Medical Sciences

Improving Bone Formation in Osteoporosis Through In Vitro Mechanical Stimulation Compared to Biochemical Stimuli

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Study Design: Laboratory investigation. Objectives: To determine and compare the ability of cellular mechanical stimulation to enhance the production of osteogenic precursor cells (OPCs) from osteoporotic bone relative to stimulation with chemical factors. Summary of Background Data: Abnormalities in the number and function of bone-forming osteoblasts play a central role in the pathophysiological processes leading to osteoporosis. Normal osteoblast production and function are regulated by chemical growth factors and mechanical signals. Nevertheless, the responses of OPCs from osteoporotic bone to mechanical signals remain poorly understood. Methods: Transpedicular aspiration of 5 cc of vertebral body bone marrow was performed in osteoporotic patients undergoing instrumented spinal fusion. Mesenchymal stem cells (MSCs) were isolated and subjected to either mechanical stimulation (4000 με elongation) or biochemical stimulation with BMP-2or PDGF at three different concentrations (0.0001, 1, and 100 ng/mL). MSC proliferation was assessed using the alamarBlue assay and the quantity of OPCs was estimated by measuring alkaline phosphatase (AP) activity and normalized to DNA content. Results: A total of 4 osteoporotic patients (3F:1M, average DXA=-2.9) were enrolled. Although there was a trend toward improved osteoporotic MSC proliferation after biochemical stimulation in a dose-dependent manner compared to mechanical stimulation, this only reached statistical significance for PDGF at its highest concentration ($p<0.05$). With regard to the quantity of OPCs within this cell population, mechanical stimulation on average resulted in an approximately 50% improvement in normalized AP activity compared to MSCs stimulated with BMP-2 ($p<0.05$) and 130% compared to PDGF ($p<0.0005$) at the highest concentrations. Conclusion: PDGF-mediated pathways involved in the proliferation of osteoporotic MSCs may remain relatively intact compared to those involving BMP-2 or mechanical forces. However, mechanical signals may be more effective than biochemical ones in promoting osteoporotic MSCs to differentiate along an osteoprogenitor lineage. Level of Evidence: Basic Science. *Journal of Nature and Science, 1(4):e63, 2015*

Osteoporosis | mesenchymal stem cells | spinal fusion | mechanical stimulation

Introduction

Osteoporosis affects approximately 10 million men and women over the age of 50 in the United States, with an additional 34 million at risk for the disease.[1] It is characterized by reduced bone mass and microarchitectural deterioration of bone tissue leading to decreased bone strength.[2] As a result of these disturbances, the treatment of osteoporotic patients requiring spinal fusion is particularly challenging with regard to both potential implant failure as well as the achievement of a structurally sound fusion mass.[3-7] While the etiology of osteoporosis is multifactorial, abnormalities in the number and function of bone-forming osteoblasts play a central role in the pathophysiological process.[8] Osteoblast production and function are regulated by a number of growth factors, including bone morphogenetic proteins (BMPs) and platelet-derived growth factor (PDGF),[9] as well as by mechanical signals acting via mechanoreceptors.[10] These biological and mechanical signals have been shown to stimulate the proliferation,[10-13] migration,[14-16] and differentiation[10-13, 17-18] of non-osteoporotic mesenchymal stem cells (MSCs) to osteoblasts. A variety of studies have found the development of age-related osteopenia and osteoporosis to be associated with alterations in biochemical pathways,[19-21] while mechanotransduction pathways, on the other hand, remain intact.[22] Nevertheless, the responses of cells from osteoporotic bone to these signals remain poorly understood. The few studies that have assessed the potential therapeutic roles of these factors in improving osteoporotic bone characteristics have focused mainly on the ability of biochemical signals to enhance the production of osteoblasts by stimulating the proliferation and osteogenic differentiation of MSCs from osteoporotic bone.[1, 23]No study, however, has assessed the responses of these MSCs to mechanical stimulation. As the effective recruitment of functionally capable osteoblasts from MSCs within osteoporotic bone is central to reducing implant failure and achieving a timely, biomechanically sound spinal fusion, understanding the capacity, and limitations, of biochemical growth factors and mechanical signals to enhance these properties is crucial to developing new strategies to improve outcomes after spinal instrumentation in the growing osteoporotic population.

