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Nanofibrous peptide hydrogel elicits angiogenesis and neurogenesis without drugs, proteins, or cells

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ABSTRACT

The design of materials for regenerative medicine has focused on delivery of small molecule drugs, proteins, and cells to help accelerate healing. Additionally, biomaterials have been designed with covalently attached mimics of growth factors, cytokines, or key extracellular matrix components allowing the biomaterial itself to drive biological response. While the approach may vary, the goal of biomaterial design has often centered on promoting either cellular infiltration, degradation, vascularization, or innervation of the scaffold. Numerous successful studies have utilized this complex, multicomponent approach; however, we demonstrate here that a simple nanofibrous peptide hydrogel unexpectedly and innately promotes all of these regenerative responses when subcutaneously implanted into the dorsal tissue of healthy rats. Despite containing no small molecule drugs, cells, proteins or protein mimics, the innate response to this material results in rapid cellular infiltration, production of a wide range of cytokines and growth factors by the infiltrating cells, and remodeling of the synthetic material to a natural collagen-containing ECM. During the remodeling process, a strong angiogenic response and an unprecedented degree of innervation is observed. Collectively, this simple peptide-based material provides an ideal foundational system for a variety of bioregenerative approaches.

1. Introduction

Biomaterials for regenerative medicine are designed to effectively integrate into the host tissue and accelerate or augment the body's natural healing ability while evoking a minimal inflammatory response. In order to achieve the necessary bioactivity to promote healing, these materials are frequently designed to deliver small molecule drugs, cells, or proteins. Serving as a delivery agent, the biomaterial provides controlled release and/or localization of bioactive components in order to drive a specific biological response. Commonly delivered small molecules include antibiotic [1,2], anti-inflammatory [3], and anti-cancer drugs [4], while a variety of cell types have been delivered to regenerate tissues such as skin and bone [5]. Proteins such as vascular endothelial growth factor (VEGF), β-nerve growth factor (β-NGF), and bone morphogenetic protein-2 (BMP-2) are frequently incorporated to promote angiogenesis, axon sprouting, and bone mineralization respectively [6]. Developed as an alternative approach to exogenous protein loading, many materials are covalently modified with short biomimetic peptides. These peptides are typically derived from growth factors or critical components of the extracellular matrix (ECM), and they are specifically designed to activate important regulation cascades by binding the same receptor as their natural counterpart. The most commonly utilized biomimetic peptide, "RGD", is a simple tripeptide which promotes cell attachment, spreading, and proliferation by mimicking a binding site on fibronectin [7]. More recently, "QK," a short peptide mimic of VEGF, has been shown to drive angiogenesis and improve material vascularization [8,9]. In order to achieve the desired biological response, many tissue regeneration strategies require the incorporation of multiple bioactive components. Collectively, these design methodologies have resulted in a wide range of impressive results in tissue regeneration [10–12].

However, multicomponent systems are complex, often leading

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to challenges in both design and synthesis. Additionally, the multiplicity of components makes it difficult to predict the biological response to the material, and, further, raises regulatory barriers for translation to the clinic. Thus, an ideal material for tissue regeneration exerts the necessary bioactive effects without depending upon the delivery of exogenous bioactive components. In this report we describe a simple, single component multidomain peptide (MDP) hydrogel with the amino acid sequence $K_2(SL)_6K_2$ which does not contain small molecule drugs, cells, nor proteins and does not present any known bioactive sequence. Despite its simplicity, we will show that this syringe deliverable peptide hydrogel 1) is rapidly infiltrated by host cells, 2) provokes an inflammatory response which resolves over a few days, 3) does not undergo fibrous encapsulation, thereby allowing communication between the implant and the host body, 4) results in a dense, mature vascular network, 5) recruits neural fascicles, and 6) predictably degrades over approximately six weeks. Analysis of the cellular infiltrate and its secretome shows the production of critical cytokines and growth factors such as monocyte chemoattractant protein-1 (MCP-1) and interleukin-4 (IL-4) to recruit and convert monocytes to a "prohealing" phenotype; VEGF and platelet-derived growth factor (PDGF) to drive angiogenesis; ciliary neurotropic factor (CNTF) and β-NGF to recruit Schwann cells and neural fascicles; and matrix metalloproteinase-8 (MMP-8) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) to mediate material remodeling. These results suggest that the nanofibrous MDP hydrogel creates an optimal healing environment for a wide variety of applications through the use of the simple peptide hydrogel alone.

