A Novel Method to Manipulate Osteoblastic Differentiation

Daniella Batarseh*, Alyssa Calabro**, Craig Queenan**, and Donna Leonardi*

* Bergen County Academies, Cell Biology Lab, 200 Hackensack Avenue, Hackensack, NJ 07601
** Bergen County Academies, Nano-Structural Imaging Lab, 200 Hackensack Avenue, Hackensack, NJ 07601

Introduction

Osteoporosis is the thinning of bone tissue and loss of bone density over time, leading to an increased risk of fracture. This prevalent bone disease results when bone, a living tissue, fails to form enough new bone while the existing bone is reabsorbed by the body at a greater rate. Since mammals have limited regenerative ability, the lack of production of new bone as a result of this disease is extremely problematic. Primary osteoporosis is genetic, but secondary osteoporosis can result from smoking, alcohol, lack of exercise, intake of steroids and certain medications.

Of interest in developing a cure for osteoporosis, is the ability to manipulate osteoblast differentiation. Osteoblasts are specialized mesenchymal cells that are responsible for bone formation through their differentiation process. The remodeling and maintenance of the bone tissue is reliant on the balance between the processes of bone resorption and bone formation. Osteoblasts are responsible for the formation of bone, while osteoclasts are responsible for reabsorption of bone [1].

Osteoblast differentiation *in vivo* occurs in three steps. The first step is cell proliferation; the second step is matrix maturation; and the final step is matrix mineralization. Alkaline phosphatase (ALP) levels reach their peak at the maturation phase of the differentiation process. This characteristic indicates that a decrease in ALP represents the movement from maturation to either proliferation or mineralization. The proliferation and mineralization phases of the differentiation process are characterized by the presence of certain proteins; procollagen I, TGF-beta, and fibronectin in the proliferation stage and osteocalcin, bone sialoprotein, osteopontin and inorganic mineral hydroxylapatite in the mineralization phase [2]. TGF-beta acts as a mitogen for osteoblast cells, aiding in the prompting of cells to go through mitosis [3].

Since osteoblasts are responsible for bone formation, both the proliferation and mineralization phases of the differentiation process are essential to strengthening of bone in patients with osteoporosis. Thus, manipulation of this differentiation process could provide mechanisms for reverting differentiation back to the proliferation stage, or continuation to the mineralization stage to further strengthen bone development.

It has been shown that zeolite, a microporous mineral with a variety of potential functions, has the ability to stimulate proliferation and differentiation in osteoblasts *in vitro* [4]. Prompting of the differentiation process by treatment with zeolite would therefore enhance bone formation. Although differentiation is essential to cell function, manipulation of the process, or the dedifferentiation of osteoblasts would allow for a possible mechanism for regeneration of bone tissue by returning cells to their proliferative state.

Dedifferentiation is defined as a cellular process in which a partially or completely differentiated cell reverts to an earlier developmental stage. This progression is often found in basal life forms and plants as a method of regeneration. Mammals have very limited ability to regenerate bone, however, other vertebrates, such as zebrafish, have the ability to entirely restore amputated bony structures through dedifferentiation of mature osteoblasts [4]. Since osteoblast dedifferentiation has the ability to allow for regeneration of bone in zebrafish, it is conceivable that manipulation of the osteoblast differentiation process, via treatment of osteoblasts with zeolite, could have implications for human bone repair.

The purpose of this *in vitro* experiment was to determine the effect of the chelator, zeolite, on osteoblasts with regard to differentiation and analyze the significance of this research for the treatment of osteoporosis by means of osteogenesis and regeneration of bone tissue.

Methods

Cell Culture

Mouse MC3T3-E1 Subclone 24 cell line was grown in Alpha Minimum Essential Medium supplemented with 10% fetal bovine serum. The cells were incubated at 37˚C with 5% CO_2 and 21% O_2. Cells were sub-cultured when confluent using 0.05% trypsin.

MTS Assay for Cell Viability

The effects of zeolite on cell proliferation was measured using CellTiter 96® AQueous One Solution Cell Proliferation Assay. After trypsinization, cells were seeded in 96-well plates at a density of 15,000 cells/well in 100 µL medium. Cells were incubated for 24 hours at 37˚C, 5% CO_2, and 21% O_2. Following incubation, cells were treated with 0.1 to 100 µg/mL zeolite. After an additional 24 hours, 15 µL of CellTiter 96®AQueous One Solution Reagent was added to each well. Cells were incubated for 1 hour at 37˚C, then absorbance was measured at 490 nm with a BioTek ELx808 microplate reader.

