Osteoblast Differentiation Is Enhanced in Rotary Cell Culture Simulated Microgravity Environments

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Purpose: **As the aging population increases, more people will become reliant on regenerative dental medicine for implant therapy. The objective of this study was to test the hypothesis that 3D rotary cell culture (RCC) environments created by simulated microgravity would enhance osteogenic gene expression using integrin mediated pathways.**

Materials and Methods: **Human embryonic palatal mesenchymal (HEPM, ATCC 1486) pre-osteoblasts were cultured in either RCC to create 3D environments or in 2D monolayers for 72 hours. Gross phenotypic analysis was performed using Alizarin Red S staining for calcium and microscopy. Real-time PCR analysis was used to detect differences in osteoblast gene expression. Aggregates developed in 3D RCC environments were treated with or without antibody to the collagen-I integrin receptor $\alpha 2\beta 1$ to determine whether this molecular pathway might contribute to the development of a mineralized matrix.**

Results: **Microscopic analysis demonstrated that RCC environments promoted 3D aggregate formation by 72 hours without any scaffold. The mass appeared osseous-like with a white, shiny, translucent surface. The center was amorphous with areas of vacuolization, tubule-like structures, and fibrous-like extensions. Real-time PCR data showed that 3D environments enhanced osteogenic gene expression as compared with 2D monolayer culturing conditions. At 72 hours, changes in levels of osteogenic gene expression were noted. Cbfa1, a necessary transcription factor for osteoblast differentiation, was expressed 33% higher ($p = 0.26$); Collagen 1, 69% higher ($p = 0.05$); Osterix, 49% higher ($p = 0.001$); and BSPII, 54% higher ($p = 0.001$) than osteoblasts cultured for 72 hours in standard 2D monolayer conditions. When cultured in the presence of collagen $\alpha 2\beta 1$ integrin receptor antibody, 3D aggregates had decreased levels of mineralization as compared with non-treated aggregates.**

Conclusion: **RCC enhances osteoblast differentiation using integrin mediated pathways.**

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INDEX WORDS: tissue engineering, dental implants, osseointegration, bone, rotary cell culture

AS THE aging population increases, more people will become reliant on regenerative dental medicine. More people are being defined as partially edentulous, and their treatment options have expanded to include the use of dental implants. For the best biologic, biomechanical, and esthetic results of these implant rehabilitations, proper implant placement is essential. This type of therapy requires special consideration with respect to the quality and quantity of bone available at the surgical site. The placement of an implant into a defective osseous site not only prevents adequate positioning of the final prosthetic restoration, but also results in poor osseointegration and subsequently, a poor prognosis for the therapeutic outcome.

Bone augmentation is a subject of intensive investigation in dentistry. Current approaches in bone reconstruction use biomaterials, autografts, or allografts, although restrictions on all these

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Accepted March 21, 2006.

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1059-941X/07

doi: 10.1111/j.1532-849X.2007.00204.x

techniques exist. These restrictions include donor site morbidity and donor shortage for autografts,[1] immunologic barriers for allografts, and the risk of transmitting infectious diseases.[2] Numerous artificial bone substitutes containing metals, ceramics, and polymers have been introduced to maintain bone function;[3] however, each material has specific disadvantages, and none can perfectly substitute for current autograft therapies.

An alternative to current autograft procedures is osseous tissue engineering. Living cells may be exploited for tissue engineering of bone for the restoration, maintenance, or enhancement of tissue function; however, current protocols require various amounts of time. We have observed that some in vitro 2D monolayer osteoblasts cultures may take 3 to 4 weeks to mineralize. Expedition of this culture time will be needed for future dental implantation and other types of craniofacial repair if in vitro engineered bone is to be used as an autograft. A theoretical way to achieve this goal would be to provide a "stimulatory" environment to the bone cells to expedite tissue engineering of bone. One possible method may be to grow pre-osteoblasts in a microgravity environment to enhance the rate of osteoblast differentiation and mineralization.

