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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 ELISA's were also performed on the membrane anchoring protein vinculin and α 6 β 4 integrin. LIPUS stimulated activation of ERK 1/2, JNK, P38 and vinculin in traditional 2 dimension (2-D) culture. Calcium release from the scaffolds was partially involved in the activation of ERK 1/2 when cell response was compared between culture on 2-D surfaces and 3 dimension (3-D) HA and TCP scaffolds. Effects of calcium extracted media from scaffolds alone could not account for the full activation of JNK, P38, AKT, vinculin and α 6 β 4 integrin. LIPUS stimulation further increased ERK activity on TCP scaffolds corresponding with an increase in both vinculin and α 6 β 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.

1. Introduction

Therapeutic ultrasound has proven 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 3 dimensional (3-D) environments.

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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 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 HA and 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 micro particle 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. 3% 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 hours. 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

Calcium phosphate scaffolds were observed by scanning electron microscopy. Calcium release profiles were generated by time immersion into 250 μ L phosphate buffered saline (PBS) at 37 °C, 5% CO₂ by calcium reduction to a colored product. A 10 μ L volume of saline was removed

and added to 240 μ L 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 550nm on a Beckman Coulter AD34C plate reader (Fullerton, CA). An initial release rate was identified by measuring calcium every 3 minutes up to 15 minutes with direct media replacement.

2.3. HEPM cell culture

Human embryonic palatal mesenchyme cells (HEPM, CRL-1486, ATCC, Manassas, VA), an osteoblast precursor cell line, were cultured in Dulbecco's modified eagle medium (DMEM, Invitrogen, Carlsbad, CA) supplemented with 7% fetal bovine serum (FBS, Invitrogen) and 1% antibiotic/antimycotic (PSA, 100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B, MP Biomedicals, Solon, OH) at 37 °C with 5% CO₂. Cells were maintained in culture flasks with media changes every two 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 hours 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 hours 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 low intensity pulsed ultrasound (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- μ s pulse modulated at 1 kHz using an output intensity of 30mW/cm². 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 hour cell attachment time, ultrasound groups were stimulated for 20 minutes and given a 30 minute recovery time before test completion.

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

Cells were attached to all surfaces and scaffolds for 6 hours and 50 minutes followed by washing twice with ice-cold PBS. Fixation was performed with 4% Carson's Millonig formaldehyde in PBS for 30 minutes at room temperature and endogenous peroxidase activity was quenched using 0.3% H₂O₂ in PBS - 0.1% Triton X-100 (PBS-T) for 30 minutes. 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 hour. 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-PBS-T and incubated overnight at 4 °C with 20 rpm rocking. Dilutions of primary antibodies were set at 1:1000 except for P38, at 1:500. As reported previously signal inhibitors for ERK1/2, P38, JNK, and AKT included PD98059 (50 μ M), SB203580 (50 μ M), SP600125 (10 μ M), Wortmannin (2 μ M), respectively, were used to check validity of signal generation and were added 30 minutes before test completion. Following primary antibody incubation, cells were washed three times with PBS-T for 5 minutes and incubated with secondary anti-rabbit IgG, HRP-linked antibody (horseradish peroxidase) with a 1:1000 dilution in 5% FCS-PBS-T for 1 hour at room temperature with rocking. The cells and scaffolds were then washed

three times with PBS-T and twice with PBS for five minutes. One-Step Ultra TMB substrate (Pierce Biotechnology, Rockford, IL) was added to the wells and color developed for 30 minutes at room temperature in the dark. The oxidation reaction was stopped and color changed and stabilized using an equal volume of 2M H₂SO₄. Volumes of 100µl of the colored product were transferred to new 96 well plates and absorbance measured at 450 nm with reference at 620 nm on a plate reader.

