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Induction of Cyclooxygenase-2 mRNA and Protein Expression by Dentin Bonding Agents in Human Gingival Fibroblasts

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Abstract: An ideal dentin bonding agent should be nonirritating to surrounding tissues. Unfortunately, all histological investigations have demonstrated that dentin bonding agents can induce mild to severe inflammatory alterations. However, there is little information on the precise mechanisms about dentin bonding agents-induced inflammatory reaction. Cyclooxygenase-2 (COX-2) is an inducible enzyme believed to be responsible for prostaglandin synthesis at the site of inflammation. The aim of the present study was to investigate the effects of three dentin bonding agents, Clearfil SE Bond, Prime & Bond NT, and Single Bond on the expression of COX-2 mRNA gene and protein in cultured human gingival fibroblasts. The exposure of quiescent human gingival fibroblasts to dentin bonding agents resulted in the induction of COX-2 mRNA expression. The investigations of the time-dependent on COX-2 mRNA expression in dentin bonding agent-treated human gingival fibroblasts revealed different patterns. The influence of COX-2 mRNA depended on the tested materials. In addition, all dentin bonding agents also induced COX-2 protein expression in human gingival fibroblasts. Taken together, the activation of COX-2 expression may be one of the potential mechanisms of dentin bonding agent-induced gingival inflammation. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 70B: 297–302, 2004

Keywords: dentin bonding agents; human gingival fibroblasts; inflammation; cyclooxygenase-2

INTRODUCTION

Dentin bonding agents are designed to form strong bonding with dentin. These agents are used to improve the bonding strength between resin and the tooth structure, increase the retention of restoration, and reduce the microleakage across the dentin–resin interface and scatter the occlusal stress. They usually keep in close contact with living dental tissue over a long period of time. An ideal dentin bonding agent requires biocompatibility.

The irritative effects of dentin bonding agents have been evaluated by histopathological examinations of the tissue responses. Unfortunately, all histological investigations demonstrated that dentin bonding agents could induce mild to severe inflammatory reactions in gingiva[2,3] as well as pulp tissue.[4,5] However, there is little information on the precise mechanisms about dentin bonding agent-induced inflammatory reaction.

Prostaglandins (PGs) are inflammatory mediators implicated in all stages of inflammation. Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase, is a key enzyme in PG biosynthesis. COX is a dual enzyme and catalyses two enzymatic reactions: (a) to convert arachidonic acid into PGG_2 by its COX activity; and (b) to reduce PGG_2 to PGH_2 by its peroxidase activity.[6] Recently, it has been demonstrated that there are two isoenzymes of COX (COX-1 and COX-2). These two isoenzymes are encoded by different genes. Whereas COX-1 is regularly expressed in various tissues,[7] COX-2 is induced after the action of cells by various inflammatory agents.[8] In addition, COX-2 is at low or undetectable levels in healthy tissues and is upregulated in inflamed tissues.[9,10]

To the best of the authors' knowledge, the effect of dentin bonding agents on human gingival fibroblasts with special reference to COX-2 expression has not been reported previously. In light of these findings, the present investigation was undertaken to study whether different dentin bonding agents could induce the expression of COX-2 mRNA and protein in human gingival fibroblasts *in vitro*.

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TABLE I. Principal Components of the Dentin Bonding Agents Tested

Material	Abbreviation	Components	Manufacturer
Clearfil SE Bond	CB	Bis-GMA HEMA MDP CQ	Kuraray Co., Osaka, Japan
Prime & Bond NT	PB	UDMA PENTA Resin R5-62-1 T-resin Cetylamine hydrofluoride	Dentsply De Trey Konstanz, Germany
Single Bond	SB	Bis-GMA HEMA polyacrylic acid CQ	3M, St. Paul, MN

MATERIALS AND METHODS

The dentin bonding agents tested in this study are listed in Table I. In order to evaluate the effects of the photo-polymerized dentin bonding agents, 60 × 10-mm cellulose strips were exposed to UV light for 30 min in order to prevent bacterial contamination. On the strips, 10 μL of each dentin bonding agent was applied and light cured for 20 s. After polymerization, each test specimen was eluted in 8 mL of serum-free culture medium at 37°C for 2 days in a 5% CO_2 air atmosphere as described previously.[11] Extracts were directly diluted in serum-free culture medium and the final concentration of dilution was 1:2. The extraction media were then collected into sterile syringes at the end of this period and passed through a 0.22-μm filter. Subsequently, these extraction media were prepared to be used in this study.

