A Characterization of Three Groups of MC3T3-E1 Pre-Osteoblastic Cells to Aid in Testing of Polyurethane-Bone Scaffolds for Wound Healing

Anna C. Brakefield, E. Margarita Prieto, and Scott A. Guelcher

KEYWORDS: Osteoblasts, alkaline phosphatase, polyurethane-bone scaffolds

BRIEF. This study characterized three groups of MC3T3-E1 preosteoblastic cells with different culturing history, focusing on proliferation rates and alkaline phosphatase levels.

ABSTRACT. Research in the field of synthesizing biomaterials for bone tissue engineering relies heavily on MC3T3-E1 pre-osteoblastic cells for testing. This cell line is commonly used for studies of osteoblast differentiation due to its ability to form extracellular matrix and collagen *in vitro* under suitable conditions. However, in order to use these cells for testing, researchers must know their behavior under culturing conditions, what markers of differentiation they express, and if they will successfully proliferate on biomaterials. Three groups of MC3T3-E1 cells with different culturing history were characterized, focusing on growth rates and alkaline phosphatase expression. These variables were monitored on cells being cultured on tissue-culture plastic. Cell numbers were used to model growth rates during culture, and alkaline phosphatase, an early marker of osteoblast differentiation, was tested to ensure the cells were appropriately proliferating and differentiating. The cells' passage number was found to greatly affect proliferation and alkaline phosphatase expression, and the appropriate density at which to split MC3T3-E1 cells to encourage the continuation of desirable growth patterns was determined. This characterization will allow researchers to draw more accurate conclusions about the success of biomaterials by fully understanding normal behavior of MC3T3-E1 cells.

INTRODUCTION.

When a soldier is injured by shrapnel from an exploding mine, the injury often involves large areas, and damage to skin, muscle, nerves, and bone. In tragic car wrecks, similar injuries can also occur. In both instances, the victims of such terrible injuries want to find the best way to help restore their body to conditions as similar to before the accident as possible. However, the current methods in place for dealing with these injuries are often not ideal. Injuries in which large portions of bone are missing are typically treated with amputation or implants made of bone cement. Amputation clearly has very few redeeming qualities, and bone cements are not an ideal treatment either. One of the disadvantages of these cements is that their mechanical properties cannot be easily manipulated, so they often weaken the surrounding bone because they are too strong and do not require the host's bone to provide any support. However, a new approach to bone implants with potential for success, polyurethane-bone composites, is being introduced.

Polyurethane-bone composite scaffolds are a mixture of ground allograft bone and a polyurethane made from the reaction of a polyol with manipulative molecular weight, a prepolymer, and a catalyst. When combined in a specific order and mixed well then cured, these components interact to form a solid, nonporous structure. These scaffolds are simple to make, can be implanted or injected into a wound site, can easily be prepared differently to accommodate for different areas of the body, slowly degrade into non-toxic products after being placed in the body, and help facilitate wound healing and infiltration by the body's natural cells. Their qualities make polyurethane –bone composites easy to introduce into hospitals, because doctors or technicians will quickly be able to master the simple skills needed to mix and mold these composites. These reasons make polyurethane-bone composites a strong substitute for previous treatment methods for bone wounds [1].

To test these scaffolds, researchers use MC3T3-E1 preosteoblastic cells. These cells are an established mouse calvarial cell line at the early stages of differentiation to a full osteoblast cell. They are used to test the materials because, ideally, the cells will represent conditions the materials will be exposed to in the body, and the cells will proliferate and differentiate on the materials. Testing ensures that the materials are actually capable of supporting cell growth and differentiation, and that the interactions between the cells and materials happen at an appropriate rate. In order to fully understand the testing done on the materials, we must first understand the cells we are using. These cells express several markers of osteoblast differentiation which can show the extent to which the differentiation process can be completed on the materials [2]. One of the early markers of osteoblast differentiation that MC3T3-E1 cells exhibit is alkaline phosphatase. This enzyme is present as soon as the cells begin to differentiate, and can be observed to ensure the cells have normal function on the materials. If the cells do not properly express alkaline phosphatase, it is an indicator that the materials are not an appropriate medium for cell regeneration, as this means they will not become osteoblasts and bone will not grow on the materials.

