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Induction of Interleukin-6 and Interleukin-8 Gene Expression by Root Canal Sealers in Human Osteoblastic Cells

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Abstract

Histological investigations have demonstrated that root canal sealers can induce mild to severe inflammatory alternations. However, there is little information on the precise mechanisms about root canal sealers-induced inflammatory reaction. Dysregulated cytokine productions at local disease sites have been considered to be major contributors to the development of inflammatory diseases. Interleukin (IL)-6 and IL-8 released have been reported to play an important role in the pathogenesis of inflammation. The aim of this study was to investigate the effects of root canal sealers N2 (zinc-oxide eugenol based) and AH Plus (epoxy resin based) on the expression of IL-6 and IL-8 mRNA gene in human osteoblastic cell line U2OS cells. The levels of mRNAs were measured by the semi-quantitative reverse-transcriptase polymerase chain reaction analysis. The exposure of quiescent U2OS cells to N2 and AH Plus resulted in the induction of IL-6 and IL-8 mRNA gene expression ($p < 0.05$). The intensity of IL-8 mRNA gene was found to be significant higher than IL-6 mRNA gene ($p < 0.05$). Taken together, the activation of IL-6 and IL-8 mRNA gene expression may be one of the pathogenesis of zinc oxide-eugenol based and epoxy resin based root canal sealers-induced periapical inflammation.

Key Words

Root canal sealers, inflammation, interleukin-6, interleukin-8

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An important requirement for dental materials is biocompatibility because the toxic components present in these materials could produce irritation or even degeneration of the surrounding tissues. Good tissue compatibility is decisive for root canal sealers because they may come into direct contact, especially when extruded, with the periapical tissues.

The use of root canal sealers in clinical cases with apical lesions requires special attention to avoid possible interference and/or delay of the healing processes. Unfortunately, several studies have shown that most commercially available root canal sealers can induce mild to severe inflammatory alternation within apical tissues (1–7). Recently, our studies have shown the activation of cycloxygenase-2 expression is one of the important pathways of root canal sealers-induced periapical inflammation (8, 9). However, there is little information about the cytokine expression in root canal sealers-induced periapical inflammatory reaction.

Cytokines released in periapical lesion have been reported to play an important role in the process of inflammation. The cytokine IL-6 is a major mediator of the host response to tissue injury and infection (10, 11). IL-6 plays a major role in B cell differentiation in the immune system. It is also accepted that IL-6 has multiple biological activities, such as the acceleration of bone resorption (12). It is, therefore, generally recognized that IL-6 is involved in the pathogenesis of pulpal inflammation (13, 14). IL-8 is a chemokine produced by a variety of tissue and blood cells and is a potent inducer of neutrophil chemotaxis and activation (15). Aberrant and persistent production of IL-8 has been demonstrated in various inflammatory diseases, including pulpal inflammation (16–19). Moreover, several reports have suggested a relationship between the progression of periapical lesions and expression of IL-6 and IL-8 in periapical tissues (20–23).

In this study, we evaluated IL-6 and IL-8 mRNA gene expression in human osteoblastic cells stimulated with epoxy resin based and zinc oxide-eugenol based root canal sealer by the semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) assay.

Materials and Methods

Sample Preparation

The test materials, product names, manufacturers, and ingredients are listed in Table 1. Under aseptic condition, the sealers were mixed according to the manufacturer's instructions. Samples were prepared as described previously (18). Briefly, freshly mixed materials were filled in glass rings (4-mm height and 10 mm in diameter) and set for 24 h at 37°C in a humidified chamber. Each test specimen was eluted in 10 ml of culture medium at 37°C for 7 days in a 5% CO_2 air atmosphere. The extraction media were then collected into sterile syringes at the end of this period and passed through a 0.22 μm filter. Subsequently, N2 (final dilution: 1:8) and AH Plus (final dilution: 1:4) of these extraction media were prepared to be used in this study.

