the two recombinant proteins. The results indicated that CBD, rather than TIMP, is necessary for the binding of recombinant BDNF to collagen.

3.3. Recombinant Protein CBD-TIMP-BDNF Responds to MMP to Release BDNF

To evaluate the specific regulatory effect of MMPs on the release of BDNF by CBD-TIMP-BDNF, we conducted in vitro release experiments in the presence of MMP-2. As shown in Figure 4, during the intervention of MMP-2, the release amount of BDNF gradually increased. Both in the absence of MMP-2 and under the condition of MMP-2 inhibition, the release of BDNF by CBD-TIMP-BDNF was significantly lower than that in the presence of MMP-2, indicating that the system can specifically respond to MMP-2 and release BDNF as a drug molecule through cleavage. The release profile of CBD-BDNF on collagen in the presence of MMP-2 was further evaluated to confirm the release of BDNF through the hydrolysis of substrate peptides by MMP-2. The results suggest that BDNF can be actively released from CBD-TIMP-BDNF in response to MMPs, which may allow for an on-demand release of BDNF consistent with the degree of tissue injury.

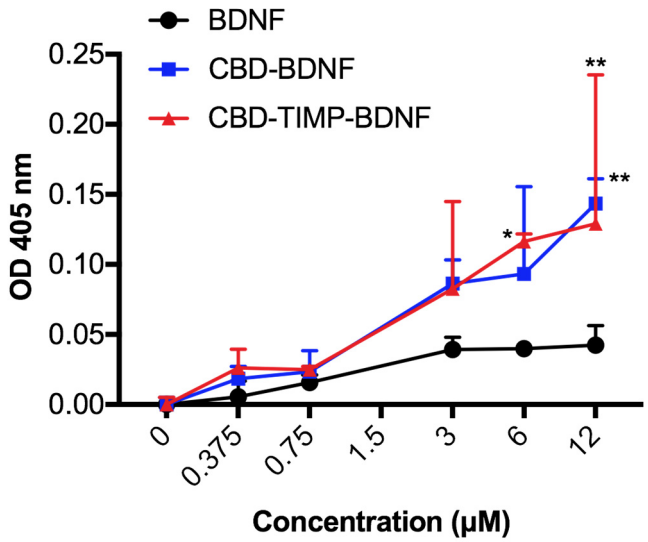

Figure 3. Detection of the binding abilities of BDNF, CBD-BDNF, and CBD-TIMP-BDNF to collagen (* $p < 0.5$, ** $p < 0.01$).

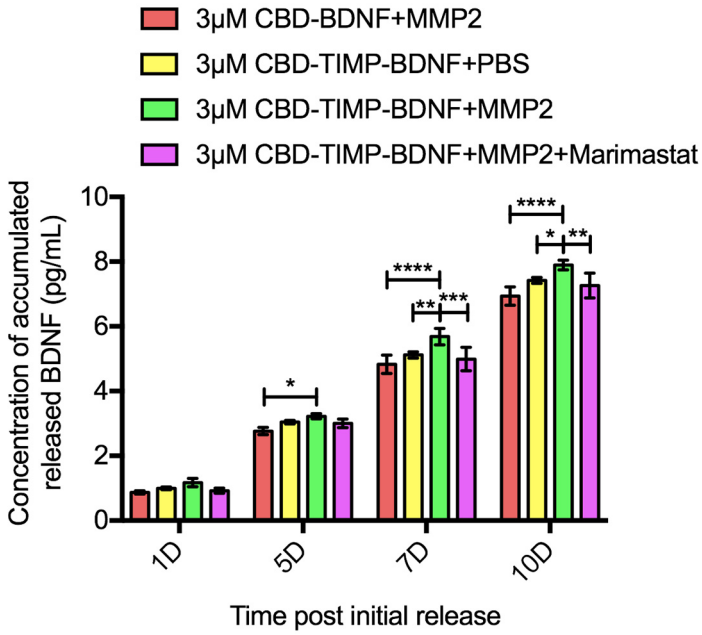

Figure 4. Cumulative release of BDNF mediated by MMP-2 at different time points (* $p < 0.5$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

3.4. The Fused Expression of CBD-TIMP Does Not Alter the Biological Activity of BDNF

The biological activity of BDNF was evaluated based on its ability to stimulate axon growth in dorsal root ganglia (DRG). First, primary DRG were isolated. The DRG of newborn mice were isolated and stripped of membranes under a dissecting microscope. The DRG were then seeded onto a well plate and incubated in DMEM/F12 medium containing 10% FBS with 0.01 μmol/L of CBD-BDNF or CBD-TIMP-BDNF added to each well. The control group received the same volume of PBS. The cells were cultured in a cell incubator for 48 h. Fluorescence staining was performed, and the length of DRG axons was statistically analyzed. The results showed that both CBD-BDNF and CBD-TIMP-BDNF could effectively promote axon growth (Figure 5), indicating that the recombinant proteins CBD-BDNF and CBD-TIMP-BDNF possess biological activity.

