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Acta Biomaterialia 5 (2009) 23–28
Acta BIOMATERIALIA
www.elsevier.com/locate/actabiomat

Sustained release of BMP-2 in a lipid-based microtube vehicle

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Received 28 May 2008; received in revised form 2 September 2008; accepted 5 September 2008
Available online 18 September 2008

Abstract

Sustained release systems have been developed for the use of growth factors in tissue engineering applications. However, many of these systems continue to have limitations associated with low loading efficiencies and reduced biological activity after release. In this paper, we utilized a lipid-based microtube system for the sustained release of BMP-2. The lipid microtubes were fabricated using a self-assembly method, in order to avoid the use of harsh organic solvents that may damage the protein. BMP-2 was loaded into the microtubes by rehydrating dried microtubes in the protein solution. The loading efficiency and release kinetics of BMP-2 in the microtubes were measured using in vitro immunoassays. Loading efficiency was found to be dependent on microtube concentration. The potential for this system to deliver biologically active BMP-2 was assessed using the alkaline phosphatase assay and von Kossa staining on human mesenchymal stem cell cultures. The results demonstrate that the lipid microtube system is able to provide sustained delivery of biologically active BMP-2 and thereby induce osteogenic differentiation.

Keywords: BMP-2; Microtubes; Sustained release

1. Introduction

Grafting procedures are presently the most common treatment used to restore function to damaged or degenerated tissue [1,2]. However, these procedures are limited by the amount of graft material, and have resulted in high rates of failure as well as donor site morbidity [1,2]. As a result, there is a great need for new tissue engineering strategies that aim to restore, maintain or remodel natural tissues at damaged or degenerated sites [3–5]. Tissue engineered constructs that include cell, protein or gene delivery have shown potential to serve as graft substitutes.

Protein or growth factor therapies have shown promise because they have highly specific actions [2,3]. However, these protein therapies often require frequent injections of high doses to overcome the poor stability of the protein, making these treatments very expensive and more likely to cause side effects in the patient [2,4]. To address these limitations, a sustained delivery system that is able to optimally control the rate and location of growth factor release while also maintaining a blood concentration level within therapeutic limits is needed [4]. The existing growth factor delivery systems include lipid-based and polymeric vehicles. However, the fabrication processes of many of these systems often involve the use of harsh organic solvents, which can denature and deactivate sensitive proteins, resulting in low efficiencies [4,6,7].

Bone morphogenetic proteins (BMPs) have been utilized widely in bone tissue engineering because of their ability to initiate and maintain the entire bone formation cascade – chemotaxis, proliferation and differentiation [2–4,8–12]. However, delivery of BMPs alone is suboptimal because of the extremely high doses needed to overcome the short half-life. Sustained BMP-2 activity has previously been achieved by incorporating the protein in a degradable

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doi:10.1016/j.actbio.2008.09.001

carrier and co-delivery with other factors to promote bone formation. The current carrier materials include collagen, tricalcium phosphate, demineralized bone matrix, hydrogels and synthetic polymers [3,4,8,13]. The delivery of BMP-2 in an absorbable collagen sponge is FDA approved for use in long bone fractures and spinal fusions. However, this system still requires large doses of BMP-2 at high costs and has unpredictable pharmacokinetic profiles [8,13]. Therefore, development of carrier systems that are able to reduce cost and have predictable release kinetics is needed. BMP-2 has been shown to have temporal expression during fracture healing, and with higher concentrations after chondrogenesis has occurred [10,11,14]. However, the exact period when BMP-2 is most effective is still unclear. A well-designed carrier system could provide sustained delivery of BMP-2 within the therapeutic limits throughout the course of tissue regeneration, ensuring that BMP-2 is present during the optimal time for activity [13,15].

