In vitro bone formation using muscle-derived cells: a new paradigm for bone tissue engineering using polymer–bone morphogenetic protein matrices

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Abstract

Over 800,000 bone grafting procedures are performed annually in the United States, creating a demand for viable alternatives to autogenous bone, the current standard in osseous repair. The objective of this study was to examine the efficacy of a BMP-polymer matrix in inducing the expression of the osteoblastic phenotype and in vitro bone formation by muscle-derived cells. Specifically, we evaluated the ability of bone morphogenetic protein-7 (BMP-7), delivered from a poly(lactide-co-glycolide) (PLAGA) matrix, to induce the differentiation of cells derived from rabbit skeletal muscle into osteoblast-like cells and subsequently form mineralized tissue. Results confirmed that muscle-derived cells attached and proliferated on the PLAGA substrates. BMP-7 released from PLAGA induced the muscle-derived cells to increase bone marker expression and form mineralized cultures. These results demonstrate the efficacy of a BMP-polymer matrix in inducing the expression of the osteoblastic phenotype by muscle-derived cells and present a new paradigm for bone tissue engineering.

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Over 800,000 bone grafting procedures are performed annually in the United States [1]. There is a significant demand for viable alternatives to autogenous bone grafts, the current standard in osseous repair. Despite a clinical success rate of 80–90%, autografts require a second surgical procedure and are associated with donor site pain and morbidity [1,2]. Moreover, these grafts are limited in supply and are often structurally incompatible with the tissue to be replaced. Other biological bone grafts such as allografts and xenografts carry the additional risks of implant rejection as well as transmission of blood-borne diseases from the donor to the recipient [3]. Synthetic bone grafts have good short-term results, but often fail at the long term due to fatigue and mismatch in mechanical properties with native bone [1–4]. In the past decade, in order to circumvent limitations associated with existing biological and synthetic grafts, bone tissue engineering has emerged as an alternative approach in the formation of viable bone grafting systems [1–4].

Tissue engineering can be defined as the application of biological, chemical, and engineering principles towards the repair and restoration of living tissues, using cells, biological factors, and biomaterials, alone or in combination [2]. The ideal tissue engineered bone graft should be biocompatible, biodegradable, osteoconductive, and osteogenic in nature [2]. The poly-α-hydroxyesters, namely poly(lactide acid (PLA), polyglycolic acid (PGA) and copolymers, poly(lactide-co-glycolide)
diffusion chambers containing bone seous tissue in vivo [18–22]. In 1975, Nogami and Urist subsequently shown by many to induce the formation of os-

demineralized bone matrix, and they have been subse-

quent to demonstrate in 1965 that demineralized bone matrix induced render the scaffold osteoinductive [18]. Urist demon-

strated the importance of the polymeric delivery substrate in facilitating osteogenesis. The use of an alternative cell source and osteoin-
ductive factors, in conjunction with a tissue engineered scaffold for bone regeneration, may be particularly ef-

fective in bone healing [3]. The objective of this study was to examine the efficacy of a BMP-polymer matrix in inducing the expression of the osteoblastic phenotype by muscle-derived cells. Our approach was to induce the differentiation of primary rabbit muscle cell tissue into osteoblast-like cells utilizing bone morphogenetic protein 7 (BMP-7) delivered through a biodegradable polymeric matrix of poly(lactide-co-glycolide) (PLAGA). Specifically, we will examine the ability of rabbit muscle cell tissue to differentiate into osteoblast-like cells when cultured on a PLAGA matrix containing BMP-7. The hypothesis is that in the presence of BMP-7 deliv-
ered by the PLAGA matrix under controlled culturing conditions, primary muscle cell tissue can differentiate into osteoblast-like cells and form a mineralized matrix.

**Materials and methods**

Fabrication of PLAGA-bone morphogenetic protein matrices. Hu-

man recombinant osteogenic protein 1 (OP-1), also known as bone morphogenetic protein 7 (BMP-7, Creative Biomolecules, Hopkinton, MA), was reconstituted in a solution containing 47.5% ethanol (Al-
drich, Milwaukee, WI), 0.01% trifluoroacetic acid (Fluka, Steinheim, Switzerland), to yield a final BMP-7 concentration of 0.1 µg/µl solution.

