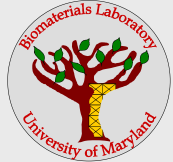
Scaffold Facilitated Osteoblastic Differentiation for Orbital Bone Repair

Sang W. An[1], Andrew D. Thompson[2], Martha W. Betz[3],
John F. Caccamese[4], Domenick P. Coletti[4], John J. Sauk[5], and John P. Fisher[1,3]

[1]Department of Chemical and Biomolecular Engineering, [2]Department of Cell Biology and Molecular Genetics,
[3]Bioengineering Graduate Program University of Maryland, College Park, MD
[4]Department of Oral and Maxillofacial Surgery, [5]Department of Pathology, University of Maryland Dental School, Baltimore, MD



ABSTRACT

Orbital floor injuries are a devastating form of craniofacial trauma. Current clinical treatments, including implantation of plastics or metals, allografts, and autografts are often inadequate due to the loss of function as well as poor aesthetics. These concerns have led our laboratory to investigate a novel tissue engineering approach for the treatment of orbital bone defects. Engineered bone grafts are often fabricated by encapsulating osteoprogenitor cells within a hydrogel scaffold. However, recent studies have found that by engineering a scaffold to mimic the natural cellular environment, osteoblastic differentiation may be promoted.

This work investigates the effects of two extracellular matrix molecules, fibronectin and hyaluronic acid, on osteogenic differentiation. Osteoprogenitor cells were isolated from bone marrow of young rats. After preculture, osteoprogenitor cells were encapsulated in alginate beads with varying concentrations of hyaluronic acid and fibronectin included as interpenetrating molecules. These constructs were cultured for 1, 4, and 8 days. At the defined time points, total RNA was isolated from the encapsulated cells. The RNA was reverse transcribed to cDNA and real time polymerase chain reaction was performed. The two genes of interest were bone morphogenetic protein-2, a known osteogenic signaling molecule, and osteocalcin, a late osteoblastic marker. These genes were chosen for their relationship to osteodifferentiation. In this experiment BMP-2 was chosen to represent increased cellular signaling as a result of augmenting the environment with extracellular matrix molecules. Then to determine if this increased signaling affected osteodifferentiation, a late marker for differentiation, osteocalcin, was used.

Bone morphogenetic protein-2 data show an increasing trend in the level of expression between the control and experimental groups on days 1 and 8. Osteocalcin data show little or no increase in gene expression between days 1 and 4, but show an increase of expression in the hyaluronic acid and fibronectin groups compared to the negative control on day 8. This experimental data indicates that extracellular matrix molecules can be used to modify cellular signaling and subsequently osteodifferentiation.

ORBITAL BONE

Craniofacial injury is a form of facial trauma and can significantly diminish a person's quality of life. One of the most common injuries is orbital fractures. Forceful impact to the skull can cause fractures along the weak point of the orbit, especially at the floor where it is thinnest. Unfortunately the body's response to orbital fractures is not adequate to reform bone with proper function and appearance. The orbital floor is opposed by the maxillary and ethmoid sinuses which are air filled and therefore there is no vascularized tissue to support regeneration of large defects. Furthermore, when the orbital floor is broken it generally shatters and does not contain sufficient bony edges to support normal primary and secondary healing. Instead, a fibrous scar forms that lacks the load bearing properties of bone. Orbital floor fractures therefore present an ideal situation for using a tissue engineered bone graft. We propose inserting a scaffold specifically designed to promote osteodifferentiation that contains an osteoprogenitor cell population as shown in Figure 1.

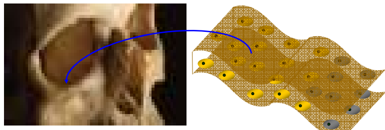


Figure 1: Diagram showing insertion of a scaffold containing an osteoprogenitor cell population

OBJECTIVE

Determine the effect of hyaluronic acid and fibronectin on osteoprogenitor cell diffentiation through the expression of bone morphogenetic protein-2 and osteocalcin by quantitative reverse transcription-polymerase chain reaction.

METHODS

Cell Harvest and Culture

Osteoprogenitor cells were aseptically obtained from young male rats of the Wistar Hannover GALAS strain (Taconic, Germantown, NY) weighing 100-125 g. The femurs and tibias were isolated, cleaned of soft tissue and passed rinsed in three media washes containing 10% penicillin/streptomycin for 10 minutes. The bones were clipped at the epiphyseal plates and the marrow flushed out using 10 mL of media expelled from a 10 mL syringe with an 18 gauge needle. The cell solution was passed through a 70 µm strainer and centrifuged at 300g for 8 minutes. The pellet was resuspended in 10 mL culture media and plated onto a T-75 flask. Cells were precultured through 2 passages with media changes every 2-3 days.

Culture Media

Standard cell culture media consisting of α -minimal essential medium (α -MEM) (Gibco, Carlsbad, CA) was used. We supplemented the α-MEM with 10% (v/v) fetal bovine serun (Gibco), 0.2mM ascorbic acid (Sigma, St. Louis, MO), and 1% (v/v) penicillin/streptomycin antibiotics (Gibco). This was designated as the control media. For a positive control, we further supplemented the control media with 10 mM Na-β-glycerophosphate (Sigma) and 10^{-8} M dexamethasone (Sigma). This was designated as osteogenic media.

