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Mesenchymal Stem Cell (MSC) Spheroid Constructs Containing Microfluidic BMP2-PLGA Microcapsules in Bone Tissue Engineering.

Bhavendrasinh Sayania
University of Pennsylvania, bsayania@gmail.com

** FULL THESIS WILL BE UPLOADED ONCE INVENTION DEVELOPMENT/ PATENT APPLICATION HAS BEEN SUBMITTED AND PROCESS HAS BEEN COMPLETED

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Bhaven V. Sayania

Research Mentor: Dr. Hyun Duck Nah

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ABSTRACT

Recombinant human bone morphogenetic protein-2 (rhBMP-2) is a clinically available osteogenic growth factor. In its current form, approved for clinical use, however, the growth factor is delivered in excessively high doses, resulting in unpredictable bone growth and unwanted clinical side effects. In this study, we evaluated a novel system, slow-release hybrid delivery system for PLGA- rhBMP-2 microcapusles in combination with BM MSC spheroids, and assess if it can lead to improved BMP-derived bone formation. The first aim of this study is to evaluate the osteogenicity of 3D cell spheroids as compared to 2D cultured cells, as we used primary cells that harvested from transgenic GFP mice. Second, we evaluated the effect of rhBMP2/PLGA microcapsules embedded within 3D cell spheroid constructs. Lastly, we combined the delivery of rhBMP2/PLGA microcapsule-containing spheroids and Matrigel to assess the bone formation in an in vivo model, as the combined effect of BMP microcapsules and spheroids has not been studied before. We found that by culturing BM MSCs in the spheroid form increases the osteogenic potential versus culturing the cells in a 2D monolayer both in vitro and in vivo. Further we concluded that incorporating BMP microcapsules in our BM MSC

spheroid construct further increases the osteogenic potential compared to spheroid constructs which did not contain BMP microcapsules. Our results suggest that the BM MSC spheroid constructs containing microfluidic BMP2-PLGA microcapsules were effective in bone regeneration even when BMP was used at a low dosage in the construct and therefore this model could be a viable model for bone tissue engineering

INTRODUCTION

Craniofacial skeletal defects result from trauma, disease, or congenital deformities and, left untreated, can lead to disfigurement and loss of normal function.[1] Bone tissue repair is possible for small defects, but in defects beyond a critical size, bone grafts are often necessary. While autogenous bone grafts remain the "gold standard" of treatment, the procedure is often accompanied by non-trivial post-operative pain and donor site morbidity, not to mention increased costs associated with the additional operating site.[2, 3] Limited donor site accessibility compounds the severity of this type of invasive procedure. These concerns have led to investigations into alternate graft sources and more effective bone tissue engineering.[2]

One approach to bone tissue engineering is protein-mediated therapy, involving the delivery of an osteoinductive growth factor to the site of the defect. A widely studied family of growth factors are bone morphogenetic proteins(BMPs), which are cytokines that play a key role bone formation and repair and have been evaluated for use in the treatment of craniofacial defects.[2, 4, 5] BMP-2, BMP-4, BMP-7, and BMP-9,members of the transforming growth factor beta (TGF-β) superfamily, have demonstrated the capacity to induce progenitor cells from mesenchymal sources to differentiate into osteoblasts.[4] Furthermore, BMPs play a role in osteogenesis, chondrogenesis, angiogenesis, and mesenchymal stem cell chemotaxis.[4, 6]

However, BMP is rapidly metabolized and, once entering the circulatory system, is cleared from the bloodstream within minutes. leaving insufficient levels of protein to act at the intended site, which can lead to unpredictable results.[7, 8] To avoid unnecessary side effects, an effective means of delivering the growth factor to the site of repair has yet to be determined.

Currently, BMP is approved for clinical use in a few select applications. The INFUSE Bone Graft , an absorbable collagen sponge (ACS) loaded with human recombinant BMP-2 (rhBMP-2), has been approved by the U.S. Food and Drug Administration (FDA) for over two decades as a medical device for use in spinal fusions, sinus lift procedures, and repair of alveolar defects after dental extractions.[8] Approval for the clinical delivery of rhBMP-2 with INFUSE was first granted by the FDA in 2002 for application in spinal fusions.[8] There are, however, drawbacks of this application; the most striking of which is the extreme, supraphysiologic dose of rhBMP-2 required for the desired osteogenic effect .[9, 10] The amount of rhBMP-2 present in one INFUSE dose for a single spinal fusion exceeds the amount of endogenous BMP naturally present in 1000 humans.[10] Retention of rhBMP-2 at the site of a skeletal defect is very poor, reflected by the protein's short 2 to 3 day half-life after implantation.[8] Nonetheless, rhBMP-2 is still regularly combined with adsorbable collagen sponges for clinical use.