In this study, we set out to determine the responsiveness of MSCs derived from osteoporotic human bone to both biochemical and mechanical signals. We hypothesized that mechanical signals are more effective at enhancing the production of osteoblasts from osteoporotic MSCs than are biochemical factors alone.

Materials and methods

Study Population

Institutional Review Board approval was obtained. Four patients (3F:1M, average age 75 years, range 69-79 years) with osteoporosis undergoing instrumented pedicle screw-rod lumbar fusion for instability degenerative scoliosis were included in this study and informed consent was obtained. Osteoporosis was defined as a T-score less than -2.5 on pre-operative dual energy x-ray absorptiometry (DXA). Average DXA T-score was -2.9 (range -2.5 to -3.3). Patients were excluded from this study if they had a history of malignancy, previous radiation, or were on chronic steroid medication or chemotherapy.

Sample collection and cell isolation

Immediately following preparation of the pedicle for screw insertion, bone marrow aspirates (5ml) were collected from the mid-vertebral body in a transpedicular fashion just prior to pedicle screw insertion using a standard cannulated Jamshidi biopsy needle (Medtronic, Memphis, TN) under fluoroscopic guidance.

Bone marrow aspirates were processed for cell extraction within 10 minutes of collection. Samples were diluted 1:1 with phosphate buffered saline (PBS) and layered onto the ficol. Tubes were centrifuged at 400g for 50 minutes at room temperature. Monolayer containing mesenchymal stem cells (MSC) was collected and

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washed twice with PBS. The cell pellet was resuspended and plated in the flask with Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY) growth media containing 10% fetal calf serum, 1% L-Glutamine and 1% antibiotic and antimycotic (Invitrogen, Grand Island, NY). Cells were cultured at 37ºC with 5% CO2 in air. After 3 days in culture, non-adherent cells were aspirated and fresh media was added. Media was changed every 2-3 days. At passage 3, stem cells were harvested and 100,000 cells/well was used for chemical and mechanical stimulation.

For all analyses, three parallel test series were performed with pooled cells from different donors, with each test series done in triplicate. Pooled cells without growth factors or mechanical stimulation were used as controls.

Chemical and mechanical stimulation

For chemical stimulation, MSCs were plated in 6-well plates. BMP-2 and PDGF (R&D Systems Inc., Minneapolis, MN) were added to MSCs in concentrations of 0.001, 1 and 100 ng/ml respectively. Cells with growth factors were cultured for an additional 3 days, with fresh medium and growth factor added every other day.

The in vitro application of mechanical loading was performed according to previously published methods[18]. Briefly, cells were plated in BioFlex collagen coated plates and cyclic strain was induced using the Flexcell FX-5000 Tension System (Flexcell Corp., Hillsborough, NC), which is a computer-regulated bioreactor that applies cyclic or static tensile strains to cultured cells in vitro. We delivered 4000με elongation, at 1 Hz frequency during 300 cycles/d for a total of 2 weeks. Media was changed twice a week.

Cellular content

Total cellular content was determined by measuring the deoxyribonucleic acid (DNA) content using the PicoGreen fluorometric method (Invitrogen, Grand Island, NY).

Analysis of Proliferation

Cell vitality and proliferation was assessed using the alamarBlue (AB) Cell Viability assay (alamarBlue, Invitrogen, Grand Island, NY). For the assay, 10% of alamarBlue was added to the cells and incubated for 2h at 37ºC. The fluorescence was measured with a plate reader at 560nm excitation and 590nm emission, as directed by the manufacturer, and expressed as a percentage of AB detected for control groups.

Analysis of Osteogenic Differentiation of MSCs

The catalytic activity of alkaline phosphatase (AP) was determined using the colorimetric Alkaline Phosphatase kit (Abcam, Cambridge, MA) as directed by the manufacturer. Final values of AP were normalized to DNA amounts and were used to indicate the relative quantity of OPCs within the stimulated MSC population.

