EDTA Treatment for Sodium Hypochlorite–treated Dentin Recovers Disturbed Attachment and Induces Differentiation of Mouse Dental Papilla Cells

Kentaro Hashimoto, DDS, Nobuyuki Kawashima, DDS, PhD,* Shizuko Ichinose, PhD,† Keisuke Nara, DDS,* Sonoko Noda, DDS,* and Takashi Okiji, DDS, PhD**

Abstract

Introduction: The disturbance of cellular attachment to dentin by sodium hypochlorite (NaOCl) may hamper pulp tissue regeneration. The aims of this study were to examine the recovering effect of EDTA on the attachment/differentiation of stemlike cells and to address the mechanisms of EDTA-induced recovery under the hypothesis that attachment to the exposed dentin matrix and the subsequent activation of integrin/phosphatidylinositol 3-kinase (PI3K) signaling play a crucial role. **Methods:** Mouse dental papilla (MDP) cells were cultured on bovine dentin disks treated with NaOCl (0%, 1.5%, or 6%) followed by EDTA (0%, 3%, or 17%). Cell attachment was evaluated by cell density, viability, and scanning and transmission electron microscopy. Odonto-/osteoblastic gene expression in attached MDP cells was analyzed with or without a pan-PI3K inhibitor (LY294002) using real-time polymerase chain reaction. **Results:** NaOCl treatment (1.5%, 10 minutes) significantly diminished attached MDP cells ($P < .00001$), but EDTA treatment (3% and 17%, ≥10 minutes) of NaOCl-pretreated dentin induced a significant increase in attached cells ($P < .05$). Ultrastructurally, MDP cells on EDTA-treated dentin showed attachment to exposed collagen fibers. MDP cells cultured on EDTA-treated disks (with or without 1.5% NaOCl pretreatment) showed significant up-regulation of alkaline phosphatase, dentin matrix protein 1, and dentin sialophosphoprotein messenger RNAs ($P < .05$). Alkaline phosphatase expression was down-regulated by LY294002 ($P < .05$). **Conclusions:** Under the present experimental conditions, 10 minutes of EDTA treatment was sufficient to recover attachment/differentiation of MDP cells on 1.5% NaOCl-pretreated dentin. EDTA-induced exposure of collagen fibers and subsequent activation of integrin/PI3K signaling may contribute, at least partly, to the recovery. *(J Endod 2017;■:1–7)*

Key Words

Cell attachment, cell differentiation, dentin surface, EDTA, integrin signaling, sodium hypochlorite

Significance

The requirement of dentin surface properties suitable for attachment/differentiation of recruited/applied stem cells is essential for regenerative endodontics, and EDTA treatment achieved the exposure of collagen on NaOCl-pretreated dentin, which works as scaffolds for cell attachment and further induces differentiation via integrin/PI3K signaling.

Pulp tissue regeneration is gaining much attention based on the notion that preservation of the pulp is critical for tooth longevity because of its crucial role in the maintenance of tooth homeostasis and strength. Thus, revitalization of immature pulpless teeth, involving intentional overinstrumentation to induce bleeding and deliver stem cells, has been conducted with considerable success (1) although this procedure does not generate the pulp-dentin complex (2). Attempts to engineer the pulp have also been conducted by the implantation of stem cells, scaffolds, and/or growth factors with promising results (3).

For regenerative endodontic procedures, the acquirement of dentin surface quality suitable for cellular attachment is crucial, in addition to complete disinfection of the root canal system. Sodium hypochlorite (NaOCl) is popularly used as a root canal irrigant of choice because it dissolves organic substances nonspecifically and efficiently kills intracanal bacteria (4, 5). However, concentrated NaOCl may hamper regenerative procedures because it induces denaturation of the dentin wall, which negatively influences the attachment and differentiation of stem cells (6, 7).

EDTA is a typical chelating agent used to remove the smear layer, which may contain dentin debris, bacterial products, and residual pulp tissues (8). In regenerative endodontics, EDTA irrigation is recommended as the final active rinse before the application of stem cells (9) because it may recover the NaOCl-induced disturbance of cell attachment and differentiation (5, 6, 10). One possible mechanism of the recovery is that EDTA releases growth factors from the dentin, which promote migration, proliferation, and differentiation of stem cells (4, 11, 12). Thus, the long-term application of highly concentrated EDTA may be favorable for the release of a sufficient amount of growth factors (4). However, the optimal concentration and application time of EDTA to NaOCl-treated dentin is still unclear.

From the *Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, and †Research Center for Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan.

Address requests for reprints to Dr Nobuyuki Kawashima, Department of Pulp Biology and Endodontics, Division of Oral Health Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan. E-mail address: kawashima.n.endo@tmd.ac.jp

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Mesenchymal stem cells are adhering cells, and attachment to the extracellular matrix (ECM) is essential for their proliferation and differentiation (13, 14). Moreover, direct contact to the EDTA-treated dentin surface is considered necessary for odonto-/osteoblastic differentiation of dental pulp stem cells (15). Thus, we hypothesized that the promotion of cellular attachment to dentin ECM is caused by the favorable effect of EDTA irrigation. We also hypothesized that integrins are involved in this mechanism because EDTA exposes integrin ligands, such as collagen type I, on the dentin surface (16), and subsequent binding of integrins may initiate integrin signaling, including phosphatidylinositol 3-kinase (PI3K) signaling (17), and activate several intracellular pathways.

Taken together, the first aim of this study was to examine a suitable concentration and application time of EDTA treatment of NaOCl-pretreated dentin to recover the attachment/differentiation of stemlike cells cultured on dentin *in vitro*. The second aim was to address the mechanisms of EDTA-induced recovery under the hypothesis that cellular attachment to the exposed dentin matrix and subsequent activation of integrin signaling play a crucial role. Thus, we examined the ultrastructure of stemlike cells attached to EDTA-treated dentin and analyzed the effect of EDTA treatment on the odonto-/osteoblastic marker expression of these cells with or without a PI3K inhibitor.