The BMP-7 delivery system was based on poly(lactide-co-glycolide) 85:15 (PLAGA) and fabricated by a solvent-casting method. First, PLAGA was dissolved in methylene chloride (Fisher Scientific, Fair Lawn, NJ) and a glass pipette was used to transfer 2 ml of the dissolved polymer solution into a Teflon-coated well in a 6-well tissue culture polystyrene plate (Falcon 3046, Becton-Dickinson, Franklin Lakes, NJ). Four drops of 50 µl each of the reconstituted human recombinant BMP-7 solution were subsequently transferred into the well containing the polymer. The BMP-7 was slowly mixed with the polymer solution, to ensure homogeneous distribution of the protein within the solution. The 6-well plate was then covered and left in a −20 °C freezer where the

(PLAGA), approved by the FDA for use in a number of applications have been widely investigated for tissue engineering applications [5–9,48]. In addition to being biocompatible, PLA, PGA, and PLAGA have been utilized successfully as bone fixation pins and screws, suggesting that they are osteoconductive and able to support bone formation [10–14]. Laurencin and co-

workers [15] examined the behavior of osteoblasts on polymeric matrices and have reported the attachment, proliferation, and differentiation of these cells on these surfaces. As these polymers are biodegradable, bone grafting systems based on these materials have the prospect of being replaced by host tissue, thus elimi-
nating the long-term disadvantages associated with non-
dergradable synthetic materials. The utilization of a scaffolding material or synthetic substrate is often neces-

sary in orthopaedic tissue engineering, as initial functionality of the replaced tissue must be established at the onset of implantation to ensure long-term repair [16]. These scaffolds can serve as a structural component and guide the development of new tissue [16].

Cell selection is another essential parameter govern-
ing the rate and reliability of ex vivo bone tissue syn-

thesis. Functional osteoblasts are obtained usually through bone or bone marrow biopsies, which can be difficult and painful for the patient. Moreover, these cells are limited in supply. Hence, alternative cell sources must be identified in order to produce copious amount of bone tissue for realistic clinical applications. Tissue engineering using connective tissue from skeletal muscle is attractive, as harvesting of cells could be performed using a percutaneous method that is relatively easy to perform and less traumatic or painful for the patient. Muscle cells are also more readily available and ob-
tainable. There is evidence to suggest that muscle con-
nective tissue, when provided with osteogenic stimuli, can produce osseous-like tissue. Ono et al. [17,42–47] implanted diffusion chambers containing purified BMPs in abdominal muscle of Sprague–Dawley rats and found that outside the diffusion chamber, cartilage morpho-
genesis was observed at one to two weeks post-implan-

tation, while bone formation was found two to three weeks later.

Addition of osteogenic growth factors such as bone morphogenetic proteins to the biocompatible, biode-
gradable, and osteoconductive polymeric substrate can render the scaffold osteoinductive [18]. Urist demon-

strated in 1965 that demineralized bone matrix induced new bone formation in vivo at an ectopic site and he later named the functioning agent bone morphogenetic protein (BMP) [18]. BMPs are members of the transforming growth factor-β superfamily, first isolated from demineralized bone matrix, and they have been subse-

quently shown by many to induce the formation of os-

seous tissue in vivo [18–22]. In 1975, Nogami and Urist [23] reported that diffusion chambers containing bone matrix gelatin could induce cartilage and bone mor-

phogenesis in surrounding tissues. Wozney et al. [22] and Wang et al. [19] isolated and purified BMPs, and identified them as the factors that induced ectopic bone formation when implanted into muscular sites in vivo. The use of bioactive factors with osteoinductive properties, in conjunction with a tissue engineered scaffold for bone regeneration, may play an important role in bone healing [3]. The delivery of growth factors from polymer matrices has been explored by many re-

searchers [24–29,49]. Laurencin and co-workers dem-

onstrated that incorporation of soluble substances containing BMPs in degradable poly(anhydride) matrices stimulated both chondrogenesis and osteogenesis [28]. However, when the soluble substances were ad-

ministered without the polymeric carrier, no cartilage or bone formation was observed [28]. These results em-

phasized the important role of the polymeric delivery substrate in facilitating osteogenesis.
solvent was slowly evaporated. At 24 h prior to the experimentation, the BMP-7/PLAGA thin films were gelled at 4 °C. On the day of the experiment, the polymer protein film was bored to yield matrices of 1.05 cm in diameter and 0.015 cm in thickness.

Control polymer matrices without BMP-7 were also fabricated by solvent-casting. Briefly, 3.40 g PLAGA was first dissolved in 40 ml of methylene chloride and the solution was then slowly poured into a Teflon mold, covered with a glass plate, and placed in a ~20 °C freezer to allow slow evaporation of the solvent. After solvent evaporation, each film was bored to yield matrices of 1.02 cm in diameter and 0.10 cm in thickness. Prior to cell seeding, all discs were UV-sterilized for 5 min on each side.