2.6. Adhesion protein immuno-detection

Similar to PACE testing, cells and scaffolds were prepared for immuno-detection of membrane proteins using mouse primary antibodies against vinculin (Sigma, St Louis, MO) and $\alpha_6\beta_4$ integrin (Chemicon Int, Inc., Temecula, CA). Primary antibodies were added in 5% FCS-PBS-T and incubated over night at 4°C with 20 rpm rocking. Dilutions of primary antibodies were set at 1:1000 for vinculin and $\alpha_6\beta_4$ integrin and were established from antibody titer-controls with maximum signal to noise ratio using cells cultured on tissue culture treated plastic. Secondary anti-mouse IgG HRP linked antibody was used with the One-Step Ultra TMB substrate to generate color for detection.

2.7. Statistical analysis

Statistical calculations were performed with SigmaStat software (Systat, Point Richmond, CA). Validity of signal inhibition with each material, and ultrasound stimulations on culture plastic were compared by paired t-test. Significance between media and scaffold groups were analyzed by two-way ANOVA using material and condition as factors with Tukey multiple comparison procedure. Significance levels were set at $p < 0.05$.

3. Results

3.1. Material characterization

Calcium phosphate surfaces and scaffolds imaged by SEM are shown in Fig. 1(a-d) demonstrating the micro structure of HA, Fig. 1a, and TCP, Fig. 1b surfaces and the overall architecture and rounded triangular strut configuration of the scaffolds, Fig. 1(c, d). No significant morphological difference was identified between HA and TCP materials in terms of bulk design, however, micro and nano scale differences do exist between these materials as previously reported [15], with HA crystals significantly smaller than TCP creating a greater number of grain boundaries available for calcium release. Additionally, TCP surfaces also contained small micro-pores. Overall scaffold arrangement was designed with complete channel interconnections throughout the scaffold design.

Calcium release behavior from HA and TCP scaffolds was characterized over 2 days with attention to the initial release rate from the scaffolds. As shown in Fig. 2, HA scaffolds initially released a greater amount of calcium into PBS at physiological conditions. The initial release rates were 1.22 and 0.64 µg calcium per mg scaffold per mL PBS per hour, respectively. After two days the dissolved calcium reached maximum values in both HA and TCP materials corresponding to 1.94 and 0.66 mM, respectively.

3.2. Ultrasound stimulations of cellular protein markers in 2-D

Cells cultured on tissue culture treated plastic after 6 hours were exposed to ultrasound for 20 minutes and given a 30 minute recovery time. Control time-experiments identified 30 minutes as a minimum time necessary for activation of intracellular proteins studied in this experiment. As shown in Fig. 3, significant increases were identified in pERK 1/2, pJNK, pP38, and vinculin proteins ($p < 0.05$), however, the activations of the stress activated protein kinases; JNK and P38 were extremely low. No differences were found for pAKT and $\alpha_6\beta_4$ integrin.

3.3. Activation of cell protein markers with scaffold extract media, cell-scaffolds and LIPUS

The activation of the stress signal mediators ERK 1/2, P38, JNK, and AKT were evaluated in response to cells exposed to scaffold media extracts, HA and TCP scaffolds directly and with applied LIPUS, Fig. 4. Media extracts were prepared from culturing control scaffolds in media for the same time period as remaining groups. This provided a control condition where the variability and effect of soluble calcium release from scaffolds could be identified.

Phospho-activation of ERK 1/2 demonstrated equal activation between HA scaffold extracts and cell-scaffold groups. Application of LIPUS significantly increased ERK 1/2 activation only on the TCP scaffold. No differences were identified within the HA group, Fig. 4a, and both TCP scaffolds experienced significantly greater ERK activity compared to HA ($p < 0.05$). Phospho-activation of the JNK stress mediator is shown in Fig. 4b. In contrast to the ERK activity, JNK was minimal from media supplementation demonstrating differences in both scaffold groups. One between group difference was identified between HA and TCP scaffolds not exposed to ultrasound, with TCP greater than HA ($p < 0.05$). The same trends were observed with phospho-activation of the P38 mediator with the exception that TCP was significantly less than the corresponding HA scaffold value ($p < 0.05$), Fig. 4c. The final phospho-protein selected for study, AKT, also a multifunctional anti-apoptosis signal, demonstrated significant increases in response to ultrasound in HA but not TCP. Similar to ERK and JNK activation, AKT was higher on TCP compared to HA scaffolds ($p < 0.05$), Fig. 4d.

