Effect of Bioaggregate on Mineral-associated Gene Expression in Osteoblast Cells

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Abstract

Introduction: This study investigated the cytotoxicity of bioaggregate (BA) and the effect of BA on mineral associated gene expression in osteoblast cells. **Methods:** The cytotoxicity of BA to mouse MC3T3-E1 osteoblast cells was evaluated via the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay after 1, 2, and 3 days of culture. The expression of mineral associated genes (collagen type 1, osteocalcin, and osteopontin) was assessed by quantitative real-time polymerase chain reaction (qRT-PCR) and compared expression after exposure to BA or mineral trioxide aggregate (MTA). The data were analyzed by one-way analysis of variance and the Tukey test. **Results:** BA was essentially nontoxic to osteoblast cells. The expression of collagen type 1, osteocalcin, and osteopontin genes significantly increased in the BA group compared with that in the MTA group on the second or third day of culture. **Conclusion:** BA appears to be a novel nontoxic root-end filling biomaterial and be able to induce mineralization-associated gene expression in osteoblast cells. *(J Endod 2010;36:1145–1148)*

Key Words

Bioaggregate, cytotoxicity, mineral trioxide aggregate, mineralization, osteoblast cells, real-time polymerase chain reaction

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To achieve a satisfactory endodontic therapy, an ideal root-end filling material has to meet the following requirements: biocompatibility with normal tissues, high sealing ability, desirable ability of periapical tissue regeneration, effective inhibition of pathogenic microorganisms, sufficient radiopacity to distinguish the material from surrounding tissue, and excellent workability and handing properties (1-3). Mineral trioxide aggregate (MTA) (Dentsply, Tulsa, OK), which was first introduced as a root-end filling material by Torabinejad et al (4) in 1993, has been widely used in endodontic therapy (5). Compared with other root-end filling materials, MTA displays significant advantages in terms of its biocompatibility and osteoconduction ability (1, 6-9). Now MTA is considered as gold standard repair material for several clinical applications including the repair of perforation, root-end filling, the repair of root resorption, apexification, and pulp capping.

Bioaggregate (BA) (Innovative Bioceramix, Vancouver, BC, Canada), a white nanoparticle ceramic cement, is a novel root-end filling material composed primarily of calcium silicate, calcium hydroxide, and hydroxyapatite (10). De-Deus et al (11) found that BA displayed similar biocompatibility to that seen for MTA when cultured with primary human mesenchymal cells. However, there have not yet been any reports on potential cytotoxicity of BA on osteoblast cells or BA's effects on mineralization-associated gene expression in these cells.

The purpose of the current study was to test the following hypotheses: (1) BA has the same cytotoxicity to osteoblasts as MTA, and (2) BA has the equal potential to MTA to promote the mineral-associated gene expression including collagen type 1 (*col1*), osteocalcin (*ocn*), and osteopontin (*opn*) in osteoblasts.

Materials and Methods

Cell Culture

MC3T3-E1 cell line (American Type Culture Collection) was cultured in Dulbecco modified Eagle medium (DMEM, Hyclone Thermo Scientific, Logan, UT) containing 10% fetal bovine serum (FBS, Hyclone), 100 U/mL of penicillin, and 100 μg/mL of streptomycin, and the medium was refreshed every 3 days. Hereby the medium was termed as "growth medium." For mineralization experiments, 50 μg/mL of ascorbic acid (Sigma-Aldrich, St Louis, MO) and 10 mmol/L β-glycerophosphate (Sigma-Aldrich) were added to the culture medium as described previously (12–14). Hereby the medium was termed as "osteogenic medium."

Preparation of BA and MTA

BA and MTA were prepared according to manufacturer's instruction under sterile conditions. The materials were packed in paraffin wax molds (diameter, 3 mm; height, 1 mm) to solidify at 37°C, 96% humidity for 4 hours before exposure to MC3T3-E1 cells.