Statistical Analysis

The results of the experimental groups were normalized to the results of the control group. Parametric and nonparametric data were compared using the unpaired Student *t* test and paired *t* test, respectively. Differences were deemed significant when $p < 0.05$. Analysis was performed using SPSS software version 13.0 for Windows (SPSS Inc., Chicago, IL).

Results

Cellular Proliferation after Biochemical vs. Biomechanical Stimulation

Compared to control groups, osteoporotic MSCs subjected to stimulation with either PDGF or BMP-2 tended to demonstrate increased AB activity in a dose-dependent manner, although this only reached statistical significance for PDGF at the highest concentration tested (100 ng/mL) in which MSC proliferation was increased by 149 ± 27% ($p<0.05$) compared to control (Figure 1).

Compared to control groups, cellular biomechanical stimulation under the conditions studied did not result in a statistically significant increase in AB activity (100± 4%, p=NS) (Figure 1).

MSC Osteogenic Differentiation after Biochemical vs. Biomechanical Stimulation

The quantity of OPCs within osteoporotic MSCs stimulated with either BMP-2 or PDGF at 100ng/mL was not statistically significantly different, as measured by AP activity ($3.98X10^{-6} \pm 2.95X10^{-6}$ U/ng DNA and $1.8X10^{-6} \pm 1.08X10^{-6}$ U/ng DNA, respectively, $p>0.05$) (Figure 2). On the other hand, biomechanical stimulation resulted in an approximately 50% increase in OPCs compared to BMP-2 ($p<0.05$) and 130% compared to PDGF stimulation ($p<0.0005$) (AP activity = $6.0x10^{-6} \pm 4.1X10^{-6}$ U/ng DNA) (Figure 2).

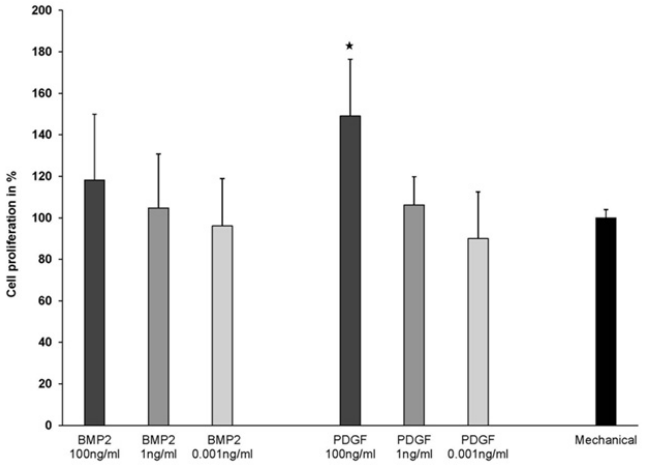

Figure 1. Cellular proliferation normalized to the control group. There was a trend toward increased proliferation in a dose-dependent manner in the biochemical stimulation group, however, this only reached significance for PDGF at 100 ng/ml. Mechanical stimulation did not result in a statistically significant increase in cellular proliferation. Statistical bars represent standard deviation.

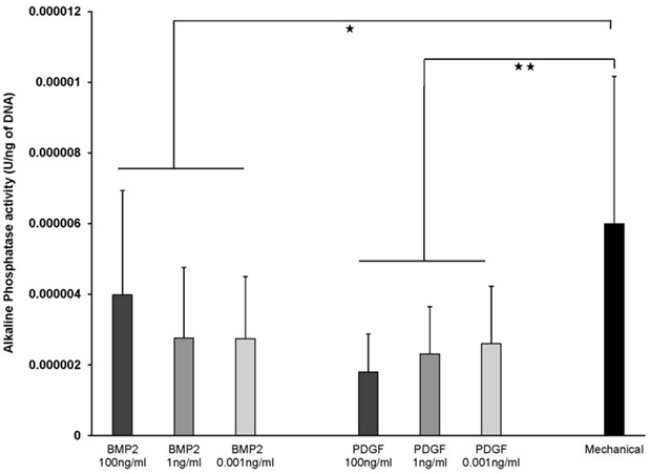

Figure 2. Osteogenic differentiation between biochemical versus mechanical stimulation groups. Mechanical stimulation results in statistically significant increases in osteogenic differentiation over BMP-2 ($p<0.05$) and PDGF ($p<0.0005$). Statistical bars represent standard deivation.

