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Bone regeneration with low dose BMP-2 amplified by biomimetic supramolecular nanofibers within collagen scaffolds

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) is a potent osteoinductive cytokine that plays a critical role during bone regeneration and repair. In the extracellular environment, sulfated polysaccharides anchored covalently to glycoproteins such as syndecan and also non-covalently to fibronectin fibers have been shown to bind BMP-2 through a heparin-binding domain and regulate its bioactivity. We report here on a synthetic biomimetic strategy that emulates biological BMP-2 signaling through the use of peptide amphiphile nanofibers designed to bind heparin. The supramolecular nanofibers, which integrate the biological role of syndecan and fibronectin, were allowed to form gel networks within the pores of an absorbable collagen scaffold by simply infiltrating dilute solutions of the peptide amphiphile, heparan sulfate, and BMP-2. The hybrid biomaterial enhanced significantly bone regeneration in a rat critical-size femoral defect model using BMP-2 amounts that are one order of magnitude lower than required for healing in this animal model. Using micro-computed tomography, we also showed that the hybrid scaffold was more effective at bridging within the gap relative to a conventional scaffold of the type used clinically based on collagen and BMP-2. Histological evaluation also revealed the presence of more mature bone in the new ossified tissue when the low dose of BMP-2 was delivered using the biomimetic supramolecular system. These results demonstrate how molecularly designed materials that mimic features of the extracellular environment can amplify the regenerative capacity of growth factors.

1. Introduction

Bone is a dynamic tissue that continuously regenerates and has the ability to heal following fractures [1]. However, sufficiently large defects resulting from traumatic bone loss, tumor resections, or infection cannot heal without intervention, and thus the treatment of these skeletal complications presents difficult challenges in orthopaedic medicine [2]. Bone grafting is widely used in clinical procedures to promote healing of non-unions and large defects. In this strategy the iliac crest autologous graft has been the gold standard due to its osteogenic, osteoinductive, and osteoconductive capacity [3]. However, certain drawbacks including limited tissue availability, pain, and donor-site morbidity have limited the use of iliac crest grafts [4]. Allogeneic bone grafting is an alternative, but this strategy has limitations that include sub-optimal osteoactivity compared to autografting, donor incompatibility, and an increased risk of disease transmission [2,3]. With the limitations of graft-based approaches, there have been many efforts to develop synthetic scaffolds to stimulate the natural healing process of bone. A variety of scaffolds have been investigated, including polymer and oligopeptide-based hydrogels [5], porous scaffolds with controlled architecture [6], and polymer-netted hydroxyapatite microcrystals [7,8] (for reviews, see [9–11]). One of the main problems with previously developed scaffolds [12,13] is the fact that they often contain components that will be difficult to resorb and remodel and thus they will be replaced by hybrids of bone and

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residual minerals in their original formulation. The field of bone regeneration would benefit from biomaterials that will disappear completely and be replaced by pure bone only.

Another emerging alternative to grafting is therapies based on delivery of growth factors such as bone morphogenetic proteins (BMPs) [14,15]. Recombinant human BMP-2 and BMP-7 delivered within an absorbable collagen sponge have been used clinically, and have demonstrated improved bone healing at a therapeutic dose as high as 12 mg per treatment [16,17]. However, the collagen scaffold has no specific binding affinity for BMPs, resulting in 30% burst release upon initial implantation [15]. In recent clinical reports, the supraphysiological amount of the exogenous protein required for improved healing has demonstrated issues with generalized hematomas in soft tissue [18], exaggerated inflammatory response in proximal humeral fractures [19] and unicameral bone cysts [20], as well as infections due to an elevated anti-BMP-2 antibody level in open tibial fractures [21]. In order to improve the efficacy of BMP-based therapies and limit adverse responses, new types of protein carriers must be able to prolong protein retention and in turn reduce the therapeutic dose of growth factors [15,22–24].

Inspired by biological systems, several biomaterials have been developed to contain key components involved in receptor–ligand interactions such as heparin [25] and fibronectin [26]. In the extracellular environment, sulfated macromolecules bind and localize cytokines and control their signaling [27]; heparan sulfate-like glycosaminoglycans potentiate BMP signaling by facilitating the BMP–BMP receptor complexation [28–30]. Benoit *et al.* modified fluvastatin-releasing poly(ethylene glycol) (PEG) hydrogels with heparin to sequester endogenous BMP-2 produced by human mesenchymal stem cells (hMSCs) in response to the released drug, resulting in augmented expression of osteogenic proteins *in vitro* [25]. In addition, fibronectin consists of several domains for binding to other fibronectin molecules, integrin, heparin, as well as growth factors [31,32]. Martino *et al.* investigated a multifunctional recombinant fragment of fibronectin that contained both integrin and growth factor binding domains, and a fibrin matrix with the recombinant fragments covalently attached exhibited increased bone regeneration in a rat critical-size calvarial defect model with small quantities of BMP-2 and platelet-derived growth factor (PDGF) [26].

