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Polymer-Ceramic Composite Scaffold Induces Osteogenic Differentiation of Human Mesenchymal Stem Cells

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Abstract—**One of the design goals of the ideal tissue-engineered bone graft is ostoinductivity, the ability to induce the osteogenic differentiation of mesenchymal stem cells and progenitor cells. In this study, we evaluated the osteoinductive potential of a polymer-ceramic composite in vitro. This composite has been shown to be biodegradable, osteoconductive, and osteointegrative in previous studies. It is hypothesized that this composite will enhance osteoblastic differentiation in human mesenchymal stem cells (hMSCs), and that this inductive potential is substrate-dependent. Human MSCs were cultured on PLGA-BG composite scaffolds and their growth and differentiation were assessed over a four-week period. Composite scaffolds of PLGA and hydroxyapatite (HA), and hMSC cultures treated with osteogenic medium served as controls. It was found that hMSCs grown on PLGA-BG composite scaffolds expressed osteogenic markers without osteogenic media stimulation. In addition, alkaline phosphatase (ALP) activity peaked significantly earlier on the PLGA-BG composite compared to that on the PLGA scaffolds. The findings of this study collectively demonstrate the osteoinductivity of the PLGA-BG composite and its potential as a bone tissue engineering scaffold.**

I. Introduction

BONE is the most commonly replaced organ of the body [1]; of the 1-2 million fractures treated each year in the United States, over 275,000 require bone grafting [2]. Due to limitations associated with allografts and xenografts, autografts have been the clinical gold standard for bone grafting. However, autografts are limited in supply and tissue harvesting often leads to donor site morbidity. Therefore, there is significant interest in a tissue-engineered substitute for autografts. The design criteria for an ideal bone graft are as follows: the substrate should be biocompatible and biodegradable, it must exhibit similar mechanical properties as bone tissue, be osteoconductive and osteoinductive to induce and support new bone formation, osteointegrative to provide a biological fixation of scaffold to bone, and angiogenic in order to extend long term functionality of the graft [3]-[6].

Material selection is critical for bone tissue engineering; thus far no single biomaterial is able to meet all of the criteria for an ideal bone graft. We have elected to focus on polymer–ceramic composites, which can be designed to exhibit a higher number of these properties than any single-material-based scaffolding system. Specifically, we have investigated a polylactide-*co*-glycolide (PLGA) and 45S5 bioactive glass (BG) composite which combines the advantages of the parent phases while minimizing their respective disadvantages. The PLGA phase of the composite is biodegradable and has been FDA-approved for biomedical applications, while the BG phase of the composite has long been recognized as the most osteointegrative material available [17]. This composite has been shown in our previous studies to be ostoconductive and osteointegrative, and possesses increased mechanical strength and structural integrity compared to polymer-only controls [7]-[15]. The PLGA-BG composite scaffold is designed to maintain physiological pH through neutralization of acidic and basic degradation products of each phase.

We have shown previously that the PLGA-BG composite supported the growth and matrix production of human osteoblast-like cells [18] and enhanced their production of type I collagen [9]. As both osteoblasts and stem cells are important in the bone healing process [16], the objective of this study is to evaluate the osteoinductive potential of PLGA-BG composites *in vitro*. Specifically, adult human mesenchymal stem cells (hMSCs) were cultured on PLGA-BG scaffolds and cell growth and differentiation were determined over time. Scaffolds of PLGA and hydroxyapatite (HA) as well as hMSC cultures with or without osteogenic medium served as controls. It is hypothesized that the PLGA-BG composites will support the growth and osteogenic differentiation of hMSCs.

II. Materials and Methods

A. Scaffold Fabrication

The composite microspheres were fabricated via a water-oil-water emulsion method [9]. Briefly, particulates of 45S5 bioactive glass (BG, 20wt%) or hydroxyapatite (HA, 20 wt%) were suspended in a polylactide-*co*-glycolide 85:15 (PLGA) solution. This suspension was mixed in 1% poly vinyl alcohol to form microspheres. The composites (PLGA-BG, PLGA-HA) were fabricated by sintering the

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microspheres past the polymer Tg for 6 hours. The resulting cylindrical scaffolds were 2.17 ± 0.35 mm in height and 6.33 ± 0.09 mm in diameter. The scaffolds were sterilized by ethanol and UV irradiation prior to cell seeding.