When administered in a bolus, rhBMP-2 can cause undesirable side effects such as local inflammation, soft tissue edema, ectopic or heterotopic bone formation, and seromas.[11-13] It has been suggested that the potential adverse side effects of the protein are more common with its off-label use[13] in the repair of alveolar clefts or cranial vault reconstructions[14] or when supraphysiologic doses are delivered.[15] Due to transient bioactivity, adverse side effects, and inconsistent clinical performance, the application of rhBMP-2 for clinical procedures requires further optimization.[8]

Requirements for BMP delivery systems

The efficacy of BMP is highly dependent on its mode of delivery, and for clinical purposes, the delivery system should be relatively simple, inexpensive, and sterilizable.[16] Ideally, they should be reliable and consistent in form, yet also malleable—with the capacity to be shaped to fit a wide variety of defects.[6] Materials should be porous, allowing the infiltration of mesenchymal cells and blood vessels; yet they should also be mechanically stable, with the ability to withstand both compressive and tensile forces.[16] The rate of resorption of the carrier itself should aim to mirror the rate of formation of the new bony tissue.[9] In addition, osseoregeneration should not be affected by inflammatory or foreign material reactions to the carrier substance itself.[9]

The biologic action of BMPs can be shaped by their carrier and the manner in which they are delivered. The carrier has the ability to alter the local retention of the protein, the release kinetics and mechanism of release, and the overall dose of the protein necessary for osteoinduction and bone formation.[2]

Site of action

Most critically, the carrier should stabilize the protein, retaining it in place at the site of intended bone formation for time enough to allow for the migration of tissue forming cells to the site, and their subsequent proliferation and differentiation.[2] If delivered systemically, rhBMP-2 is rapidly cleared from the bloodstream; therefore, delivery of BMP directly to the site of desired bone formation, and in a carrierthat maintains adequate protein levels, is imperative.[2, 6] Early studies illustrated that a significantly larger percentage of the delivered dose of BMP is retained

at the delivery site when the protein is administered in a gelatin or collagen-like substance as compared to in a purely soluble form.[17] By retaining the protein at the delivery site, the pharmacokinetics of BMPs can be altered: Chen and Mooney[11] note that delivery methods successful in sequestering growth factors at the site of action result in enhanced bone formation and require an overall lower dose of the growth factor.

rhBMP-2 Release kinetics

There has been much investigation into the optimal release profile for rhBMP-2; yet there does not appear to be a clear consensus as to one particular profile that consistently leads to enhanced bone formation. Studies have indicated that the extremes of protein release are not ideal: i.e. bolus delivery or incredibly low level, slow release do not improve results.[16] In fact, much work has suggested that combining an initial burst release—in order to bring in cell mediators and trigger vasculogenesis—with a slower, long-term delivery of the growth factor can lead to increased osteogenesis.[18] Determining an ideal release kinetic profile is further complicated by the fact that in vivo factors can alter the functioning of the growth factor and its delivery system. When delivered in vivo, the growth factors are subject to biochemical factors that are not necessarily present in vitro. For instance, bodily fluids, local proteins and enzymes, temperature, pH, and the local microenvironment can all affect the kinetics of release of rhBMP-2, as well as its subsequent bioactivity.[2]

Protein dosage/concentration

As suggested above, due to the inconsistent release profiles of rhBMPs, when used clinically, they are often delivered in supraphysiologic doses. This ensures that a critical

threshold of protein is isolated at the implant site and is maintained there for a sufficient period of time.[2] Delivery systems that have the capacity to deliver the protein at a sustained release rate have the potential to decrease the overall dose of BMP necessary for bone regeneration at a defect site.[7] Studies have indicated that by implementing a long-term, slow release delivery system, therapeutic results are enhanced, compared to a similar dose administered via short-term delivery.[7, 19] A lower dose of rhBMP-2 would lead to not only decreased overall cost, but also potentially fewer adverse side effects associated with the delivery of excessive amounts of the growth factor.

Delivery systems for rhBMP-2

With the aforementioned guidelines, many approaches have been made to engineer delivery systems for the optimal, sustained-release delivery of rhBMP-2. There are four major categories of materials that have been examined for their use in growth factor delivery, each with associated advantages and disadvantages. These include natural polymers, such as collagen or fibrin, inorganic materials, such as calcium phosphate cements or hydroxyapatite, and synthetic polymers, the most common of which are the poly(α-hydroxy acids).[2, 4, 6] Poly(α-hydroxy acids) are approved by the FDA, and are a common component of resorbable surgical sutures.[20] Composites of the above materials have also been evaluated.[6] In this study, we evaluate the effectiveness of a polymer-based delivery vehicle.