ALP Staining

TRACP & ALP double-stain kit was used to measurelevels of ALP after treatment with various concentrations of zeolite. Cells were cultured in a 96-well plate, seeded at 15,000 cells/well in 100 µL medium. Cells were incubated for 24 hours at 37˚C, 5% CO_2, and 21% O_2. Following incubation, cells were treated with 0.1 to 100 µg/mL zeolite. After an additional 24 hours, each well was treated with 50 µL fixation solution and 250 µL sterile distilled water. The substrate solution was added at 50 µL per well. Cells were incubated at 37˚C for 45 minutes. Solution was discarded from the wells which were then washed three times with sterile distilled water. Samples were then examined by fluorescence microscopy. Plate was read at 405 nm with a BioTek ELx808 microplate reader.

Preparation of Lysates

Cells were cultured in a T75 culture flask seeded at 3,000,000 cells in 10 mL medium. Cells were incubated for 24 hours at 37˚C, 5% CO_2, and 21% O_2. Following incubation, cells were treated with 0.1 to 100 µg/mL zeolite. After an additional 24 hours of incubation, cells were trypsinized and transferred to 15 mL centrifuge tubes. Cells were then centrifuged at 1,200 rpm for 7 minutes at 25˚C. The cells were then washed with 1.5 mL cold 1X phosphate-buffered saline (PBS) and centrifuged again at 1,200 rpm for 7 minutes. PBS was decanted and the cells were treated with 1 mL 1X lysis buffer and placed on ice for 10 minutes. Cells were then centrifuged at 13,000 rpm for 15 minutes at 25˚C. Supernatants were collected into pre-chilled 1.5 mL microcentrifuge tubes and stored at -80˚C.

Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA (Detector HRP ELISA kit) was used to measure levels of TGF-beta and osteopontin after zeolite treatment, following manufacturer's instructions. Briefly, 96-well ELISA plates were coated with cell lysates at 100 µL/well. Plates were then incubated at 4˚C for 24 hours. Plates were blocked for 15 min and 100 µL/well of primary polyclonal TGF-beta mouse antibody or primary polyclonal osteopontin mouse antibody was added to the wells. Following 1 hour incubation and washings, a secondary HRP-labeled antibody was applied for 1 hour. All antibodies were diluted at 1:500 in 1X BSA Diluent/Blocking Solution. After addition of secondary antibody, plates were washed and 100 µL/well ATBS substrate was added to each well. Absorbance was measured at 405 nm in a microplate-reader after 30 minutes.

Light Microscopy

A Nikon Eclipse TS100 Microscope was used to examine both zeolite-treated and untreated osteoblasts. The NIS Elements Basic Research Imaging Software was used to obtain images of the cultured experimental cells and compare these images with those of untreated cells.

Scanning Electron Microscopy (SEM) / Energy-Dispersive X-Ray Spectroscopy (EDS)

SEM was used to analyze the structure of the zeolite particles. Zeolite was placed onto double-stick conductive carbon tape on an aluminum SEM pin and sputter coated with approximately 1 nm of gold. Sample was imaged at 30 kV with an FEI Quanta 200 3D scanning electron microscope and elemental analysis was performed with Oxford INCA energy-dispersive X-ray spectroscopy.

Transmission Electron Microscopy (TEM)

TEM was used to visualize ultrastructure of osteoblasts. Treated and untreated cells were fixed in 5% glutaraldehyde, 4% formaldehyde, 5% calcium chloride in 0.2 M sodium cacodylate buffer, pH 7.4, scraped from the wells with Teflon® and gently spun down to form a pellet. The pellet was post-fixed in 2% osmium tetroxide, dehydrated in a graded series of acetone, infiltrated and cured in epoxy resin. 100 nm sections were collected onto 200 mesh copper grids and post-stained with 2% uranyl acetate and 0.5% lead citrate. Samples were imaged with a JEOL JEM-2100 transmission electron microscope at 200 kV.

Data Analysis

Data were analyzed using Microsoft Excel 2013. Samples were evaluated with sample size of n=5 and assays were performed at least twice. The Student's T-test was used to analyze statistical significance. The unpaired, two-tailed test was defined by an alpha value of 0.05.

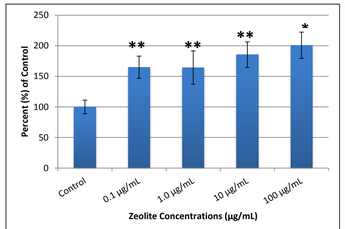

Figure 1. Proliferative effect of zeolite on MC3TC-E1 subclone 24 cells. Viability was measured after 24 hours incubation. Data represented as means ± SD. Asterisk denotes statistically significant difference from control p<0.05* or 0.01**.

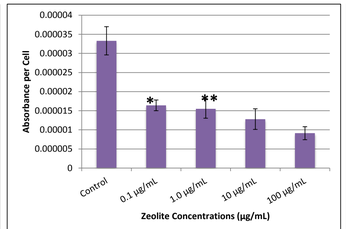

Figure 2. Levels of alkaline phosphatase in zeolite-treated osteoblasts. ALP levels were measured by absorbance at 405 nm. Data represented as means ± SD. Asterisk denotes statistically significant difference from control p<0.05* or 0.01**.

