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Enhanced proliferation of neural stem cells in a collagen hydrogel incorporating engineered epidermal growth factor

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ABSTRACT

Neural stem cells (NSCs) have received much attention in cell-transplantation therapy for central nervous disorders such as Parkinson's disease. However, poor engraftment of transplanted cells limits the efficacy of the treatments. To overcome this problem, collagen-based hydrogels were designed in this study to provide microenvironments for embedded cells to survive and proliferate. Our approach was to incorporate epidermal growth factor (EGF), known as a mitogen for NSCs, into a collagen hydrogel. For the stable binding of EGF with collagen under mild conditions, EGF was fused with a collagen-binding polypeptide domain by recombinant DNA technology. A cell population containing NSCs was derived from the fetal rat brain and cultured in the composite hydrogels for 7 d followed by analysis for cell proliferation. It was shown that the number of living cells was significantly higher in hydrogels incorporating collagen-binding EGF. This effect is largely owing to the collagen-binding domain that serves to sustain presentation of EGF toward cells within the hydrogel. It is further revealed by gene expression analysis that cells proliferated in the EGF-incorporating collagen hydrogel contained subpopulations expressing the marker of stem cells, neurons, astrocytes, or oligodendrocytes.

1. Introduction

Stem cell based therapy for the regeneration of the central nervous system has attracted much attention since the discovery of neural stem cells (NSCs) in mammals including human [1,2]. Somatic NSCs obtained from the fetus or adult brains or neural precursor cells derived from embryonic stem cells have been used for transplantation in animal brain tissues with promising outcomes [3]. However, current methodologies have limited effects on the restoration of brain functions primarily due to poor survival of transplanted cells in host tissues [4,5]. For instance, it was reported that less than 4% of embryonic ventral mesencephalon cells [6–10] or NSCs [11–13] survived initial few weeks after allogeneic transplantation. The poor survival is primarily because of accelerated apoptosis as well as necrosis induced by inflammatory reactions [14,15].

To overcome these limitations, we are involved in developing a type I collagen-based hydrogel to be used as a carrier for embedding neural stem cells [16]. This is based on our hypothesis that a hydrogel serves to prevent infiltration of inflammatory cells into transplanted cells leading to improved engraftment. Brännvall et al. [17] and Watanabe et al. [18] have also used type I collagen hydrogels as carriers for neural progenitor cells. However, collagen is intrinsically inert for NSCs [19–22], probably due to the fact that this protein contains no sequences presenting trophic signals or providing anti-apoptotic effects. Therefore, further modification of collagen is needed for promoting the engraftment of donor cells.

In this study, epidermal growth factor (EGF) was incorporated into a type I collagen hydrogel. EGF is known to promote proliferation of NSCs in *in vitro* culture [23,24] as well as *in vivo* environments [25]. We expect that NSCs temporally receive trophic signals within the collagen-EGF composite hydrogels, which serves to prevent cell loss and thus bring about an increased therapeutic effect. In order to incorporate EGF into a collagen hydrogel, we used here the genetically engineered EGF that was fused with a collagen-binding domain derived from von Willebrand factor (CBD) [26,27]. This strategy requires no toxic reagents and enables EGF conjugation *via* mild reactions without the loss of its biological activity. This is a large contrast to the case of covalent conjugation through conventional condensation reactions [28]. Our previous study [29] using transmission electron microscopy combined with a gold nanoparticle-labeling technique showed that CBD served to densely display its fusion partner, EGF, on the surface of type I collagen fibrils in consequence of specific recognition under physiological conditions.

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The present study was undertaken to examine especially the biological functions of the EGF-CBD chimeric protein specifically bound to collagen networks. First 2-dimensional cell culture assays were performed with NSCs on collagen-coated glass substrates onto which EGF-CBD was immobilized. Then NSCs were cultured in the collagen-EGF-CBD composite hydrogels to investigate the trophic effect of incorporated EGF.

