Abstract

To investigate the stimulating effect of endodontic medications on the mRNA expression of some osteogenesis-related genes associated with reparative dentinogenesis and hard-tissue formation, human dental pulp cells (D824 cells) were treated with calcium hydroxide (Ca (OH)$_2$), formocresol, or guaiacol. The effect on growth was determined by growth curves of D824 cells treated for 1–3 days with 0.03–0.3 mM Ca (OH)$_2$, 0.0007%–0.0014% formocresol, or 0.24–2.43 mM guaiacol. The mitotic activity of individual cells and the mRNA expression of the osteogenesis-related genes for alkaline phosphatase (\textit{ALP}), type I collagen (\textit{COL-1}), and bone sialoprotein (\textit{BSP}) in the cells treated for 24 h with the same concentrations of the medications as described above were determined by colony-forming efficiency and by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis, respectively. Cellular growth and mitotic activity were scarcely affected by Ca (OH)$_2$, but were significantly reduced by formocresol or guaiacol. The mRNA expression of the osteogenesis-related genes was little affected by Ca (OH)$_2$ or formocresol, but was significantly enhanced by guaiacol. The results indicate that guaiacol may stimulate the mRNA expression of genes associated with reparative dentinogenesis and hard-tissue formation in human dental pulp cells, suggesting that the novel property of guaiacol provides new insights into the utilization of guaiacol in endodontic therapy.

Key words Guaiacol · Human dental pulp cells · Osteogenesis-related gene expression · Reparative dentinogenesis

Introduction

The induction of reparative dentin formation or apical hard-tissue formation is one of the important actions of endodontic medications used for pulp capping or root canal filling, respectively. Various pulp-capping agents have been used in the vital pulpotomy of primary teeth. Calcium hydroxide (Ca (OH)$_2$) is still the best agent available for pulp capping, and is the gold standard. Formocresol is also used in pulpotomy of primary teeth with high clinical success rates. Ca (OH)$_2$ and formocresol are applied for root canal filling and/or root canal disinfection as well.

Although the mechanism is not fully elucidated, it is well known that Ca (OH)$_2$ induces reparative dentin formation and apical hard-tissue formation. Goldberg et al. have suggested that osteogenesis-related proteins, including alkaline phosphatase (\textit{ALP}), type I collagen (\textit{COL-1}), and bone sialoprotein (\textit{BSP}) are synthesized during reparative dentinogenesis. \textit{ALP} is an essential factor in dentin mineralization and in the formation of acellular cementum. \textit{COL-1} constitutes 90% of the dentin matrix. The collagen matrix provides not only the scaffold to promote and develop a mineralized tissue but also an excellent natural support for noncollagenous proteins such as \textit{BSP} and dentin sialoprotein (\textit{DSP}). \textit{BSP} is a major constituent of the bone matrix. The expression of \textit{BSP} is highly specific for mineralizing tissues including bone and dentin, although the expression level in dentin is much lower than in bone. The expression of \textit{DSP}, a specific marker of dentin, is observed in the odontoblast layer of primary dentin and odontoblast-like cells of the reparative dentin, whereas \textit{BSP} is strongly expressed in the odontoblast-like cells of reparative dentin but barely expressed in the odontoblast layer of primary dentin. These previous findings suggest the possible involvement of the expression of \textit{ALP}, \textit{COL-1}, and \textit{BSP} genes in reparative dentinogenesis. In addition, reparative dentin may have both dentinogenic and osteogenic properties.

To examine the ability of endodontic medications to stimulate the expression of \textit{ALP}, \textit{COL-1}, and \textit{BSP}, which might be associated with reparative dentinogenesis, human
dental pulp cells were treated with Ca (OH)₂, or formocresol, and the mRNA levels of these genes were determined by real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis. Guaiacol (2-methoxyphenol) was also examined because guaiacol itself and the mixture with formalin (formalin-guaiacol) are used as a root canal medication. Guaiacol is sometimes used as a sedative for pulp and a disinfectant for cavity preparations as well.