3.4. Stimulation of adhesion proteins

The stimulation of the cytoskeleton attachment and adhesion proteins vinculin and $\alpha_6\beta_4$ integrin were evaluated with cells exposed to scaffold media extracts, HA and TCP scaffolds directly and with applied LIPUS, Fig. 5. Increases in vinculin were identified for cells cultured on scaffolds compared to 2-D surfaces in both HA and TCP. Ultrasound significantly increased vinculin levels in TCP but not HA scaffolds ($p < 0.05$), Fig. 5a. An identical trend was observed with $\alpha_6\beta_4$ integrin levels with high levels in scaffolds with and without ultrasound only significantly affecting the TCP group Fig. 5b.

4. Discussion

The clinical application of ultrasound to damaged bone has found application to accelerate healing time and prevent loss of bone during the healing period [28]. As these systems are increasingly used clinically, knowledge of the biological effects of ultrasound is required to identify safe guidelines for use and to help develop these systems for effective mechanisms of action. In this study, the importance of material and 3-D design in conjunction with LIPUS was explored on the regulation of stress, apoptosis and adhesion mediators with an osteoblast precursor cell line.

4.1 Scaffold design and calcium release

The structural composition of natural bone consists of an apatite mineral with strut or rod-like organization in trabecular bone. Calcium phosphate scaffolds fabricated with similar material and structural characteristics make cell behavior studies more reflective of an *in-vivo* environment. The scaffold architecture employed in this study was carefully matched to the histological parameters of the human lumbar spine. This arrangement of surface curvature provides characteristics not represented in 2-D that may alter signaling patterns reported using traditional cell culture. Specifically, 3-D structure may influence integrin adhesion and subcellular protein distribution thus changing biological response [29]. Calcium release profiles demonstrated greater levels in HA compared to TCP scaffolds, a result contrary to the known high dissolution behavior of TCP, however, equal sintering temperatures were used in this study resulting in small tightly bound HA crystals as shown in Fig. 1a. Crystal size of TCP

was approximately 10X larger, Fig. 1b, resulting in fewer grain boundaries and lower calcium release over the short time period of this study. Calcium release has been implicated in the activation of many biological pathways including the MAPK cascade [30] and in the course of this study did not exceed 2mM, well below the cytotoxic safe limit [31]. The preparation of media extracts from control scaffolds also allowed for the identification of calcium effects on cell signaling and adhesion behavior. An additional characteristic not normalized in this study was the effect of micropores in TCP materials creating a different surface profile compared to HA.

4.2 Ultrasound stimulation of HEPM cells in 2-D

Low intensity pulsed ultrasound (LIPUS) using a commercially available product already applied in a clinical setting supplied the acoustic stimulations of 30mW/cm² in this study. Application of ultrasound to HEPM cells in 2-D traditional culture stimulated the activation of pERK 1/2, pJNK, pP38 and production of the cell membrane cytoskeleton protein vinculin over control non-stimulated HEPM cells. These findings, with respect to ERK activity, match recently reported observations of a Gαi protein-ERK-Runx2-osteocalcin pathway involved in osteogenic transition behavior [32]. While the SAPK's; JNK and P38 demonstrated low activity in 2-D, ultrasound was able to significantly increase these values demonstrating that other MAPK members may be involved in this signal transduction. While no significant change was identified with α6β4 integrin activity in response to ultrasound, the membrane cytoskeleton protein vinculin was increased. A previous reported study identified mechanosensitive molecules including integrin activation occurring with physical stimulation of the membrane [33]. Activation of these proteins demonstrates that multiple mechanosensitive pathways can be engaged during membrane stimulation.

4.3 Scaffold and ultrasound stimulation of HEPM cells

HEPM cells were cultured in 2-D with calcium media extracts prepared from scaffolds to serve as a calcium control for the variable release behavior of HA and TCP scaffolds. Signaling from cells exposed to extracts demonstrated a substantial level of ERK activity independent of HA scaffold configuration or ultrasound. This effect was only identified for ERK and was completely absent on the other MAPK members; JNK, P38 and the cell survival signal AKT. In TCP scaffolds, ultrasound significantly increased the activation of ERK over media control. In all other measured signals, calcium media extracts with cells in 2-D showed minimal activation with both scaffold groups in HA and TCP significantly greater than controls. In addition to the ERK activation on TCP scaffolds, ultrasound stimulated an increase in pAKT on HA scaffolds and both vinculin and α6β4 integrin expression on TCP scaffolds.