B. Cell Culture and Scaffold Seeding

Human mesenchymal stem cells (hMSCs, Cambrex, Walkersville, MD) were expanded in fully-supplemented Dulbecco's Modified Eagle's Medium (F/S DMEM; 10% FBS, 1%PS, 1%NEAA), and seeded on each substrate at a density of 3100 cells/cm^2. Specifically, the hMSCs were cultured on four substrate groups: PLGA-BG, PLGA-HA, PLGA, and tissue culture polystyrene (TCP). One half of the samples were cultured in control media (F/S DMEM) while the other half were treated with osteogenic media. The osteogenic media [19] consisted of F/S DMEM supplemented with 50μg/mL ascorbic acid, 0.1μM dexamethasone, and 10mM β-glycerophosphate. The samples were maintained at 37°C and 5% CO_2, and medium was changed regularly. The samples were harvested at 1, 7, 14, 21, and 28 days, and cell viability, growth and differentiation were assessed.

C. Cell morphology and Viability

Cell viability was assayed using the LIVE/DEAD® Kit (Invitrogen) following the manufacturer's suggested protocol. The samples were rinsed twice with phosphate buffered saline (PBS) and then incubated for 30 minutes in the calcein AM/ethidium homodimer-1 solution. After a PBS wash, the samples were imaged by fluorescent microscopy.

D. Cell Proliferation

Cell proliferation was determined by measuring total DNA per scaffold using the Picogreen® dsDNA assay (Molecular Probes, Eugene, OR) following the manufacturer's protocol. The samples (n=6) were harvested with 0.1% Triton X and stored at -20°C until the day of the assay.

E. ALP Activity

Sample ALP activity (n=6) was quantified by an enzymatic assay, based on the hydrolysis of p-nitrophenyl phosphate (pNP-PO_4) to p-nitrophenyl (pNP) [20]. The cell lysate was mixed with pNP-PO_4 for 20 min at 37°C. The reaction was terminated by 0.1 N NaOH. The absorbance was measured at 405nm, and a pNP standard curve was generated.

F. Gene Expression

Gene expression of osteocalcin, osteopontin, and osteonectin was measured by RT-PCR. Total RNA was isolated using the Trizol® (Invitrogen) extraction method. The isolated RNA was reverse transcribed into cDNA using the SuperScriptTM First-Strand Synthesis System (Invitrogen). All genes were amplified for 40 cycles in a thermal cycler (iCycler, Bio-Rad, Hercules CA).

G. Statistical Analysis

Data in the graphs are presented as mean±standard deviation, with *n* equal to the number of samples. In the case of multiple comparisons, one-way and two-way analyses of variance (ANOVA) were performed (JMP IN®, SAS Institute, Cary, NC), and the Tukey-Kramer HSD test was used to compare between the means. Significance was attained at $p<0.05$.

III. Results

A. Cell Proliferation and Morphology

The hMSCs attached to and proliferated on all substrates tested (Fig. 1). Visually, more cells were observed on PLGA as compared to PLGA-BG scaffolds. After 7 days, all cells grown in osteogenic media exhibited cuboidal morphology similar to those of osteoblast-like cells. Cells grown in control media on both PLGA and TCP maintained a spindle-like morphology found typically in undifferentiated hMSCs. Morphology of the cells grown on the PLGA-BG scaffolds resembled those of cells in osteogenic media after 7 days.

