Comparison of the Osteogenic Potential of Mineral Trioxide Aggregate and Endosequence Root Repair Material in a 3-dimensional Culture System

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Abstract

Introduction: The ability to promote osteoblast differentiation is a desirable property of root-end filling materials. Several in vitro studies compare the cytotoxicity and physical properties between mineral trioxide aggregate (MTA) and Endosequence root repair material (ERRM), but not their osteogenic potential. Three-dimensional cultures allow cells to better maintain their physiological morphology and better resemble in vivo cellular response than 2-dimensional cultures. Here we examined the osteogenic potential of MTA and ERRM by using a commercially available 3-dimensional Alvetex scaffold. Methods: Mandibular osteoblasts were derived from 3-week-old male transgenic reporter mice where mature osteoblasts express green fluorescent protein (GFP) driven by a 2.3-kilobase type I collagen promoter (Col(I)−2.3). Mandibular osteoblasts were grown on Alvetex in direct contact with MTA, ERRM, or no material (negative control) for 14 days. Osteoblast differentiation was evaluated by expression levels of osteogenic genes by using quantitative polymerase chain reaction and by the spatial dynamics of Col(I)−2.3 GFP-positive mature osteoblasts within the Alvetex scaffolds by using 2-photon microscopy. Results: ERRM significantly increased alkaline phosphatase (Alp) and bone sialoprotein (Bsp) expression compared with MTA and negative control groups. Both MTA and ERRM increased osxterix (Osx) mRNA significantly compared with the negative control group. The percentage of Col(I)−2.3 GFP-positive cells over total cells within Alvetex was the highest in the ERRM group, followed by MTA and by negative controls. Conclusions: ERRM promotes osteoblast differentiation better than MTA and controls with no material in a 3-dimensional culture system. Alvetex scaffolds can be used to test endodontic materials. (J Endod 2016;42:760–765)

Key Words
Alvetex, Endosequence root repair material, mineral trioxide aggregate, osteoblast differentiation, 3-dimensional culture

Root-end filling materials should be minimally cytotoxic, bactericidal, or bacteriostatic, with good scaling ability, and be bioactive to promote bone/cementum formation. Mineral trioxide aggregate (MTA) has been the prototype for root-end filling materials because of its excellent biocompatibility, sealing capability, and ability to enhance cell proliferation and promote mineralization (1, 2). Endosequence root repair material (ERRM), a bioceramic material, is manufactured as a premixed material to allow easier handling than MTA. In vitro testing by using 2-dimensional (2D) cell cultures has shown comparable cytotoxicity and sealing ability between ERRM and MTA (3–6). However, few in vitro studies compare their osteogenic potential.

Endodontic materials are mostly tested by using 2D culturing techniques. Because data from in vitro studies are the basis to more expensive and time-consuming in vivo studies, it is significant to establish an in vitro culture model that can better mimic the physiological environment. Three-dimensional (3D) culturing systems allow cells to have morphologies more characteristic of cells in living tissues (7). Therefore, cells in 3D cultures maintain homeostasis more closely than those cultured in 2D systems (8–10). We chose to use the non-degradable 3D Alvetex scaffold (Reinervate Ltd, Durham, UK) in this study. It is a highly porous polystyrene scaffold with a thickness of 200 μm and pore sizes of 36–40 μm, allowing for efficient exchange of nutrients, gases, and waste products. It is chemically inert, easy to handle, and relatively inexpensive and can be conveniently analyzed by a variety of techniques (11).

We hypothesized that both MTA and ERRM will promote osteoblast differentiation in a 3D Alvetex culture system. To visualize mature osteoblasts, we used osteoprogenitors that express green fluorescent protein (GFP) only when they become mature osteoblasts or osteocytes. These cells were derived from transgenic mice where GFP was driven by a 2.3-kilobase type 1 collagen promoter (Col(I)−2.3 GFP) (12, 13). Using different promoter fragments for driving stage-specific transgenes has been
widely used to monitor cell lineage status in brain, gut, and bone (13–15). Although Col(I) is ubiquitously expressed, Col(I)-2.3 GFP is strongly correlated to osteoblast differentiation and is limited to mature osteoblasts capable of forming bone or osteocytes in vitro and in vivo (13, 16–18). We incorporated the use of 2-photon microscopy to appreciate the spatial dynamics of Col(I)-2.3 GFP-positive mature osteoblasts. Long excitation wavelengths of the 2-photon microscope allow for deep penetration and 3D visualization of cells within the scaffold in real time (19). The combination of a 3D culture model and labeled osteoblasts will allow us to more effectively evaluate the dynamics and maturation of cells.