In this study, growth rates and alkaline phosphatase expression of MC3T3-E1 cells were characterized. The characterization was done on three groups of MC3T3-E1 cells with different culturing histories to identify which group would be the most successful, and to determine how poorly the oldest group of cells was performing compared to the other, newer cells. Culturing history was believed to be a significant factor on the cells' behavior, according to data from former research [3], but this study was designed to the difference between the cell groups. This characterization study was done in T75 cell culture flasks to ensure that growth was occurring and to get a growth curve model. Simultaneously, the cells were analyzed for alkaline phosphatase levels. Once this model was achieved, further studies were carried out using only the most ideal group of cells to determine the proper method for testing the cells on the materials. This characterization will allow researchers to more effectively analyze the results of their materials testing studies, because they will understand behavior of the cells when they are grown under controlled conditions.

MATERIALS AND METHODS.

Cell groups.

This cell characterization study was performed using three groups of MC3T3-E1 pre-osteoblastic cells, each with different culturing histories. The first group, hereafter referred to as the "old" cells, was frozen for a year, and analyzed in this study at passage 10 since thawing. These cells had an unknown culturing history before the time they were frozen, and were the most widely used for materials testing in the lab. However, they had been proliferating slowly and inconsistently, which was not ideal for materials testing. The second group, hereafter referred to as the "thawed" cells, was also frozen for a year and had an unknown prior culturing history, but was analyzed in this study at passage two since thawing. These cells were thawed in the hopes of providing an alternative to the "old" cells. The final group, the "new" cells, was received fresh from the Vanderbilt Bone Center, had never been frozen, and was analyzed at passage three. These cells were obtained as another potential alternative to the "old" cells. In these descriptions, a passage is defined as any time the cells were split, and does not include when media was changed in the flasks or any other cell culture process.

Cell Culture.

The three groups of cells were cultured in T75 flasks following normal protocol for MC3T3-E1 cells. They were kept in αMEM media and at days 1, 3, 4, and 7, the media in the flasks was removed, the cells were washed with phosphate buffered saline (PBS) and fresh media was added to the flasks. The "old" cells were seeded at $3x10^5$ in four separate flasks, and the "new" cells were also seeded at this density in four flasks. The "thawed" cells were seeded at this density in only three flasks, due to lower than expected cell numbers the day of seeding. The 11 flasks were seeded on the same day, Day 0. At days 1, 3, 4, and 7, one flask from each group of cells was detached by adding trypsin to the cells and incubating them to remove the cells from the surface of the flask. Then, the cells were centrifuged, resuspended in media, and counted in a hemocytometer. This process was performed for each group of cells at each time point to obtain a growth curve for each group. The "thawed" cells were detached at Days 1, 4, and 7, not 1, 3, 4, and 7 as the other groups were.

Alkaline Phosphatase testing.

After being counted, the cells were centrifuged again, the media was removed, and Triton X was added to the cells. The cells in Triton X were then frozen at -80° C. Alkaline phosphatase levels at each time point for the different cell groups were analyzed using an alkaline phosphatase kit (Sigma Diagnostics Procedure No. 104). The cells were subjected to three cycles of freezing at -80° C for 30 minutes and then thawing in an incubator for 30 minutes to lyse the cell membranes. After lysing, 20 µL of the samples were plated in a 96 well plate with 100 µL of the alkaline phosphatase buffer, which contains 1.5 M Alkaline Buffer pH 10 (Sigma A9226), deionized water, Magnesium Chloride, and Alkaline Phosphatase Substrate (Sigma P4744). A Sigma alkaline phosphatase calibration curve was prepared and plated as well. After three hours, absorbance was read at 405 nm using a µQuant machine, and the samples were plated in the 96-well plate at the same time to ensure no unnecessary variability occurred. Results were normalized to a total protein measurement using the BCA protein assay kit. The absorbance readings of the calibration curves and the samples were used to calculate the amount of alkaline phosphatase at each time point.