Cell Culture

U2OS cells (American Tissue Type Collection HTB 96) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Sigma Chemical Co., St. Louis, MO), 100 μg/ml of strep-

TABLE 1. Composition of the tested root canal sealers as given by the manufacturers

Material	Type	Manufacturers	Ingredients
AH Plus	Epoxy resin-based	De Trey Dentsply, Switerland	***Paste A*** Epoxy resin Calcium tungstate Zirconium oxide Aerosil Iron oxide ***Paste B*** Adamantane amine N,N`-Dibenzoyl-5-oxanonane -diamine-1,9-TCD-diamine Calcium tungstate Zirconium oxide Silicone oil Aerosil
N2	Zinc xide-eugenol based	Indrag-Agsa, Losone, Switzerland	***Powder*** ZnO Bismuth nitrate Bismuth carbonate Paraformaldehyde TiO ***Liquid*** Eugenol Peanut oil Rose oil Lavender oil

tomycin, 100 mg/ml of penicillin at 37°C in humidified incubator under ambient pressure air atmosphere containing 5% CO_2. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. The cells were subcultured at 1:4 splits every third day.

Treatments

Cells arrested in G0 by serum deprivation (0.5% FCS; 48 h) were used in the experiments. Before treatment, the cells were washed with serum-free DMEM and immediately thereafter exposed to the elutes of N2 and AH Plus for the indicated incubation times. The viability of cells exposed to these elutes were in general cytoatatic to U2OS cells during 24-h culture period (24). Total RNA was isolated at 1, 3, 6, 8, and 24 h, respectively. In addition, cultures without FCS were used as negative control.

RNA Isolation

Total RNA was prepared using TRIzol reagent (Gibco) following the manufacturer's instructions. The concentration of RNA solution was quantified by spectrophotometry with an absorbance wavelength of 260 nm, and the purity was assessed by the OD_{260}/OD_{280} ratio.

Reverse-Transcriptase Polymerase Chain Reaction

Complementary DNA was synthesized from RNA in a 15 μl reaction mixture containing 100-mg random hexamer and 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for polymerase chain reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and two units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for IL-6 or IL-8 in a thermal cycle. Each cycle consisted of 1 min of denaturation at 94°C, 1 min of annealing at 57°C, and 1 min of extension at 72°C. The sequences of primers used were as follows:

A) GAPDH Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′
Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′

B) IL-6 Forward: 5′- ATGAACTCCTTCTCCACAAGCGC-3′
Reverse: 5′- GAAGAGCCCTCAGGCTGGACTG -3′
C) IL-8 Forward: 5′- CGATGTCAGTGCATAAAGACA-3′
Reverse: 5′- TGAATTCTCAGCCCTCTTCAAAAA -3′

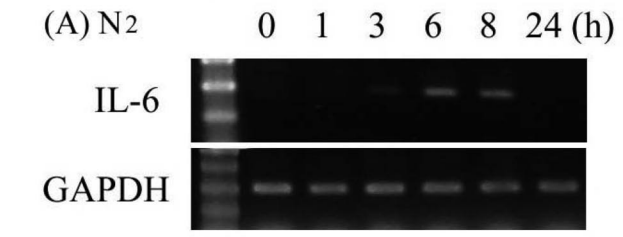
When the cells were probed for IL-6 and IL-8 mRNA production by RT-PCR, a 628 bp band for IL-6 and a 225 bp band for IL-8 were noted. These bands were consistent with the size as designed by primers. When the band densities were measured and compared with the density of the band obtained for the housekeeping gene GAPDH, relative proportions of mRNA synthesis could be determined within each experiment. The intensity of each band after normalization with GAPDH mRNA was quantified by the photographed gels with a densitometer (AlphaImager 2000; Alpha Innotech, San Leandro, CA). Each densitometric value was expressed as the mean $\pm$ SD.

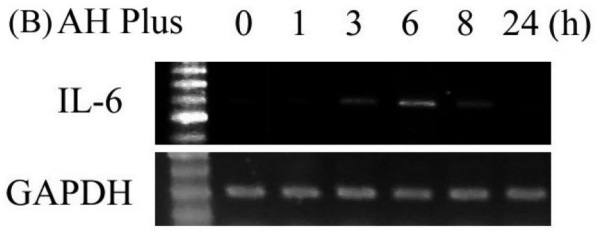
Statistical Analysis

Triplicate or more experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. Statistical analysis was conducted by one-way ANOVA. Tests of differences of the treatments were analyzed by Duncan's test and a value of $p < 0.05$ was considered statistically significant.

