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Engineered stem cell mimics to enhance stroke recovery

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ABSTRACT

Currently, no medical therapies exist to augment stroke recovery. Stem cells are an intriguing treatment option being evaluated, but cell-based therapies have several challenges including developing a stable cell product with long term reproducibility. Since much of the improvement observed from cellular therapeutics is believed to result from trophic factors the stem cells release over time, biomaterials are well-positioned to deliver these important molecules in a similar fashion. Here we show that essential trophic factors secreted from stem cells can be effectively released from a multi-component hydrogel system into the post-stroke environment. Using our polymeric system to deliver VEGF-A and MMP-9, we improved recovery after stroke to an equivalent degree as observed with traditional stem cell treatment in a rodent model. While VEGF-A and MMP-9 have many unique mechanisms of action, connective tissue growth factor (CTGF) interacts with both VEGF-A and MMP-9. With our hydrogel system as well as with stem cell delivery, the CTGF pathway is shown to be downregulated with improved stroke recovery.

1. Introduction

Stroke has devastating consequences for survivors and their caregivers [1]. Stroke's societal cost of greater than $100 billion per year is also staggering [2]. Despite this, no medical treatments exist for stroke recovery. Stem cell transplantation is a promising stroke therapy showing efficacy in animal models and in multiple early phase clinical trials [3–7]. Human neural progenitor cells (hNPCs) are a type of stem cell derived from embryonic or fetal cells which are predisposed to a neural fate; they have shown promise in stroke recovery [8].

Unfortunately, several drawbacks remain to developing long-term, cell-based therapies. First, typically only 1–8% of transplanted stem cells survive, due in part to the highly hypoxic and inflamed post-stroke environment [9–11]. Second, the time, cost, and infrastructure required to prepare an adequate number of stem cells for transplantation limits the ability to maintain a large-scale, stable product. Finally, the theoretical safety risks associated with transplantation of stem cells requires the development of rigorous protocols to insure cell homogeneity, quality assurance, and absence of tumorgenicity.

Bioengineering cell-free, stem cell mimics offers a solution to these limitations. Stem cells are thought to enhance stroke recovery largely through trophic factor release [12,13]. A polymeric system is well suited to deliver factors without the restrictions of cell-based therapies. Recently developed, multi-component hydrogel systems are promising for biocompatible, controlled molecule delivery into biologic systems [14].

Based upon analysis of trophic factors produced by hNPCs, hNPC-secreted VEGF-A and MMP-9 were found to be important for hNPC-enhanced stroke recovery [12,13]. If given at early time points after stroke, VEGF-A, a factor involved in angiogenesis and neural recovery, weakens blood vessels and is detrimental to stroke recovery [15–17]; but if delivered in a sustained manner, VEGF-A improves healing [13,16,18]. Similarly, MMP-9, a molecule that

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increases active VEGF during angiogenesis and impacts extracellular matrix remodeling, is detrimental in the acute period of stroke [19]; but if delivered in the subacute timeframe (>7 days) is beneficial [20,21]. The dosing of these molecules is also critical, as higher concentrations can be detrimental to recovery [22]. It is thought that stem cells react to cues in the post-stroke environment to deliver these molecules over time [23,24]. Given the importance of timing and dose, a controlled-release system is necessary to design an acellular therapy with trophic factor effects for stroke recovery.

In our experiments, we utilize a composite, protein-based hydrogel system to incorporate VEGF-A and MMP-9 [14,25]. We use this polymeric system to release the factors in a pre-clinical stroke model and investigate the effect on functional recovery. The hydrogel-based treatments delivering VEGF-A, MMP-9, and VEGF-A with MMP-9 (VEGF-A + MMP-9) were compared to animals with embryonic stem cell-derived hNPC treatment. By controlling the release profiles of VEGF-A and MMP-9, we observed similar stroke recovery to animals receiving stem cell transplantation. These studies demonstrate the feasibility of creating a polymeric, stem cell mimic.

2. Materials and methods

2.1. Hydrogel preparation

The C7 recombinant protein and PEG-peptide polymers were synthesized and purified as reported previously [26]. Phosphate buffered saline (PBS) solutions of both components were prepared at a concentration of 13.3 wt% for C7 and 6.7 wt% for PEG-peptide. 30 μL hydrogel was created by mixing 15 μL C7 with 15 μL PEG-peptide to achieve a final polymer concentration of 10 wt% and a C:P ratio of 1:1. Hydrogel delivering VEGF-A was prepared by encapsulating recombinant human VEGF-A-165 (Novoprotein). VEGF-A-165 was first mixed with the PEG-peptide component and subsequently with C7. The final concentration of VEGF-A within the hydrogel was 1 μg per 30 μL gel. Similarly, hydrogel delivering MMP-9 was prepared by encapsulating MMP-9 (Biolegend) at a concentration of 1 μg per 30 μL gel.

