Medical Sciences

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

Frank L. Acosta, Jr.1, Martin Pham1, Yalda Safai2, Zorica Buser3

1Department of Neurological Surgery, University of Southern California. 2Department of Surgery, Cedars Sinai Medical Center, Los Angeles, CA. 3Department of Orthopaedic Surgery, University of Southern California, Los Angeles, CA, USA

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 μ elongation) or biochemical stimulation with BMP-2 or 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 ficoll. Tubes were centrifuged at 400g for 50 minutes at room temperature. Monolayer containing mesenchymal stem cells (MSC) was collected and

Conflict of interest: No conflicts declared.

Corresponding Author: Frank L. Acosta, Jr., MD, Associate Professor of Neurosurgery, University of Southern California, 1520 San Pablo Street, HCC 2, Suite 3800, Los Angeles, CA 90033 Phone: 323-442-6720; Fax: 323-442-7611 Email: frank.acosta@med.usc.edu

© 2015 by the Journal of Nature and Science (JNSCI).
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 37°C 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 passage 3, stem cells were harvested and 100,000 cells/well was added. Media was changed twice a week. 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.

**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 37°C. 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 MSC’s**

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 (3.98X10⁶ ± 2.95X10⁶ U/ng DNA and 1.8X10⁶ ± 1.08X10⁶ 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 = 6.0x10⁴± 4.1X10⁴ U/ng DNA) (Figure 2).

**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 prevalence 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 prevalence 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
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 bone density and volume with mechanical stimulation 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 postmenopausal women. Our experimental population of osteoporotic MSCs with 4000cycles 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.

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, 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.

Acknowledgements
Supported by Scoliosis Research Society, Small Exploratory Research Grant