Encapsulation of drug within a polymer material typically occurs through the formation of emulsions.[21] Emulsions are created when mixing two or more immiscible liquids.[22] Due to the liquids' hydrophobic properties, the drops remain distinct from one another. Conventionally, encapsulation is performed by a solvent extraction/evaporation method in which microspheres are produced en-mass, resulting in a poly-disperse microsphere sample.[23] Additionally, when

proteins are encapsulated in such a manner, encapsulation efficiency is low, and protein loss is excessive.[24, 25]

Microfluidics have made it possible to improve greatly upon the conventional method, both in terms of uniformity and encapsulation efficiency.[21,22,26,27] A flow-focusing microfluidic device allows the fabrication of identical emulsions one at a time, thus producing a monodisperse sample.[21, 22, 28] Double emulsions are created when a hydrophobic inner phase, containing a protein for encapsulation, is combined with a hydrophobic middle phase (PLGA), all within a hydrophilic outer phase. When the solvent from the middle phase evaporates, monodisperse microcapsules (MC) result.[21] When proteins are encapsulated, there is virtually no loss of the inner layer protein.[22]

In the case of PLGA microcapsules, a water-in-oil-in-water (w/o/w) double emulsion is created to encapsulate rhBMP-2 in a PLGA shell. Hollow, thin-walled microspheres (microcapsules)[21] containing proteins, growth factors, or drugs have the ability to provide sustained, slow release of the encapsulated materials.[11] PLGA microcapsules degrade over time by hydrolysis into their component monomers, lactic and glycolic acid. RhBMP-2 is released by diffusion through intact PLGA or through openings in ruptured microcapsules.[11]

Mesenchymal Stem Cells

A high volume of research in bone tissue engineering has been devoted to adult stem cells, which can be isolated from tissues such as a bone marrow or adipose tissue. The term stem cell has generally been use for those cells possessing the ability to self-replicate and give rise to daughter cells which undergo an irreversible, terminal differentiation process.[29]Stem cells in adults have been studied extensively from the epidermis, gastrointestinal epithelium, and the

hematopoietic compartment of bone marrow. Hematopoietic stem cells are perhaps the best characterized and are noted for their ability to give rise to multiple cellular phenotypes through lineage progression of daughter progenitor cells.[30,31,32] In particular, bone marrow-derived mesenchymal stem cells (BM-MSCs) are attractive candidates due to their high osteogenic capacity.[33] MSCs in the bone marrow cavity can also differentiate into cartilage, fat, and bone cells (mesoderm) and into several other cell types.

The incorporation of MSCs into bone tissue engineering bio-materials is a widely studied strategy for accelerated bone formation and osteointegration during bone defect repair and regeneration. Mechanisms by which enhanced bone regeneration occurs involves directly providing MSCs for osteogenic differentiation and bone formation, as well as enhanced osteoinductivity of the biomaterial via the release of osteogenic growth factors and stimulation of the migration and differentiation of host osteoprogenitors.[34] In addition, pre-differentiating MSCs into the osteogenic lineage before implantation has been shown to further accelerate defect repair and osteointegration of the construct in vivo by delivering a more mature osteogenic population capable of immediate bone formation.

Osteoblast recruitment

It has been well understood and studied now that MSC can give rise to osteoblast. One aspect of bone formation that has not been been studied thoroughly is the recruitment of osteogenic cells to site of bone regeneration. The current knowledge suggests that osteoprogenitors and endothelial cells may be attracted to sites where vascularized bone is to be formed by common chemotactic stimuli and/or osteo-angiogenic coupling factors. In vitro, several factors with chemotactic potential towards osteoblast and their precursors have been identified including many constituents of the bone matrix such as fragments of collagen,

osteocalcin, inflammatory cytokines and growth factors such as vascular endothelial growth factor (VEGF), homologous placental growth factor (PIGF), and transforming growth factor-B (TGF-B). [35,36]

The ability for mesenchymal cells to migrate in vitro appear to change during osteogenic differentiation: migration is generally highest at early differentiation stages and gradually decreases during later differentiation while adhesiveness increased.[37] This is essentially in line with the aforementioned in vivo study using stage-specific osteoblast lineage cell tracing in which specifically osteoblast precursors, and not mature osteoblasts, were found to migrate from the perichondrium to the developing bone center.[38]

Three-Dimensional Cell Culture

MSCs are commonly cultured as a two-dimensional (2D) monolayer using conventional tissue-culture techniques. These 2D-monolayer techniques inadequately reproduce the in-vivo microenvironment of stem cells, established by extrinsic and intrinsic cellular signals and have a profound influence on their biological functions. [39] Culturing multipotent MSCs in a 2D adherent monolayer can alter their normal physiological behavior, resulting in the loss of replicative ability, colony-forming efficiency, and the differentiation capabilities over time.[40] Replication of this complex in-vivo microenvironment in-vitro requires highly sophisticated cell-culture systems.

