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Induction of heme oxygenase-1 expression by dentin bonding agents in human pulp cells

Abstract

Aim The purpose of this study was to investigate the effects of dentine bonding agents on the expression of HO-1 in human pulp cells.

Methodology Set specimens from Clearfil SE Bond (CB), Prime & Bond 2.1 (PB), and Single Bond (SB) were eluted with culture medium. Cytotoxicity and Western blot assays were used to investigate the effects of human primary pulp cells exposed to dentine bonding agents.

Results Our data showed that CB, PB, and SB were cytotoxic to pulp cells in a concentration-dependent manner ($p<0.05$). The exposure of quiescent pulp cells to CB, PB, and SB resulted in the induction of HO-1 protein expression in a time-dependent manner ($p<0.05$). The influence of the cytotoxicity and HO-1 expression depended on the materials tested.

Conclusion Taken together, HO-1 expression might be one signal transduction pathway linked to the induction of stress-inducible protein by dentine bonding agents.

Key words: dentin bonding agents; heme oxygenase-1; pulp cells

Introduction

Dentine bonding agents are designed to form strong bonding with dentine. 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 dentine-resin interface and scatter the occlusal stress. They usually remain in close contact with living dental tissue over a long period of time. Ideally, dentine bonding agents should be biocompatible.

Monomers eluted from dentine bonding systems and bacterial microleakage have been implicated as possible causes of pulpal irritation after composite resin restoration (Akimoto *et al*. 1998, Cox *et al*. 1998). Investigators have measured cell growth, cytotoxicity, and genotoxicity as indicators of cellular response to dentine bonding agents using murine fibroblast cell line L929 (Schedle *et al.* 1998, Hashieh *et al*. 1999, Kaga *et al*. 2001), mouse odontoblast-like cell line MDPC-23 (de Souza Costa *et al*. 1999), human gingival fibroblasts (Szep *et al*. 2002, Huang *et al.* 2003) and human pulp cells (Huang & Chang 2002, Huang *et al*. 2005a). However, the intracellular mechanisms altered by dentine bonding agents are still not clear.

Heme oxygenase (HO) is originally identified as an enzyme that catalyzes the initial reaction in heme catabolism the oxidative cleavage of the α-meso carbon bridge of b-type heme molecules to yield equimolar quantities of biliverdin IXa,

cabon monoxide (Kageyama *et al.* 1992). HO has long been known to undergo adaptive regulation in response to heme. There are two major isoforms: HO-1 and HO-2. HO-2 is the major isoform that presents under physiological conditions, localized in microsomes, and the stress-inducible isoform HO-1 is localized in mitochondria. HO-1 expression is very sensitive to stress, and is induced by many stimuli, including heme, heavy metal, heat shock, endotoxin, inflammatory cytokines, prostaglandins, and oxidative stress (Keyse *et al.* 1990, Savdana *et al.* 1992, Lautier *et al.* 1992).

Intra-orally, cells are exposed to many stressors from dental materials or bacteria. HO-1 functions as an anti-oxidant enzyme because locally produced bilirubin works as an efficient scavenger of reactive oxygen species (Vogt *et al.* 1995). However, there is little information about the ability of dentine bonding agents to modulate the expression of HO-1. We hypothesized that if dentin bonding agents stress cells, this stress may be manifest by the induction of HO-1 expression. To investigate this hypothesis, we exposed human pulp cells to dentin bonding agents by using cytotoxicity and Western blot assays.

Materials and methods

Sample preparation

Three dentine bonding agents were evaluated: Clearfil SE Bond (CB), Prime & Bond 2.1 (PB), and Single Bond (SB) (Table 1). In order to evaluate the effects of the photo-polymerized dentine bonding agents, 60 mm x 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 dentine bonding agent was applied and light-cured for 20 s. After polymerization, each test specimen was eluted in 8 ml of culture medium at 37℃ for 2 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, various dilutions (1:1, 1:2, and 1:4) of these extraction media were prepared to be used in this study.

