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Ultrasound effect on osteoblast precursor cells in trabecular calcium phosphate scaffolds

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Abstract

This study investigated the *in vitro* effect of low-intensity pulsed ultrasound (LIPUS) on human embryonic palatal mesenchyme cells (HEPM, CRL-1486, ATCC, Manassas, VA), an osteoblast precursor cell line, during early adhesion to calcium phosphate scaffolds. Hydroxyapatite (HA) and β-tricalcium phosphate (TCP) ceramic scaffolds were produced by a template coating method. Phospho-specific antibody cell-based ELISA (PACE) technique was utilized on stress activation proteins, including the extracellular signal-regulated kinase (ERK1/2), P38, c-Jun N-terminal kinase (JNK) and the anti-apoptosis mediator protein kinase B (PKB/AKT). Cell-based ELISAs were also performed on the membrane anchoring protein vinculin and $\alpha 6\beta 4$ integrin. LIPUS stimulated activation of PERK 1/2, PJNK, PP38 and vinculin in traditional two-dimensional (2-D) culture. Calcium release from the scaffolds was partially involved in the activation of PERK 1/2 when cell response was compared between culture on 2-D surfaces and three-dimensional (3-D) HA and TCP scaffolds. Effects of calcium extracted media from scaffolds alone could not account for the full activation of PJNK, PP38, PAKT, vinculin and $\alpha 6\beta 4$ integrin. LIPUS stimulation further increased PERK activity on TCP scaffolds corresponding with an increase in both vinculin and $\alpha 6\beta 4$ integrin levels. It was concluded from this study that LIPUS treatment can significantly affect stress signaling mediators and adhesion proteins in osteoblast precursor cells during the early cell-attachment phase to trabecular patterned scaffolds.

Keywords: Scaffold; Ultrasound; Calcium phosphate; Osteoblast precursor cells; Cell signaling

1. Introduction

Therapeutic ultrasound has proven to be a valuable tool for the treatment of pathological and trauma fractures particularly with the development of low-intensity pulsed ultrasound (LIPUS) [1]. Ultrasound has been defined as a pressure or sound wave with the ability to transfer mechanical energy into biological tissues [2]. This acoustic energy has demonstrated improved fracture healing with *in vivo* studies [3,4] and in controlled clinical trials [5]. However, identification of the cellular signals stimulated by ultrasound still remains to be fully understood especially with respect to three-dimensional (3-D) environments.

Bone cells react to mechanical forces by mechanotransduction of biological signals linking environmental forces with genetic regulation and cellular adaptation [6]. At the cell surface, integrins mediate these events through tyrosine phosphorylation of signaling proteins [7] forming focal adhesions. Inside the cell, these focal points recruit a variety of structural proteins such as vinculin connecting integrins with signaling pathways such as the mitogen-activated protein kinase (MAPK) cascade [8,9]. The MAPK pathway also has a role during the transition of mesenchyme stem cells into the osteogenic lineage [10]. Within this family are three sub-pathways: the extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal

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kinases (JNK) and P38 kinases [11], the latter two termed the stress-activated protein kinases (SAPKs). Cell stress and survival signaling also involves a mediator termed AKT/PKB, protein kinase B. AKT regulates an assorted set of cell functions including survival, glycogen synthesis, glucose transport [12] and can inhibit apoptosis [13,14].

Ultimately, investigations of cell signaling behavior have clinical application, and in the case of bone repair, regeneration is often assisted by scaffold grafts. Identification of signaling mechanisms can be profoundly affected by 3-D culture with surface area and shape influencing cell stress responses and differentiation [15,16]. Ceramic scaffolds have provided an excellent platform for bone regeneration in recent studies [17–23]. Scaffolds prepared from calcium phosphate (CP) ceramics permit a stable platform for cell adhesion, migration and proliferation [24] with surface properties very similar to natural bone apatite. In addition to exhibiting negligible immunoreactivity, these materials induce direct binding to the cell-collagen matrix creating a strong mechanical interlock between bone and implant [25]. Scaffolds offer a reproducible stage for the identification of specific biological pathways as well as their use in regenerative medicine [26,27]. Careful attention to material and architectural properties permits investigation of cell signaling patterns in an environment analogous to natural tissue.

In the present study, the effect of LIPUS treatment on osteoblast precursor cell signaling and adhesion behavior was explored in 3-D culture on hydroxyapatite (HA) and β-tricalcium phosphate (TCP) scaffolds. The identification of both environment and ultrasound induced changes in MAPK and AKT activation as well as the membrane-associated proteins vinculin and $\alpha 6\beta 4$ integrin was performed using *in vitro* culture of human embryonic palatal mesenchyme cells (HEPM, CRL-1486, ATCC, Manassas, VA) on HA and TCP trabecular patterned scaffolds.