Indeed, our laboratory (as well as other researchers) has been investigating the effects of simulated microgravity on osteoblast differentiation as a means to study cellular and molecular mechanisms associated with 3D osseous tissue growth.[4-7] Several studies have analyzed the effect of simulated microgravity on osteoblast differentiation using established osteoblast cell lines.[5,6,10] These studies demonstrated increased cellular aggregation and enhancement of osteoblast differentiation. The conclusions drawn from these studies were that reduced gravity may activate compensatory effects on the osteoblasts as a result of the negative loading and thus lead to enhanced osteoblast differentiation.[8-10] It has been reported that HEPM preosteoblast cells cultured in simulated microgravity conditions as compared with standard tissue culture plastic conditions had an increased rate of mineralization.[10]

In normal osteoblast cell biology, cell membrane receptors called integrins serve to adhere cells to their surrounding extracellular matrix, and when doing so, activate internal signaling cascades leading to cell growth and differentiation.[11]It has been suggested that integrin adhesion and signaling pathways may contribute to the ability of osteoblasts to mediate the initiation of the mineralization phenotype biologically.[12] Thus, the objective of this current study was to test the hypothesis that 3D rotary cell culture (RCC) environments, created by simulated microgravity, would enhance osteoblast differentiation and osteogenic gene expression using cell adhesion/integrin mediated pathways.

Materials and Methods

Cell Culture

Human embryonic palatal mesenchymal (HEPM 1486; ATCC) pre-osteoblasts were cultured in either RCC to create 3D environments or in standard 2D monolayers on tissue culture plastic for 72 hours for comparison. HEPM pre-osteoblasts were cultured in triplicate on 2D tissue culture plastic (50,000 cells/10μL) or suspended in a Synthecon RCCS D-410 3D RCC vessel system (10×10^6 cells/10 ml) using freshly prepared EMEM media treated with β-glycerophosphate (5 mM) and ascorbate (50 μg/ml), or untreated (no additive). Using previously described methods,[10] all air bubbles were removed from the 3D culture chamber, and the chamber was initially rotated at 15 rpm. Rotational speed was increased after 24 hours to 30 rpm to maintain the cell aggregates in suspension using rotary motion.

Gross Phenotypic Analysis

Cell aggregates were collected from the 3D chamber and desiccated under vacuum at room temperature for 1 week. The samples were then observed using either a Stemi 2000-C Stereo Dissecting Light Microscope (Carl Zeiss Light Microscopy, Göttingen, Germany).

Cell Proliferation

Cell proliferation was evaluated by counting cells collected from the 2D and 3D cultures at 0 and 72 hours. Cells were collected from each group and dissolved in trypsin/EDTA solution (3 ml) for 45 minutes to disaggregate the 3D mass into single cells. A Hemocytometer and Z2 Coulter Counter (Beckman Coulter, Inc., Fullerton, CA) were used for cell counting. Numbers were compared to the 0 hour baseline for either the 2D and 3D samples to determine percent increase or decrease.

Real-Time PCR Analysis

RNA isolation was performed at 0 and 72 hours using an RNA-easy Stabilization and Total RNA Isolation Collection System (Qiagen Inc., Valencia, CA) per manufacturer's instructions. RNA extracts were normalized

for PCR analysis using a spectrophotometer. Real-time PCR analysis was performed using previously described methods.[20] Briefly, normalized RNA was reverse transcribed into cDNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA). Real-time PCR primers and probes for our targeted osteogenic genes included Cbfa1 (Forward: CAACAAGACCCTGCCCGT; Reverse: TCCCATCTGGTACCTCTCCG; Probe: CTTCAAGGTGGTAGCCC), Osterix (Undisclosed sequences, Applied Biosystems), BSPII (Undisclosed sequences, Applied Biosystems), and Col I (Forward: ACAGCCGCTTCACCTACAGC; Reverse: GTTTTGTATTCAATCACTGTCTTGCC; Probe: TGTCGATGGCTGCACGAGTCACAC), as well as 18S-rRNA for our control (Undisclosed sequences, Applied Biosystems). These were designed with Primer Express software (Perkin Elmer, Waltham, MA). Real-time PCR multiplex reactions were performed in 96-well Optical Reaction Plates (Applied Biosystems) in an ABI PRISM 7700 Sequence Detection System (Perkin Elmer) at the University of Iowa DNA facility using previously described real-time PCR protocols.[20]