**Materials and Methods**

**Primary Mouse Mandibular Osteoblasts Cultured in Alvetex and 2D Culture Plates**

The animal protocol was approved by the Institutional Animal Care and Use Committee of the University of Connecticut Health (Protocol number: 100782-1116). Mandibles from 3-week-old male mice expressing Col(I)-2.3 GFP reporter were enzymatically digested with 0.05% trypsin (Life Technologies Corporation, Norwalk, CT) and 0.15% type II collagenase (Life Technologies) for 4 cycles (20 minutes per cycle) at 37°C. Cells were first cultured in growth medium (Dulbecco modified Eagle medium containing 10% fetal bovine serum [Hyclone, Logan, UT]), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Life Technologies) for 5–7 days until confluent and were then maintained in differentiation medium (α-minimal essential medium containing 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin). Ascorbic acid (50 μg/mL) and β-glycerophosphate (4 mmol/L) (Sigma-Aldrich, St Louis, MO), required for osteoblast differentiation in 2D cultures, were omitted in a subgroup of the 3D cultures when testing the osteogenic potential of materials by using Alvetex scaffolds. Mouse mandibular osteoblasts (mOBs) were plated at a density of 10,000/cm² in 2D culture plates. For the 3D culture system, an Alvetex scaffold (Reinervative) was first wetted with 70% ethanol, followed by 2 washes with growth medium. Cells were plated at densities of 0.3 × 10⁵ cells/well and 2.5 × 10⁵ cells/well on 96-well and 24-well of Alvetex scaffolds, respectively.

**Fluorescent Microscopy and Imaging**

Col(I)-2.3 GFP cells in culture were imaged by using an Axiozoom MRc digital camera and Zen software (Zeiss, Oberkochen, Germany) with a Zeiss Observer Z.1 inverted microscope.

**Methyl-thiazol-tetrazolium Assay, Cell Counting, Alkaline Phosphatase Staining**

Cell viability was evaluated at days 1, 3, 5 and 12 after seeding mOBs on 96-well 2D culture and Alvetex plates by using a colorimetric methyl-thiazol-tetrazolium (MTT) assay (American Type Culture Collection [ATCC]) according to the manufacturer’s recommendations. The

![Figure 1](image_url)

**Figure 1.** Characterization of mOB cultures. (A) Col(I)-2.3 GFP expression increases during osteoblast differentiation. Fluorescence and bright field images of mOBs cultured for 0, 7, 14, and 21 days in differentiation medium. Mosaic images were composed of 12 images to display a larger view of cultures. Scale bar = 500 μm. (B) Expression of osteogenic genes in 2D cultures of mOBs for 7, 14, and 21 days in differentiation medium shown by qPCR. 18S serves as internal control. Data presented as mean ± standard deviation. *Significant difference by 1-way ANOVA (P < .05). (C) ALP staining at days 7, 14, and 21 in differentiation medium. Three wells per time point were shown.
570-nm absorbance was read in a microtiter plate reader (Safire2; Tecan, Männedorf, Switzerland). Results from technical triplicates of days 3, 5, and 12 were normalized to those of day 1 to represent the fold changes of viable cells. MTT assays were repeated 4 times. For cell counting, mOBs were grown in 24-well plates at a density of 20,000 cells/well. At each time point, cultures were trypsinized and counted by using a hemacytometer. Cell viability was determined by trypan blue dye exclusion. Alkaline phosphatase (ALP) staining was performed by using a commercially available ALP kit (Sigma-Aldrich) as described previously (20).