Cell Culture

Human gingival fibroblasts were cultured by using an explant technique as described previously.[11,12] Gingival connective tissues from crown-lengthening surgery were used to culture gingival fibroblasts. All patients were duly informed of the nature and extent of the study, and their informed consent was obtained according to the Helsinki Declaration. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Gibco, Grand Island, NY) and antibiotics (100 units/mL of penicillin, 100 μg/mL of streptomycin and 0.25 μg/mL of fungizone). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent cells were detached with 0.25% trypsin and 0.05% EDTA for 5 min, and aliquots of separated cells were subcultured. Cell cultures between the third and eighth passages were used.

Treatments

Cells arrested in G0 by serum deprivation (0.5% FCS; 48 h) were used in the experiments.[13] For gene-expression studies, nearly confluent monolayers of gingival fibroblasts were washed with serum-free DMEM and immediately thereafter exposed at the indicated incubation times to dentin bonding agents. Total RNA was isolated at 1, 3, 6, 8, and 24 h. For Western blot analysis, cell lysates were collected at 2, 4, 8, and 24 h. In addition, cultures with 0.5 and 10% FCS were used as negative and positive control, respectively.

Reverse-Transcriptase Polymerase Chain Reaction

Total RNA was prepared with the use of TRIzol reagent (Gibco, Grand Island, NY) in accordance with the manufacturer's instructions. Single-stranded 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, Grand Island, NY). The reaction mixture was diluted with 20 μL of water, and 3 μL of the diluted reaction mixture was used for polymerase chain

TABLE II. Nucleotide Sequence and Size of the Expected PCR Products for Oligonucleotide Primers Used for RT-PCR

Gene	Sequence	PCR Product (bp)
(A) GAPDH	Forward: 5′-TCCTCTGACTTCAACAGCGACACC-3′ Reverse: 5′-TCTCTCTTCCTCTTGTGCTCTTGG-3′	207
(B) COX-2	Forward: 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′ Reverse: 5′-AGATCATCTCTGCCTGAGTATCTTT-3′	232

reaction (PCR). PCR reaction mixture contains 10 pmol of forward and reverse primers and 2 units of Tag DNA polymerase. Amplification was performed at 25 cycles for GAPDH and 30 cycles for COX-2 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 employed are listed in Table II. The PCR products were analyzed by agarose gel electrophoresis. 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 ± SD.

Western Blot

For Western blot analysis, cell lysates of human gingival fibroblasts were collected as described previously.[14] Briefly, cells were solubilized with SDS-solubilization buffer (5m*M* EDTA, 1m*M* $MgCl_2$, 50m*M* Tris-HCl, pH 7.5 and 0.5% Trition X-100, 2m*M* phenylmethysulfonyl fluoride, and 1m*M* N-ethylmaleimide) for 30 min on ice. Then, cell lysates were centrifuged at 12,000 g at 4°C and the protein concentrations determined with Bradford reagent with the use of bovine serum albumin (BSA) as standards. Equivalent amounts of total protein per sample of cell extracts were run on a 10% SDS-PAGE and immediately transferred to nitrocellulose membranes. The membranes were blocked with PBS containing 3% BSA for 2 h, rinsed, and then incubated with primary antibodies anti-COX-2 diluted 1:1000 in PBS containing 0.05% Tween 20 for 2 h. After three washes with Tween 20 for 10 min, the membranes were incubated for 1 h with biotinylated secondary antibody diluted 1:2000 in the same buffer, washed again as described above and treated with 1:2000 streptavidin-peroxidase solution for 30 min. After a series of washing steps, the reactions were developed with the use of diaminobenzidine (DAB, Zymed). The intensities of the obtained bands were determined with the use of a densitometer (AlphaImager 2000). Each densitometric value was expressed as the mean ± SD.

