Biomaterials 33 (2012) 283–294

Contents lists available at SciVerse ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Effects of osteogenic growth factors on bone marrow stromal cell differentiation in a mineral-based delivery system

Linh N. Luong[a], Janani Ramaswamy[a], David H. Kohn[a,b,*]

[a] Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA
[b] Biologic & Materials Sciences, University of Michigan, Ann Arbor, MI 48109, USA

ARTICLE INFO

Article history:
Received 26 August 2011
Accepted 21 September 2011
Available online 19 October 2011

Keywords:
Coprecipitation
Biomineralization
Biomimetic material
SBF (simulated body fluids)
BMP
FGF

ABSTRACT

Delivering growth factors from bone-like mineral combines osteoinductivity with osteoconductivity. The effects of individual and sequential exposure of BMP-2 and FGF-2 on osteogenic differentiation, and their release from apatite were studied to design a dual delivery system. Bone marrow stromal cells were seeded on TCPS with the addition of FGF-2 (2.5, 10, 40 ng/ml) or BMP-2 (50, 150, 450 ng/ml) for 6 days. DNA content and osteogenic response were examined weekly for 3 weeks. FGF-2 increased DNA content; however, high concentrations of FGF-2 inhibited/delayed osteogenic differentiation, while a threshold concentration of BMP-2 was required for significant osteogenic enhancement. The sequence of delivery of BMP-2 (300 ng/ml) and FGF-2 (2.5 ng/ml) also had a significant impact on osteogenic differentiation. Delivery of FGF-2 followed by BMP-2 or delivery of BMP-2 followed by BMP-2 and FGF-2 enhanced osteogenic differentiation compared to the simultaneous delivery of both factors. Release of BMP-2 and FGF-2 from bone-like mineral was significantly affected by the concentration used during coprecipitation. BMP-2 also demonstrated a higher "burst" release compared to FGF-2. By integrating the results of the sequential delivery of BMP-2 and FGF-2 in solution, with the release of individual growth factors from mineral, an organic/inorganic delivery system based on coprecipitation can be designed for multiple biomolecules.

1. Introduction

The clinical basis for bone regeneration is the correction of bone defects caused from trauma, congenital malformations, and progressively deforming skeletal disorders. Bone tissue engineering provides an alternative to bone grafting and direct usage of growth factors to regenerate bone. Bone tissue engineering uses engineering design to strategically integrate cells, an extracellular matrix (ECM) analog, and signaling molecules to induce bone regeneration in a controlled and predictable manner [1]. An ideal bone tissue engineering approach would incorporate osteoinductivity and osteoconductivity into the design of the supporting biomaterial, as well as biocompatibility, degradability, mechanical integrity, and the ability to support cell transplantation.

Osteoinductive properties can be integrated into a tissue engineering system by immobilization of biomolecules to a biomaterial surface, or encapsulation within a biomaterial. Employing an inductive approach to bone regeneration in the form of growth factors can regulate cellular responses (proliferation, migration, differentiation), and have either synergistic or antagonistic effects on other growth factors. Although the activation of a single growth factor can have an impact on several signaling pathways, in the cellular environment, signaling is not limited to a single growth factor but a multitude of growth factors at different locations and times. Therefore, in developing a delivery system to better simulate the microenvironment that cells are subjected to *in vivo*, exposure to multiple biological cues with spatial and temporal gradients would be advantageous. For example, vascular endothelial growth factor (VEGF) alone is not sufficient to heal critical size defects, however combining VEGF with bone morphogenetic protein 4 (BMP-4) enhances healing [2]. In some instances, delivering multiple factors together may not be sufficient to affect a biological response [3]. Simultaneous exposure of rat BMSCs to insulin growth factor-I (IGF-I) and BMP-2 does not increase alkaline phosphatase expression or calcium secretion, but exposure of cells to BMP-2 followed by IGF-I does increase osteogenic differentiation, demonstrating the importance of timing and the sequence of delivery of the growth factors on cell response [4].