2. Materials and methods

2.1. Sample preparation

Scaffolds were prepared from microparticle HA and TCP (TAL Materials, Ann Arbor, MI) as previously reported using a template coating technique [15]. Briefly, polyurethane sponges (EN Murray, Denver, CO) were coated with HA or TCP distilled water-based slurry containing 1% v/v ammonium polyacrylate dispersant and 3% v/v *N*,*N*-dimethylformamide drying agent during initial mixing and overnight vacuum drying. Three percent high molecular weight polyvinyl alcohol and 1% v/v carboxymethylcellulose binders were added to provide specific temperature burn out stages during sintering. Scaffolds were twice coated with CP slurry and heat-sintered to 1230 °C for 3 h. Purity of the composition was validated using X-ray diffraction analysis. Scaffold dimensions for all *in vitro* studies were diameter and length of 5 mm. Samples were placed into non-binding 96-well plates (Corning, Acton, MA) and ethylene oxide gas sterilized before testing.

2.2. Sample characterization

CP scaffolds were observed by scanning electron microscopy. Calcium release profiles were generated by time immersion into 250 μL phosphate-buffered saline (PBS) at 37 °C, 5% CO_2 by calcium reduction to a colored product. A 10 μL volume of saline was removed and added to 240 μL of calcium reagent based on the 2-cresolphthalein complexone method (Raichem Hemagen, Columbia, MD). Determinations were obtained from a standard curve generated using a calcium standard with absorption measurements made at 550 nm on a Beckman Coulter AD34C plate reader (Fullerton, CA). An initial release rate was identified by measuring calcium every 3 min up to 15 min with direct media replacement.

2.3. HEPM cell culture

HEPM cells (CRL-1486, ATCC, Manassas, VA), an osteoblast precursor cell line, were cultured in Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA) supplemented with 7% fetal bovine serum (Invitrogen) and 1% antibiotic/antimycotic (PSA, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B; MP Biomedicals, Solon, OH) at 37 °C with 5% CO_2. Cells were maintained in culture flasks with media changes every 2 days and passaged at confluence using trypsin (Invitrogen). Cell passages 10–12 were used in the course of this experiment. HEPM cells were counted using a coulter counter (Beckman Coulter Z2, Fullerton, CA), and 20,000 cells were seeded onto tissue culture treated plates or CP scaffolds. Calcium medium extracts were prepared by incubating scaffolds in culture media for 6 h with an identical volume to that used for cell studies. Extracts were removed from scaffolds ($n = 6$) and used to resuspend cells at the same start time used for direct cell-scaffold studies. Early attachment time was identified from a previously reported study with a time point of 6 h identified where cells equally attached to HA and TCP materials with greater than 90% cell binding.

2.4. Stimulation with ultrasound

To stimulate cells on tissue culture plastic and scaffolds the Exogen LIPUS system was utilized (Smith & Nephew, Memphis, TN). This system is configured with six independent ultrasound transducers that each produce a 1.5-Mhz ultrasound wave, 200-μs pulse modulated at 1 kHz using an output intensity of 30 mW/cm^2. Ultrasound transducers were fitted on a plastic frame to stimulate specific regions of a 96-well plate where samples were located. Coupling gel was placed between the transducer and culture plates. Untreated controls were maintained in identical conditions to ultrasound stimulated plates. Following a 6-h cell attachment time, ultrasound groups were stimulated for 20 min and given a 30-min recovery time before test completion.

2.5. Phospho-specific antibody cell-based ELISA (PACE)

Cells were attached to all surfaces and scaffolds for 6 h and 50 min followed by washing twice with ice-cold PBS. Fixation was performed with 4% Carson's Millonig formaldehyde in PBS for 30 min at room temperature and endogenous peroxidase activity was quenched using 0.3% H_2O_2 in PBS-0.1% Triton X-100 (PBS-T) for 30 min. PACE testing was performed as previously reported [15]. Briefly, cells and scaffolds were washed three times with PBS-T, followed by blocking with 10% fetal calf serum (FCS, Invitrogen) in PBS-T for 1 h. Primary rabbit antibodies against phospho-p44/42 (ERK1/2, Thr202/Tyr204), phospho-P38 (Thr180/Tyr182), phospho-SAPK/JNK (Thr183/Tyr185) and phospho-AKT (Ser 473, Cell Signaling Technology, Danvers, MA) were added in 5% FCS-PBST and incubated overnight at 4 °C with 20 r.p.m. rocking. Dilutions of primary antibodies were set at 1:1000 except for PP38, at 1:500. As reported previously, signal inhibitors for PERK1/2, PP38, PJNK and PAKT included PD98059 (50 μM), SB203580 (50 μM), SP600125 (10 μM) and Wortmannin (2 μM), respectively, were used to