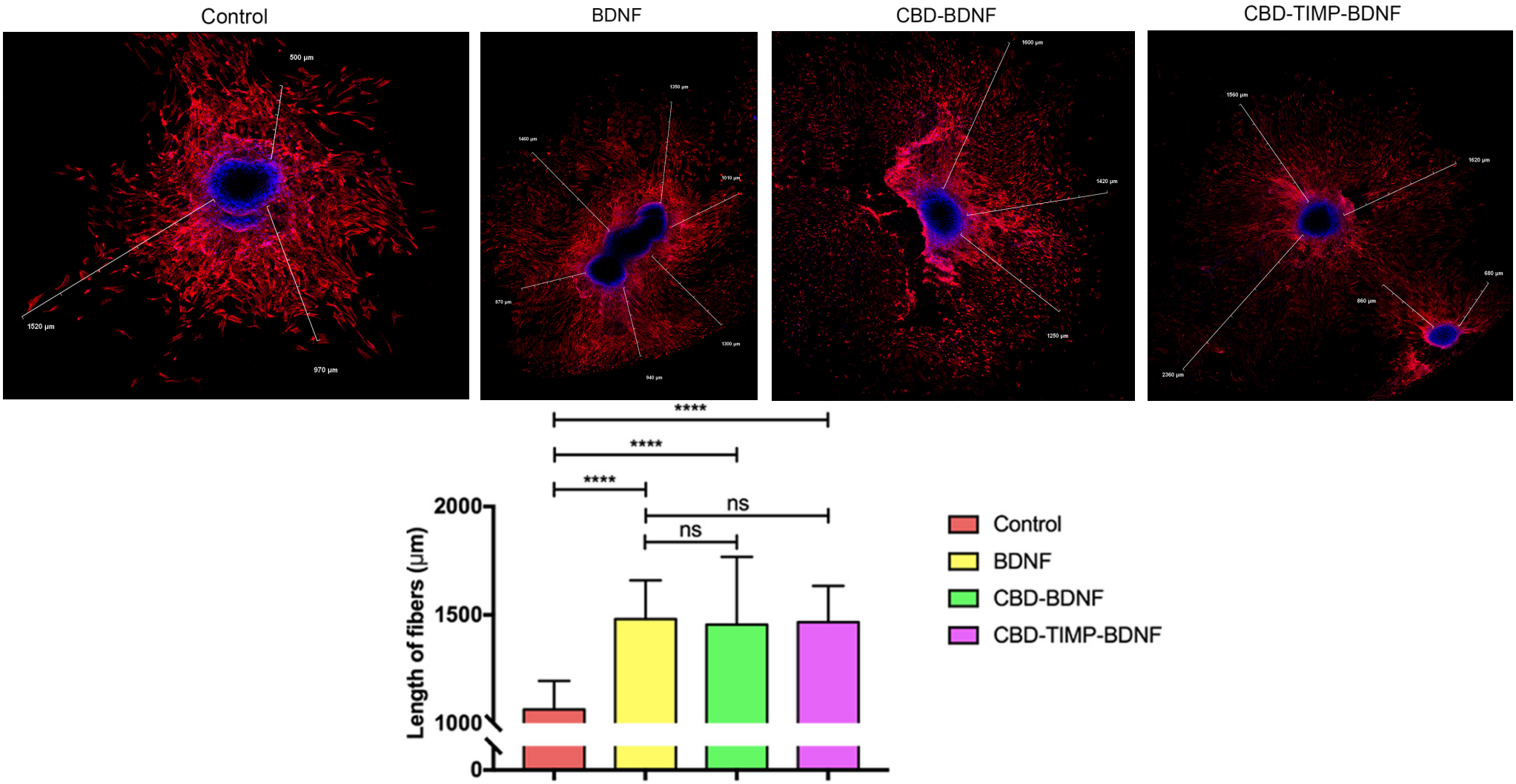

Figure 5. Biological activity of CBD-TIMP-BDNF. In the fluorescence image, blue represents the cell nucleus, which is the location of the DRG, and red represents the nerve fibers. The lengths of nerve fibers in different directions were measured using software rulers and labeled in the figure (with white lines and length numbers). Scar bar represents 1000 μm. **** $p < 0.0001$. "ns" represents "no significant difference".