Cell culture

Cell culture

Human pulp cells were cultured using an explant technique as described previously (Huang *et al*. 2004, Huang *et al*. 2005b). Briefly, impacted third molars were obtained from healthy patients of the Oral Medicine Centre (Chung Shan Medical University Hospital, Taichung, Taiwan) with the informed consent. Teeth were sectioned horizontally below the cementoenamel junction with a number 330

high-speed bur with water spray. The pulp tissue was removed aseptically in lamina flow, rinsed with Hanks' buffered saline solution, and placed in a 60 mm dish. Pulp tissue was minced with a blade into small fragments and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % foetal calf serum (FCS) and antibiotics (100 U/ml penicillin, 100 μg/ml 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 in this study.

Cytotoxicity assay

A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay was developed to monitor mammalian cell survival and proliferation in vitro. Briefly, 2×10^4 cells per well were seeded to 96 well plate and left overnight to attach. Serial dilutions of various elute in 100 µl volumes were added, and cells were treated for 24 h. After treatment, 50 µl of MTT (Sigma, St. Louis, MO) solution (1mg/ml in PBS) was added to each well and incubated for another 4 hours at 37°C. To each well, 150 µl of dimethyl sulfoxide was added. Plates were then shaken until crystals were dissolved. Reduced MTT was then measured spectrophotometrically in a dual beam microtiter plate reader at 570 nm with a 650

nm reference. The percentage of the dehydrogenase activity at each material, compared with that of the control, was calculated from the absorbance values.

HO-1 mRNA gene expression analysis

Cells arrested in G_0 by serum deprivation (0.5 % FCS for 48 h) were generally used in these experiments. Prior to treatment, the cells were washed with serum-free DMEM and immediately exposed for the indicated incubation times (1, 2, 4, 8, and 24 h) to various extraction media. The viability of cells exposed to these elutes were in general cytoatatic according to the MTT assay.

Total RNA was prepared using TRIzol reagent (Gibco Laboratories, Grand Island, NY, USA) following 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 Laboratories, Grand Island, NY, USA). The reaction mixture was diluted with 20 μl of water and 3 μl of the diluted reaction mixture was used for the polymerase chain 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 HO-1 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 (Quan *et al*, 2004):

A) GAPDH Forward: 5'-TCCTCTGACTTCAACAGCGACACC-3'

Reverse: 5'-TCTCTCTTCCTCTTGTGCTCTTGG-3'

B) HO-1 Forward: 5'-CAGGCAGAGAATGC TGAGTTC-3'

Reverse: 5'-GATGTTGAGCAGGAACGCAGT-3'

The PCR products were analyzed by agarose gel electrophoresis and a 550 bp band for HO-1 was noted. 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, USA).

Statistical analysis

Three replicates of each concentration were performed in each test. All assays were repeated three times to ensure reproducibility. Statistical analysis was by one-way analysis of variance (ANOVA). Tests of differences of the treatments were analyzed by Duncan's test.

Results

As shown in Fig. 1, the cytotoxicity of elutes of dentine bonding agents were evaluated using MTT assay in pulp cells. The results showed that CB, PB, and SB were cytotoxic to pulp cells in a concentration-dependent manner during 24-h incubation period ($p<0.05$). The toxicity decreased in an order of PB>SB>CB. In vivo acute toxicity is usually not associated with the clinical use of a dental material. Thus, the sublethal doses of all dentine bonding agents (1:4 dilution) were selected in the following experiments.

Investigations of the time dependence of HO-1 expression in CB-treated cells revealed a rapid accumulation of the transcript, a significant signal first detectable after 1 h of exposure and remained elevated throughout the 24-hour incubation period (Fig. 2). Moreover, the peak of HO-1 level induced by CB was at 4 h (Fig. 2). The quantitative measurement was made by the AlphaImager 2000. The levels of the HO-1 increased about 1.7, 4.6, 5.9, 4.0, and 2.2 fold after exposure to CB for 1, 2, 4, 8, and 24 h, respectively (Fig. 2).

The induction of HO-1 expression by PB in pulp cells was similar to that of CB. A significant signal first detectable after 1 h of exposure and remained elevated throughout the 24-hour incubation period (Fig. 3). The peak of HO-1 level induced by PB was at 4 h. The quantitative measurement was made by the AlphaImager 2000.

The levels of the HO-1 increased about 2.9, 2.8, 3.4, 1.9, and 1.5 fold after exposure to PB for 1, 2, 4, 8, and 24 h, respectively (Fig. 3).