METHODS

Bead Formation

These experiments were performed in alginate beads, a known naturally derived water-soluble polysaccharide. In addition, alginate lacks cellular recognition proteins and therefore the encapsulated cell populations have limited attachment to the polymer. After preculture, the cells were lifted with trypsin, counted and suspended in 1.2% w/v alginate solution and dropped from an 18 gauge needle into a 50 mM $CaCl_2$ solution, forming spherical beads. The cell density of the beads were approximately 50,000 cells per bead (see Figure 2). The first experimental group was modified with 1 mg hyaluronic acid per 1 mL alginate solution. The second experimental group was modified with 20 µg of fibronectin per 1 mL alginate solution. The positive and negative control groups were unmodified. The beads were cultured in a 12-well plate, each well receiving 10 beads for a total of 500,000 cells per well. All experiments were performed in triplicate.

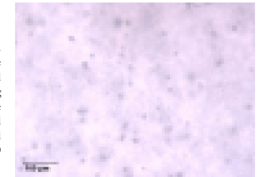


Figure 2: Osteoprogenitor cells encapsulated within an alignate bead.

Cell Recovery and RNA Isolation

The study was performed over an 8 day period. At days 1, 4, and 8, cells were recovered from the beads and their RNA extracted. Cells were recovered by disrupting the alginate network with 1.25 mL of 50 mM ethylenediamine tetraacetic acid (EDTA, Sigma) and 0.25 mL of Trypsin per well. The cell solution was centrifuged down to a pellet and washed with phosphate buffered saline (PBS, Gibco). The PBS cell solution was again centrifuged down to a pellet and the RNA was isolated using an Rneasy mini kit (Qiagen, Valencia, CA).

QUANTITATIVE REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

Quantitative reverse transcriptase- polymerase chain reaction (qRT-PCR) is an *in vitro* method to quantify gene expression. First the isolated total RNA was reverse transcribed using High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The PCR was performed using specific primers and probes designed by our laboratory from the published sequences of the gene of interest (see Table 1). We analyzed bone morphogenetic protein-2 and osteocalcin, an osteogenic signaling molecule and late osteoblastic marker, respectively. The genes were cloned using Taqman chemistry.

Protein	Segment	Sequence
BMP-2	Forward Primer	TGCCCCCTAGTGCTTCTTAGAC
	Reverse Primer	CCCGGCCACCATGGT
	Probe	ACTGCGGTCTCCTAAA
Osteocalcin	Forward Primer	GGCTTCCAGGACGCCTACA
	Reverse Primer	GGGCAACACATGCCCTAAAC
	Probe	CGCATCTATGGCACCAC

Table 1: Primer and probe sequences used for osteocalcin and BMP-2 analysis

The relative standard curve method was used to analyze the relative quanitation of BMP-2 and osteocalcin expression. In order to standardize the amount of mRNA for each experiment 18s was used (the sequences are proprietary). The gene expression of BMP-2 and osteocalcin was normalized to 18s and was calibrated to one sample to obtain gene fold expression.

RESULTS

BMP-2 Expression

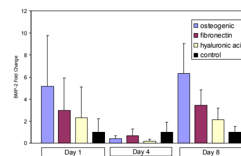


FIGURE 3: BMP-2 expression on days 1, 4 and 8 of the negative control group, positive control group in osteogenic media and the two experimental groups supplemented with fibronectin and hyaluronic acid as measured by RT-PCR. There is an slight increasing trend of expression on day 1 from the control group to the two experimental groups and the positive control. Day 4 does not show any significant differences. Day 8 shows a trend similar to Day 1. The mean and standard deviations for all groups are reported (n=3-9).

Osteocalcin Expression

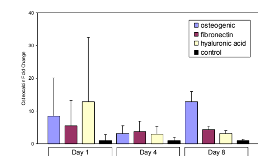


FIGURE 4: Osteocalcin expression on days 1, 4 and 8 of the negative control group, positive control group in osteogenic media and the two experimental groups supplemented with fibronectin and hyaluronic acid as measured by RT-PCR. Days 1 and 4 do not show statistically significant differences between groups. Day 8 shows a significant increasing trend of the fibronectin and hyaluronic acid groups expression as compared to the negative control, but not as high as the osteogenic control. The mean and standard deviations for all groups are reported (n=3-9).

SUMMARY

BMP-2 expression was measured on days 1, 4 and 8 for all groups. Days 1 and 8 showed a slight increasing trend of the hyaluronic acid and fibronectin supplemented groups over the negative control. This increase in BMP-2, a signaling molecule for osteodifferentiation, increased differentiation of osteoprogenitor cells. This increase of differentiation was measured by osteocalcin, and known late marker of osteodifferentiation. This increase is statistically present in the day 8 data showing higher levels of expression in the two experimental groups as compared to the negative control.

ACKNOWLEDGEMENTS

This work was supported by University of Maryland, College Park, MD., the Minta Martin Foundation, and the National Science Foundation.