Biodentine Induces Immortalized Murine Pulp Cell Differentiation into Odontoblast-like Cells and Stimulates Biomineralization

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Abstract

Introduction: Biodentine (Septodont, Saint Maur des Faussés, France), a new tricalcium silicate-based cement, has recently been commercialized and advertised as a bioactive material. Its clinical application and physical properties have been widely described, but, so far, its bioactivity and biological effect on pulp cells have not been clearly shown. Thus, the aim of this study was to evaluate the biological effect of Biodentine on immortalized murine pulp cells (OD-21). **Methods:** OD-21 cells were cultured with or without Biodentine. Cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay after 2, 3, and 5 days of stimulation. The expression of several biomolecular markers was analyzed to screen differentiation pathways, both on a gene level with Real-time reverse transcription polymerase chain reaction and on a protein level by measuring alkaline phosphatase activity. Alizarin red staining was used to assess and quantify biomineralization. **Results:** The expression patterns of several genes confirmed the differentiation of OD-21 cells into odontoblasts during the period of cell culture. Our results suggest that Biodentine is bioactive because it increased OD-21 cell proliferation and biomineralization in comparison with controls. **Conclusions:** Because of its bioactivity, Biodentine can be considered as a suitable material for clinical indications of dentin-pulp complex regeneration, such as direct pulp capping. *(J Endod 2012;38:1220–1226)*

Key Words

Bioactivity, Biodentine, biomineralization, odontoblast differentiation

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For many years, the physiopathology of the dentin-pulp complex has been widely investigated (1). Three types of dentinogenesis are commonly described in the literature, primary, secondary, and tertiary (2), although tertiary dentinogenesis only occurs under pathological conditions (3).

Under severe pulp exposure resulting in the destruction of the underlying odontoblast layer, the dentin-pulp complex regenerates through progenitor cell recruitment and differentiation into secreting cells and the stimulation of reparative dentinogenesis (3). The main objective of this healing process is to form a barrier of mineralized tissue to protect the underlying pulp from bacterial or toxin leakage (4). Clinically, the objectives of treatments such as direct pulp capping, Cvek pulpotomy, or the stepwise technique are to seal the pulp wound, induce odontoblast-like cell differentiation, and stimulate dentin secretion and mineralization in order to build a dentin bridge (5).

To improve the clinical outcome, knowledge of the molecular mechanisms involved in pulp healing must be better understood, and new biomaterials should be developed based on these results. Several aspects of the molecular mechanisms, including the progenitor cells and the odontoblast differentiation pathways, are still unclear. Many published articles stress that a parallel exists between the initial development and tissue regeneration, especially in the regeneration of the dentin-pulp complex (6, 7). Runx2 is required for tooth development, especially for odontoblast differentiation, which are neural crest–derived cells (8). Runx2-deficient mice show impaired tooth formation in the cap/early bell stage (9). Runx2 is also greatly implicated in osteoblast differentiation but with an inverse time-dependent expression (Fig. 1) (10). In summary, Runx2 determines the lineage of osteoblasts and odontoblasts from mesenchymal cells (11, 12). Furthermore, Msx2 regulates osteoblast/odontoblast lineage differentiation by repressing the expression of markers of terminal differentiation, such as Runx2 for the osteoblast lineage and osteocalcin (OC) for the odontoblast lineage (13, 14). The analysis of the expression and regulation of these genes and several other genes reveals the stage of differentiation of specific cells.

The second field of research in dentin-pulp complex regeneration is the bioactivity of biomaterials. An ideal biomaterial should stimulate and modulate the healing process to properly seal the pulp wound to prevent bacterial leakage (15). So far, several materials have been investigated for their potential to stimulate tertiary dentinogenesis. Calcium hydroxide has been the gold standard as a pulp-capping material for a long time. Histologically, newly secreted dentin is generally porous according to Horsted et al (16), and the gap between this barrier and the dentinal wall is considered responsible for bacterial leakage, leading to pulp inflammation and/or necrosis. For these reasons, among others, the clinical results remain inconsistent (4).

Mineral trioxide aggregate (MTA), which was first introduced by Torabinejad in 1993 as a root-end filling material for endodontic surgery, is today indicated in many clinical settings. The benefits of MTA as a pulp-capping material have been shown in several animal models such as rhesus monkeys (17), dogs (18), rats (19), and mice (20). Nair et al (21) later confirmed these interesting preliminary results in humans and stressed that based on the results of their randomized clinical study, MTA should be considered the new gold standard for pulp-capping treatment (22, 23). Biodentine (Septodont), a new tricalcium silicate–based cement, has recently been commercialized and advertised as a bioactive material and pulp-capping agent. Its

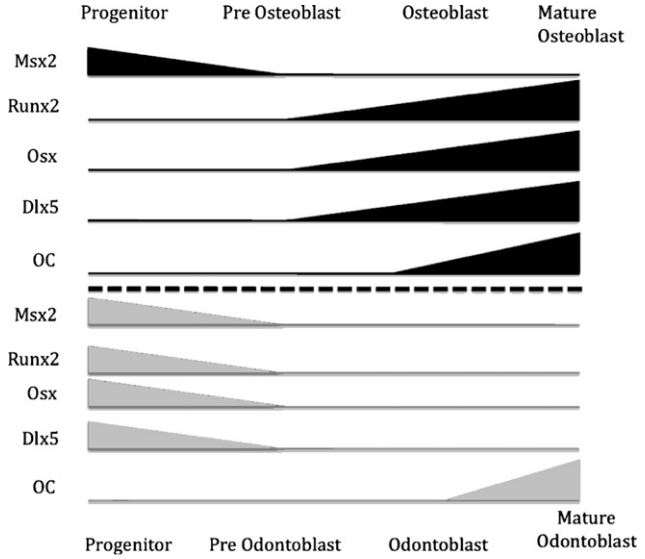

Figure 1. Expression patterns of several genes during osteoblast (*in gray*) and odontoblast (*in black*) differentiation (summarized from the literature [10–14]).