Investigations of the time dependence of HO-1 expression in SB-treated cells is shown in figure 4. It demonstrated, a significant signal first detectable after 2 h of exposure. The peak of HO-1 level induced by SB was at 8 h. The quantitative measurement was made by the AlphaImager 2000. The levels of the HO-1 increased about 1.1, 2.0, 5.4, 6.3, and 1.4 fold after exposure to SB for 1, 2, 4, 8, and 24 h, respectively (Fig. 4).

Discussion

Many cell culture techniques have been applied to assess the biocompatibility of dental materials. These methods are based on cell cultures with established or diploid cell lines and tissue explant techniques. However, an increasingly number of authors has stated that in vitro biocompatibility tests should be performed with the most appropriate cells (i.e. cells homologous to the human tissues of ultimate concern) (Feigal *et al.* 1985). Any material used for restorative will come into contact with, or close proximity to the dental pulp. Thus, the effects of dentine bonding agents on this cell type may have clinical significance.

In the present study, the cytotoxicity of elutes of three dentine bonding agents was evaluated using MTT assay in pulp cells. It was found that all dentine bonding agents were cytotoxic to pulp cells. Data suggest substantial differences in cytotoxicity among three dentine bonding agents. Although the experimental conditions in this study differed from these used in other studies, our results were in agreement with previous studies (Schedle *et al.* 1998, Hashieh *et al.* 1999, de Souza Costa *et al.* 1999, Kaga *et al.* 2001, Szep *et al.* 2002, Huang & Chang 2002, Huang *et al.* 2003, Huang *et al.* 2005a) that CB, PB, and SB tested were cytotoxic.

HO-1 is known as a stress-inducible protein. HO-1 expression is very sensitive to stress, and is induced by many stimuli. Detectable amounts of HO-1, however, can

be seen in oral cells, such as gingival fibroblasts (Huang & Chang in press) and pulp cells (Min *et al*. 2006). In this study, HO-1 was first found to be upregulated in human pulp cells stimulated with dentine bonding agents. Recently, similar results were reported that HO-1 was found to be upregulated by resin based dental materials by human gingival fibroblasts (Huang & Chang in press). Thus, HO-1 expression might be one signal transduction pathway linked to the induction of stress response protein by dentine bonding agents.

HO-1 may be induced by a variety of non-heme products, such as reactive oxygen species, endotoxin, inflammatory cytokines, and nitric oxide (Choi *et al*. 1996). The functional significance of HO-1 induction is not well understood, as HO-1 activity can result in either cell protection or cell injury depending on the experimental setting (Da Silva *et al*. 1996). Keyse & Tyrrell (1989) have demonstrated that HO-1 is involved in the general cellular defense mechanisms against oxidative injury. Thus, this enzyme is believed to play an important role in maintaining cellular homeostasis in response to oxidant injury by dentine bonding agents.

Conclusions

HO-1 is known as a stress-inducible protein and functions as an antioxidant enzyme. Little is known about the induction of cellular signaling events after cell exposure to dentine bonding agents. In summary, the current study has shown that dentine bonding agents can significantly upregulated the expression of HO-1 at sublethal concentrations in human pulp cells. The response can be upregulated, depending on the material tested and time after stimulation.

ACKNOWLEDGMENTS

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

Material	Manufacturer	Components
Clearfil SE Bond (CB)	Kuraray Co, Osaka, Japan	Bis-GMA HEMA MDP CQ
Prime & Bond 2.1 (PB)	Dentsply De Trey Konstanz, Germany	Bis-GMA UDMA PENTA Cetylamine hydrofluoride
Single Bond (SB)	3M, St. Paul, MN, USA	Bis-GMA HEMA polyacrylic acid CQ