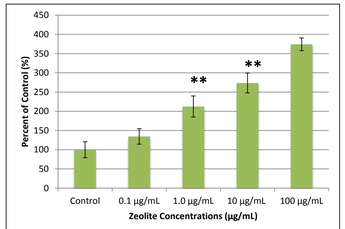

Figure 3. Effect of zeolite on TGF-beta expression in osteoblasts. Zeolite demonstrated a dose-dependent stimulation of TGF–beta expression. Data represented as means ± SD. Asterisk denotes statistically significant difference from control p<0.05* or 0.01**.

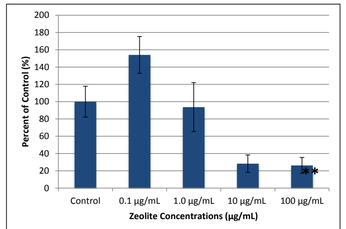

Figure 4. Effect of zeolite on osteopontin expression in osteoblasts. Zeolite administration resulted in a dose-dependent decrease in osteopontin expression. Data represented as means ± SD. Asterisk denotes statistically significant difference from control p<0.05* or 0.01**.

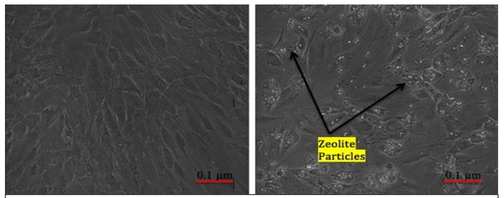

Figure 5. Light microscopy images acquired at 1000X. Left: Untreated control osteoblasts. Right: Experimental, zeolite-treated osteoblasts.

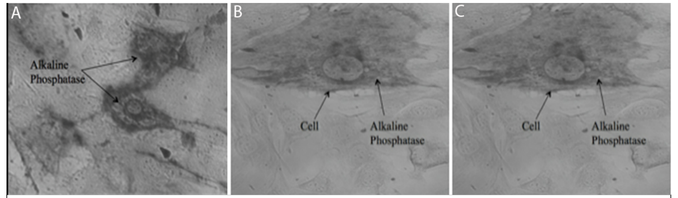

Figure 6. ALP staining of osteoblasts; LM images recorded at 400X magnification. A) Zeolite concentration 0.1 µg/mL. B) Zeolite concentration 10.0 µg/mL. C) Zeolite concentration 100.0 µg/mL.

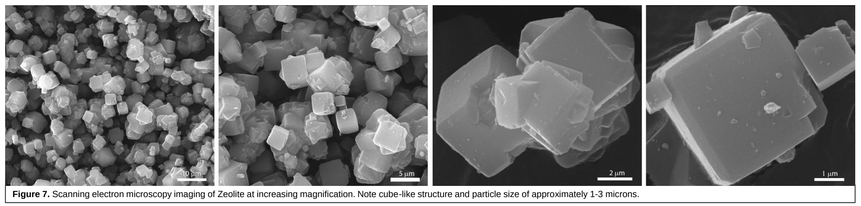

Figure 7. Scanning electron microscopy imaging of Zeolite at increasing magnification. Note cube-like structure and particle size of approximately 1-3 microns.

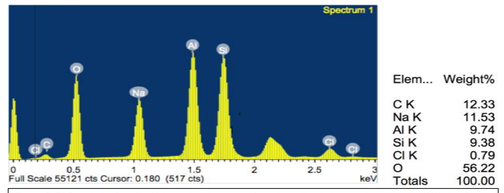

Figure 8. EDS analysis of zeolite. Unlabeled peaks account for gold coating during sample preparation. Majority of composition by weight consists of oxygen, carbon, and sodium; smaller amounts of silicon and aluminum; traces of chlorine.

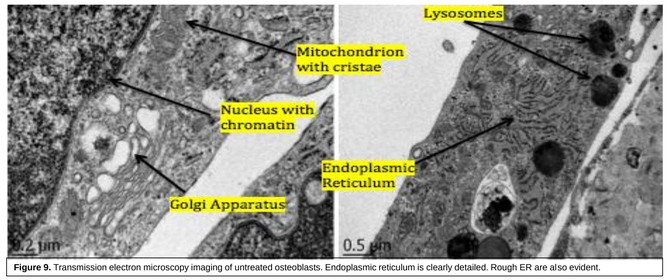

Figure 9. Transmission electron microscopy imaging of untreated osteoblasts. Endoplasmic reticulum is clearly detailed. Rough ER are also evident.