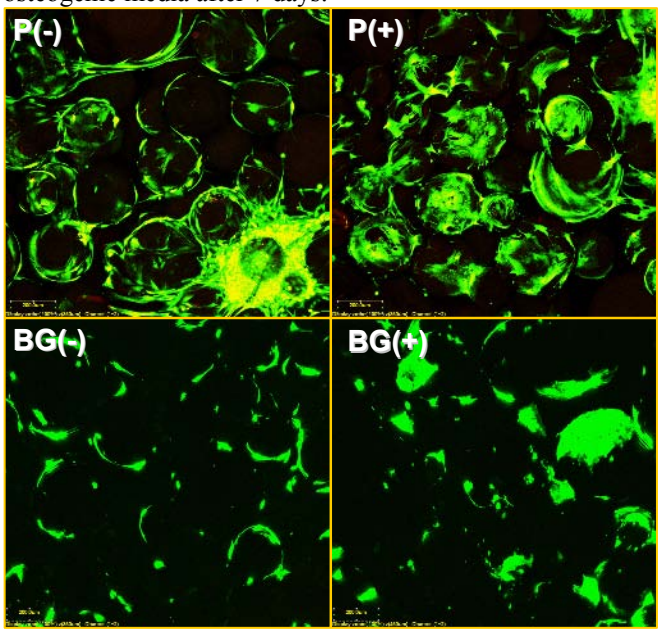

Fig. 1. Viability of hMSCs on PLGA and PLGA-BG scaffolds on Day 21,10x. The samples were cultured in control media [P(-), BG(-)] and in osteogenic media [P(+), BG(+)]. The cells were viable on all substrates tested.

Cell proliferation was measured over time, and groups cultured in control media generally had more cells compared to groups cultured in osteogenic media (Fig. 2). The samples grown on PLGA scaffolds and in control media measured the highest cell number throughout the experiment compared to all other groups. On day 14, PLGA scaffolds cultured in control media had significantly higher cell number compared to PLGA-BG groups ($p<0.05$). On day 21, significantly higher cell number was found on PLGA scaffolds compared to PLGA-BG, when both scaffold types were cultured in osteogenic media. At day 28, PLGA-BG

plus osteogenic media had significantly fewer cells compared to all other groups.

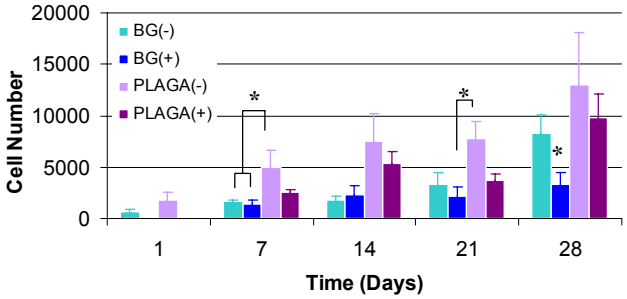

Fig. 2. Cell proliferation on PLGA and PLGA-BG scaffolds in control (-) and osteogenic (+) media. The hMSCs attached to and proliferated on all scaffolds. On day 14, the cell number on PLGA scaffolds in control media was significantly greater than the number of cell found on the PLGA-BG scaffolds (*:p<0.05).

B. ALP Activity

As shown in Fig. 3, the ALP activity on the PLGA scaffolds in control media remained relatively low and constant throughout the experiment, which was similar to that of TCP controls. For the PLGA scaffolds treated with osteogenic media, ALP activity peaked at day 21, which is also similar to that found in the treated TCP monolayer. The highest ALP activity on the PLGA-BG scaffolds cultured in control media was measured at day 7, and decreased thereafter. For PLGA-BG scaffolds treated with osteogenic media, ALP activity level was comparable to that of the untreated PLGA-BG, and remained relatively constant throughout the 4 weeks. A significant difference in ALP activity was found between the treated and untreated PLGA-BG scaffolds only at day 28. In the absence of osteogenic media, the ALP activity on the PLGA-BG scaffold was consistently higher compared to the untreated PLGA group during the first two weeks of culture. Afterwards, the ALP activity on the treated PLGA scaffold became significantly higher when compared to the PLGA control.

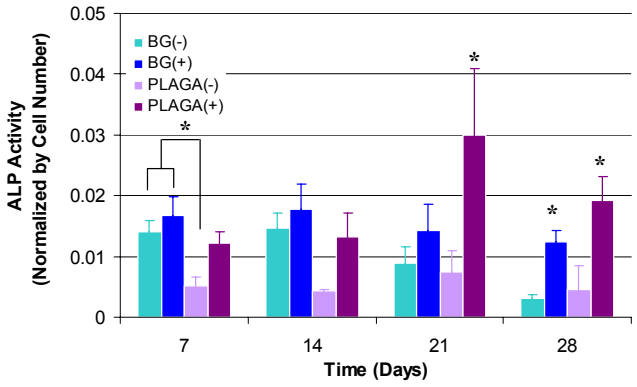

Fig. 3. Normalized ALP activity for hMSCs cultured on PLGA and PLGA-BG scaffolds in control (-) and osteogenic (+) media, n=5, p<0.05.