2. Materials and methods

2.1. Chimeric proteins

The structure and nomenclature of chimeric and control proteins used in this study are shown in Fig. 1A. A chimeric protein which encompassed EGF and CBD derived from von Willebrand factor (EGF-CBD-His) was expressed in *Escherichia coli* (*E. coli*) as reported before [29]. The hexahistidine sequence (His) was also fused for the purification of the protein by nickel chelate chromatography. As a control, EGF-deficient CBD (CBD-His) and CBD-deficient EGF (EGF-His) were prepared as reported elsewhere [16,29]. In brief, plasmids encoding these three proteins were constructed by standard recombinant DNA technology and transfected into *E. coli*. The proteins were expressed in *E. coli* as inclusion bodies. The proteins were solubilized in 8 M urea solution and purified using a His Trap column (Amersham Biosciences Corp., New Jersey, USA). Then, proteins were refolded by step-wise dialysis.

The purity and molecular size of these proteins were analyzed by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue staining.

To assess secondary structure, far-UV circular dichroism (CD) spectrum was recorded using JASCO J-805 spectropolarimeter for these proteins dissolved in phosphate buffered saline (PBS, pH 7.4) to a concentration of 150 μg/mL. Spectra were recorded in a 0.1 cm path length cell at 20 °C, a response time of 0.5 s, a bandwidth of 1 nm, and a scan speed of 100 nm/min with an accumulation of 8 scans. Pure PBS was used as reference. To evaluate the structural integrity of EGF-CBD-His, a geometric average of the spectra for EGF-His and CBD-His was determined using the following equation:

$$\theta_{\mathrm{EGF-His/CBD-His}}(\lambda) = [\theta_{\mathrm{EGF-His}}(\lambda) \times 61] + [\theta_{\mathrm{CBD-His}}(\lambda) \times 197]/(61 + 197)$$

where $\theta_{\mathrm{EGF-His/CBD-His}}(\lambda)$, $\theta_{\mathrm{EGF-His}}(\lambda)$, and $\theta_{\mathrm{CBD-His}}(\lambda)$ are the molar ellipcity of EGF-His/CBD-His (synthetic spectrum), EGF-His, and CBD-His (experimental spectra), respectively, as a function of wavelength, λ. The numbers, 61 and 197, are the number of amino acid residues in EGF-His and CBD-His, respectively. The obtained $\theta_{\mathrm{EGF-His/CBD-His}}(\lambda)$ was compared with the spectrum of EGF-CBD-His (experimental spectrum), under the assumption that the C-terminal LEHHHHHH sequence (duplicated in the synthetic spectrum) had a negligible contribution.

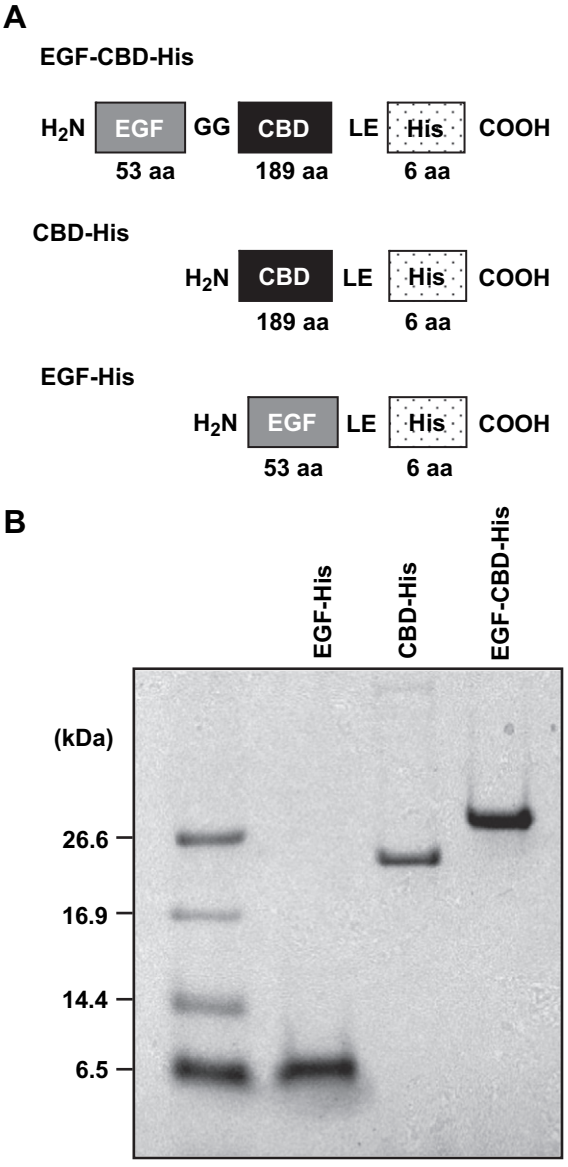

Fig. 1. (A) Structure and nomenclature of expressed proteins. (B) The result of SDS-PAGE analysis for proteins expressed in *E. coli* and purified by nickel chelate chromatography. aa: amino acid residues.