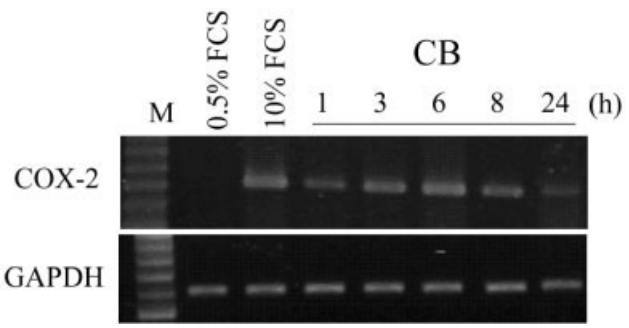

Figure 1. Expression of COX-2 mRNA gene in CB-treated human gingival fibroblasts by RT-PCR assay. A DNA ladder of known base pairs was used for identification of PCR products. GAPDH gene was performed in order to monitor equal RNA loading. FCS samples (0.5 and 10%) serve as controls.

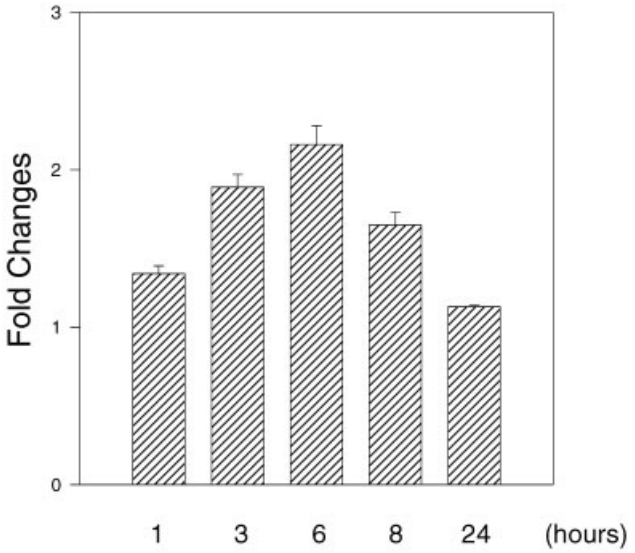

Figure 2. Levels of COX-2 mRNA gene treated with CB were measured by AlphaImager 2000. The relative level of COX-2 gene expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the mean ± SD.

Statistical Analysis

Experiments were performed three or more times throughout this study. All assays were repeated three times to ensure reproducibility. The significance of the results obtained from control and treated groups were statistically analyzed by paired Student's *t* tests.

RESULTS

To examine the effect of dentin bonding agents on the COX-2 expression, human gingival fibroblasts were treated with elutes of dentin bonding agents in this study. For a reference, the response of COX-2 in human gingival fibroblasts treated with dentin bonding agents was compared with their induction in cells cultured with serum-free medium. The effects of dentin bonding agents on the COX-2 gene expression in three different cell strains were similar, and their intracellular variations were limited.

As shown in Figure 1, the investigations of the time dependent on COX-2 mRNA expression in CB-treated cells revealed a rapid accumulation of the transcript, a significant signal first detectable after 1 h of exposure and diminished to the nearly control level after 24 h. The quantitative measurement was made by the AlphaImager 2000. The levels of the COX-2 mRNAs increased about 1.4-, 2.0-, 2.2-, and 1.7-fold after exposure to CB for 1, 3, 6, and 8 h, respectively (Figure 2). However, cells resting in 0.5% FCS did not express detectable levels of COX-2.

Expression of COX-2 mRNA gene in SB-treated human gingival fibroblasts was shown in Figure 3. It demonstrated a rapid accumulation of the transcript, a significant signal first detectable after 1 h of exposure and diminished to the control

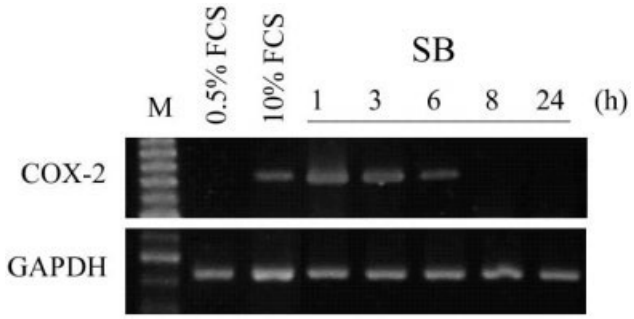

Figure 3. Expression of COX-2 mRNA gene in SB-treated human human gingival fibroblasts by RT-PCR assay. A DNA ladder of known base pairs was used for identification of PCR products. GAPDH gene was performed in order to monitor equal RNA loading. FCS samples (0.5 and 10%) serve as controls.

