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Covalent growth factor tethering to direct neural stem cell differentiation and self-organization

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ABSTRACT

Tethered growth factors offer exciting new possibilities for guiding stem cell behavior. However, many of the current methods present substantial drawbacks which can limit their application and confound results. In this work, we developed a new method for the site-specific covalent immobilization of azide-tagged growth factors and investigated its utility in a model system for guiding neural stem cell (NSC) behavior. An engineered interferon-γ (IFN-γ) fusion protein was tagged with an N-terminal azide group, and immobilized to two different dibenzocyclooctyne-functionalized biomimetic polysaccharides (chitosan and hyaluronan). We successfully immobilized azide-tagged IFN-γ under a wide variety of reaction conditions, both in solution and to bulk hydrogels. To understand the interplay between surface chemistry and protein immobilization, we cultured primary rat NSCs on both materials and showed pronounced biological effects. Expectedly, immobilized IFN-γ increased neuronal differentiation on both materials. Expression of other lineage markers varied depending on the material, suggesting that the interplay of surface chemistry and protein immobilization plays a large role in nuanced cell behavior. We also investigated the bioactivity of immobilized IFN-γ in a 3D environment *in vivo* and found that it sparked the robust formation of neural tube-like structures from encapsulated NSCs. These findings support a wide range of potential uses for this approach and provide further evidence that adult NSCs are capable of self-organization when exposed to the proper microenvironment.

Statement of Significance

For stem cells to be used effectively in regenerative medicine applications, they must be provided with the appropriate cues and microenvironment so that they integrate with existing tissue. This study explores a new method for guiding stem cell behavior: covalent growth factor tethering. We found that adding an N-terminal azide-tag to interferon-γ enabled stable and robust Cu-free 'click' immobilization under a variety of physiologic conditions. We showed that the tagged growth factors retained their bioactivity when immobilized and were able to guide neural stem cell lineage commitment in vitro. We also showed self-organization and neurulation from neural stem cells *in vivo*. This approach will provide another tool for the orchestration of the complex signaling events required to guide stem cell integration.

1. Introduction

Signaling proteins are used in a wide variety of tissue engineering strategies, both in soluble and immobilized form, to guide stem cell behavior [1]. In the adult mammalian central nervous system (CNS), which does not innately regenerate following injury [2], it is particularly important to provide the proper cues and microenvironment so that functional integration can occur. Immobilizing signaling proteins can improve the duration and level of signaling while spatially sequestering them within the material, affording greater control over local cellular behavior [3]. Within the CNS, injuries result from mechanical damage and begin a secondary injury cascade, which can last for months [4,5]. The transplantation of neural stem cells (NSCs) has been investigated as a potential therapy for CNS regeneration, with a wide variety of outcomes [6]. However, simply transfusing NSCs by themselves is

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insufficient, as the cells must be provided with the right cues and microenvironment to differentiate and functionally integrate with existing tissue. Encapsulating NSCs within a biomimetic hydrogel conduit can provide protection, locate the cells within the injury site, control lineage specification, and guide neurite outgrowth [7]. To guide the differentiation of encapsulated NSCs, the material can be functionalized with lineage directing (signaling) proteins. In fact, platelet-derived growth factor-AA (PDGF-AA) can be used to specify oligodendrocytes [3,8], bone morphogenetic protein-2 (BMP-2) to specify astrocytes [3], and interferon-γ (IFN-γ) to specify neurons [9]. Ideally, and for enhanced precision and latency, signaling proteins would be site-specifically tagged for immobilization, rather than simply sequestered within a matrix. Proteins can be site-specifically labeled through enzymatic means [10,11] or by the incorporation of non-canonical amino acids [12]. Unlike non-canonical amino acids, which require nonsense suppression and carefully controlled synthetic tRNAs, enzymatic labeling offers a straightforward method for controlled, site-specific protein modification. It is also important to provide a permissive microenvironment to encourage the self-organization of encapsulated NSCs, with the eventual goal of generating functionally integrated tissue.

The material and immobilization chemistry employed have large implications both for efficacy and translational considerations. The material itself should be biomimetic and neuroinductive. Recapitulating moieties and repeat units found within the CNS and carefully controlling stiffness can strongly influence the behavior of encapsulated cells [13,14]. A wide variety of materials have been investigated for this purpose, such as hyaluronan/methyl-cellulose blends [15], collagen [16], fibrin [17], and chitosan [7]. An immobilization chemistry that is stable and bio-orthogonal should be employed to afford greater control over the presentation of signaling proteins and improve the potential for clinical translation, as well as reduce the number of factors which could affect cellular behavior. Strain-promoted alkyne-azide cycloaddition (SPAAC) has been used in many biological systems to tag biomolecules, including proteins and nucleic acids [18]. However, its use for the guidance of stem cell behavior has not been extensively investigated.