Materials and methods

Human dental pulp cells (D824 cells) derived from the dental pulp tissue obtained from a lower third molar extracted from a woman (22 years old) were grown as described previously. All experiments were carried out using D824 cells at 10–15 passages. Calcium hydroxide (Ca (OH)₂; 99.9% pure) and guaiacol (>99% pure) were obtained from Wako Pure Chemical (Osaka, Japan). Ca (OH)₂ was dissolved at 30 mM in glycerol at 65°C and filter-sterilized after being diluted with culture medium to the desired concentrations. Formocresol was prepared by mixing formalin (37% formaldehyde solution; Wako Pure Chemical), tricresol (98% pure; Koso Chemical, Tokyo, Japan), and ethanol (99.5% pure; Wako Pure Chemical) and used as a 100% solution. Formocresol and guaiacol were diluted with culture medium to the desired concentrations and applied to D824 cells. The pH range of culture media containing the test medications was approximately 7.2–7.5 as determined by the color of phenol red as a pH indicator added to the culture media.

Growth curve

D824 cells (1.6 × 10⁵) in the logarithmic growth phase were plated on 60-mm dishes (Costar, Corning, NY, USA). After overnight incubation, the cells were treated with varying concentrations of test medications for 1–3 days. The number of cells per 60-mm dish was determined after harvesting with 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA). Cell counts are presented as the mean ± SD from three dishes per counting point (1, 2, and 3 days after start of treatment).

Mitotic activity

The mitotic activity of individual cells was determined by the colony-forming efficiency of D824 cells treated with test medications. Cells (500) were plated, in triplicate, on 60-mm dishes, incubated overnight, and treated with varying concentrations of test medications for 24 h. The cells were then washed twice with 2 ml fresh medium and incubated for 13 days to form colonies. The colonies formed were fixed with absolute methanol and stained with 3% Giemsa solution.

The number of colonies with more than 50 cells was then counted and the percent mitotic activity was expressed as the number of colonies in the treated dishes divided by the number in control dishes × 100.

Determination of ALP, COL-1, and BSP transcript levels by qRT-PCR analysis

Cells (1.5 × 10⁶) were plated into 75-cm² flasks (Costar) and incubated for 3 days until they reached confluence. The cells were treated with test medications for 24 h. This treatment time was chosen because ALP and BSP play an important role in the early formation of dentin. Total cellular RNA was isolated with the RNaseasy mini kit® (Qiagen, Tokyo, Japan) according to the manufacturer’s instructions. cDNA was synthesized from 2 μg total RNA, using the high capacity RNA-to-cDNA kit® (Applied Biosystems, Foster City, CA, USA). Then qRT-PCR was performed using TaqMan® gene expression assays and a StepOnePlus® RT-PCR system on human target genes, ALP, COL-1, and BSP, and eukaryotic 18SrRNA (18S) (assay IDs: Hs01029144 m1, Hs01076746 g1, Hs00173720 m1, and Hs99999901 s1, respectively; Applied Biosystems). The 18S rRNA was selected as an endogenous control gene based on preliminary experiments, indicating its higher stability of expression by D824 cells as compared to β-actin or glyceraldehyde-3-phosphate dehydrogenase. The relative quantity was determined by the standard curve method, in which a relative standard curve of known dilutions (1×, 1:10, 1:100, 1:1000, 1:10000) was run with the unknown samples. The relative quantity of each unknown was determined by plotting a standard curve (threshold cycle (Ct) versus starting quantity), and calculating from the Ct of each sample the amount amplified. The normalized value was determined by dividing the relative quantity of the target genes for each sample by the relative quantity of 18S for that sample. Each of three separate experiments was performed in triplicate.

Statistical analysis

Statistics were assessed using Student’s t-test, assuming double-sided independent variance and with P < 0.05 considered to be significant.

Results

The concentration-dependent effects on cellular growth of treatment with test medications for 1–3 days were examined (Fig. 1A). In D824 cells treated with Ca (OH)₂, at 0.03–0.3 mM, little effect on cellular growth was observed. However, growth was reduced by treatment with formocresol at 0.0007%-0.0014% or guaiacol at 0.24–2.43 mM, in a concentration-dependent manner. The effect of the test medications on the mitotic activity of individual cells was determined by the colony-forming efficiency of D824 cells following treatment with the medications for 24 h (Fig. 1B).