To provide the design parameters for the development of a delivery system for bone engineering, BMP-2 and fibroblastic

* Corresponding author. Biomedical Engineering, University of Michigan, Ann Arbor, MI 48109, USA. Tel.: +1 734 764 2206; fax: +1 734 647 2110.
E-mail address: dhkohn@umich.edu (D.H. Kohn).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.09.052

growth factor 2 (FGF-2) were chosen due to their important roles in osteogenic differentiation. BMPs regulate the growth and differentiation of cells in the osteoblast lineage. *In vitro*, rhBMP-2 not only induces differentiation of osteoblastic precursors [5], but it also inhibits myogenic differentiation [6]. BMP-2 is one of the earliest genes that is induced in fracture healing, with a second peak occurring late in the period of osteogenesis [7], which suggests that a simple burst release or sustained release may not be optimal to elicit an osteogenic response.

Fibroblast growth factors promote cell growth, induce a mitogenic response, stimulate cell migration, and induce differentiation [8]. FGF-2 is a well known angiogenic factor, and at lower concentrations, it also plays a role in osteogenic differentiation. FGF-2 can stimulate the replication of osteoprogenitor cells, which then further differentiate into an osteoblastic phenotype. However, FGF-2 can also inhibit bone formation at specific concentrations (100 ng/ml) and durations (24–96 h) [9]. Because both BMP-2 and FGF-2 regulate osteogenesis, they have been used in combination. FGF-2 stimulates cell growth and osteoblastic differentiation of dexamethasone treated MSCs, and upon exposing cells to both BMP-2 and FGF-2, bone formation is enhanced more than either growth factor individually [10], confirming the importance of temporal gradients.

Osteoconductivity is derived from surface and bulk properties of synthetic or natural materials, which allow the recruitment of targeted host cells while preventing unwanted cells from entering the cellular microenvironment. To ideally deliver multiple biomolecules, an osteoconductive/osteoinductive delivery system must be developed that allows for temporal control over release. Polymeric dual delivery systems have been developed that are based on the fusion of a polymer containing one biomolecule with microspheres containing a second biomolecule, where release is controlled by polymer degradation [11]. Another approach is to coat a scaffold with hydrogel copolymers containing biomolecules, where the release of biomolecules is based on diffusion through the different hydrogel layers [12]. However, protein aggregation within the hydrogel can occur, resulting in incomplete release [12]. A third approach uses a peptide-modified alginate hydrogel, where multiple factors are delivered in conjunction with cells to induce ectopic bone formation. However, the sequence of delivery is not controlled [13].

An alternative to polymeric systems is the coprecipitation of proteins with biomimetic apatite onto an implant or scaffold surface. In addition to providing spatial-temporal control over delivery like many polymer systems, bioceramic coatings provide a high degree of osteconductivity. The formation of a bone-like mineral layer *in vivo* leads to interfacial bonding between implants and bone [14]. The synthesis of a bone-like mineral layer may enhance the conduction of host cells into scaffolds [15], in addition to inducing osteogenic differentiation of cells transplanted [16]. Additionally, the apatite increases stiffness, a design parameter not provided by polymer systems capable of temporal delivery. Substrate stiffness can have effects on DNA uptake, cell structure, and protein expression [17,18]. An important advantage to coprecipitation is the ability to produce calcium phosphate coatings at a physiological temperature [19,20], minimizing conditions that would alter the biological activity of the factors [21]. Biomolecules can be incorporated at different stages of the deposition of the calcium phosphate coatings [21], which spatially localizes the biomolecule through the apatite thickness [22], thus impacting release. Delivering the growth factors from a mineralized substrate is a tissue engineering approach that combines osteoinductivity, provided by the inclusion of the growth factors, and osteoconductivity, provided by the presence of biomimetically precipitated apatite.