Integrin Antibody Perturbation and Alizarin Red S Staining

Mouse monoclonal antibodies against $\alpha 2\beta 1$ integrin receptor (Chemicon International, Temecula, CA) were added to 3D cell cultures at 0, 24, and 48 hours (50 μg/ml, respectively) as described in previous studies.[12] Another group was cultured without the antibody treatment to serve as control. After the 72-hour culture, cell aggregates from each group were collected, fixed for 10 minutes in 10% formalin, rinsed with Nanopure water, and stained with 2% Alizarin Red-S for 10 minutes at room temperature. After rinsing the wells four times with Nanopure water, we evaluated the intensity of the AR-S staining to detect changes in levels of mineralization.

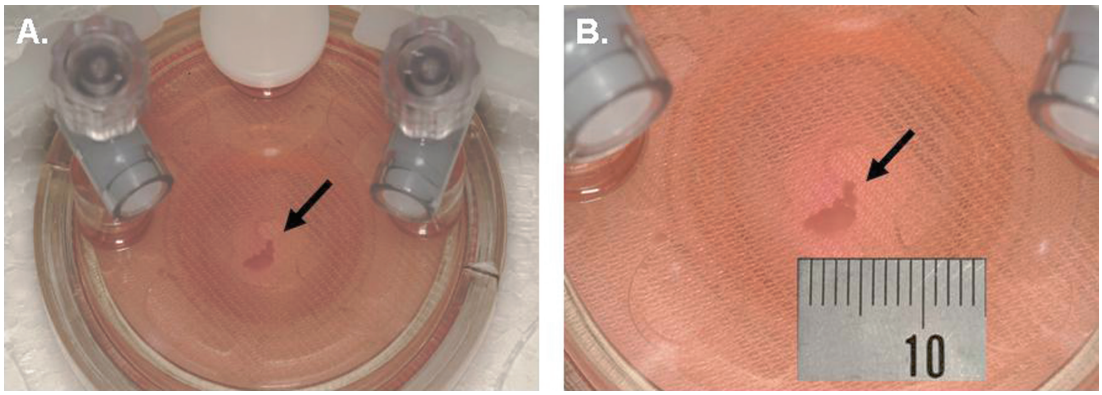

Figure 1. 3D RCC chamber aggregate at 72 hours (*A, arrow*). Note the large cell aggregate which measured 4 mm across (*B, arrow*).

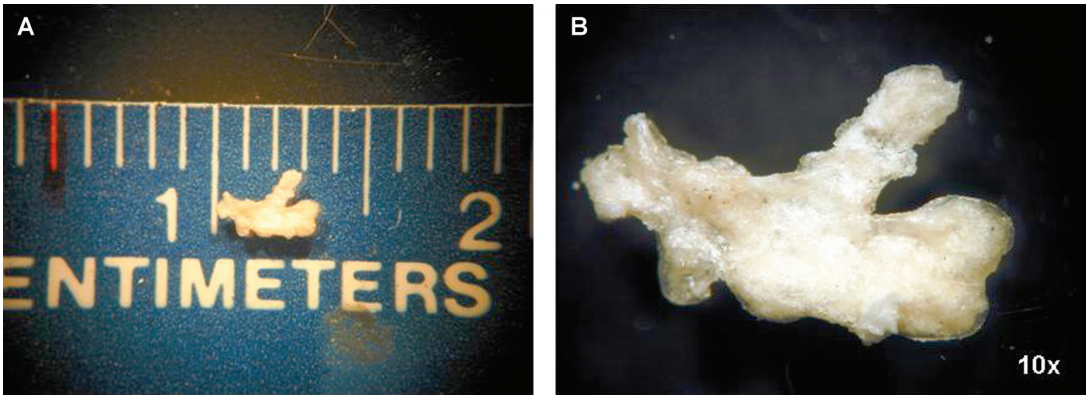

Figure 2. Light microscopic analysis of 72 hour 3D aggregates after desiccation. The mass was nearly 4 mm in length (*A*, magnification 4×) and appeared tissue-like with a white, shiny, translucent surface, similar to that of bone (*B*, magnification 10×).