Cell isolation and cell culture. Rabbit muscle cell tissue was isolated from the quadriceps and triceps muscles of 1.2 kg New Zealand white rabbits (Covance, PA). Briefly, the muscle bundles of the rabbit were excised from the animal, and the outer sheath surrounding the muscle bundles was removed and the remaining tissues were minced. The muscle pieces were seeded as explants and cultured in Ham’s F-12 medium with l-glutamine (Cellgro, VA) supplemented with 10% fetal bovine serum (Cellgro, VA), and 1% antibiotics (Cellgro, VA). The muscle fragments underwent successive digestions at 37 °C in a 1 mg/ml collagenase solution. Cell pellets were collected every 30 min and plated into flask containing the supplemented Ham’s F-12 medium. The Trypan blue dye exclusion test was used to determine cell viability. All cultures were maintained at 5%/CO2 and 37 °C. The Trypan blue dye exclusion test was used to determine cell viability. All cultures were maintained at 5%/CO2 and 37 °C.

Cell proliferation was quantified using Picogreen dsDNA assay (Molecular Probes, Eugene, OR) specific for actin. The cytoskeletal organization of cells from rabbit muscle was examined on PLAGA and PLAGA-BMP substrates. Confluent cultures from New Zealand white rabbit muscle (passage 1) were washed twice with Hank’s balanced salt solution, trypsinized, and collected via centrifugation. The cells were resuspended in the supplemented Ham’s F-12 media and plated on the thin film matrices at 5 x 10⁴ cells/cm². In order to promote attachment, cells were allowed to adhere to the scaffold for 30 min prior to the addition of media. One ml of the supplemented Ham’s F-12 media was later added to each well and the plates were cultured under humidified conditions, at 5%/CO2 and 37 °C. At a week after cell seeding, mineralization medium supplemented with 10 μg/ml ascorbic acid and 3.0 mM β-glycerophosphate was added to the cultures. Cell attachment adhesion, proliferation, and phenotype expression were examined during three weeks of culture.

Cell adhesion and proliferation on PLAGA and PLAGA-BMP substrates. Using immunofluorescence staining for actin, the cytoskeletal organization of cells from rabbit muscle was examined on PLAGA and PLAGA-BMP substrates at 4 and 10 days. Briefly, the samples were first washed in phosphate-buffered solution (PBS), fixed, and stained with a rhodamine-phalloidin antibody (Molecular Probes, Eugene, OR) specific for actin. Samples were then visualized using a Zeiss Axiosvert fluorescence microscope (Zeiss, Thornwood, NY) at an excitation specific for actin. Samples were then visualized using a Zeiss Axiovert 200M epifluorescence microscope (Zimmer).

Cell morphology and the formation of mineralized matrices were examined using scanning electron microscopy (SEM, JEOL JSM 6300, operated at 10 keV) combined with energy dispersive X-ray analysis (EDX, JEOL JSM 6300, operated at 10 keV). The scanning electron microscope preparative procedure consisted of a fixation process followed by alcohol dehydration. At the desired time point, the samples were washed with 0.2 M sodium cacodylate buffer and then fixed for 24 h in Karnovsky fixative, and the specimens were dehydrated using a graded-ethanol series. The samples were then left to dry overnight in a critical point dryer (Sigm−Aldrich, St. Louis, MO). Prior to image analysis, each sample was coated with carbon to eliminate any charging effects. Mineralization was further confirmed using alizarin red S staining specific for calcium, combined with a quantitative calcium concentration assay, following a method developed by Jacobs et al. [31].

Statistical analysis. Data are presented here as means ± SD. A one-way analysis of variance (ANOVA) was used to compare the proliferation data, alkaline phosphatase synthesis, and calcium concentration data as a function of culturing time. Comparison between the two means was determined using the paired t test, with statistical significance evaluated at p < 0.05.

Results

Cell adhesion and growth on PLAGA and PLAGA-BMP substrates

Scanning electron microscopy images, shown in Fig. 1, indicated that muscle-derived cells adhered and grew on the PLAGA-BMP (Fig. 1A) and PLAGA (Fig. 1B) matrices. After 18 days of culture, the cells covered the polymer surface and formed extensive cell sheets on both experimental and control surfaces. In terms of cytoskeletal organization, cells assumed spindle-shaped, elongated morphology on both PLAGA and PLAGA-BMP substrates, as seen in Fig. 2. The cytoskeletal organization key protein, actin, was expressed by the adhered cells over time on the PLAGA and PLAGA-BMP surfaces, demonstrating cellular structure integrity and framework maintenance. On BMP-containing matrices, a higher number of cells appeared to exhibit full cytoskeletal organization, representing movement and spatial control. Qualitatively, this degree of organization was less evident on PLAGA matrices without BMP.