Preparation of Root-end Filling Materials

Materials, approximately 2 mm in thickness, were dispensed into 24-well plates. ERRM (Brasseler, Savannah, GA), a premixed putty, was directly condensed into the wells. ProRoot MTA (Dentsply Tulsa Dental Specialties, Tulsa, OK) was mixed with sterile water following the manufacturer’s recommendations. The MTA and ERRM were then incubated at 37°C with 5% CO2 for 48 hours to ensure adequate setting before the placement of Alvetex scaffolds with mOBs. Alvetex and mOBs in contact with the polystyrene culture plates without any dental materials served as negative controls.

RNA Analysis

Total RNA from cultured cells was prepared with TRIzol (Life Technologies), followed by treatment of DNaseI (Life Technologies). The cDNA was synthesized by using Superscript II reverse transcriptase (Life Technologies), and quantitative polymerase chain reaction (qPCR) was performed by using SYBR Green Supermix with ROX (Applied Biosystems, Foster City, CA) in an ABI-7300 instrument (Applied Biosystems). PCR efficiency was optimized and primer specificity was analyzed by using a melting curve analysis. Relative quantification of gene expression was determined by using the \( \Delta\Delta C_t \) method. Mouse 18S RNA served for normalization. Marker genes during osteoblast differentiation were analyzed, including alkaline phosphatase (Alp), bone sialoprotein (Bsp), and osterix (Osx). The melting temperature for all examined genes was 60°C. All qPCR experiments were performed in triplicate. The primer sequences are listed in Supplemental Table S1 (Supplemental Table S1 is available online at www.jendodon.com).

Two-Photon Imaging

Alvetex membranes with mOBs cultured on MTA, ERRM, or polystyrene for 14 days were fixed with 4% paraformaldehyde for 20 minutes. After permeabilization, the membranes were stained with FITC-conjugated secondary antibodies. The images were acquired using a two-photon laser scanning microscope (FV1000, Olympus, Japan).

### Table 1. Quantitative PCR Assays of mOBs Grown in Alvetex and in Contact with Different Materials for 14 Days

<table>
<thead>
<tr>
<th></th>
<th>Alp</th>
<th>Bsp</th>
<th>Osx</th>
</tr>
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<tbody>
<tr>
<td>Ctl (+), with AA and β-GP</td>
<td>0.81 ± 0.3</td>
<td>0.97 ± 0.29</td>
<td>1.52 ± 0.4</td>
</tr>
<tr>
<td>Ctl (-), without AA and β-GP</td>
<td>1.37 ± 0.99</td>
<td>1.04 ± 0.28</td>
<td>0.99 ± 0.46</td>
</tr>
<tr>
<td>MTA, without AA and β-GP</td>
<td>0.96 ± 0.24</td>
<td>1.09 ± 0.23</td>
<td>15.09 ± 14.23*</td>
</tr>
<tr>
<td>ERRM, without AA and β-GP</td>
<td>8.2 ± 6.87*</td>
<td>1.73 ± 0.08*</td>
<td>19.69 ± 2.58*</td>
</tr>
</tbody>
</table>

AA, ascorbic acid; β-GP, β-glycerophosphate.

Quantitative PCR data were normalized to housekeeping gene 18S. Fold differences were compared with negative control groups (Ctl (-)). Ctl (+), positive control; Ctl (-), negative control. Data are presented as mean ± standard deviation. Statistical analysis: 1-way ANOVA.

*P < .05.
10 minutes, and nuclei were stained with Hoechst 33342 (Life Technologies). Images were obtained by using a 2-photon microscope (Ultima IV; Prairie Technologies, Middleton, WI) with a water immersion lens (XLUMPlanFL 20x/0.95 W; Olympus, Tokyo, Japan) in phosphate-buffered saline as previously described (19). Z-stacks were obtained in 1-μm increments for approximately 100 μm into Alvetex scaffolds directly under the control of the acquisition software (Prairie Viewer; Prairie Technologies). Reconstruction and quantification assays were completed by using Fiji software. Osteoblast differentiation was evaluated by the percentage of GFP-positive cells over total cells stained with Hoechst 33342 (Supplemental Fig. S1). (Supplemental Figure S1 is available online at www.jendodon.com.) Images were taken from 3 spots of each membrane and 3 Alvetex membranes of each group.

Statistical Analysis

One-way and 2-way analysis of variance (ANOVA) in Prism software (GraphPad Software, La Jolla, CA) was used to conduct statistical analysis. A P value <.05 is considered statistically significant.