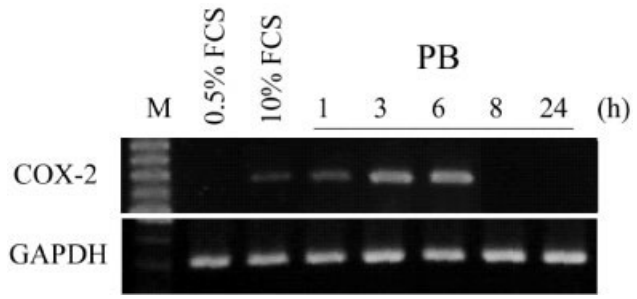

Figure 5. Expression of COX-2 mRNA gene in PB-treated human gingival fibroblasts by RT-PCR assay. A DNA ladder of known base pairs was used for identification of PCR products. GAPDH gene was performed in order to monitor equal RNA loading. FCS samples (0.5 and 10%) serve as controls.

level after 8 h. The quantitative measurement was made by the AlphaImager 2000. The levels of the COX-2 mRNAs increased about 3.2-, 2.9-, and 1.4-fold after exposure to SB for 1, 3, and 6 h, respectively (Figure 4).

The induction of COX-2 mRNA expression by PB in human gingival fibroblasts was shown in Figure 5. A significant signal was first detectable after 1 h of exposure and diminished to the control level after 8 h (Figure 5). The quantitative measurement was made by the AlphaImager 2000. The levels of the COX-2 mRNAs increased about 1.2-, 4.3-, and 4.6-fold after exposure to PB for 1, 3, and 6 h, respectively (Figure 6).

Expression of COX-2 protein in human gingival fibroblasts challenged with dentin bonding agents was directly assessed in cell lysates using Western blot analysis. Figure 7 shows that human gingival fibroblasts resting in 0.5% FCS did not express COX-2. In addition, CB, SB, and PB were found to induce COX-2 protein expression in human gingival fibroblasts (Figure 7). The kinetics of this response affected by CB, SB, and PB showed that COX-2 was detectable at 2, 4, and 8 h, respectively, and remained elevated throughout the 24-h incubation period.

The quantitative measurement of COX-2 protein was made by the AlphaImager 2000 (Figure 8). The levels of the COX-2 protein increased about 1.2-, 1.3-, 2.33-, and 3.2-fold after exposure to CB for 2, 4, 8, and 24 h, respectively. The levels of the COX-2 protein increased about 1.7-, 1.8-, and 3.1-fold after exposure to SB for 4, 8, and 24 h, respectively. The levels of the COX-2 protein increased about 1.8- and 3.4-fold after exposure to PB for 8 and 24 h, respectively.

DISCUSSION

Murine fibroblast cell line L929,[15–17] mouse odontoblast-like cell line MDPC-23,[18] human pulp cells,[19] and human gingi-

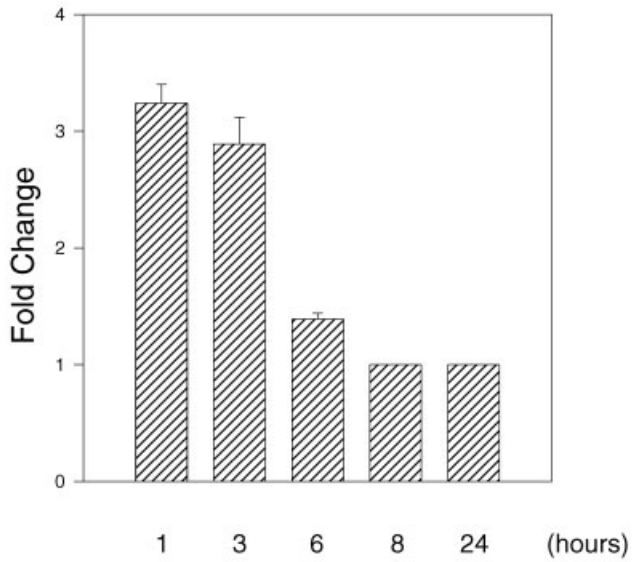

Figure 4. Levels of COX-2 mRNA gene treated with SB were measured by AlphaImager 2000. The relative level of COX-2 gene expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the mean ± SD.