SEM Analysis

Cell aggregates from the rotary cell cultures were prepared for scanning electron microscopy by fixing them in glutaraldehyde in a phosphate buffer. After osmification in 1% OsO_4 the samples were dehydrated in ascending ethanol for 24 hours followed by drying in a critical point dryer. The samples were then mounted on stubs with copper tape and sputter-coated with gold and viewed with an 1820 digital scanning electron microscope (SEM) (Amray, Bedford, MA).

Statistical Analysis

Statistical analysis ($N \geq 3$) was performed by analysis of variance (ANOVA) with a Tukey's Multiple Comparison Test to a confidence level of $p < 0.05$.

Results

Osteoblasts Aggregate to Form Tissue-Like Masses in 3D Rotary Cell Culture

Compared with pre-osteoblasts cultured in 2D tissue culture plastic environments, cells grown in 3D simulated microgravity environments (Fig 1A) show a very distinctive pattern of cell growth. By 24 hours, cells grew in monolayers under 2D conditions while osteoblasts grown in 3D cultures started to aggregate. These 3D small aggregates coalesced into larger aggregates of approximately 4 mm in size by 72 hours (Fig 1B). Gross light microscopic analysis of aggregates isolated from 3D RCC revealed a mass that appeared tissue-like with a white, shiny, translucent surface, similar to that of bone (Fig 2A and B). SEM analysis revealed that the tissue-like aggregates appeared to have a solid, smooth, irregular outer surface (Fig 3A); however, in cross-section, the middle of the aggregate appeared to be amorphous, with areas of vacuolization and fibrous-like extensions (Figs 3B–D).

Osteoblast Gene Expression Is Enhanced in 3D Rotary Cell Culture Environments

Our real-time PCR data suggest that 3D environments may enhance osteogenic gene expression as compared with 2D monolayer culturing