The overall goal of this research was to develop a sustained release system for bone tissue engineering applications, using a lipid-based microtube vehicle previously described for delivery of neural growth factor [6], transforming growth factor-β [16] and brain-derived neurotrophic factor [17]. The self-assembly method used to fabricate this carrier system avoids the use of organic solvents, heat and other harmful procedures [6,18]. These microtubes are also beneficial because they can be injected for minimally invasive procedures and are unlikely to be engulfed by cells due to their size [6,17]. We hypothesized that the lipid microtubes will provide sustained release of BMP-2, enhancing bone regeneration. To test this hypothesis, the system was analyzed using in vitro assays to monitor the loading efficiency, release kinetics and the bioactivity of the released BMP-2.

2. Materials and methods

2.1. Preparation of lipid microtubes

Lipid microtubes were prepared using a self-assembly method described by Meilander et al. [6]. First, 10 mg of $DC_{8,9}PC$ lipid (Avanti Polar Lipids, Alabaster, AL, USA) was dissolved in 10 ml of 70% ethanol at 55 °C. The lipid solution was held at 55 °C for 6 h and then slowly cooled to 25 °C in a microprocessor-controlled refrigerated water bath (Isotemp Refrigerated Circulator, Fisher Scientific, Waltham, MA, USA). The microtubes were then stored at room temperature until further use.

Before using the microtubes, the cryoprotectant trehalose (Sigma, St. Louis, MO, USA) was added to the solution at a concentration of 50 mM to preserve the tubular structure during the drying process. The microtubes were then centrifuged (1500 rpm, 10 min) and the supernatant was removed. Any remaining ethanol solution in the microtubes was removed by lyophilization.

2.2. Microtube characterization

An aliquot of microtubes was placed on a glass slide with a cover slip to create a single layer of microtubes. The microtubes were imaged using a Zeiss Axiovert light microscope connected to an AxioCam digital camera (Carl Zeiss, Inc., Oberkochen, Germany) and desktop computer running AxioVision software. The lengths of microtubes in at least five fields of view were traced from the captured images using the microscope software. The yield of microtubes was calculated from a known dilution of microtubes counted on a hemocytometer.

To monitor the degradation, microtubes were incubated at 37 °C in 500 μl of phosphate-buffered saline (PBS) or minimal essential medium (α-MEM; Invitrogen, Carlsbad, CA, USA) with 15% fetal calf serum (FBS; Atlanta Biologicals, Atlanta, GA, USA), 2 mM L-glutamine, 100 U of penicillin and 100 μg of streptomycin (Invitrogen). At various time points microtube samples were collected and their lengths measured using the light microscope.

2.3. Protein loading

The microtubes were loaded with recombinant human BMP-2 (R&D Systems, Minneapolis, MN, USA) by rehydrating the dried microtubes in 40 μl of protein solution per mg of lipid. The microtubes were allowed to incubate in the protein solution overnight. Unencapsulated growth factor was collected by centrifugation (1500 rpm, 10 min).

To measure the loading efficiency, microtubes were counted on a hemocytometer and diluted to known concentrations. The microtubes were then incubated overnight in BMP-2 solution at a concentration of 2 μg ml^{-1}. The unencapsulated protein was collected by centrifugation and assayed with an enzyme-linked immunosorbent assay (ELISA; R&D Systems).

To monitor the release kinetics, BMP-2 loaded microtubes were incubated in 500 μl of PBS. The microtubes were then separated by centrifugation and the supernatant was collected and stored at −20 °C until assay. Microtubes were resuspended in 500 μl of fresh PBS at each time point. The amount of BMP-2 released was measured using an ELISA.

2.4. Bioactivity assay

The bioactivity of released BMP-2 was analyzed by determining its ability to increase alkaline phosphatase activity in human mesenchymal stem cells (hMSCs). Human mesenchymal stem cells, kindly provided by Tulane University, were harvested from bone marrow aspirates. Cells were passaged twice in α-MEM containing 15% FBS, 2 mM L-glutamine, 100 U of penicillin and 100 μg streptomycin. The hMSCs were subcultured at a low density (500 cells cm^{-2}) and not allowed to expand past 70% confluency. The hMSCs were then trypsinized, counted, and seeded in 24-well tissue culture treated plates at a density of 10,000 cells cm^{-2}. The cells were allowed to reach

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