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Growth factor gradients via microsphere delivery in biopolymer scaffolds for osteochondral tissue engineering

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ABSTRACT

Temporally and spatially controlled delivery of growth factors in polymeric scaffolds is crucial for engineering composite tissue structures, such as osteochondral constructs. In the present study, microsphere-mediated growth factor delivery in polymer scaffolds and its impact on osteochondral differentiation of human bone marrow-derived mesenchymal stem cells (hMSCs) was evaluated. Two growth factors, bone morphogenetic protein 2 (rhBMP-2) and insulin-like growth factor I (rhIGF-I), were incorporated as a single concentration gradient or reverse gradient combining two factors in the scaffolds. To assess the gradient making system and the delivery efficiency of polylactic-co-glycolic acid (PLGA) and silk fibroin microspheres, initially an alginate gel was fabricated into a cylinder shape with microspheres incorporated as gradients. Compared to PLGA microspheres, silk microspheres were more efficient in delivering rhBMP-2, probably due to sustained release of the growth factor, while less efficient in delivering rhIGF-I, likely due to loading efficiency. The growth factor gradients formed were shallow, inducing non-gradient trends in hMSC osteochondral differentiation. Aqueous-derived silk porous scaffolds were used to incorporate silk microspheres using the same gradient process. Both growth factors formed deep and linear concentration gradients in the scaffold, as shown by enzyme-linked immunosorbent assay (ELISA). After seeding with hMSCs and culturing for 5 weeks in a medium containing osteogenic and chondrogenic components, hMSCs exhibited osteogenic and chondrogenic differentiation along the concentration gradients of rhBMP-2 in the single gradient of rhBMP-2 and reverse gradient of rhBMP-2/rhIGF-I, but not the rhIGF-I gradient system, confirming that silk microspheres were more efficient in delivering rhBMP-2 than rhIGF-I for hMSCs osteochondrogenesis. This novel silk microsphere/scaffold system offers a new option for the delivery of multiple growth factors with spatial control in a 3D culture environment for both understanding natural tissue growth process and *in vitro* engineering complex tissue constructs.

1. Introduction

Growth factors are polypeptides that can either stimulate or inhibit cellular proliferation, differentiation, migration, adhesion, and gene expression. Growth factor effects are concentration-dependent, often in a complex non-monotonic way [1]. Due to their control of many biological processes, growth factors are finding wide-spread use in the regeneration of many tissue types, such as musculoskeletal, neural, hepatic, and vascular systems [1,2]. Typically, recombinant types of growth factors are delivered in the culture medium to regulate cellular processes in the field of tissue engineering. For clinical therapies, these factors are administered either systemically or via direct injection into the tissue site of interest. However, the short half-lives, relatively large size, slow tissue penetration, and potential toxicity at the systemic level have hindered many applications for these bioactive compounds [3].

One option to enhance the in vitro and in vivo efficacy of growth factors is to incorporate them into polymeric biomaterials in order to maintain their stability and control their release kinetics. Growth factors can be incorporated directly into a polymeric scaffold to be used for tissue formation either during or after scaffold fabrication [4–6]. The release of these factors is then controlled by diffusion and/or scaffold erosion or degradation mechanisms. Growth factor delivery can also be accomplished in the form of microparticles, nanoparticles or related material formats incorporated into the scaffold [7–9], or via growth factor-secreting natural or genetically engineered cells harbored within the scaffolds [10,11].

One important application for growth factor delivery is in bone and cartilage tissue engineering. Degenerative diseases such as osteoarthritis, and traumatic injuries, are both prominent causes of cartilage defects. Due to the avascular nature, adult human cartilage has a

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limited capacity for regeneration. Therapies such as osteochondral grafting, chondroplasty, and prosthetic joint replacement have found only partial or temporary success due to inadequate donor tissue availability, donor site morbidity, the risk of infection, abrasion of the cartilage surface, loosening of implants, and limited durability of prosthetics [12]. Tissue engineering provides a promising alternative therapy, such as through engineering an osteochondral tissue that has the same structural and mechanical properties as a native cartilage-bone plug for subsequent implantation in vivo.

However, the fabrication of such a scaffold to control the formation of a composite bone and cartilage architecture remains a significant challenge. Since human mesenchymal stem cells (hMSCs) can differentiate into multiple tissue-forming cell lineages, such as osteoblasts, chondrocytes, adipocytes, tenocytes, and myocytes, under the stimulation of growth factors, a useful strategy is to immobilize specific growth factors in the scaffold such that the hMSCs are guided toward different tissue types with spatial control or patterning [13,14]. To date, the most commonly used strategy to engineer osteochondral construct is to fabricate a polymer scaffold consisting of two layers of materials with distinct properties, such as porosity, mechanical strength and material microstructure to mimic the natural ECM environment for bone and cartilage development. The scaffolds seeded with either hMSCs or pre-differentiated chondrocytes and osteoblasts can then be used for *in vitro* or *in vivo* studies [15–18]. Growth factors were added to the medium if the constructs were cultured *in vitro*. In some studies, growth factors have been incorporated into polymer scaffolds and the sustained release of growth factors facilitated bone and cartilage regeneration *in vitro* and *in vivo* [4–9,19–21]. However, the dose and spatial distribution of growth factors in these scaffold systems was not controlled.