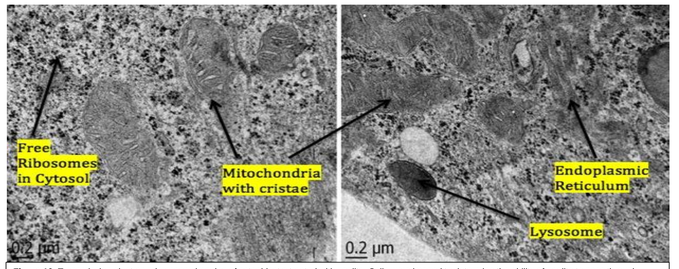

Figure 10. Transmission electron microscopy imaging of osteoblast s treated with zeolite. Cells were imaged to determine the ability of zeolite to pass through plasma membrane into the cell, and to analyze any morphological changes induced by treatment. Endoplasmic reticulum and the ribosomes are more diffuse throughout the cell; ribosomes are not bound clearly to the ER, as seen in the control images.

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Results & Discussion

Due to the lack of literature concerning zeolite, the effects of this particle on osteoblasts remain largely unknown. In this study, zeolite was identified as a potent mitogen. Osteoblast production was stimulated by zeolite and was correlated with increased proliferation (Figure 1). In order to determine the effect of zeolite on osteoblastic differentiation, ALP staining was performed. Since ALP is an enzymatic marker of bone formation in the mineralization phase, a decrease in ALP levels as concentrations of zeolite increase indicates a movement form the maturation phase back to the proliferation phase (dedifferentiation). Since the maturation phase is categorized as having the maximum levels of ALP, the decrease of ALP levels indicates that the osteoblasts are in movement from the maturation phase to the proliferation phase or from the maturation phase to the mineralization phase (Figure 2). The decrease in alkaline phosphatase levels can also been seen qualitatively by light microscopy (Figure 6).

In order to confirm these results, ELISAs were conducted to measure proteins found specifically at the proliferation and mineralization phases: TGF-beta and osteopontin, respectively. These results showed a specific threshold concentration, 0.1 µg/mL, above which TGF-beta levels increased (Figure 3) and osteopontin levels decreased (Figure 4), indicating the occurrence of dedifferentiation, sending the osteoblasts back to the proliferative stage.

In light microscopy images, the zeolite particles can clearly be seen layering the surface of the osteoblasts (Figure 5). This suggests that zeolite may be initiating a signal transduction pathway that begins extrinsically. It has been previously shown that zeolite is an adsorbent medium for phospholipids. Based on this property, it is hypothesized that there is a pathway which provoked a cellular response due to this zeolite binding. This extrinsic response that prompts dedifferentiation of the osteoblasts is reasoned to have regenerative ability similar to regeneration of bony fin structures in zebrafish.

After determining that zeolite had the ability to dedifferentiate osteoblasts, the mechanism by which zeolite interacted with osteoblasts was studied. First, SEM imaging was used to analyze the size of the zeolite particles relative to the osteoblasts (Figure 7). Since the zeolite particles were approximately 1-3 µm in size, it was hypothesized that the particles would be too large to enter the cell through the cell membrane.

The composition of the zeolite is important to the understanding of how this particle plays a role as an adsorbent to the cell membrane. Elemental analysis provides quantitative data, amount of each element in the compound as well as qualitative data, which elements are present in the compound. It was found that the majority of composition by weight consisted of oxygen, carbon, and sodium; smaller amounts of silicon and aluminum; and traces of chlorine (Figure 8). The elements that make up the zeolite can be used in future research to determine a possible mechanism by which there is a stimulation of a signal-transduction pathway as a result of the binding of the zeolite to the cell membrane.

TEM imaging of the cross sections of both treated and untreated osteoblasts was used to further analyze zeolite's effect on osteoblast differentiation (Figure 9). It was concluded that the zeolite used in this study were not taken up by the cells (Figure 10). However, there were significant differences in the morphology of the treated and untreated cells. In the control sample, there were far fewer free floating ribosomes and the endoplasmic reticulum was very clearly visible. In the experimental sample, the endoplasmic reticulum was substantially more diffuse and there were notably more free ribosomes in the cytosol.

Based on the data presented here, it can be determined that the osteoblasts transitioned from the maturation stage to the proliferation stage. This is especially supported because levels of TFG-beta, a marker protein of the proliferative stage, increased as zeolite concentrations increased, indicating dedifferentiation. Manipulation of the differentiation process from maturation to proliferation allows the osteoblasts to multiply before mineralization occurs. This has important implications in osteoporosis research because the ability of the cells to proliferate can lead to greater osteogenesis through the increased presence of osteoblasts and can influence possible regenerative mechanisms of bone. Although zeolite does not enter into the cell, it can be deduced that zeolite does extrinsically induce an intracellular response through its potential binding to the osteoblast cell membrane.

References

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