2.2. Cell isolation and culture

The striatum was isolated from fetuses (embryonic day 16) of EGFP-transgenic Sprague Dawley rats and dissociated into single cells by trypsinization. Cells from EGFP-positive and EGFP-negative fetuses were separately cultured in suspension for 4 d in DMEM/F12 (1:1) medium (Invitrogen Corp., California, USA) containing 2% (v/v) B27 (Invitrogen), 20 ng/mL basic fibroblast growth factor (Invitrogen), and 20 ng/mL EGF (Invitrogen), 5 μg/mL heparin, 100 unit/mL penicillin, and 100 μg/mL streptomycin to obtain neurospheres that contained NSCs at 50–60% of total cells [29]. All animal experiments were carried out according to the guidelines of the Animal Experimentation Committee of the Institute.

2.3. Cell culture on collagen-coated surfaces

Each well (φ = 4 mm) of a microwell glass slide (Matsunami Glass Ind., Ltd., Osaka, Japan) was exposed to 0.1 mg/mL type I collagen solution (Cellmatrix type I-A, Nitta Gelatin, Inc., Osaka, Japan) for 2 h to adsorb collagen onto the glass surface. After washing the surface with PBS, an aliquot of PBS containing one of the chimeric proteins (10 μM) was pipetted to the wells and kept for 2 h to bind these proteins onto pre-coated collagen. Then, the surface was extensively washed with PBS to remove unbound proteins. Neurospheres from EGFP-nonexpressing cells (passage 2) were dissociated into single cells by trypsinization. The cells were suspended in DMEM/F12 (1:1) containing 2% (v/v) B27, 5 μg/mL heparin, 100 unit/mL penicillin, and 100 μg/mL streptomycin and pipetted to each well of the microwell glass slide at a density of 5.0×10^4 cells/cm^2. After 2-h incubation at 37 °C under 5% CO_2 atmosphere, the microwell glass slide was immersed in a culture medium and incubated at 37 °C under 5% CO_2 atmosphere.

Cells were cultured for 3 d and fixed with 4% (w/v) paraformaldehyde solution, and cell membranes were permeabilized with 0.05% (v/v) Triton-X solution. After blocking the cells to prevent nonspecific protein adsorption, cells were bound with primary antibodies to nestin (1:200, mouse monoclonal Rat 401, BD Biosciences, California, USA) and β-tubulin III (1:500, rabbit polyclonal, Convance, New Jersey, USA) followed by binding of secondary antibodies including Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 594 anti-mouse IgG (both from Molecular Probes, Inc., Oregon, USA) at a dilution of 1:500. Cells were observed with a fluorescent microscope BX51 TRF (Olympus, Tokyo, Japan).

2.4. Cell culture in collagen hydrogels

Type I collagen solution (Cellmatrix type I-A, Nitta Gelatin, Inc., Osaka, Japan) was neutralized by mixing with a medium [5-fold concentrated DMEM/F12 (1:1) containing 25 μg/mL heparin, 500 unit/mL penicillin, 500 μg/mL streptomycin] and a reconstitution buffer solution [50 mM NaOH, 260 mM $NaHCO_3$, and 200 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] at a volume ratio of 7:2:1. Then the mixture was supplemented with 2% (v/v) B27, and placed on ice to delay gelation. The final concentration of collagen was adjusted to 1.0 mg/mL in most experiments. However, when the effect of collagen concentration was studied, the concentration was adjusted to 0.75–1.5 mg/mL. To this mixed solution, PBS containing CBD-His, EGF-His, or EGF-CBD-His was added to the concentration of 5.4 nM and kept on ice for 30 min to allow for binding of these proteins to collagen. As a control, pure PBS was added. When we examined the effect of chimeric proteins concentrations, EGF-His and EGF-CBD-His were added to a collagen solution to the concentrations ranging from 0 to 8.9 nM (compositions are shown in Supplementary Table S1). Then, dissociated neurosphere-forming cells were dispersed in the mixed solution at