With the amino acid sequence $K_2(SL)_6K_2$, the simple peptide described in this study is a member of the broader class of self-assembling amphiphilic peptides termed MDPs (Fig. 1A) [13]. MDPs consist of an alternating sequence of hydrophilic and hydrophobic amino acids flanked by charged residues at each peptide termini (Fig. 1B). As a consequence of this design, MDPs exhibit a β-sheet secondary structure, high solubility, and supramolecular assembly in aqueous solution to form nanofibers with structural similarity to the native ECM (Fig. 1C&D) [14,15]. Previously, we have demonstrated that MDP hydrogels can be loaded to effectively deliver bioactive molecules including drug molecules [16–18], growth factors [19–22], and cytokines [19,23]. The robust mechanism governing self-assembly and hydrogelation of MDPs allows for significant customization of the peptide sequence. Taking advantage of this, we have incorporated bioactive sequences into the MDP to create a tissue engineering scaffold capable of driving specific cellular processes. These sequences include the RGD motif to support cell adhesion [20,24–26], LRG to accelerate enzymatic degradation of the peptide by matrix metalloproteinase 2 (MMP-2) [20,24–26], and the "QK" sequence derived from VEGF to drive angiogenesis [22,27].

Previous studies performed by our laboratory have used the MDP $K_2(SL)_6K_2$ as a baseline self-assembling system and in vitro studies showed no special bioactivity yielding modest results in cell viability, spreading, and morphology when compared to related hydrogels with designed bioactivity [24,25]. This had led us to focus on delivering biologically active molecules using MDP hydrogels, or modifying the MDP hydrogel with biomimetic peptide sequences to gain bioactivity. Those hydrogels which were deemed to be successful in in vitro experiments progressed to more complex in vivo experiments, while peptides showing less success in vitro were eliminated from consideration for further studies. Therefore, before the current study we had not tested $K_2(SL)_6K_2$ in vivo. However, there is a wide and growing consensus in the scientific community that in vitro results often fail to accurately predict the in vivo effect of a material, largely due to the absence of systemic processes [28]. In this report, we show that the MDP $K_2(SL)_6K_2$ supports this hypothesis, as in vivo results described here contrast sharply with predictions based on our in vitro work.

In this study, we use a subcutaneous injection model to examine the physiological response to the MDP $K_2(SL)_6K_2$. This in vivo model has many advantages in determining the interaction between a biomaterial and a living system. First, it is a relatively simple and inexpensive experiment to carry out. Second, this system allows interpretation of biomaterials properties outside of the complicating influences of injury or disease (Fig. 1E). Despite its simplicity, this model offers valuable insight into the interaction of the MDP with systemic processes such as the immune, vascular, and nervous systems which are impossible in in vitro systems. As with any biomaterial, the inflammatory response triggered by the MDP is critical in defining its success or failure, and this response can be determined through examination of the response to subcutaneous injection. Because this model is performed on healthy tissue, the ideal result is ultimately degradation of the MDP and remodeling into native tissue. Until complete degradation is achieved, the physiological response to the MDP can be evaluated.

2. Materials and methods

2.1. Peptide synthesis

$K_2(SL)_6K_2$ was synthesized using a Focus XC Automated Peptide Synthesizer with N-terminus acetylation and C-terminus amidation, by methods previously reported by our laboratory [14]. Successful synthesis was confirmed through MALDI-TOF MS (SI Fig. 1). After cleavage of the peptide from the synthesis resin, the peptide was dialyzed against H_2O for one week to remove any remaining cleavage scavengers. Tubing with a MWCO of 100–500 Da was used for dialysis, and daily exchanges of the dialysis bath were performed. The peptide solution was then sterilized using a 0.2 μm filter, and lyophilized to yield a peptide powder. The peptide powder was redissolved to a concentration of 20 mg/mL in sterile H_2O containing 300 mM sucrose, and hydrogelation was induced by mixing this solution 50:50 with 1X HBSS. The final concentration of peptide in the hydrogel used for experimentation was therefore 1 wt%.

2.2. Subcutaneous injection

All experiments were performed in accordance with an IACUC protocol approved by Rice University. Wistar rats weighing 226–249 g (Charles River Laboratories) were given 200 μl subcutaneous injections on the dorsal aspect. The animals were anesthetized using isoflurane (3% carried by oxygen) and maintained on a nose cone (2% isoflurane). Prior to injection, hair was clipped with a power trimmer, and the injection sites were sterilized with an alcohol swab. After 3, 7, 14, 21, or 42 days, the animals were euthanized by CO_2 overdose, and the dorsal tissue was harvested and fixed in 4% paraformaldehyde for histological analysis.

2.3. Histology & immunohistochemistry

All samples were processed, embedded, and sectioned at Baylor College of Medicine's Breast Cancer Pathology core, and all staining was performed on 5 μm thick tissue sections. Hematoxylin and eosin and Masson's trichrome staining were performed according to manufacturer's instructions. For immunostaining, tissue sections were deparaffinized and rehydrated through xylene and ethanol washes. Slides were boiled in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 min for antigen retrieval. Slides were allowed to cool to room temperature, and 0.5% Triton-X