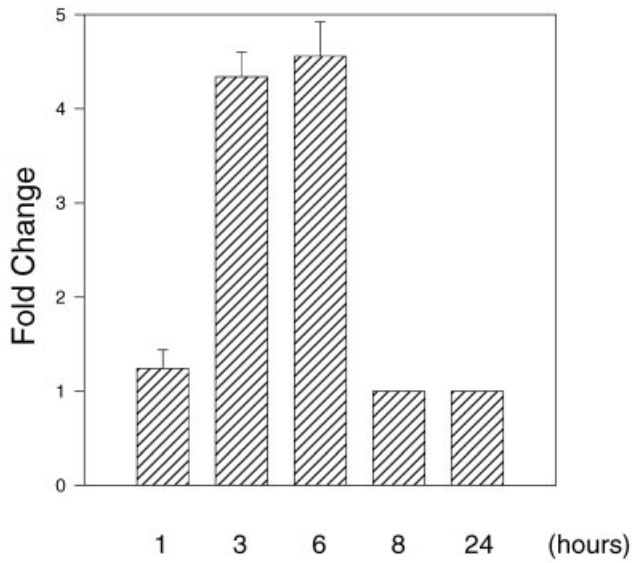

Figure 6. Levels of COX-2 mRNA gene treated with PB were measured by AlphaImager 2000. The relative level of COX-2 gene expression was normalized against GAPDH mRNA signal and the control was set as 1.0. Optical density values represent the mean ± SD.

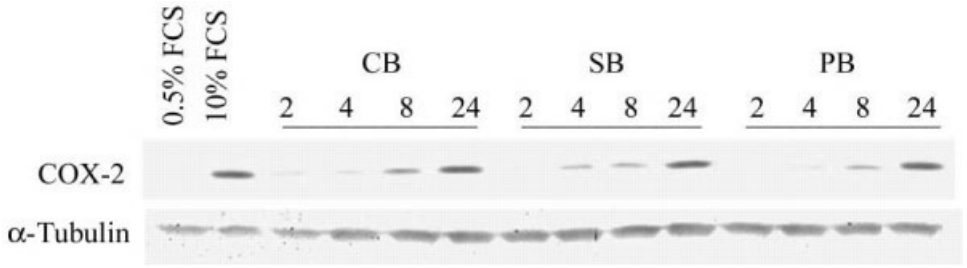

Figure 7. Expression of COX-2 protein in dentin bonding agent–treated human gingival fibroblasts by Western blot. Lanes 1 and 2 represent controls: 0.5% and 10% FCS. Lanes 3–5 represent CB: 2, 6, and 24 h, respectively. Lanes 6–8 represent SB: 2, 6, and 24 h, respectively. Lanes 9–11 represent PB: 2, 6, and 24 h, respectively. α-tubulin was performed in order to monitor equal protein loading.

val fibroblasts[11,20] have been applied to evaluate the biocompatibility of dentin bonding agents. Although culture systems vary, most employ cells that are transformed or established cell lines as the model for cell response. However, normal diploid cells differ from established or transformed cells in many ways.[21] Normal diploid cells from primary culture have a more normal phenotype and they correlate to *in vivo* response more accurately.[22,23] Human gingival fibroblasts were obtained as primary culture from explants of biopsy. The use of human gingival fibroblasts permits enhanced relevance, and it is frequently argued that dentin bonding agents that come into direct contact with the gingiva may have the potential to damage it.[2,3]