The effects of individual and sequential exposure of BMP-2 and FGF-2 on osteogenic differentiation of murine BMSCs were examined in conjunction with the individual release kinetics of BMP-2 and FGF-2 coprecipitated within biomimetic apatite, to build toward the long term objective of utilizing biomineralization to deliver multiple growth factors in a controlled manner. It was hypothesized that low concentrations of FGF-2 would increase cell number while high concentrations of BMP-2 would enhance osteogenic differentiation. It was also hypothesized that the sequence of FGF-2 followed by BMP-2 would best enhance the osteogenic activity of the BMSCs compared to the delivery of each of the growth factors independently. DNA content, alkaline phosphatase (ALP) activity, osteocalcin (OCN) serum content, and mineralization were analyzed over 3 weeks. To determine growth factor concentrations needed to promote BMSC growth and differentiation, cells were seeded on TCPS with the addition of FGF-2 (0, 2.5, 10, 40 ng/ml) or BMP-2 (0, 50, 150, 450 ng/ml). To determine the effects of sequential administration on BMSC growth and differentiation, 2.5 ng/ml of FGF-2 and 300 ng/ml of BMP-2 were used. Coprecipitation schemes were then designed to mimic the concentration and sequence of FGF-2 and BMP-2 that optimally differentiated the cells. BMP-2 and FGF-2 were incorporated within apatite during the coprecipitation process and the release of these factors was examined using ELISA. It was hypothesized that the release profiles of BMP-2 and FGF-2 would be dependent on the growth factor concentration in supersaturated ionic solution during coprecipitation.

2. Materials and methods

2.1. Murine bone marrow stromal cell extraction and cell culture

Six-week-old C57BL6 mice were utilized for BMSC extraction. Freshly extracted long bones (6 per mouse) were suspended in Hank's Balanced Salt Solution (HBSS). The metaphyses of each bone were cut, and 2 bones were placed into a 200 μl pipette tip which was placed in a microcentrifuge tube. Tubes were spun for 8–12 s up to a maximum speed of 2000 rpm [23]. An 18 gauge needle was used to gently agitate the cell pellets. Cell pellets were then pooled and split into T75 flasks. Media was exchanged and non-adhesive cells were removed after 5 days. Media was then exchanged every 3 days until cells reached confluency, at which time cells were split 1:3 and replated. After reaching confluency, cells were counted and replated in 24 well-plates.

Growth medium for primary cell culture and plating was composed of 10% FBS, 1% penicillin-streptomycin, and MEMα. During growth factor administration, the growth medium was supplemented with 10^{-8} M dexamethasone for 6 days. After this time period, osteogenic medium was utilized, which was growth medium supplemented with dexamethasone, 50 mg/L L-ascorbic acid-phosphate, and 10 mM β-glycerol phosphate disodium salt hydrate.

2.2. Growth factors and heparin

The growth factors rhBMP-2 and rhFGF-2 were obtained from Peprotech (Rocky Hill, NJ). FGF-2 was reconstituted in 5 mM Tris buffer containing bovine serum albumin (BSA). BMP-2 was reconstituted in sterile water containing BSA. Heparin sodium salt derived from porcine intestinal mucosa was purchased from Sigma–Aldrich (St. Louis, MO).

2.3. Single growth factor optimization

Cells were plated into 24 well-plates ($n = 4$ per group) at a density of 40,000 cells per well and allowed to attach for 24 h. Medium was then removed and replaced with growth medium containing dexamethasone and growth factor (FGF-2 at 0, 2.5, 10, and 40 ng/ml or BMP-2 at 0, 50, 150, and 450 μg/ml), which was designated as Day 1. The media containing growth factor was replaced on Day 4. On Day 7, and every 3 days thereafter, the medium was replaced with osteogenic medium.

2.4. Multiple growth factor optimization

Cells were plated into 24 well-plates ($n = 4$ or 5 per group) at a density of 50,000 cells per well and allowed to attach for 24 h. Medium was then removed and replaced with growth medium containing dexamethasone and growth factors on Days 1 and 4 (Table 1). On Day 7, and every 3 days thereafter, the medium was replaced with osteogenic medium.