RESULTS.

Culturing history affects MC3T3-E1 cell growth rates and patterns.

I analyzed the cell numbers recorded at each time point- Day 1, Day 3, Day 4, and Day 7- for the three groups of cells and modeled their growth rates. The growth curves showed a significant difference in the three groups. The "thawed" and "new" cells showed a growth curve characteristic of MC3T3-E1 preosteoblastic cells, with the numbers doubling each day to form an exponential growth trend (Figure 1). However, the "thawed" cells' numbers were lower at each time point than the "new" cells (Fig. 1). The "old" cells did not show this exponential curve, but instead had a linear trend, and the growth curve never showed a significant peak in cell growth (Figure 1). By the Day 7 time point, the cells' growth was leveling off, not increasing as the others had done, and at all of the time points, the cell numbers were much lower than the "new" or "thawed" cells.

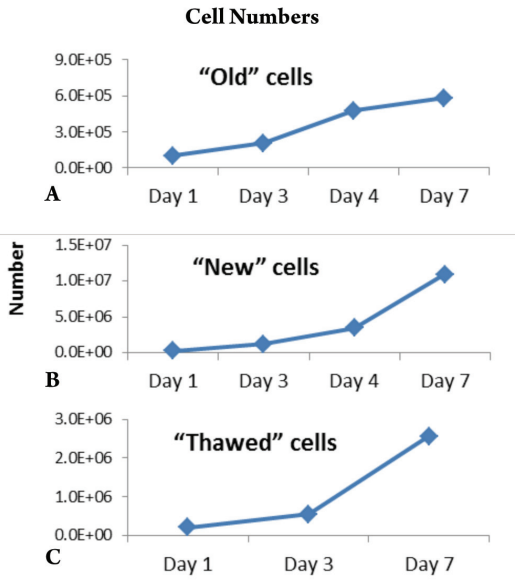

Figure 1. The cell numbers for each group of cells. Notice the "old" cells (**A**) have a linear growth rate and much lower numbers than the other groups. Also notice the "new" (**B**) and "thawed" (**C**) cells have an exponential trend, although the numbers at each time point are higher for the "new" cells.

Culturing history also affects alkaline phosphatase expression.

After reaching confluence, cells will begin to differentiate. Because alkaline phosphatase is an indicator of osteoblast differentiation, I expected to see a peak in alkaline phosphatase levels at or after the point when the cells reached confluence in the flasks. With the "new" and "thawed" cells, this trend was very evident. The "new" cells had a steady level of alkaline phosphatase at the Day 1 and Day 3 time points, and a major spike at the Day 4 time point which continued in the Day 7 time point (Figure 2). The "thawed" cells had a similar trend, with almost no expression at Days 1 and 3, and a spike before Day 7 (Figure 2). The "old" cells, however, did not have this trend. This group had very low levels at the first time point, Day 1, but the spike occurred before Day 3, and continued to increase slightly at Day 4 and Day 7 (Figure 2).

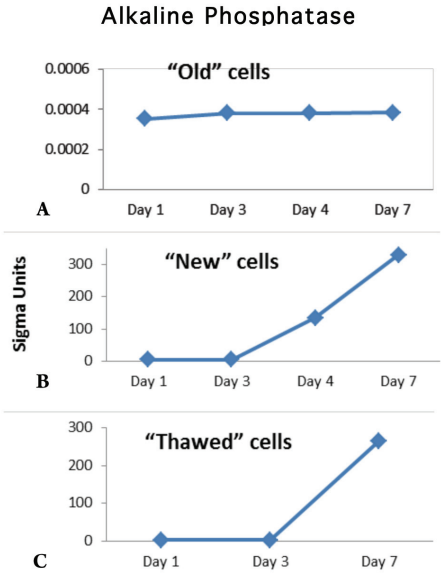

Figure 2. Alkaline phosphatase expression for each group of cells. Notice the "old" cells (**A**) have no change in expression, while both the "new" (**B**) and the "thawed" (**C**) cells have low levels of expression followed by a spike in alkaline phosphatase expression which corresponds to the point at which they reached confluency.