Materials and Methods

Preparation and Treatment of Dentin Disks

Dentin disks, approximately 1-mm thick, were prepared from the coronal to middle portion of bovine incisor roots. After soft tissue removal by mechanical separation and dissolution by NaOCl (Nippon Shika Yakuhin, Yamaguchi, Japan; 1% for 10 minutes), the roots were sliced perpendicular to the tooth axis with a low-speed saw (IsoMet; Buehler, Lake Bluff, IL).

The dentin disks were treated for 10 minutes with NaOCl (1.5% or 6%) or phosphate-buffered saline (PBS, 0.01 mol/L) followed by EDTA (3% or 17%; Dojindo Laboratories, Kumamoto, Japan) or PBS for 1 to 60 minutes. Each treatment was performed at room temperature in an ultrasonic cleaner (40 Hz, 160 W [Branson 5800; Branson, Danbury, CT]) followed by washes with PBS 3 times for 1 minute each.

Analysis of Cell Attachment

For stemlike cells, we used originally cloned mouse dental papilla (MDP) cells, which were established from incisor apical buds of Institute of Cancer Research mice and possess the potential for odonto-/osteogenic differentiation (18, 19). MDP cells were cultured in alpha-modified minimum essential medium (Wako Pure Chemical, Osaka, Japan) containing 10% fetal bovine serum (GE Healthcare, Buckinghamshire, UK) and an antimicrobial solution (Penicillin-Streptomycin-Amphotericin B Suspension, Wako Pure Chemical) at 37°C, 5% CO_2, and 100% humidity. Enhanced green fluorescent protein (EGFP)-expressing MDP cells were constructed by transfection of an EGFP-expressing mammalian protein expression vector (pcDNA3.1+) using the Neon Transfection System (Thermo Fisher Scientific, Waltham, MA).

MDP cells (2×10^4 cells/well) were seeded on dentin disks placed in 48-well plates (TPP Techno Plastic Products, Trasadingen, Switzerland) filled with alpha-modified essential medium containing 10% fetal bovine serum and the antimicrobial solution and were cultured in a CO_2 incubator for 24 hours. Images of EGFP-expressing MDP cells on the dentin disk were taken by a fluorescence microscope (Axio Observer Z1; Carl Zeiss, Oberkochen, Germany) and analyzed using image analyzing software (Image J; National Institutes of Health, Bethesda, MD) (20). The viability of cells on the disks was measured using a water soluble tetrazolium salt assay (Cell Counting Kit-8, Dojindo Laboratories).

Ultrastructural Analysis

MDP cells (1×10^4 and 1×10^5 cells/well for scanning electron microscopy [SEM] and transmission electron microscopy [TEM], respectively) were seeded on dentin disks in 48-well plates and cultured for 24 hours. The specimens were fixed in 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer for 2 hours and post-fixed with 1% OsO_4 (TAAB, Aldermaston, UK) buffered with 0.1 mol/L phosphate buffer for 2 hours. For SEM, the specimens were dehydrated, dried in a critical point drying apparatus (HCP-2; Hitachi, Tokyo, Japan), spatter coated with platinum, and examined using a scanning electron microscope (S-4500, Hitachi). For TEM, the specimens were dehydrated, embedded in an embedding resin (Epon 812, TAAB), cut in 90-nm-thick sections, double stained with uranyl acetate and lead citrate, and observed using a transmission electron microscope (H-7100, Hitachi).

Analysis of Odonto-/Osteoblastic Gene Expression

MDP cells (1×10^5 cells/well) seeded on dentin disks placed in 48-well plates were cultured for 48 hours. MDP cells (5×10^3 cells/well) seeded in blank wells (without disks) were used as a control. The total RNA extracted using the RNAqueous Micro kit (Thermo Fisher Scientific) was reverse transcribed into complementary DNA (RevertAid H Minus Reverse Transcriptase, Thermo Fisher Scientific), and a quantitative polymerase chain reaction assay was performed to determine the expression level of alkaline phosphatase (*Alp*), dentin matrix protein 1 (*Dmp1*), and dentin sialophosphoprotein (*Dspp*) using the CF96 real-time polymerase chain reaction detection system (Bio-Rad, Hercules, CA) with specific primers (Table 1). In some experiments, MDP cells on dentin disks were cultured with or without LY294002 (a PI3K inhibitor [21], 10 μmol/L; Cayman Chemical, Ann Arbor, MI) for 48 hours.

Statistical Analysis

Data were analyzed using statistical software (Prism 6; GraphPad, San Diego, CA). Attached cell numbers, viability, and gene expression were analyzed by 1-way analysis of variance with the Tukey post hoc test ($P < .05$). Gene expression with or without the PI3K inhibitor was analyzed by the Student *t* test ($P < .05$).

TABLE 1. Primer Sequences

Genes	Upper primers	Lower primers	Gene bank	Size (bp)
β actin	5′-GTAAAGACCTCTATGCCAACACAGT-3′	5′-AATGATCTTGATCTTCATGGTGCTA-3′	NM_007393	98
Alp	5′-GATTACGCTCACAACAACTACCAG-3′	5′-GGAATGTAGTTCTGCTCATGGAC-3′	NM_007431	156
Dmp1	5′-CGTTCTGAGGAAGACAGTGACTC-3′	5′-TTAGTTTCCTACTGTCAGCTTCCAT-3′	NM_016779	106
Dspp	5′-AAGGATAGCAGTTCTGACAGCAG-3′	5′-AATCATCACTGGTTGAGTGGTTACT-3′	NM_010080	112