Discussion

A continuous decrease in bone mass and density occurs during aging, and will result in osteoporosis in one of three women and one of eight men over 50 years of age[24-26]. The age-matched prevalance of osteoporosis is 17-20% of women over 50 years old, 26% over 65 years old, and 50% over 85 years old. In patients who underwent spinal surgery over the age of 50 years old, the prevalance of osteoporosis was 14.5% in women and 51.3% in men[27]. Multiple studies have shown that for elderly patients who have spinal stenosis and instability, decompression with instrumented fusion produce favorable outcomes[28-33]. The ability to attain successful fusion in elderly osteoporotic patients, however, can be challenging due to the

reduced bone quality and the delayed time to bony fusion from negative bone remodeling[33-35]. Because the ability to form bone is key to the prevention of instrumentation failure and pseudarthrosis, this investigation sought to determine the potential effects of mechanical versus biomechanical stimulation on the osteoblastogenesis of human mesenchymal stem cells in osteoporotic patients.

Mesenchymal stem cells (MSCs) have been demonstrated as a promising source of expandable and pluripotent cells for both tissue engineering and regenerative medicine[36-37]. The stromal component of bone marrow is known to contain mesenchymal stem cells capable of differentiating into adipocytes, chondrocytes, myoblasts, and osteoblasts. During aging and in patients with osteoporosis, the number of these osteoprogenitor cells is maintained along with their proliferative capacity and osteogenic potential. Stenderup et al. obtained osteoporotic osteoprogenitor cells and demonstrated growth and functional characteristics comparable to age-matched controls[38]. A prior study confirmed similar proliferative and differentiation abilities of bone marrow stromal cells upon stimulation by growth hormone and calcitriol between osteoporotic patients and age-matched controls as well[39]. Osteoblasts obtained from patients with osteoporosis show normal proliferative capacity and biological characteristics. However, taking advantage of this latent capacity becomes problematic due to the variable effectiveness of exogenous induction from either biological or mechanical pathways.

Much of the research to date has been focused on the biological pathways and signals that effect terminal differentiation of progenitor cells into an osteogenic lineage. The sensitivity of these biological pathways, however, is affected as we age. In patients with osteoporosis, the premature expression of multiple factors causes an auto-inhibition that reduces the proliferation of osteoblasts[24]. SOST expression in mesenchymal stem cells code for osteocyte-specific protein Sclerostin that inhibits proliferation of MSCs and osteoblasts, blocks osteogenic differentiation, and even induces apoptosis in osteoblasts[40-41]. There also appears to be reduced effectiveness in BMP-signaling in advance age and primary osteoporosis[42]. Also observed were the enhanced gene expression leading to osteoclast precursor differentiation and activation that would lead to enhanced bone resorption seen in osteoporosis alongside a reduction in proliferative activity of osteoporotic osteoblasts[24, 43-44]. We found that compared to control MSC populations, biochemical stimulation with either BMP-2 or PDGF weakly trended towards improved cell viability and proliferation and only reached statistical significance for PDGF at the highest concentration used (100 ng/mL). We did see a response to BMP-2 and PDGF induction with regards to osteoblast differentiation, but this was significantly less than our mechanically stimulated population. With age-related osteopenia and osteoporosis affecting the efficacy of these pathways, investigations are needed to explore mechanotransduction pathways that remain intact in the face of these pathophysiological processes[19, 21-22, 45]. The application of specific mechanical stimuli would then be used to help direct MSCs down osteogenic cell lineages.