DISCUSSION.

"New" cells should be used for future testing and studies.

The "new" cells have the most consistent growth rates and alkaline phosphatase expression. These cells can be expected to grow at a continuous rate, and will be used for all future studies. The "thawed" cells grew similarly to the "new" cells, but without achieving the same level of cell numbers as the "new" cells. The "old" cells were shown to have grown even more slowly than expected, and results from materials testing in which these cells were used should be discredited until the results have been validated using the "new" cells.

MC3T3-E1 cells in good condition will proliferate consistently when grown on tissue culture plastic.

The "new" cells were shown to have a consistent growth rate when seeded and cultured in a T75 cell culture flask. This curve is exponential, and increases in a consistent manner at each time point measured. This growth curve can be compared to growth curves of the same cells on polyurethane-bone composites to test the efficacy of the biomaterials as a method of promoting bone regeneration.

MC3T3-E1 cells in good condition will also show expected levels of alkaline phosphatase expression when grown on tissue culture plastic.

The "new" cells and the "thawed" showed the expected alkaline phosphatase

expression. Because this is a marker of osteoblast differentiation, it is expected that the levels will peak as the cells reach confluency in the cell culture flask. These groups of cells did have the peak at the same time as they were reaching confluency, which is the expected behavior. The "old" cells had a peak at the beginning, and then the alkaline phosphatase expression levels plateaued, which leads to the conclusion that these cells had reached confluency and begun to differentiate at some previous point in their culturing history. Due to the "old" cell's linear growth rate and early peak in alkaline phosphatase levels, it can be concluded that these cells are no longer in the exponential growth phase of cell growth and are now in the linear growth phase.

These MC3T3-E1 cells should be split at or before day 3 or day 4 to maintain proper growth rates and alkaline phosphatase expression.

Because the cells' levels of alkaline phosphatase expression peaked at Day 3, and on this day they also reached confluence in the flask, we can conclude that the cells will begin to differentiate as soon as they reach confluence. To ensure the cells maintain proper growth rates and alkaline phosphatase expression in the future, the cells should be split before this point. The optimal density for splitting is at approximately 60% confluence, when the cells are nearing complete confluence, but have not yet begun to differentiate.

MC3T3-E1 cells are consistent enough to allow for comparisons between growth on tissue culture plastic and growth on polyurethane-bone composites.

Because the "new" cells have shown consistency in both growth rates and alkaline phosphatase expression, they can be compared to behavior on the composites. The same trends should still be evident when the cells are seeded on and cultured on the composites.

In conclusion, MC3T3-E1 cells when cultured under controlled conditions will have a consistent growth rate and levels of alkaline phosphatase expression. When properly cultured, these cells will show an exponential growth rate characteristic of these cells. Culturing history has a significant effect on the behavior of these cells. For future testing, the "new" cells should be used. The characterization of these cells will provide researchers with a tool to better understand their research and their results.

ACKNOWLEDGMENTS. We would like to acknowledge Dr. Jonathan Creamer and the School for Science and Math at Vanderbilt for support throughout the research process. We would also like to thank everyone in Dr. Scott Guelcher's lab and the Chemical and Biomolecular Engineering Department at Vanderbilt University.

REFERENCES.
1. A. Kumarasuriyar, *et. al.*, *Tissue Eng.* **11**, 1281 (2005).
2. D. Quarles, *et. al.*, *Endocrinology*. **128**, 3144 (1991).
3. C. Chung, *et. al.*, *Bchem. and Bphys. R. Comm.* **265**, 246 (1999).

Anna Claire Brakefield is a student at Hillsboro High School and enrolled in the School for Science and Math at Vanderbilt.