Results

As shown in Fig. 1*A*, IL-6 mRNA gene was found to be induced by N2. Investigations of the time dependence of IL-6 mRNA gene expression in N2-treated cells revealed a significant signal first detectable after 6 h of exposure and diminished to control level after 24 h (Fig. 1*A*). The quantitative measurement was made by the AlphaImager 2000. The levels of the IL-6 mRNA gene increased about 1.1-, 2.3-, and 1.9-fold after exposure to N2 for 3, 6, and 8 h, respectively (Fig. 1*C*). However, cells resting without FCS did not express any detectable levels of IL-6 mRNA gene. Investigations of the time dependence of IL-6 mRNA gene expression in AH Plus-treated cells revealed a significant signal first detectable after 3 h of exposure and diminished to control level after 24 h (Fig. 1*B*). The quantitative measurement was made by the AlphaImager 2000. The levels of the IL-6 mRNA gene





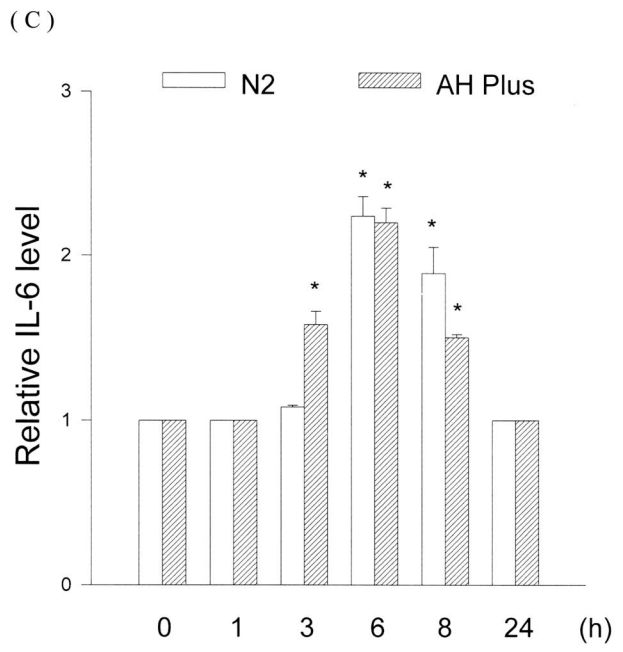
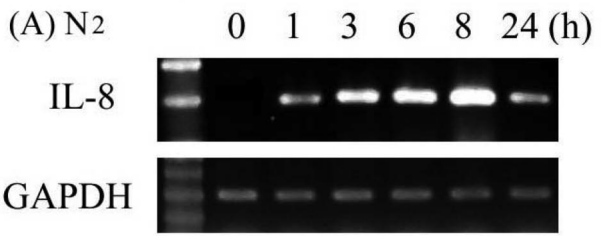
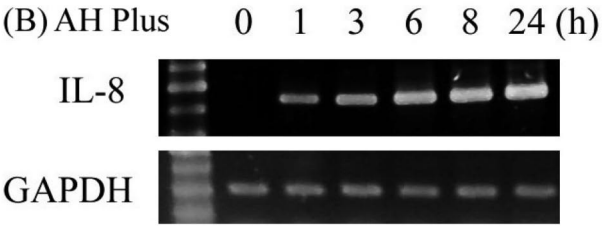


Figure 1. IL-6 expression by (*A*) N2 or (*B*) AH Plus-treated human osteoblastic cells for 0, 1, 3, 6, 8, and 24 h incubation periods by RT-PCR assay. A DNA ladder of known base pairs was used for identification of PCR products. GAPDH gene was performed to monitor equal RNA loading. (*C*) Levels of IL-6 mRNA gene treated with N2 and AH Plus were measured by AlphaImager 2000. The relative level of IL-6 mRNA gene expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the mean ± SD. The asterisk represents significant differences from control values with $p < 0.05$.