Cell Inoculation

Cells were trypsinized with 0.25% Trypsin and 1 g/L EDTA (Hyclone Thermo Scientific), and the cell density was determined by hemocytometry. For MTT experiments, the cells were seeded on 96-well plates at a density of 1,000 cells per well in 100 μL of growth medium. After 24 hours of incubation, cells were subjected to three different treatments in three groups: negative control group, MTA group, and BA group. In

the MTA group or BA group, each specimen was directly supplemented into respective groups. On the first, second, and third day, the cells were collected for MTT assay to test the cytotoxicity of BA or MTA to MC3T3-E1. To assess the effect of BA on mineralization of MC3T3-E1 cells, the cells were seeded on a 24-well plate after the passage of the cells to a density of 5×10^4 cells per well and incubated in 1 mL of osteogenic medium. On the first, second, and third day after incubation, cells were collected to examine mineralization-associated gene expression via quantitative real-time polymerase chain reaction (qRT-PCR).

MTT Assay

The cytotoxicity of BA to MC3T3-E1 osteoblast cells was assessed using an MTT Cell Proliferation Kit (Roche Applied Science, Indianapolis, IN). Briefly, three groups of cells were incubated at 37°C, 5% CO_2 for 1, 2, and 3 dayrs after cell inoculation as described previously. A 50-μL volume of MTT working solution was added to each well, and the mixture was incubated for another 4 hours. Purple crystal formazan around cells was observed under 40× magnification with microscopy. The cell medium was carefully removed, and then 100 μL of dimethylsulfoxide was added to each well to dissolve the formazan. After a further 15 minutes of incubation at 37°C to completely dissolve the formazan, the absorbance at 490 nm was measured on an enzyme-linked immunosorbent assay plate reader, and the results were expressed as optical density values.

qRT-PCR Experiment

On the first, second, and third day after cell incubation, the cells from each group were homogenized with Trizol Reagent (Invitrogen, Carlsbad, CA), and chloroform was added to the mixture. This mixture was centrifuged at 12,000*g* for 15 minutes at 4°C to partition RNA into the upper aqueous phase. The RNA was precipitated from the aqueous phase with isopropyl alcohol and then centrifuged at 12,000*g* for 10 minutes at 4°C. The supernatant was removed, and the RNA pellet was washed once with 75% ethanol followed by vortexing the sample and centrifuging at 7,500*g* for 5 minutes at 4°C. The RNA was dissolved in RNase-free water and stored at −80°C. Reverse-transcriptase PCR with a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Dalian, China) generated complementary DNA from the extracted RNA. Mineralization-related gene expression was evaluated by determining the expression of several specific marker genes: *col1, ocn,* and *opn*. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) gene was used as a control gene for normalization of RNA expression. The qRT-PCR was performed with a SYBR Green PCR kit (Invitrogen) on the ABI PRISM7500 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). Each sample was tested in triplicate. The data for gene expression were analyzed by the $\triangle\triangle$Ct method as described previously (15).

Statistical Analysis

Statistical significance was analyzed by one-way analysis of variance followed by a Tukey test. The data were expressed by mean ± standard deviation, and $p < 0.05$ was considered as statistically significant.

Results

MTT Experiment Analysis

As shown in Figure 1*A*, there was no statistical difference in cell viability or proliferation between the BA group, the MTA group, and the control group throughout the culture period.

Gene Expression Analysis

Col1. The *col1* expression level dectlined in both MTA and BA groups compared with the control group on the first day. However, gene expression recovered to the control level in both the BA and MTA groups on the second and third day. In addition, the *col1* expression in the BA group increased significantly compared with the MTA group (Fig. 1*B*).

Ocn. *Ocn* expression was substantially suppressed by BA or MTA on the first day compared with the control group. On the second day, the *ocn* expression level raised in the BA group but remained inhibited in the MTA group compared with the control group. By the third day, there were no longer any significant differences among the three groups (Fig. 1*C*).

Opn. *Opn* expression in the MTA and BA groups decreased significantly compared with the control group on the first day. By the second day, *opn* expression was similar among all groups. By the third day, *opn* expression in the BA group was clearly enhanced compared with the other two groups (Fig. 1*D*).