Dynamic light scattering microrheology was performed using a Malvern Zetasizer ZS (630 nm laser) and analyzed using custom software, as previously described [27]. Briefly, 1.0 μm diameter polystryene beads (Polysciences #08226-15) functionalized with poly (ethylene glycol) were dispersed into each hydrogel component at a final concentration of 0.25% w/v. 15 μL of hydrogel was prepared in a quartz cuvette (Malvern ZEN2112) by mixing each component in situ. After 15 min of incubation at 37 °C, raw scattering autocorrelation functions were collected in back-scatter detection for 15 min at a fixed measurement position of 4.2 mm, followed by a sweep over measurement positions ranging from 3.6 mm to 5.2 mm in 0.1 mm increments to obtain the ensemble-averaged scattering intensity. The particle mean-squared displacement was extracted from the raw scattering autocorrelation function and the ensemble-averaged scattering intensity according to the previously described broken-ergodicity correction procedure [27].

The particle mean-squared displacement was transformed to the frequency-dependent complex shear modulus $G^*(\omega)$ according to the generalized Stokes-Einstein relation [28], $G^*(\omega) = \frac{k_B T}{\pi a \, i\omega \Delta r^2(\omega)}$, where k_B is Boltzmann's constant, T is the absolute temperature, a is the particle diameter, i is the imaginary unit, and $\Delta r^2(\omega)$ is the unilateral Fourier transform of the particle mean-squared displacement. The unilateral Fourier transform of the mean-squared displacement was performed using a local power law analysis, as described previously [27,28].

2.2. Release kinetics of VEGF-A and MMP-9

Release kinetics of VEGF-A and MMP-9 were measured by first preparing 30 μL hydrogel + VEGF-A or MMP-9 in microcentrifuge tubes ($n \geq 3$). The mixture was allowed to gel for 15 min at 37 °C before adding 1 ml of PBS. Release kinetics were determined by sampling and replenishing 10 μL of the PBS supernatant over a period of 14 days. Samples were frozen at −80 °C immediately after collection and thawed prior to quantification. The amount of VEGF-A and MMP-9 present in the supernatant at each time point was quantified using Human VEGF-A Quantikine ELISA Kit (R&D Systems) and Human MMP-9 Quantikine ELISA Kit, according to the manufacturer's protocol. Results are normalized to the initial amount of protein incorporated into each hydrogel.

2.3. Cell culture

hMVECs culture: human microvascular endothelial cells (Lonza) were cultured in EBM-2 endothelial basal medium supplemented with EGM2-MV bullet kit supplements (Lonza) in a humidified incubator at 37 °C and 5% CO_2. Cells received regular media replenishment every two days and were passaged using TrypLE Express (Thermo Fisher Scientific). Passages 2–7 were used in subsequent experiments.

Human neural progenitor cell culture: All stem cell procedures were approved by Stanford's Stem Cell Research Oversight committee. As previously described [12], hNPC (passages 17–22) are derived from embryonic stem cells and then were cultured in neural maintenance media supplemented with 1× B27 and N2 along with LIF (10 μg/ml), EGF (20 μg/ml), bFGF (10 ng/ml, all Invitrogen, Waltham, MA except for EGF and LIF from Millipore, Darmstadt, Germany), and pooled human serum albumin (1%, Mediatech Inc, Pittsburgh, PA).

2.4. In vitro measurement of VEGF-A activity assay on hMVECs

To measure the *in vitro* activity of released VEGF-A (Novoprotein), 30 μL of hydrogel or 1 μg of VEGF-A encapsulated in 30 μL of hydrogel samples were prepared and incubated in 1 mL EBM-2 endothelial basal medium supplemented with 2% fetal bovine serum (Gibco) in microcentrifuge tubes ($n = 4$). 100 μL of the supernatant was collected on day 6, and replenished with equal volume of fresh EBM-2. hMVECs were seeded into a 48-well tissue culture plate in EGM2-MV and allowed to attach for 4 h. Media in each well were then replaced with 500 μL EBM-2 basal medium supplemented with 2% fetal bovine serum and 10 μL of collected supernatant. CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) was used to quantify cell metabolic activity at days 1 and 4 according to the manufacturer's protocol. hMVEC metabolic activity values were normalized to the value measured in the media control group.

2.5. In vitro MMP-9 activity test after release from hydrogel

To measure the *in vitro* activity of released MMP-9 (Biolegend), 1 μg of MMP-9 was encapsulated in 30 μL of hydrogel, allowed to gel at the bottom of microcentrifuge tubes, and incubated in 1 mL of PBS (Gibco) ($n = 3$). 100 μL of the supernatant was collected on day 6 and replenished with equal volume of fresh PBS. A 100-fold dilution of collected samples was used to measure MMP-9 activity using the Fluorokine® E Human Active MMP-9 kit (R&D Systems) according to manufacturer's protocol.

For proteolytic activity of released MMP-9, MMP-9 supernatants collected from hydrogel with MMP-9 or VEGF-A + MMP-9 at day 3 release were separated on a zymography gel (Thermo Fisher). Two