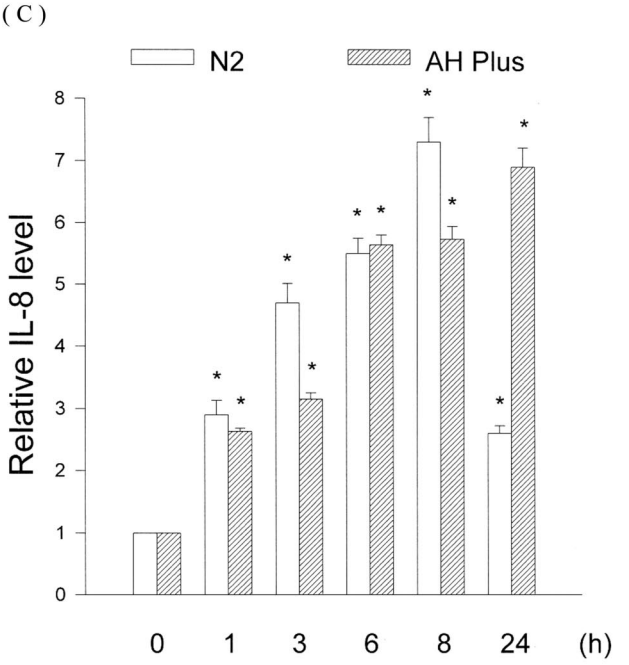


Figure 2. IL-8 expression by (*A*) N2 or (*B*) AH Plus-treated human osteoblastic cells for 0, 1, 3, 6, 8, and 24 h incubation periods by RT-PCR assay. A DNA ladder of known base pairs was used for identification of PCR products. GAPDH gene was performed to monitor equal RNA loading. (*C*) Levels of IL-8 mRNA gene treated with N2 and AH Plus were measured by AlphaImager 2000. The relative level of IL-8 mRNA gene expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the mean ± SD. The asterisk represents significant differences from control values with $p < 0.05$.

increased about 1.6-, 2.2-, and 1.5-fold after exposure to AH Plus for 3, 6, and 8 h, respectively (Fig. 1*C*).

As shown in Fig. 2*A*, the kinetics of this response showed that IL-8 mRNA gene was detectable as early as 1 h post-N2 challenge and remained elevated throughout the 24-h incubation period. The quantitative measurement was made by the AlphaImager 2000. The levels of the IL-8 mRNA gene increased about 2.9, 4.7, 5.5, 7.3, and 2.6-fold after exposure to N2 for 1, 3, 6, 8, and 24 h, respectively (Fig. 2*C*). However, cells resting without FCS did not express detectable levels of IL-8 mRNA gene. The same induction pattern was found in AH Plus-induced IL-8 mRNA gene expression (Fig. 2*B*). The quantitative measurement was made by the AlphaImager 2000. The levels of the IL-8 mRNA gene increased about 2.6-, 3.2-, 5.7-, and 6.9-fold after exposure to AH Plus for 1, 3, 6, 8, and 24 h, respectively (Fig. 2*C*).

Discussion

Osteoblasts are considered as cells primarily concerned with providing physical barriers and structural components in periapical tissues

(25). These cells may be important in the recruitment of immune cells and contribute to the inflammation. To date, the interactions of root canal sealer and human osteoblastic cells are still not fully understood. This is the reason why we chose this type cells in this study.

In vivo acute toxicity is usually not associated with the clinical use of a dental material. In this study, we chose the cytoatatic concentrations of root canal sealers in U2OS cells for evaluating IL-6 and IL-8 mRNA gene expression. Higher concentrations were not studied because they would rapidly cause cell death, making a change in secretion of inflammatory mediators unlikely. This is the reason why subtoxic concentrations were chosen to determine by RT-PCR assay for IL-6 and IL-8 mRNA gene expression according to our previous study (24).