Our previous work has demonstrated the impressive neurogenic capabilities of immobilized IFN-γ [7,3,9,19]. IFN-γ is a type-II interferon which is not natively expressed within the CNS, but nonetheless shows many potent effects [20]. In this study, our goals were to improve upon previous immobilization techniques (biotin-streptavidin conjugation), compare IFN-γ immobilization across different materials, and investigate the response of NSCs in more detail (Fig. 1), both *in vitro* and *in vivo*. As pluripotent stem cells undergo programmed neural differentiation, they organize into neural rosettes under specific conditions [21]. These clusters of cells can develop into neural tubes or even form neuroepithelium *in vitro* [22]. However, such behavior is typically only associated with pluripotent-derived NSCs and not primary adult NSCs [22]. Unexpectedly, our previous work uncovered the ability of adult rat NSCs to self-organize and form neural tube-like structures. This is a novel finding which deserves further investigation. For the current study, we hypothesized that proteins incorporating a site-specific azide-tag can be covalently immobilized under physiologic conditions to alkyne-functionalized hydrogels using SPAAC with no loss in bioactivity. To prove this, we N-terminally labeled a recombinant IFN-γ fusion protein with an azide tag through the use of N-myristoyltransferase [11]. Next, we investigated the immobilization of this protein (azIFN-γ) to a biomimetic hydrogel, methacrylamide chitosan (MAC). We demonstrated that this approach results in the stable, bio-orthogonal, covalent tethering of azIFN-γ, and can be used for immobilization to intact hydrogels or polymer in solution. We then compared the biological response between this material and methacrylated hyaluronan (MA-HA, a polysaccharide found natively in the CNS) *in vitro*, to demonstrate the utility of this approach when applied to a different hydrogel material. Lastly, we were interested to determine the extent to which our previous observations of adult NSC self-organization were affected by the presence of streptavidin, and whether we could reproduce previous results with covalent immobilization. Thus, we assessed the bioactivity of covalently immobilized azIFN-γ *in vivo* and the ability of this approach to direct the self-organization of NSCs.

2. Materials and methods

2.1. Synthesis of 12-azidododecanoic acid (12-ADA)

Synthesis of 12-ADA was performed as previously described by Heal et al. [11] Briefly, 2 g of 12-bromododecanoic acid (Sigma–Aldrich) was esterified in methanol (Sigma–Aldrich) at reflux for 3 h in the presence of concentrated sulfuric acid (EMD). The product (an oil) was then dissolved in 60 mL of dimethyl sulfoxide (DMSO,VWR) and 519 mg of sodium azide (Sigma–Aldrich) was added. This reaction was stirred at RT for 16 h, and then quenched with 1 M hydrochloric acid (EMD). The product (an oil) was then purified via flash chromatography (9:1 cyclohexane:diethyl ether

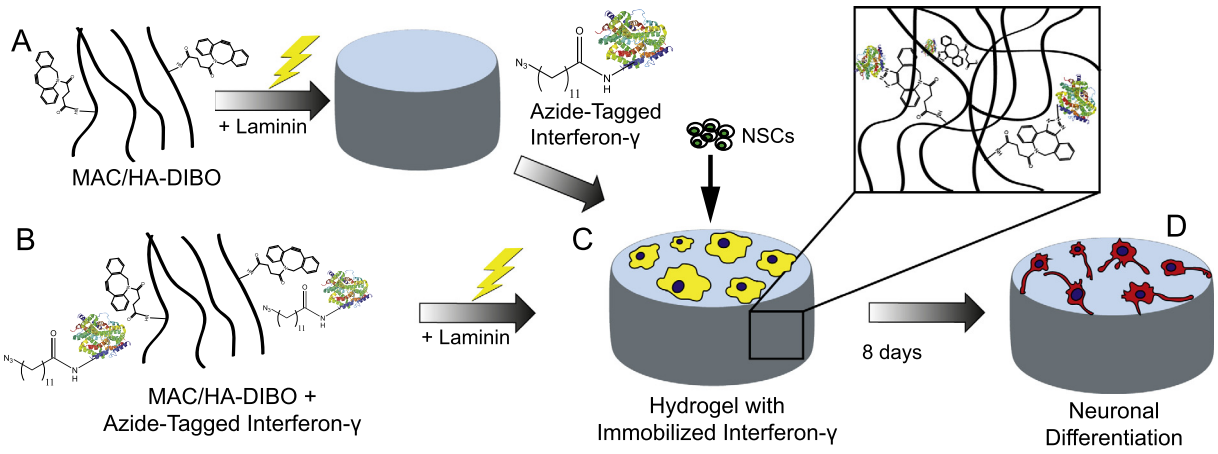

Fig. 1. Overview of azIFN-γ 'click' immobilization and *in vitro* NSC differentiation. (A) azIFN-γ was immobilized to crosslinked hydrogels (*in situ* immobilization) or (B) immobilized to polymer in solution prior to hydrogel formation (*de novo* immobilization). (C) NSCs were seeded on top of 2D azIFN-γ-functionalized hydrogels where they (D) differentiated into neurons after 8 d. Hydrogels were formed from methacrylamide chitosan (MAC) or methacrylated hyaluronan (MA-HA).