Results

Characterization of mOB Cultures

We first characterized osteoprogenitors isolated from mouse mandibles in a traditional 2D culture system before using these cells to test the osteogenic potential of root-end filling materials. The mOB cultures showed increased numbers of Col(I)-2.3 GFP cells when cultured in differentiation medium supplemented with ascorbic acid and β-glycerophosphate for 0, 7, 14, and 21 days (Fig. 1A). Increasing expression of osteogenic markers (Alp, Bsp, Osx) and ALP staining were observed as osteoblasts differentiated (Fig. 1B and C).

Osteoblast Growth and Differentiation on 3D Alvetex Culture System

We next examined whether mOBs can grow and differentiate in 3D Alvetex cultures. MTT assays showed increasing numbers of viable mOBs in 2D and Alvetex cultures grown for 1–12 days (Fig. 2A, Supplemental Fig. S2). The results of MTT assays correspond to the data from cell counting experiments (Supplemental Fig. S3). Interestingly, we detected significantly higher levels of Alp, Bsp, and Osx in Alvetex cultures than in traditional 2D cultures when cultured for 7, 14, and 21 days in differentiation medium supplemented with ascorbic acid and β-glycerophosphate (Fig. 2B). (Supplemental Figures S2 and S3 are available online at www.jendodon.com.)

To determine whether the 3D culture model alone could induce osteoblast differentiation, mOBs were grown in Alvetex scaffolds and cultured in differentiation medium with or without ascorbic acid and β-glycerophosphate. If ascorbic acid and β-glycerophosphate are...
needed for osteoblast differentiation in a 3D culture system, we would expect significant differences in the expression of osteogenic marker genes in treated cells. Surprisingly, we observed no significant increase in the gene expression of Alp, Bsp, and Osx between treated (positive control) and untreated cells (negative control) (Table 1).

**ERRM and MTA Increase Osteogenic Genes and Col(II)-2.3 GFP Expression in mOBs on Alvetex**

To better evaluate the osteogenic potential of ERRM or MTA, we decided not to add ascorbic acid and β-glycerophosphate, which are known to promote osteoblast differentiation in 2D cultures (21). We decided to examine osteoblast differentiation 14 days after culturing on Alvetex because Col(I)-2.3 GFP and osteogenic marker genes were reasonably expressed at this time point as shown in Figures 1 and 2. ERRM significantly increased gene expression of Alp, Bsp, and Osx, whereas MTA significantly increased gene expression of Osx in comparison with the negative control (no material) group (Table 1). The mRNA levels of Alp and Bsp were significantly higher in the ERRM samples compared with MTA (Table 1).

Osteoblast differentiation was also evaluated by the percentage of cells expressing Col(I)-2.3 GFP on Alvetex cultures on the basis of 2-photon images. Static images and videos of 2-photon microscopy of negative control, MTA, and ERRM groups are shown in Figure 3A and Supplemental Videos S1 and S2, respectively. Quantification of total cell numbers and the percentages of Col(I)-2.3 GFP-positive cells over total cells indicating the percentage of cells undergoing maturation are shown in Figure 3B.

The negative control group showed more total cells at almost every level of the scaffold when compared with the experimental groups; however, there was no Col(I)-2.3 GFP expression noted beyond 10 μm into the scaffold. Despite less total cell numbers in the ERRM group, the percentage of Col(I)-2.3 GFP-positive cells was significantly higher in the negative control group at every penetration depth. The MTA group showed less amounts of total cells than the ERRM group; however, the percentage of Col(I)-2.3 GFP-positive cells was higher than in the negative control group.

**Discussion**

2D cultures have demonstrated that ascorbic acid and β-glycerophosphate are required to induce in vitro osteoblast differentiation. Ascorbic acid plays an important role in the formation of the extracellular matrix and the induction of dose-dependent collagen synthesis (21). Beta-glycerophosphate is essential for osteogenesis and specifically for mineralization in vitro and therefore is directly linked to ALP activity (21). Overall, our data suggest that the 3D Alvetex cultures, with or without these 2 reagents, lead to a more robust gene expression of osteogenic markers when compared with 2D cultures. Because the Alvetex scaffold is composed of polystyrene, which is the material of 2D cell culture plates, differences in results between 2D cultures and Alvetex can be attributed to differences in dimension of culture plates. Therefore, it is possible that a 3D culture system better mimics the physiological condition and allows spontaneous differentiation of osteoblasts.