Discussion

In the present stud, BA had no cytotoxicity to MC3T3-E1 osteoblast cells, which was similar to that shown for MTA. Several previous reports comparing the cytotoxicity of MTA with other root-end filling materials have shown that MTA is not cytotoxic to MC3T3-E1 osteoblast cells (7, 16). MTA is primarily composed of calcium silicate, calcium aluminate, and calcium hydroxide. Calcium ions are released from hydrated MTA when MTA powder is mixed with water (13). The amount of calcium released has been shown to be adequate to support the proliferation and survival of osteoblast cells (17, 18). The chemical component of BA, consisting of calcium silicate, calcium hydroxide, and hydroxyapatite, is similar to that of MTA. Therefore, calcium ion released from BA may also play an important role in osteoblast cell survival.

To evaluate the effect of BA on activities of osteoblast, expression of *col 1, ocn, and opn* were measured by the method of qRT-PCR in the presence of BA. This method is a technology to monitor gene expression over time, and it is considered as the gold standard in the field of gene detection because of its sensitivity, precise gene quantification, real-time character, and time-saving characteristic in comparison to other methods such as Northern blotting, Southern blotting, and RNase protection assays (19).

The *col1* gene codes for a collagenous protein that is one of the major components of the extracellular matrix of osteoblasts. Osteocalcin, coded by *ocn,* is another noncollagenous protein that is expressed in the extracellular matrix during osteoblastic mineralization. Osteopontin, coded by *opn*, is an organic component of bone and also plays an important role in extracellular matrix mineralization (20).

In general, in the present study, both BA and MTA were found to induce osteogenic gene expression (*colI, ocn,* and *opn*). The expression level of these three genes was the lowest on the first day. It may be explained by the pH changes of the both materials during setting. When BA or MTA was mixed with sterile water, the pH of the medium increased from 7.2 to approximately 10.2 to 12.5 within 24 hours; this pH change may have an inhibitory influence on gene expression on day 1 (10, 21–23).

In the present study, BA clearly enhanced *col1* expression in osteoblast cells on the second and third day over the level induced by MTA. *Ocn* expression was inhibited by MTA, whereas it was substantially activated by BA on the second day. BA dramatically induced *opn* expression on the third day over that seen in the other two groups. The BA showed significant higher expression of the three genes compared with MTA in