clinical application and physical properties have been widely described although so far its bioactivity and biological effect have not been clearly shown. This new material was initially developed as a restorative material. The 2 main benefits for Biodentine over other products are the reduced setting time (a few minutes compared with several hours for MTA) and better mechanical properties. Moreover, its sealing ability, when in contact with dentin, has been confirmed *in vitro* with a very low silver nitrate penetration (24).

Because it contains tricalcium silicate, the Septodont Company stated that Biodentine should be bioactive. However, this bioactivity has not been clearly shown. Thus, the aim of this work was to investigate the biological effects of Biodentine on immortalized murine pulp cells (OD-21).

Materials and Methods

Cell Culture

Immortalized murine pulp cells (OD-21) (25), including controls, were cultured in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), penicillin-streptomycin (50 UI/mL; Gibco, Paris, France), ascorbic acid (50 μg/mL), and β-glycerophosphate (10 mmol/mL) in a 37°C humidified incubator in an atmosphere of 5% CO_2.

Biomaterial

Premixed Biodentine was prepared according to the manufacturer's instructions (ie, powdered and sterilized by dry heat in an oven at 180°C for 160 minutes). Various concentrations (1, 2, and 4 mg/mL) were first tested to determine the dose effect on cell viability. Based on the first data, the 1-mg/mL concentration was finally retained for the following experiments. OD-21 cells were seeded at a concentration of 30,000 cells/mL in 6-well plates and grown for 4 days until they reached 70% confluence. Then, 1 mg/mL Biodentine was added to the culture medium (Fig. 2). The time of stimulation was noted as S0 (day 4 of experiments). All experiments were run in triplicates. Untreated cells served as controls.

MTS Cell Viability Assay

Cell viability was assessed at S2, S3, and S5 with the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI). This assay quantifies the production of formazan in surviving cells, which are capable of reducing the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) tetrazolium compound in the mitochondria. The activity of the cells is proportional to the amount of reduced product quantified by measuring the optical density of the cell supernatant with a spectrophotometer (Precision Microplate Reader; Molecular Devices, Sunnyvale, CA) at 490 nm.

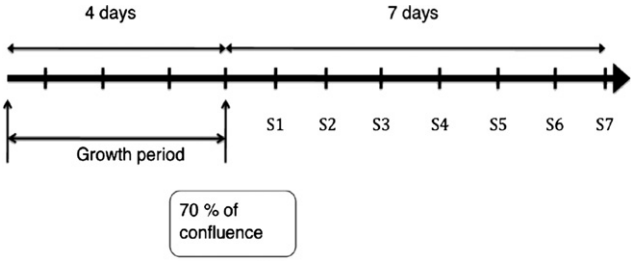

Figure 2. OD-21 cells were seeded at a concentration of 30,000 cells/mL in 6-well plates and cultured for 4 days in Dulbecco modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), penicillin-streptomycin (50 UI/mL, Gibco), ascorbic acid (50 μg/mL), and β-glycerophosphate (10 mmol/mL) in a 37°C humidified incubator in an atmosphere of 5% CO_2. Stimulation started on day 4 (S0) by adding 1 mg/mL preset Biodentine to the culture medium.

Alkaline Phosphatase Activity Assay

The protein content was estimated with the bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). The specific alkaline phosphatase (ALP) activity was assayed in cell layers by the release of p-nitrophenol from p-nitrophenolphosphate (Sigma-Aldrich, Lyon, France). The optical density was read at 410 nm with a spectrophotometer, and the enzyme activity was expressed as UI ALP/μg of protein.

Evaluation of Gene Expression by Real-Time RT-PCR

OD-21 cells were collected at S1, S2, S3, S5, and S7, and the total RNA was extracted using the TriReagent kit (Euromedex, Souffelweyersheim, France) according to the manufacturer's instructions. One microgram of total RNA of each sample was reverse transcribed into complementary DNA using 200 U Superscript II (Invitrogen) and 250 ng random primers.

Real-time reverse-transcription polymerase chain reactions (RT-PCRs) were performed with an aliquot of 1/60th of reverse transcription reaction using a MiniOpticon Real-Time RT-PCR Detection System and SYBRGreen I kit (Bio-Rad Laboratory, Hercules, CA) under the following conditions: 95°C for 10 seconds as the denaturation program followed by 40 amplification cycles (95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 45 seconds). Finally, the temperature was gradually increased from 56°C to 95°C to ensure the specificity of the reaction. The gene expression levels of Msx2, RUNX2, Osx, Dlx5, collagen I (Coll I), ALP, and OC were normalized to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1).

Quantitative Analysis of Alizarin Red Staining

Cells were fixed in 4% (w/v) paraformaldehyde for 15 minutes at room temperature and stained for 20 minutes with 2% (w/v) alizarin red stain (ARS) solution (Sigma-Aldrich) at a pH level of 4.2 under gentle agitation. The cells were washed 3 times with distilled water