Root-end filling materials are mostly tested by culturing cells directly on materials or in medium containing cement elutes. However, components in cement elutes are often unknown. Scanning electron microscopy (SEM) is often used to evaluate cell proliferation and morphology in studies culturing cells on materials (22). Calcium phosphate crystals caused by chemical reactions between MTA and SEM preparation media may lead to distorted SEM images (23). Our 3D culture system mimics the clinical setting better because osteoblasts cultured in the medium do not directly contact the root-end filling materials but were attracted toward the materials. Clinically, root-end filling materials contact the tissue fluids but not osteoblasts in the periapical lesions. In addition, the combination of several techniques makes this Alvetex culture system attractive. Alvetex scaffolds can be easily removed from the material and analyzed by a variety of methods, including histology, microscopy, and RNA or protein analysis. The use of transgenic Col(I)-2.3 GFP mOBs gives us the capability to visualize the maturation of osteoblasts. Rather than SEM or histology, 2-photon microscopy is ideal because of its ability to image scaffolds as an entire volume. The long excitation wavelengths allow for deeper penetration than conventional and confocal fluorescent microscopy (24). This allowed us to evaluate the migration and differentiation patterns of mOBs in contact with various root-end filling materials. Therefore, Alvetex 3D cultures are more convenient and physiologically representative model for testing materials.

MTA has been the gold standard of modern root-end filling materials. Bioceramics are relatively new materials and are much less studied. Several in vitro studies have shown that MTA and ERRM are comparable in their biocompatibility (4–6, 25). Modareszadeh et al (26) showed that cell viability and ALP activity were significantly increased in human osteoblast-like cells in contact with cement eluates from MTA than ERRM. We showed that ERRM increased osteoblast differentiation more than MTA by increased Col(I)-2.3 GFP-positive cell numbers and expression levels of Alp and Bsp. Differences between the results of that study and our study may be because of the different culture systems (2D versus 3D), different cell types (Saos-2 osteoblast-like cells versus primary mouse pre-osteoblastic cells), and different concentrations of cell elutes of MTA and ERRM. On the other hand, our findings are consistent with an in vitro study examining the hard tissue healing in periapical lesions of roots retrofilled with MTA and ERRM in dogs (27). Healing occurs in both MTA and ERRM groups; however, ERRM increased the formation of cementum-like, periodontal ligament–like, and bone tissue when compared with MTA (27).

Our 2-photon volumetric images indicate that ERRM and MTA were able to promote greater mOB differentiation despite having lower cell numbers than the negative control. The increased number of mOBs coupled with the low percentage of Col(I)-2.3 GFP-positive cells in the negative control group suggests that less cells undergo differentiation. On the other hand, more Col(I)-2.3 GFP-positive cells in ERRM and MTA groups suggest that these materials induce osteoprogenitors to differentiate to mature osteoblasts. Further studies will be needed to determine whether decreased cell numbers are also a result of changes in cell proliferation or apoptosis caused by MTA and ERRM. We found that Alvetex scaffolds in the MTA group have the least cell numbers and were more difficult to image because the scaffolds appeared discolored. Tooth discoloration caused by ProRoot MTA had previously been reported (28–30).

Alvetex can be used to test a wide range of dental and medical biomaterials and different types of cellular activities. Ultimately, we hope to apply this culture system to test dental material with patient-specific induced pluripotent stem cells to identify the best material for individuals. Taken together, we have isolated mouse mandibular osteoblasts and verified the use of Alvetex as a simple 3D culture system for testing the osteogenic potential of endodontic materials. ERRM promotes more osteoblast differentiation in a 3D culture system and may potentially contribute to better bone formation in vitro.

**Acknowledgments**

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Supplementary Material

Supplementary material associated with this article can be found in the online version at www.jendodon.com (http://dx.doi.org/10.1016/j.joen.2016.02.001).

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