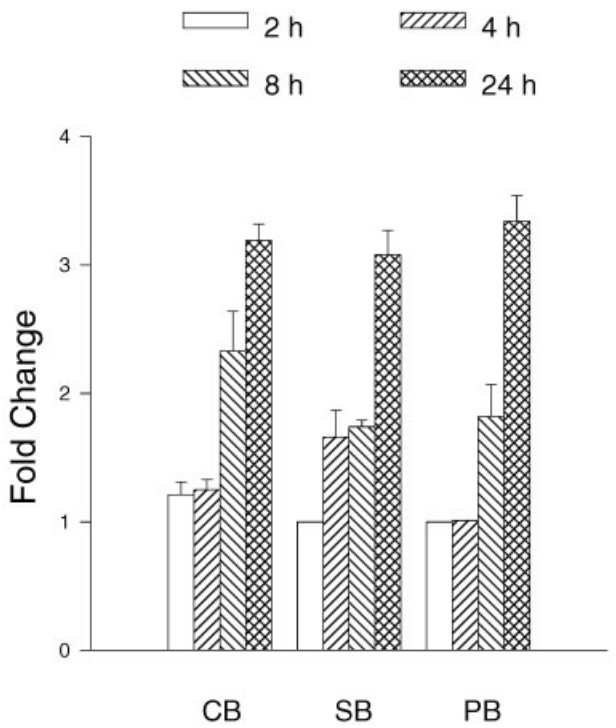

Figure 8. Levels of COX-2 protein treated with dentin bonding agents were measured by Alphalmager 2000. The relative level of COX-2 protein expression was normalized against α-tubulin signal and the control was set as 1.0. Optical density values represent the mean ± SD.

PG synthesis is initiated by the 1:1 exaction of arachidonic acid from the cell in membrane phospholipids through the action of phospholipases A2. The metabolism of arachidonic acid via the COX pathway results in the production of PG. COX is the rate-limiting enzyme in the production of PG.[8] Many of the inflammatory and inducible effects of COX are mediated through COX-2 pathway,[9,10] because the gingival injury from dentin bonding agents results in an inflammatory process.[2,3] This study focused on the effect of dentin bonding agents on human gingival fibroblasts by COX-2 expression.

This study first found that dentin bonding agents were capable of stimulating COX-2 mRNA and protein expression in human gingival fibroblasts. Recently, Morton and Dongari-Bagtzoglou[24] have shown that COX-2 may play an important role in the pathogenesis of gingival inflammation. This study leads to the suggestion that COX-2 expression may play a critical role in dentin bonding agent-induced gingival inflammation.

Various histological investigations have indicated that components leaching from dentin bonding agents may induce local inflammatory effects. In the present study, dentin bonding agents were found to induce COX-2 expression in human gingival fibroblasts. Constituents of dentin bonding agents, such as bisphenol-A-glycidyl methacrylate (Bis-GMA), triethyleneglycol di-methacrylate (TEGDMA), 2-hydroxy-ethyl-methacrylate (HEMA), and urethane di-methacrylate (UDMA), were found to leach from dentin bonding agents.[25–28] These leachable components from dentin bonding agents might cause some inflammatory effects. HEMA as well as Bis-GMA, for instance, have been associated with inflammatory pulp responses.[25] In addition, HEMA and methacrylic acid (a hydrosis product of Bis-GMA) were found to stimulate macrophages for release of tumor necrosis factor α (TNF-α).[29] HEMA and TEGDMA were found to induce inflammatory mediators TNF-α[30] and heat shock protein[31] in THP-1 human monocytes *in vitro*. Recently, Kostoryz et al.[32] have shown that dentin bonding agents can induce IL-6 release from macrophages. These results may partly explain why dentin bonding agents can induce inflammatory reactions.

The mechanism responsible for the COX-2 expression by dentin bonding agents may explain as follows. Recently,

epoxy-resin–based root-canal sealer was found to induce COX-2 expression in human osteoblastic cells.[14] Moreover, enhanced *c-fos* mRNA levels have been found in dentin bonding agent-treated human gingival fibroblasts,[11] suggesting that the AP2 region in the COX-2 promoter region might also be involved, as described by Inoue et al.[33]

As far as is known, the effects of dentin bonding agents on COX-2 expression in human gingival fibroblasts have not previously been studied. Data from these *in vitro* experiments showed that dentin bonding agents were capable of stimulating COX-2 mRNA and protein expression in human gingival fibroblasts. This suggests that one of the pathogenic mechanisms of gingival tissue inflammation *in vivo* may be the synthesis of COX-2 by resident cells in response to the dentin bonding agent challenge. COX-2 may play a pivotal role in the control of gingival inflammation. However, care should be taken to reduce the possibility of gingival irritation from dentin bonding agents in clinical application until their side effects are more thoroughly studied.

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