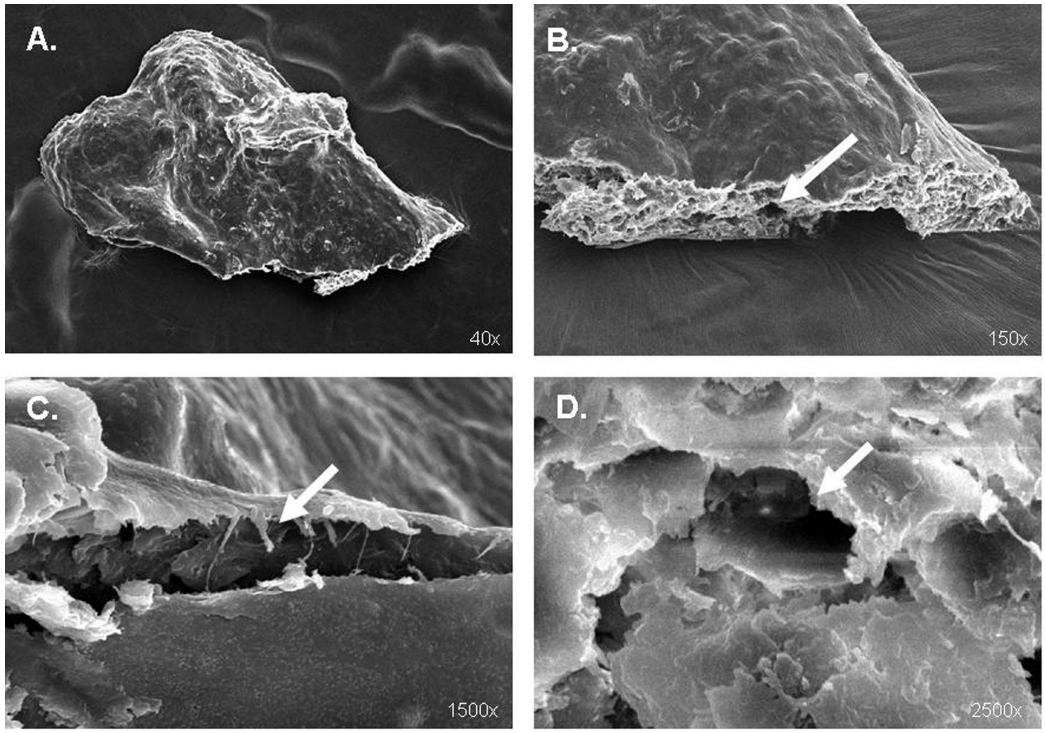

Figure 3. Scanning electron microscopy of the 72-hour 3D cultured mineralized aggregate showed a mass with a smooth and solid-like outer surface with irregular peaks and valleys (A, magnification 40×). When cross-sectioned the center appeared to be amorphous (B, arrow, magnification 150×) and have fibrous extensions (C, arrow, magnification 1500×) and vacuolated areas (D, arrow, magnification 2500×).

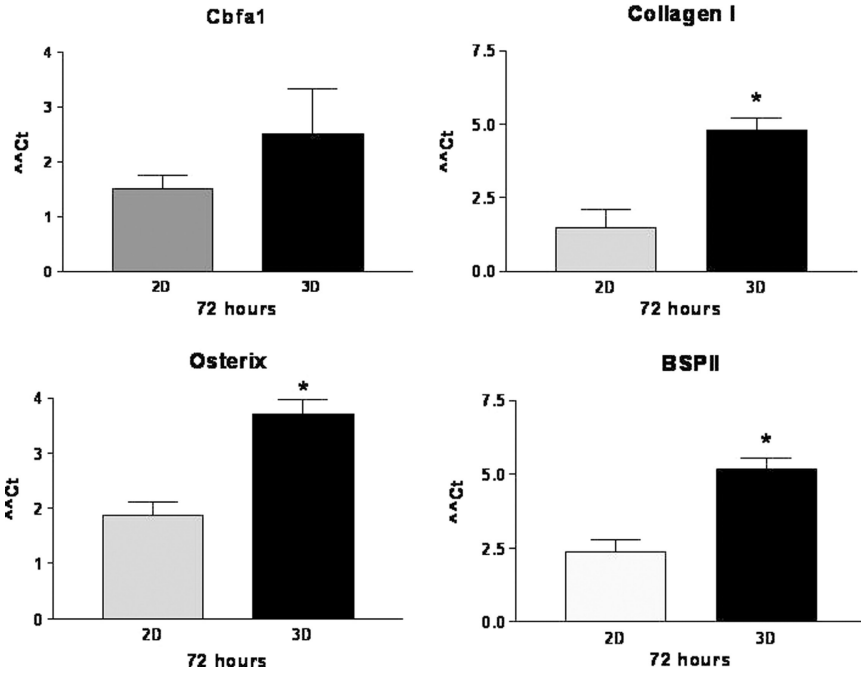

Figure 4. Real-time multiplex PCR results for various genes necessary for osteoblast differentiation and mineralization showed that Cbfa1 gene expression was increased in the 3D group compared with the 2D group, although not significantly ($p = 0.26$, N = 9). However, Collagen I ($p < 0.05$, N = 9), Osterix ($p < 0.001$, N = 9), and BSPII ($p < 0.001$, N = 9) all exhibited significantly higher gene expression in the 3D culture samples compared with 2D. (*is significant at confidence level of $p < 0.05$).

conditions. At 72 hours Cbfa1, a necessary transcription factor for osteoblast differentiation,[21] was expressed 33% more in 3D RCC, compared with 2D monolayer conditions ($p = 0.26$), (N = 9). While the Cbfa1 data was not significant at a confidence level of $p < 0.05$, other osteogenic genes were. At 72 hours, 3D osteoblast aggregates expressed Collagen I 69% higher ($p < 0.05$, N = 9); Osterix, 49% higher ($p < 0.001$, N = 9); and BSPII, 54% higher ($p < 0.001$, N = 9) than osteoblasts cultured for 72 hours in standard 2D monolayer conditions (Fig 4).