C. Gene Expression

The expression of osteonectin was found on the TCP control group, although this was not seen on cells cultured on the PLGA scaffolds. Cells cultured on the untreated PLGA-BG scaffolds expressed osteonectin, osteopontin and osteocalcin, which are consistent with osteogenic differentiation by hMSCs in the absence of osteogenic media (Fig. 4). Treatment with osteogenic media resulted in higher levels of osteonectin and osteocalcin expression on the PLGA scaffolds, while osteogenic media had no effect on gene expression levels on PLGA-BG substrates.

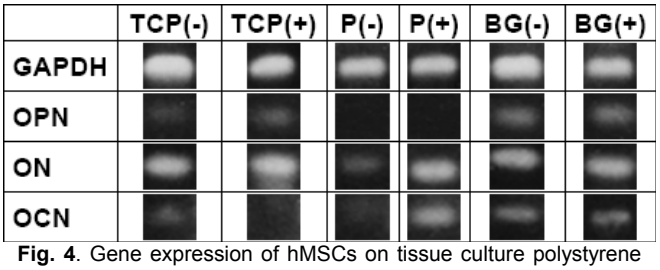

Fig. 4. Gene expression of hMSCs on tissue culture polystyrene (TCP), PLGA (P), PLGA-BG (BG) in control (-) and osteogenic (+) media. The mRNA expression for osteopontin (OPN), osteonectin (ON), and osteocalcin (OCN) were assayed on Day 7.

IV. Discussion

Our results suggest that substrate composition plays a significant role in modulating the osteogenic differentiation of hMSCs. It was found that hMSCs grown on PLGA-BG scaffolds exhibited greater osteogenic potential than those found on the polymer-only scaffolds. Observations of cell morphology, ALP activity and gene expression suggest that osteoblastic differentiation may have occurred within the first two weeks of culture on PLGA-BG scaffolds. Moreover, this response of hMSCs on PLGA-BG was independent of stimulation with osteogenic media, suggesting that the observed osteogenic responses were mediated by the scaffold alone.

Several reported studies have examined the effect of 45S5 bioactive glass on osteoblasts and mesenchymal stem cells [21]-[24]. Pajamaki *et al.* reported the bioactive glass implants accelerated new bone induction by demineralized bone matrix [25]. Yuan *et al.* found that 45S5 bioactive glass is osteoinductive in soft tissue [26], and Gatti *et al.* have shown BG to be osteoinductive in both soft and hard tissue [27]. Various reported studies have also shown that BG can enhance osteoinduction of progenitor cells *in vitro* under defined conditions [28]-[30].

The mechanism underlying the osteoinductive potential of polymer-ceramic composites is not well-understood. It is possible that changes in specific ion concentrations during the initial phase of BG surface transformation regulate hMSC metabolism and eventual differentiation. The role of released ions such as Si and Ca in modulating the bioactivity of 45S5 glass and osteoblast maturation has been reported [27], [31]-[34]. Kubo *et al.* showed that the addition of Ca, Si, and vitamin D to fibroblasts cultures induced the formation of mineralized nodules [31]. Hench *et al.* suggested that the presence of critical concentrations of Si and Ca ions may be responsible for osteoblast maturation [32]. Also, Si has been reported as an essential ion for bone formation during development [33]. It is likely the increased Si and Ca ion concentrations due to dissolution of BG are significant inducers of the osteogenic differentiation

of hMSCs observed here and this will be investigated in future studies.

V. Conclusion

In this study, it was found that hMSCs grown on PLGA-BG composite scaffolds expressed osteogenic markers without the stimulation of osteogenic media. Also, ALP activity peaked significantly earlier on the PLGA-BG composite than on the PLGA scaffolds. These results collectively suggest that PLGA-BG is osteoinductive compared to polymer-only scaffolds. Based on its documented osteointegrative, osteoconductive and osteoinductive properties, the PLGA-BG composite scaffold is a promising grafting systems for bone tissue engineering.

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