Cellular response to mechanical stimulation plays a considerably important role in the differentiation of stem cells. Mechanical load aligns collagen fibers and is important for maintaining the physiological and mechanical properties of mature bone[46], and serves a role in bone formation and bone metabolism[37, 47-48]. Low magnitude mechanical stimulation has been shown to be anabolic to bone, in which mature female sheep demonstrated a 30% increase in trabecular density and volume at the femur after one year of daily intermittent treatment[49]. This increase paralleled an increase in bone stiffness and strength as well[50]. Based on these studies, it was determined that low magnitude mechanical signals bias MSCs towards osteoblastogenesis[51]. Further studies were conducted on unloaded non-weightbearing hind limbs of experimental mice using high frequency oscillation, resulting in increased volume and stiffness of the proximal tibiae as compared to the contralateral control leg[52]. This suggests that cells can not only sense matrix distortion, but also an acceleration/deceleration motion independent of tissue environment distortion[51, 53-54]. Human clinical trials have been conducted for LMMS as well. Following a one-year trial, women who used the LMMS device for at least 2 minutes per day (n=18) showed statistically significant increases in cortical and cancellous bone of the spine compared with controls or poor compliers[55]. In our investigation, we stimulated MSCs with 4000με elongation at a 1 Hz frequency during 300 cycles per day over a total of two weeks. There was no significant difference in cell viability or proliferation when compared to controls. However, there was a statistically significant increase of an observed osteoblastic end fate as measured by AP activity in this mechanically stimulated population when compared to both BMP-2 and PDGF stimulation. This suggests that osteoporotic MSC populations are more responsive to mechanical signaling than biochemical induction with regards to osteoblastogenesis.

Prior studies have shown mechanical stimulation at frequencies between 0.1 and 1 Hz can inhibit or induce stem cell differentiation down desired paths[56-60], and BMP-2 induces osteogenic differentiation through the upregulation of Runx2 and Osx[61-62]. Khayat et al. showed in their study of murine myoblastic mesenchymal cells that low-frequency mechanical stimulation had a synergistic effect to push more cells towards their osteogenic fate in the presence of BMP-2[63]. This study demonstrates successful osteogenic differentiation via upregulation of bone marker genes with frequencies as low as 0.01 Hz. At this frequency, enhancement of osteogenic differentiation can occur at frequencies lower than that of fundamental locomotion with concomitant exposure to BMP-2. Specifically, Runx2, Osx, Alp, and Col1a1 were significantly upregulated versus controls that did not receive mechanical stimulation[63]. Our experimental population of osteoporotic MSCs was stimulated at a 1 Hz frequency. Future studies, however, can examine and investigate the lowest frequency of mechanical stimulation needed to successfully induce osteoblast differentiation.

There are several limitations to this study. This was only a small exploratory analysis with a limited number of patients and trial runs. Likewise, our use of alkaline phosphatase activity as an indicator for calcification as part of our research protocol may not be as strong as a calcification assay itself such as alizarin red, Von Kossa stain, or an mRNA profile. Although we used pooled cells without growth factors or mechanical stimulation as control groups, we did not have available non-osteoporotic cells as a further control for comparison.

To our knowledge, there has been no prior study that has examined the differentiation and proliferative responses of osteoporotic MSCs to mechanical stimulation. In addition to this, there has been no other direct comparison of both biological and mechanical pathways to produce functionally viable osteoblasts in the osteoporotic patient. We demonstrate that mechanical transduction pathways were more effective at inducing osteogenic differentiation in osteoporotic progenitor cells when compared to either biochemical stimulation with either BMP-2 or PDGF. Taking advantage of this pathway may potentially enhance fusion rates and reduce instrumentation failure in the osteoporotic patient requiring spinal surgery.

Conclusion

Our results show that mechanical stimulation of human mesenchymal stem cells obtained from osteoporotic patients was more effective in inducing osteoblast differentiation than stimulation by either BMP-2 or PDGF. Although there is increasing evidence of mechanical stimulation as a regulator of osteogenic differentiation, to our knowledge this is the first investigation of this pathway as it applies in patients with osteoporosis. Further studies are needed to examine a variety of mechanical transduction protocols as well as other biologic and biochemical agents in improving osteoblastogenesis in this patient population.

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