Quantitative cell proliferation was determined on all polymer surfaces as a function of culturing duration (Fig. 3). Compared to the polymeric substrates, tissue
culture polystyrene (TCPS) supported significantly higher cell adhesion and growth. On the other hand, a significant increase in cell number was observed at day 14 and proliferation plateaued for all groups after 21 days of culture. Direct comparison of cellular growth between PLAGA to PLAGA-BMP surfaces yielded no significant differences between the means, at $p < 0.05$.

**Differentiation of muscle explant cells into osteoblast-like cells**

In the presence of BMP-7, cells from rabbit muscle tissue were found to form mineralized matrices, as well as to express the classic markers indicative of the osteoblastic phenotype. Alkaline phosphatase was synthesized by these cells grown on the PLAGA, PLAGA-BMP, and TCPS substrates. When normalized against
cell number per substrate, the highest amount of alkaline phosphatase synthesis was measured on the PLAGA-BMP surfaces, with the lowest amount found on TCPS.

The rabbit muscle cells formed mineralized nodules on the PLAGA-BMP surfaces after culturing with mineralization media for 7 days, as shown in Fig. 1. Cells grown on control PLAGA and TCPS failed to mineralize after culturing for the same duration, under similar conditions. Qualitative analysis (Fig. 4) using Alizarin red S staining for calcium revealed a higher staining intensity on PLAGA-BMP surfaces, as compared to both PLAGA and TCPS substrates. Quantitatively, as shown in Fig. 5, the amount of calcium measured after elution of the Alizarin red S dye from the polymeric substrates indicated that Ca accumulation on PLAGA-BMP surface increased as a function of culturing time, and it was significantly higher than the control groups at all time points examined, with $p < 0.05$.

EDXA spectra, presented in Fig. 6, revealed that the nodules (10–15 μm in size) found after the culture of these cells on PLAGA-BMP surfaces were primarily composed of Ca and P, with the relative calcium to phosphorus ratio found to be less than that of bone mineral, suggesting that the calcium phosphate phase formed was likely to be amorphous in nature. Similar nodules were not observed in cases where cells were cultured on PLAGA or TCPS substrates in mineralization media, as indicated in the EDXA spectrum shown in Fig. 6B.

In addition, immunohistochemistry results suggested that cells cultured from rabbit muscle expressed osteo-
calcin, a bone-specific protein synthesized during the formation of the mineralized matrix. In Fig. 7, immuno- fluorescent images show positive osteocalcin production by cells grown on PLAGA-BMP matrices. The synthesis of osteocalcin was not observed in control group cultures. On PLAGA-BMP, it was observed that osteocalcin was found among cells located at the periphery of the PLAGA-BMP substrate and diminished towards the center of the disc. Double staining depicting both osteocalcin and actin expression on tested substrates revealed that the positive staining for osteocalcin was less evident for cells creating contact.

Discussion

The objective of this study was to examine the efficacy of a BMP-polymer matrix in inducing the expression of the osteoblastic phenotype and subsequent development of mineralized tissue using muscle-derived cells. The bone morphogenetic protein incorporated in a polymeric matrix remained bioactive and induced the differentiation of rabbit muscle explant cells into osteoblast-like cells. These results demonstrate the important role of the polymeric delivery substrate in facilitating osteogenesis. Additionally, in spite of the ability to synthesize growth factors in large quantities through recombinant technologies, BMPs remain very costly, and a large dose is often required to elicit a positive response. Use of biodegradable polymeric carriers may reduce the dosage of BMPs needed, while potentially yielding equivalent amounts of bone formation. Moreover, the PLAGA-BMP systems may provide the continuous delivery of osteoinductive protein throughout the healing process. These are some of the first studies to use a polymeric BMP delivery system to investigate the feasibility of muscle cultures as a potential cell source for bone tissue engineering. In this study, cells grown on the PLAGA substrate in the absence of BMP-7 proliferated at a faster rate than cells grown on BMP-7 containing matrices. This suggests that cells grown on the PLAGA-BMP substrate were stimulated to differentiate into osteoblast-like cells instead of proliferating rapidly. We believe the direct action of BMP-7 on cultures derived from muscle in this system may be in promoting the osteoblastic phenotype through cell differentiation instead of cell proliferation. BMPs have a significant effect on organogenesis and embryonic development. These proteins are also involved in the migration of osteoblast progenitor cells, proliferation of mesenchymal cells, cell differentiation into chondrogenic or osteoblastic phenotype, angiogenesis, and bone remodeling.