4. Discussion

Extensive research has fully revealed the potential of BDNF [9]. Following spinal cord injury, apoptotic factors in axotomized neurons are upregulated [10]. The introduction of BDNF can significantly reduce the quantity of these apoptotic molecules, even rendering them inactive, thereby significantly improving the survival rate of the organism [11,12]. This effect not only facilitates the repair of damaged neurons but also promotes their reconnection, leading to the restoration of physiological functions [13]. Additionally, BDNF's impact on neurons extends to its ability to protect cells from degenerative changes. While maintaining cellular activity, BDNF can break down diseased cytoplasm and other unnecessary components, ensuring the normal functioning of cells. However, some studies have also pointed out potential risks associated with BDNF [14]. Administered at inappropriate dosages, BDNF may exacerbate cellular degeneration or even lead to necrosis. The inflammatory response triggered by BDNF is also undesirable. Therefore, there is an urgent need for a BDNF reagent that can be released on demand and automatically quantified. Such a reagent should target specific MMPs for precise drug delivery, possess stronger protein-binding capabilities, and exhibit higher gene expression levels. To overcome the limitations of BDNF monotherapy, we can consider incorporating other compatible drugs for parallel treatment. This approach can sometimes yield multiplicative therapeutic effects.

With the in-depth research on the mechanism of spinal cord injury and treatment strategies, we have discovered an increasing number of candidate drugs. Among them, CBD-TIMP-BDNF, as a novel recombinant protein drug, exhibits broad application prospects in the treatment of spinal cord injury due to its unique ability to respond to MMPs and release BDNF. Through our activity detection, the activity of CBD-TIMP-BDNF under different conditions has been fully verified, demonstrating its high reliability. Using prokaryotic expression and purification techniques, we have ensured efficient expression of BDNF, enabling accurate analysis and rapid treatment of spinal cord injury in a shorter time frame. Additionally, the high binding affinity of CBD-TIMP-BDNF with collagen further enhances its drug release capabilities, demonstrating more significant therapeutic effects compared to CBD-BDNF. Meanwhile, as an MMP-responsive pharmaceutical product, CBD-TIMP-BDNF can autonomously regulate the amount of MMPs targeted, effectively avoiding the inefficiency issues associated with traditional drugs that require manual regulation.

More importantly, CBD-TIMP-BDNF has demonstrated excellent activity detection in complex environments, proving its high reliability and providing patients with a safer treatment option. In the future, we will continue to optimize the preparation process of CBD-TIMP-BDNF and explore the optimal application dosage and timing for the treatment of spinal cord injury. At the same time, we will also delve into the pharmacological characteristics of CBD-TIMP-BDNF, including its pharmacokinetics, biodistribution, and long-term efficacy in vivo, to comprehensively evaluate its effectiveness and safety in the treatment of spinal cord injury. In addition, we will focus on studying the biological functions of BDNF and its relationship with spinal cord injury repair, aiming to provide theoretical support for the development of more efficient and safe treatment strategies. We firmly believe that this research achievement will bring better treatment outcomes and quality of life to patients with spinal cord injury and revolutionize the medical field.

5. Conclusions

In this study, we successfully constructed the CBD-TIMP-BDNF recombinant protein responsive to MMPs and performed prokaryotic expression and purification. These processes not only improved the drug production capacity and response ability of the recombinant protein to target cells but also comprehensively analyzed its binding ability to collagen, BDNF release characteristics, and biological activity, providing a solid foundation for subsequent improvements in BDNF recombinant protein. Experimental results showed that CBD-TIMP-BDNF had the ability to specifically bind to collagen and achieved precise release of BDNF under the action of MMP-2. This characteristic ensures the high efficiency and precision of BDNF, enabling the damaged site to receive appropriate and timely

BDNF treatment. The released BDNF exhibited good biological activity and significantly promoted the growth of dorsal root ganglion (DRG) axons and cellular self-repair. This study provides a new perspective and method for the treatment of spinal cord injury and is expected to bring revolutionary treatment methods for the repair and regeneration of damaged neurons. Future research directions may focus on optimizing BDNF treatment regimens to improve efficacy, further enhancing their biological activity, and increasing sensitivity to targets, thereby achieving faster treatment responses. Looking ahead, we expect to develop BDNF molecules with better efficacy and higher activity, providing more diverse and effective treatment options for patients with spinal cord nerve injury.

Author Contributions: Conceptualization, C.F. and J.Y.; methodology, C.F., H.C. and J.H.; software, C.F.; validation, C.F., Y.W. and J.H.; formal analysis, C.F.; investigation, C.F., H.C. and J.H.; resources, J.Y. and J.H.; data curation, C.F.; writing—original draft preparation, C.F. and J.H.; writing—review and editing, C.F. and J.H.; visualization, C.F.; supervision, C.F.; project administration, C.F.; funding acquisition, C.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Shaoxing University, grant number 20235005.

Data Availability Statement: Data are available from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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