Cytokine research has become one of the most important parts in the clinical research. Measurements of cytokine production capability are expected to become new markers for diagnosis or treatment in clinical practice. RT-PCR assay for analyzing cytokine expression involves the detection of cytokine mRNA gene (26, 27). It becomes a routine laboratory examination method for new immunological markers. Quantitative measurement of cytokine mRNA gene expression is now as a useful tool for analyzing cytokine networks.

Dysregulated cytokine productions at local disease sites have been considered to be major contributors to the development of inflammatory diseases. Human periapical lesion is an inflammation disease evoked by bacterial infection in root canal system and give rise to bone destruction at the periapical tissue as the result of host-microbial interplay. Previous studies have been shown IL-6 and IL-8 expression in periapical lesions (20–23). In this study, we first found that N2 and AH Plus were capable of inducing IL-6 and IL-8 mRNA gene expression. Thus, IL-6 as well as IL-8 may play a critical role in zinc oxide-eugenol based and epoxy resin based root canal sealers-induced periapical inflammation.

At the site where inflammation and tissue destruction have occurred, cells might communicate with one another through the interaction of cytokines, Thus, it is important to elucidate completely the complex cytokine cascade flow associated with inflammation-mediated tissue destruction at the molecular level. The present study demonstrated that IL-6 and IL-8 mRNA gene expression by U2OS cells was enhanced by N2 and AH Plus. However, the induction pattern between IL-6 and IL-8 mRNA gene was different. The intensity of IL-8 mRNA gene was found to be significant higher than IL-6 mRNA gene. The difference in the specific activity could be related either to the type of receptor-molecule interactions or to a different intracellular distribution of second messenger molecules as have been suggested previously (28, 29).

The mechanism responsible for the activation of IL-6 and IL-8 mRNA gene expression by N2 and AH Plus contributes to inflammatory responses is not clear. These may be because of N2 and AH Plus were found to release formaldehyde (30, 31). Formaldehyde has been shown found to induce nonneoplastic responses, such as epithelial degeneration and a mixed inflammatory cell infiltration (32). This might be the reason why N2 and AH Plus can induce IL-6 and IL-8 mRNA gene expression. Zinc oxide-eugenol root canal sealers were attributable to free eugenol liberated from the set material (33). Eugenol is a tissue irritant that has been found to induce inflammatory reaction on oral mucosal membrane (34). This may the possible mechanism that Zinc oxide-eugenol root canal sealers can induce inflammatory reaction in vivo.

As for as we known, this is the first attempt to evaluate the role of IL-6 and IL-8 mRNA gene expression in human osteoblastic cells stimulated with N2 and AH Plus. Data from our in vitro experiments showed that N2 and AH Plus was capable of stimulating IL-6 and IL-8 mRNA gene expression in human osteoblastic cells. This suggests that one of the pathogenic mechanisms of periapical tissue inflammation induced by zinc-oxide eugenol based and epoxy resin based root canal sealers in vivo may be the synthesis of IL-6 and IL-8 by resident cells.

Recently, IL-10 was found to inhibit IL-6 and IL-8 mRNA expression in human pulp cell cultures (35). The possibility of using IL-10 to protect cells against the adverse effects of root canal sealers should be considered. In addition, the advantage of RT-PCR assay is simplicity, sensitivity, rapidity, and cheapness. This experimental system may be useful for cytokine expression profile screening of various dental materials in vitro.

Dysregulated cytokine productions at local disease sites have been considered to be major contributors to the development of inflammatory diseases. IL-6 and IL-8 released have been reported to play an important role in the pathogenesis of periapical inflammation. The results showed that both IL-6 and IL-8 mRNA gene expression were found to be induced by N2 and AH Plus in human osteoblastic cells. The activation of IL-6 and IL-8 mRNA gene expression may be one of the pathogenesis of zinc oxide-eugenol based and epoxy resin based root canal sealers-induced periapical inflammation. However, care should be taken to reduce the possibility of periapical irritations from inadvertent extrusion of the root canal sealers in clinical treatment until their side effects are more thoroughly studied.

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