Our laboratory has developed molecular building blocks known as peptide amphiphiles (PA) that are programmed to self-assemble into high-aspect-ratio nanofibers that are 6–10 nm in diameter and microns in length [33,34]. When dilute solutions of these PAs are injected into physiological environments they can form networks of nanofibers as charges on their surfaces are screened by natural electrolytes. A PA molecule typically contains two main segments, a peptide sequence covalently linked to a hydrophobic alkyl tail or other similar non-peptidic but hydrophobic segments. The self-assembly of PA molecules in an aqueous environment is driven by the hydrophobic collapse of alkyl tails and hydrogen bonding down the length of the nanofibers due to β-sheet propensity in specific domains of the peptide segment adjacent to the hydrophobic tails [35]. On the terminus of the peptide sequence away from the alkyl tail, PA molecules may contain biological signals as epitopes for receptors [36–39], peptide sequences that bind specific proteins [40], or even covalently bound therapeutic drugs that are hydrolytically released over time [41,42]. The supramolecular nanofibers are designed to present a high surface density of biological signals or a tuned concentration of signals and may therefore be described as artificial extracellular matrices (ECM) [43,44]. These nanofibers can also form self-supporting gels or infuse into porous titanium scaffolds to create bioactive implants [45,46].

In previous works we investigated non-covalent interactions between PAs and sulfated polysaccharides to form biologically functional gels [47,48]. A PA molecule was developed with a Cardin-Weintraub heparin-binding peptide domain to specifically bind to heparin with moderate affinity so it could form nanofibers that display non-adhered "loops" of the polysaccharide [48]. These chains of heparin could localize growth factors through their respective heparin-binding domains [47]. The heparin-binding peptide amphiphile (HBPA) nanofibers can entangle to form gel networks in the presence of sulfated polysaccharide chains. These nanofiber gels demonstrated prolonged release of angiogenic growth factors and induced substantial neo-vascularization in a rat cornea using only nanogram quantities of basic fibroblast growth factor (FGF-2) and vascular endothelial growth factor (VEGF) [47]. Furthermore, these HBPA gels containing heparan sulfate (HS) were found to promote *de novo* formation of a highly vascularized connective tissue with minimal inflammation when implanted subcutaneously [49]. Recently, a hierarchical membrane made of HBPA, heparin, and hyaluronic acid was shown to promote enhanced growth factor retention and increased angiogenesis in the chicken chorioallantoic membrane assay [50].

We report here on the use of these systems with an osteogenic protein such as BMP-2. Biological BMP-2 signaling involves transmembrane glycoproteins such as syndecan, which contain covalently bonded strands of heparan sulfate that localize the cytokine during its interaction with receptors [51]. At the same time, these polysaccharide strands interact non-covalently during signaling with fibronectin fibers, which in turn have both heparin- and integrin- binding domains [31,51]. In the work reported here, we have investigated the consequence to bone regeneration through BMP-2 signaling using the fibronectin-like peptide amphiphile nanofibers with a strong capacity to bind heparan sulfate chains. These nanofibers could therefore localize both BMP-2 and fibronectin fibers. The nanofibers displaying heparan sulfate strands were infiltrated in the pores of an absorbable collagen scaffold to test the regenerative capacity of this biomimetic system in a rat femoral critical-size defect model.

2. Materials and methods

2.1. PA synthesis and purification

Heparin-binding peptide amphiphiles was synthesized using solid phase peptide synthesis methods as previously reported [49]. HBPA molecules were purified using reversed phase high performance liquid chromatography (HPLC) in a water/acetonitrile gradient, each containing 0.1% v/v trifluoroacetic acid (TFA). The purified material was lyophilized and stored at −20 °C until use.

2.2. Cryogenic TEM

Cryo-TEM was performed on a JEOL 1230 microscope according to a previously described protocol [37]. HBPA was prepared at 3 wt% in sterile water, then diluted to 1 wt% for imaging.

2.3. Growth factor release kinetics

Heparan sulfate (HS) was purchased from Celsus Laboratories and BMP-2 from PeproTech. We verified the binding ability of the HBPA nanofiber-heparan sulfate complex to BMP-2 by studying the release kinetics of the cytokine from a gel formed by the nanofibers. The formation of HBPA nanofiber gel was triggered by mixing with either heparan sulfate dissolved in deionized water (HBPA + HS) or phosphate buffered saline (PBS, Hyclone) in order to form a control gel without heparan sulfate (HBPA + PBS). HBPA gels were not incorporated into absorbable collagen sponge for this assay. In order to form the two types of gels ($n = 3$), 100 ng rhBMP-2 were added to solutions of either 2 wt% HS in deionized water or 2x PBS and mixed with 3 wt% HBPA solutions. The final concentrations of HBPA and HS in the HBPA + HS gel were 1.5 wt% and 1 wt%, respectively. This gel was allowed to equilibrate for 2 h at room temperature before it was soaked with 1 mL of release media (0.1wt% albumin in PBS). Afterward, 800 μL of the supernatant was immediately removed and replenished with 800 μL fresh release media. At 1, 2, 3, 4, 5, 6, 7, and 8 day time points, 800 μL of the supernatant was similarly removed