It has been widely reported in the literature that bone morphogenetic protein 2 (BMP-2) and insulin-like growth factor (IGF-I) can induce hMSC osteogenic and chondrogenic differentiation, although the role of rhIGF-I in chondrogenesis still remains controversial [4,6,8,10,20–30]. In the present study, rhBMP-2 and rhIGF-I were microencapsulated in PLGA and silk microsphere systems which were further incorporated as a single or reverse gradient in a biopolymer scaffold comprising of either alginate or silk fibroin. With different combinations of growth factors in different carrier systems, we expected to induce hMSC osteogenic and chondrogenic differentiation in one scaffold matrix with spatial control of growth factors distribution and temporal control of their release. The finding will be useful for the future fabrication of osteochondral constructs for bone repair applications. For the polymer scaffold system incorporating microspheres and supporting tissue formation, alginate gel was used for the initial scoping studies due to its long history of use for cell encapsulation [31–33], and the ease with which gels can be formed to generate microsphere/growth factor gradients. Silk porous sponge-like scaffolds were used due to their excellent biocompatible, biodegradable, and mechanical properties for tissue engineering applications [34–40]. For the microsphere systems carrying growth factors, PLGA microspheres were selected as they have been used for encapsulating growth factors, including rhBMP-2 and rhIGF-I [41–43]. Recently, silk fibroin protein was also formed into microspheres using a novel method suitable for protein encapsulation and controlled release [44]. In the present study, in parallel with PLGA microspheres, silk microspheres were also used to encapsulate rhBMP-2 and rhIGF-I. The loading and release of growth factors from the two microsphere systems, as well as the impacts of growth factor release on hMSC differentiation in different scaffold matrices were compared.

2. Materials and methods

2.1. Materials

Cocoons of *B. mori* silkworm silk were kindly supplied by M. Tsukada (Institute of Sericulture, Tsukuba, Japan). 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). rhBMP-2 was supplied by Medtronic Inc (Minneapolis, MN) and recombinant human insulin like growth factor 1 (rhIGF-I) by Tercica (Brisbane, CA). Pysiogel® (80 mg/ml of succinylated gelatin) was from Braun Medical (Emmenbrucke, Switzerland). 3,3′5,5′ Tetramethylbenzidine (TMB) solution was purchased from BioFX laboratories (Owing Mills, MD). Horseradish peroxidase (type VI-A), low viscosity alginate, 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and other chemicals were from Sigma-Aldrich (St. Louis, MO).

2.2. Purification of silk fibroin

Silk fibroin aqueous stock solutions were prepared as previously described [45]. The final concentration of silk fibroin aqueous solution was approximately 8% (w/v).

2.3. Silk microspheres encapsulating HRP, rhBMP-2 and rhIGF-I

Two hundred mg of DOPC was dissolved in 1 ml chloroform in a glass tube and dried into a film under a flow of nitrogen gas. One ml of 8% (w/v) silk fibroin solution was mixed with (1) 125 μl of 2 mg/ml HRP, rhBMP-2 or rhIGF-I (3.125 μg growth factor per milligram silk) in the alginate gel scaffold experiment; (2) 125 μl of 2 mg/ml rhBMP-2 and 375 μl of 2 mg/ml rhIGF-I (9.375 μg growth factor per milligram silk) in the silk scaffold experiment. The mixture was added to hydrate the lipid film. The mixture of DOPC, silk and rhBMP-2 was diluted to 4 ml with water and moved to a plastic tube. The sample was frozen in liquid nitrogen for 15 min and then thawed at 37 °C for 15 min. The freeze-thaw step helped form smaller vesicles with homogeneous size distributions as well as increased the protein loading [44]. This freeze-thaw cycle was repeated 3 times and then the thawed solution was slowly pipetted into a glass beaker containing 50 ml water with fast stirring. The resulting solution was lyophilized for 3 days and stored at 4 °C. To prepare silk microspheres, the lyophilized material was suspended in 40 ml of pure methanol (MeOH) in a 50 ml plastic tube and the suspension was incubated for 15 min at room temperature followed by centrifugation at 9000 rpm for 20 min at 4 °C (Sorvall RC-5B centrifuge). MeOH was used in this case to remove the lipid templates and induce silk self-assembly (β-sheet structure) to form silk microspheres with an average diameter of about 1.6 μm [44]. The pellet obtained was dried in air and stored at 4 °C before use (Supplementary data).

2.4. PLGA microspheres encapsulating HRP, rhBMP-2 and rhIGF-I

PLGA microspheres were prepared by solvent evaporation from a water-in-oil-in-water ($W_1/O/W_2$) dispersion [46]. Details are provided in the Supplementary data.

2.5. Determination of loading and release of rhBMP-2 and rhIGF-I from silk microspheres

Silk microspheres loaded with rhBMP-2 or rhIGF-I (initial loading of 3.125 μg/ mg silk for both) were suspended in phosphate buffer, pH 7.2 to a concentration of 20 mg/ml of microspheres. The microspheres were dispersed by ultrasonication. One ml of suspension (20 mg/ml) was used for growth factor loading and release study. To determine loading, 1 ml of suspension was lyophilized and the dried material was treated with hexafluoroisopropanol (HFIP), and dissolved in 1 ml phosphate buffer at pH 7.2. rhBMP-2 or rhIGF-I content was determined using an BMP-2 or IGF-I ELISA kit (R&D systems, Minneapolis, MN). To determine rhBMP-2 or rhIGF-I release, a 1 ml silk microsphere suspension was incubated at 37 °C. At desired time points, the suspensions were centrifuged at 10,000 rpm for 2 min. The supernatant was moved to another tube and the pellet was re-suspended in 1 ml fresh buffer. rhBMP-2 or rhIGF-I content in the supernatant was determined by ELISA. The actual loading was then obtained by comparing growth factor content with the initial loading