Previous studies have compared three-dimensional (3D) over two-dimensional (2D) culture of mesenchymal stem cells, and evidence supports the hypothesis that 3D culture more closely approximates the in vivo cell microenvironment. While cells are typically maintained in 2D culture, evidence suggests that this alters the cells potential for replication, colony-forming,

and differentiation. Studies investigating 3D cell culture suggest that 3D culture better reflects the in vivo microenvironment, especially in terms of cell-cell interaction, extracellular matrix, and gene expression.[41]

There is a wide variety of techniques to culture cells in a 3D environment. These techniques can be grouped into two categories: scaffold based and non-scaffold based. Scaffold based 3D cell culture can consist of polymeric hard scaffold, biologic scaffolds, and micropatterned surface microplates. Non scaffold based 3D cell culture can consist of hanging drop microplates, microplates containing ultra-low attachment (ULA) coating, or microfluidic 3D cell cultures.

The hanging drop method relies on gravity-enforced self-assembly to produce spheroids. To make spheroids, small volumes (20-30 μL) of a cell suspension are pipetted onto the inside lid of a tissue culture plate. The lid is inverted, and the drops stay attached to the lid due to surface tension. Gravity causes the cells to settle and concentrate at the bottom of the drop, and a single spheroid is formed.[42] A variety of cell types have formed spheroids using this method including both primary cells as well as cell lines7. Different cell types can be co-cultured to form heterotypic spheroids. Spheroid size and cellular composition is controlled by adjusting the cell density in each drop. Advances into high throughput production of spheroids using the hanging drop method have been established, producing up to 384 spheroids in a single array. However, with this method, it is difficult to track these spheroids during formation and it is nontrivial to exchange media or add drugs.

The most effective and convenient technique to fabricate consistently sized spheroids has been shown when using a 96 well non adhesive culture plates. This technique has been

previously described for example for embryonic body formation from mouse embryonic stem cells by statically cultivation as well as for aggregation of human embryonic stem cells in low attachment plates by centrifugation. [43,44] The spheroids generated by cultivation of a cell suspension in 96 well non adhesive culture plates have shown to be round and tightly packed with a constant range of size. This technique has also shown to be the most efficient method to generate spheroids (95%) compared to hydrogel suspension culture 46%, hydrogel semi solid culture (45%), and 15mL polyprene tube (83%).[45]

When comparing the osteogenic potential in MSCs cultured in a 2D monolayer versus 3D microenvironment under the same conditions, 3D MSC spheroids have showed a greater osteogenic potential both in in-vitro and in-vivo models. In-vitro studies showed gene expression of osteogenic genes: *RUNX-2*, *OSX*, *OPN*, and *BSP* were upregulated in the 3D MSC spheroids. [45, 46, 47] In this work, we compared the osteogenic potential of 2D cultured cells with 3D cell spheroids in conjunction with sustained release of rhBMP-2 from microcapsules. Although there are many advantages of 3D cell culture, there are disadvantages of 3D cell cultures. Previous studies have shown that the core cells in the spheroid receive less oxygen, growth factors and nutrients from the local environment and tend to be quiescent or hypoxic state.[48] Another explanation for the hypoxia in the core of the spheroid maybe related to the lack of transport system to remove waste from the center of the spheroid.[48]

Experimental Aims

In this study, we examined a novel, slow-release hybrid delivery system for rhBMP-2 in combination with 3D cells spheroids, and assess if it can lead to improved BMP-derived bone formation. The first aim of this study was to evaluate the osteogenicity of 3D cell spheroids as compared to 2D cultured cells, as we used primary cells that harvested from transgenic GFP mice. Second, we evaluated the effect of rhBMP2/PLGA microcapsules embedded within 3D cell spheroids. Lastly, we combined the delivery of rhBMP2/PLGA microcapsule-containing spheroids and Matrigel to assess the bone formation in an in vivo model, as the combined effect of BMP microcapsules and spheroids has not been studied before.

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