Rotary Cell Culture Mineralization Is in Part Regulated by α2β1 Integrin Receptors

When cultured in the presence of the Collagen-1 $\alpha 2\beta 1$ integrin receptor antibody, 3D aggregates had decreased levels of mineralization, compared with non-treated 3D aggregates. This phenomenon was observed under the light microscopy in combination with Alizarin Red S staining (Fig 5). The $\alpha 2\beta 1$ integrin antibody-treated aggregates showed less mineralization (less red stain) (Fig 5A), compared with the non-treated control, which was darker, indicating greater levels of mineralization (Fig 5B). The result from Alizarin Red S staining implies that osteoblast differentiation in cultures maintained in 3D environments may be in part regulated by the $\alpha 2\beta 1$ integrin receptor signaling pathway.

Discussion

The horizontal rotation of an RCC system maintains cells in suspension within the vessel, but does allow the osteoblasts to adhere to the extracellular matrix via integrin receptors created within the aggregate.[7-9] This system enhances cell aggregation by maintaining the cells in a fluid orbit, which in effect keeps the cells in a constant state of freefall, hence simulated microgravity. Simulated microgravity appears to promote cell–cell association while avoiding high shear stress acting on cells.[13] Consequently, cells are suspended in continuous freefall at a terminal velocity through the medium with low shear stress force, and low turbulence.[7,14,15] This creates an opportunity to study the development of tissue in 3D environments. The main advantage of this system comes from the fact that it creates an environment that tends to aggregate individual cells into a tissue mass and allows it to differentiate at an increased rate compared with conventional tissue culture.[8,10,16]

Previous studies have found that scaffolds are needed for 3D cellular aggregation.[5] Other studies, including our current experiment, confirmed that osteoblast aggregation could occur in a 3D RCC environment without any scaffolds.[6,10]

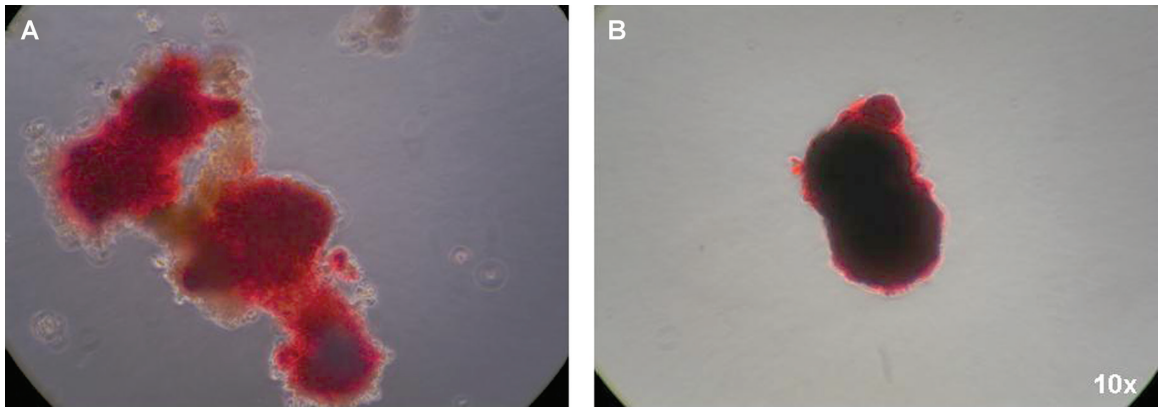

Figure 5. Alizarin Red S stain for mineralization in 72-hour 3D cell aggregates treated with (A) or without (B) $\alpha 2\beta 1$ integrin receptor antibody. Treated aggregates showed considerably lighter red appearance due to reduced levels of mineralization (A) as compared with the darker, more intense stain in the non-treated (B) control aggregates. (magnification, 10×).