It was of particular interest to note that of the MAPK proteins evaluated in this study only ERK 1/2 was significantly increased in response to ultrasound and only on TCP scaffolds. This result matched the increases in vinculin and α6β4 integrin, once again only on TCP scaffolds. This result appears in conflict with the lower calcium release from the TCP materials at this early time period, however as previously reported, TCP surfaces at the micro and nano scale have small micro-pores that may supply locally high calcium concentrations especially during ultrasound that are not detectable by bulk media testing in addition to their surface roughness and morphological differences. As LIPUS treatment has been shown to redistribute actin stress fibers and focal adhesions [34], the described results could be masked by the specific characteristics of TCP scaffolds and the selection of adhesion-phase timing chosen in this study. Direct visualization of calcium activity through the cell membrane in addition to matching the crystalline features of these materials may provide greater insight to identify the contribution of local calcium influx during LIPUS treatments.

5. Conclusion

Low intensity pulsed ultrasound stimulated phospho-activation of the MAPK proteins ERK 1/2, JNK and P38 in addition to the membrane anchoring protein vinculin. Calcium release from the scaffolds was partially involved in the activation of ERK 1/2 when cell response was compared between culture on 2-D surfaces and 3-D HA and TCP scaffolds. Effects of calcium extracted media from scaffolds alone were not responsible for activation of the stress activated protein kinases; JNK and P38. LIPUS stimulation further increased ERK activity on TCP scaffolds corresponding with an increase in both vinculin and $\alpha\beta4$ 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.

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Figure 1. SEM photographs of (a) macro calcium phosphate scaffolds representative of both HA and TCP bulk structure and (b) individual rounded triangular strut design of the scaffold.

Figure 2. Calcium release behavior from HA and TCP scaffolds. Calcium release reported at selected times after immersion in PBS at 37°C, 5% CO₂, reported as μg calcium / (mg scaffold dry weight, mL PBS). Data represents average values + S.D., n=4.

Figure 3.

Ultrasound induced increase for phospho and membrane protein markers. Immunoreactivity of six proteins measured with HEPM cells cultured on tissue culture plastic after 6 hours 50 minutes(*control white bars*) or cultured for 6 hours, stimulated with ultrasound for 20 minutes with 30 minute recovery (*black bars*). The data shown represent average values (n=6) + S.D., * p < 0.05 by paired *t*-test.

Figure 4.

Scaffold and ultrasound stimulations of intra-cellular stress signal markers. Phospho-immunoreactivity of pERK1/2, pJNK, pP38 and pAKT for HEPM cells cultured on 2D tissue culture surfaces exposed to calcium containing media from incubated control scaffolds (*control white bars*), cells cultured directly on scaffolds (*grey bars*) and cells cultured on scaffolds stimulated with ultrasound for 20 minutes with 30 minute recovery prior to test completion (*black bars*). The data shown represent average values, (n=6) + S.D., * denotes differences from control within material groups (HA or TCP), *i* denotes differences between material groups per condition, $p < 0.05$ by two factor ANOVA with Tukey pairwise multiple comparisons.

Figure 5.

Scaffold and ultrasound stimulations of cell attachment and adhesion signal markers. Immunoreactivity of vinculin and $\alpha 6 \beta 4$ integrin proteins for HEPM cells cultured on 2D tissue culture surfaces exposed to calcium containing media from incubated control scaffolds (*control white bars*), cells cultured directly on scaffolds (*grey bars*) and cells cultured on scaffolds stimulated with ultrasound for 20 minutes with 30 minute recovery prior to test completion (*black bars*). The data shown represent average values, (n=6) + S.D., * denotes differences from control within material groups (HA or TCP) by two factor ANOVA with Tukey pairwise multiple comparisons.