Osteocalcin is a bone-specific protein, synthesized by osteoblasts at the onset of mineral deposition. In this study, only the PLAGA-BMP substrate supported the synthesis of osteocalcin, suggesting that the presence of BMP-7 played the determining role in controlling the differentiation of muscle cells into osteoblasts. Immuno- fluorescence of actin allowed a direct visualization of the cytoskeletal organization of cells grown on the polymers. Double-staining depicting both osteocalcin and actin expression on tested substrates revealed that the signals obtained were found to be in cells surrounding the periphery of the matrix and less in cells
creating contact. This distribution implies that cellular differentiation of the cells derived from muscle which released osteocalcin may be dependent on the ability of the cell to maintain its differentiating potential and spatial arrangement. SEM and EDXA results indicated that the cells were able to attach and grow on the PLAGA matrices, and in the presence of BMP-7, form mineralized tissue, which primarily consisted of phosphorus and calcium deposits. EDXA results were further supported by quantitative analysis of alizarin–calcium expression, with the highest calcium concentration measured on the PLAGA-BMP substrates.

Cells derived from muscle may be an alternative cell source for bone tissue engineering. The osteogenic potential of muscle explant cells was first demonstrated in a study by Nogami and Urist [23], where muscle explants combined with a demineralized bone matrix were found to result in chondrogenesis and osteogenesis at an ectopic site. In the current study, the use of a primary rabbit muscle culture resulted in a heterogeneous culture of myoblasts, fibroblasts, and other connective tissue cells which may include osteoprogenitor cells. Based on our results, it is yet unclear which type of these cells directly responded to the osteoinductive signal delivered through the PLAGA matrix. There is extensive evidence in the literature on the ability of BMPs to induce bone formation via a variety of cells [16,38–44]. However, the effects differ as a function of the type of BMP used and culture additives. For example, bone morphogenetic protein-2 (BMP-2) has been shown not only to stimulate the maturation of osteoprogenitor cells, but also to induce osteoblast-like differentiation of pluripotent fibroblasts [43,44].

Our working hypothesis is that the connective tissue cells associated with muscle tissue contain a population of stem cells which can differentiate into osteoblasts under the influence of BMP-7. Musgrave et al. [42] established homogeneous human muscle explant cultures, using successive plating of explant cells on a collagen-coated tissue culture polystyrene surface. The authors were able to isolate specific cell populations and selected a multi-day culture population with osteogenic potential. It is believed that these were the cells within the muscle-derived cell population that responded to the BMP signal [42]. Jortikka et al. [45] examined the effects of BMPs on the osteoinductivity of L6 rat skeletal muscle myoblasts and suggested that myoblasts were target cells for the action of BMP. Using adenoviral transfection of BMP-2, Okubo et al. [46] induced C2C12 myoblasts to be differentiated into the osteoblast lineage. Katagiri et al. [43] reported that BMP-2 inhibited terminal differentiation of C2C12 myoblasts and converted their differentiation pathway into the osteoblast lineage. There are currently limited data on the effects of BMP-7 on muscle cell differentiation, although BMP-2 and BMP-7 are both members of the TGF-β superfamily of proteins. Efforts are underway in our laboratory to create homogeneous cultures and subcultures of muscle tissue-derived cells, in order to identify the precise cell type that is responding to the stimulation of BMP-7, as well as the specific action of BMP-7 with respect to muscle cell proliferation and differentiation into the osteoblastic lineage.

Conclusions

The results of this study demonstrate the efficacy of a BMP-polymer matrix in inducing the expression of the osteoblastic phenotype by muscle-derived cells. The bone morphogenetic protein incorporated in a polymeric matrix remained bioactive and induced the differentiation of rabbit muscle explant cells into osteoblast-like cells. PLAGA-BMP matrices supported the adhesion and differentiation of rabbit muscle explant cells into osteoblast-like cells, and the cultured cells formed mineralized matrices and synthesized osteocalcin. Under controlled culturing conditions, we have found these cells to be a potentially reliable cell source for bone tissue engineering. The use of tissue derived from muscle presents a new paradigm for bone tissue engineering.

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