Additionally, we were able to demonstrate that an actual tissue-like mass begins to develop as early as 24 hours. The phenotype of this tissue-like mass was analyzed through various microscopes, and it has a general appearance similar to that of a hard tissue. To confirm this cultured tissue is truly a viable tissue mass available for grafting purposes requires further in vivo investigation.

Some studies indicate that osteogenic gene expression is enhanced in 3D environments,[5,6] whereas others observed reduced gene expression.[17,18] Our observations suggest that osteogenic genes related to osteoblast differentiation and mineralization are generally expressed higher in cells cultured in 3D environments as compared with 2D. This implies that the 3D environment may enhance cell-to-extracellular matrix signaling pathways via integrin receptors that are involved in the osteoblast cell differentiation and mineralization as previously described for 2D systems.[12] We did find that adding $\alpha 2\beta 1$ integrin receptor antibody to cells maintained in 3D RCC environments led to decreased mineralization. Other studies have reported that addition of $\alpha 2\beta 1$ integrin receptor antibody to osteoblasts in 2D cultures led to decreased levels of mineralization by up to 95%.[12] Our findings, as well as others,[10] suggest that osteoblast differentiation and mineralization in 3D environments may be in part mediated through $\alpha 2\beta 1$ integrin receptor pathways.

Interaction of integrin receptors with the ECM can activate or deactivate intracellular signaling pathways, which subsequently can influence gene expression. One possible mechanism for the regulation of osteoblast differentiation seen in the 3D RCC environments may be through the enhancement of the integrin associated focal adhesion kinase's (FAK) ability to mediate signals from integrins and growth factors to MAPK signaling cascades. Indeed, studies have shown that FAK interacts with Grb2 through FAK's Y925 phosphorylated amino acid, and subsequently modulates MAPK activity.[22] In addition, MAPK pathways have been shown to activate and phosphorylate Cbfa1, a necessary transcription factor for osteoblast differentiation.[23] More recent studies report that Cbfa1 phosphorylation can be modulated by $\alpha 2\beta 1$ integrin, TGFβ, FGF, and BMP2 signals converging through the MAPK and SMAD pathways leading to regulation of mesenchymal precursor cell differentiation.[24-27]

It appears possible that osteoblasts cultured in 3D environments appear to have increased levels of gene expression necessary for differentiation and the formation of a mineralized matrix, and that this can occur by 1 week in 3D cultures, compared with 2D, which takes nearly 4 weeks. This increase in osteogenic gene expression supports the previously reported concept[10] of an increased rate of mineralization seen in cells cultured in 3D environments. Interestingly, cell proliferation analysis revealed that by 72 hours, 3D cultures had nearly a 65% decrease in cell number, compared with 2D cultures, which showed an increase of nearly 95%, yet the level of expression of genes needed for osteoblast differentiation increased in

the 3D cultures as compared with 2D. This observation may be in part due to 3D culture conditions providing a more physiological-like environment that activates more readily cellular processes associated with bone development such as anoikis, or programmed cell death.

Conclusion

Our studies suggest that RCC simulated microgravity environments enhance osteoblast aggregate formation and differentiation. At the molecular level, 3D aggregate differentiation may be regulated in part by integrin mediated pathways, in particular $\alpha 2\beta 1$. Further analysis is needed to characterize the properties of these aggregates both at the phenotypic and genotypic level to determine if they are osseous-like tissue, and if so, could they be used for grafting of osseous defects. The use of simulated microgravity environments might then translate into novel osseous tissue engineering strategies for the specialty of prosthodontics.

Acknowledgments

This work was supported by NIH grants R21-DE016677–01 (GS), P60-DE13076 (GS), University of Iowa Vice President for Research Biological Sciences Funding Program (BSFP), and the American College of Prosthodontists/ESPE Fellowship in Geriatric Prosthodontics (JK, GS). This article was awarded 2nd place in the John J. Sharry Competition at the 2005 ACP Annual Session in Los Angeles, CA.

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