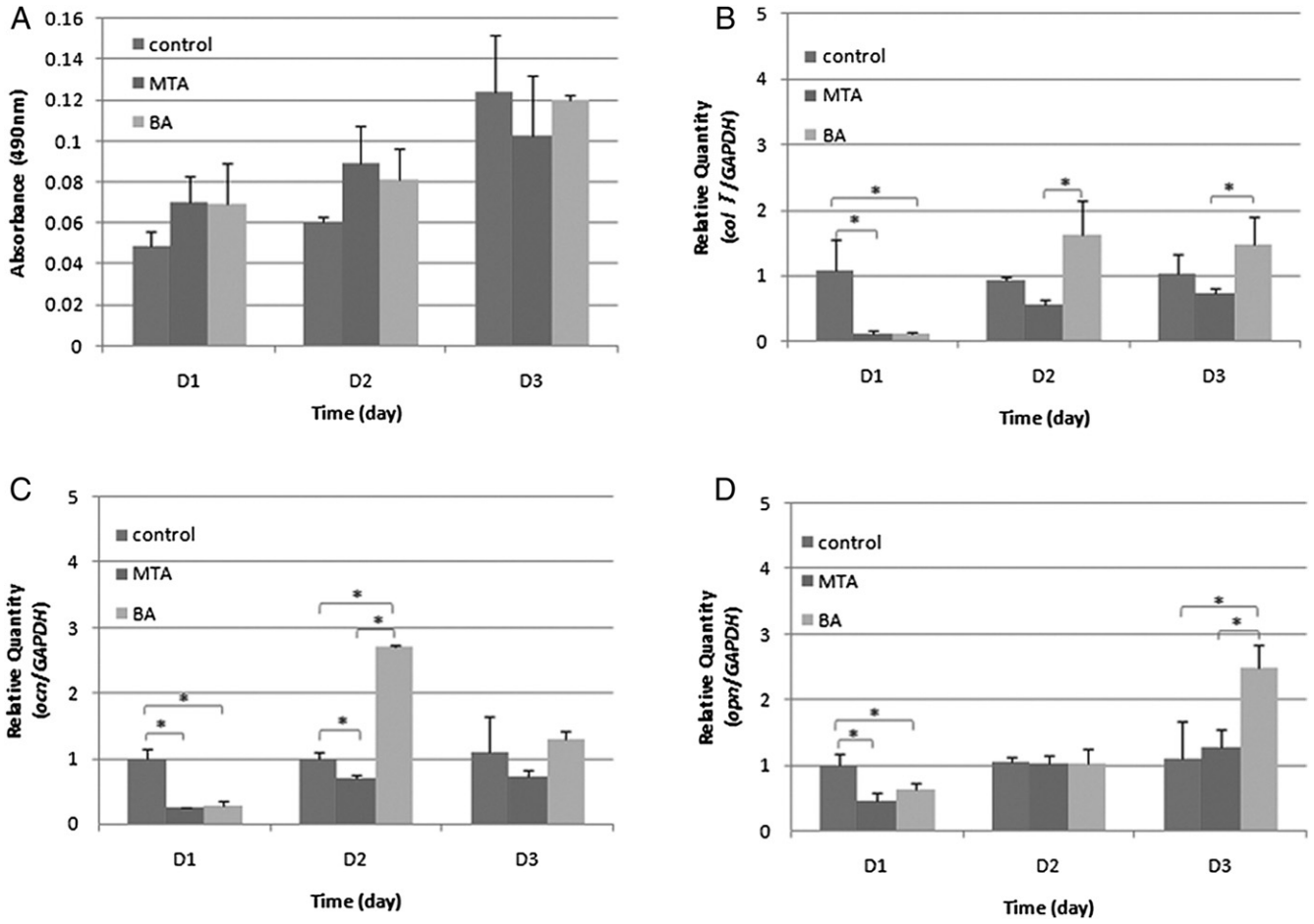

Figure 1. (*A*) The effect of BA and MTA on cell survival assayed by MTT. Results are expressed as the mean of optical density values at 490 nm (mean ± standard deviation). Groups identified by the same day are not significantly different ($p > 0.05$). (*B*) The relative expression of *col1* gene normalized against a housekeeping gene (*GAPDH*). (*C*) The relative expression of the *ocn* gene normalized against a housekeeping gene (*GAPDH*). (*D*) The relative expression of the *opn* gene normalized against a housekeeping gene (*GAPDH*). *$p < 0.05$ was considered as statistically significant.

the different day. These results quite possibly reflect the different compositions of the two aggregates. Comparing BA with MTA, hydroxyapatite was the main component in BA. Hydroxyapatite has been previously shown to enhance *col1* expression level in MC3T3-E1 cells (24, 25); Lin et al (26) found that hydroxyapatite could prominently up-regulate *col1* expression in osteoblast-like cells. Hydroxyapatite has also been shown to enhance *ocn* expression in MC3T3-E1 cells (27-30). Similarly, Chou et al (31) and Whited et al (32) reported that *opn* expression in MC3T3-E1 was significantly enhanced 3 days after the cells were seeded on a hydroxyapatite-coated scaffold, again consistent with the results presented here for osteoblasts in culture. So this component of hydroxyapatite, available in BA but not in MTA, probably contributed to increased of *col1, ocn*, and *opn* expression in osteoblasts. Meanwhile, the different effects of BA and MTA on gene expression may be related to the differences on the speed of ion release in the two aggregates during the culture period. Pharmacokinetic studies on BA and MTA are therefore required to characterize the ions released from BA or MTA.

The definite mechanism by which BA influences mineralization-related gene expression is not well understood. However, the available data now suggest that BA has an ability to enhance expression of genes involved in the mineralization of osteoblast cells. This effect may be because of the presence of hydroxyapatite in the aggregate.

Conclusion

BA was shown to be a nontoxic root-end filling material that could induce expression of genes associated with mineralization in osteoblast cells.

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