Figures for legends

Fig. 1

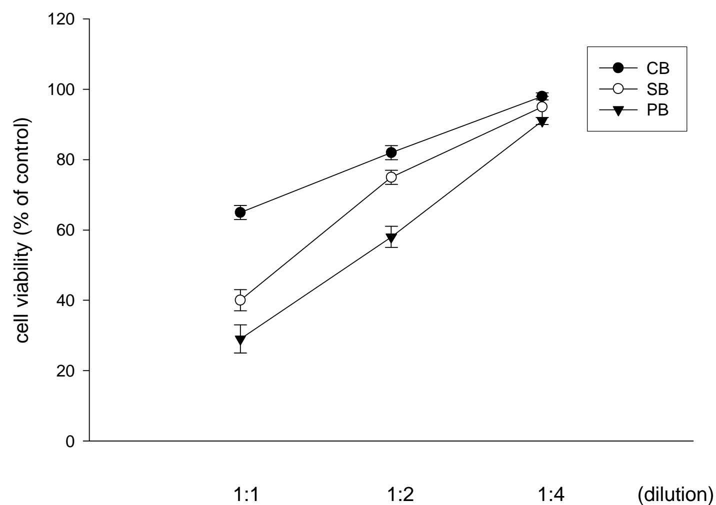

Fig. 1 Effect of elutes of CB, PB, and SB on human pulp cells by MTT assay for 24 h incubation period. Percentage of cell viability compared with that of control was calculated. Each bar represents a mean ± SD.

Fig. 2 (A)

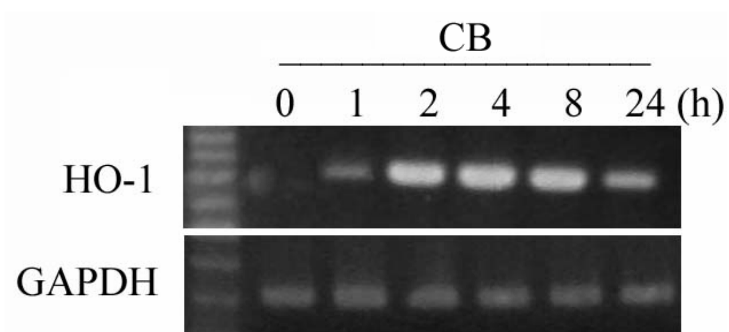

Fig. 2 (B)

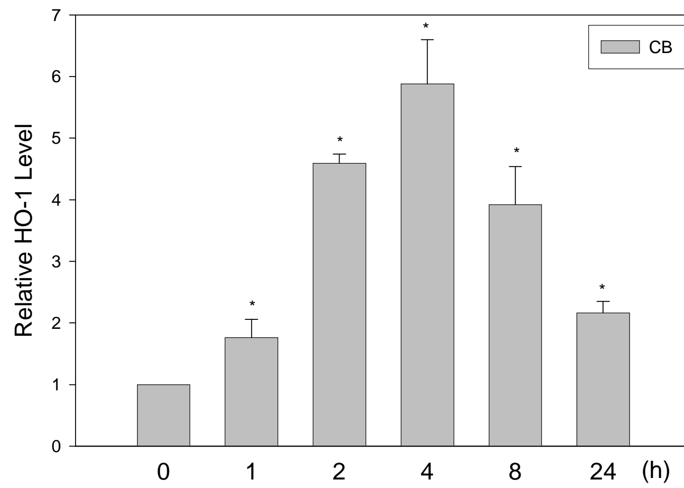

Fig. 2 (A) Kinetics of HO-1 mRNA gene expression in human pulp cells exposed to CB for 0, 1, 2, 4, 8, and 24 h, respectively. GAPDH was performed in order to monitor equal protein loading. (B) Levels of HO-1 mRNA treated with CB were measured by densitometer. The relative level of HO-1 mRNA expression was normalized against GAPDH signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p<0.05$.

Fig. 3 (A)

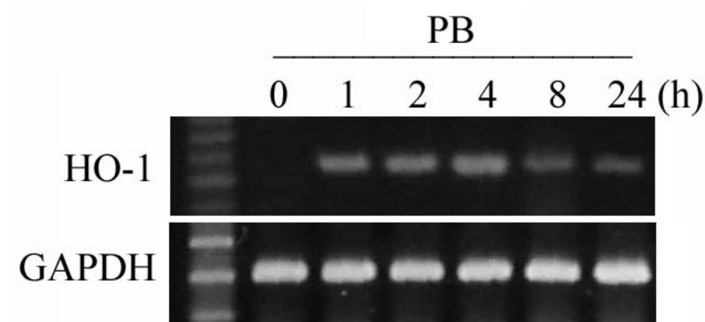

Fig. 3 (B)

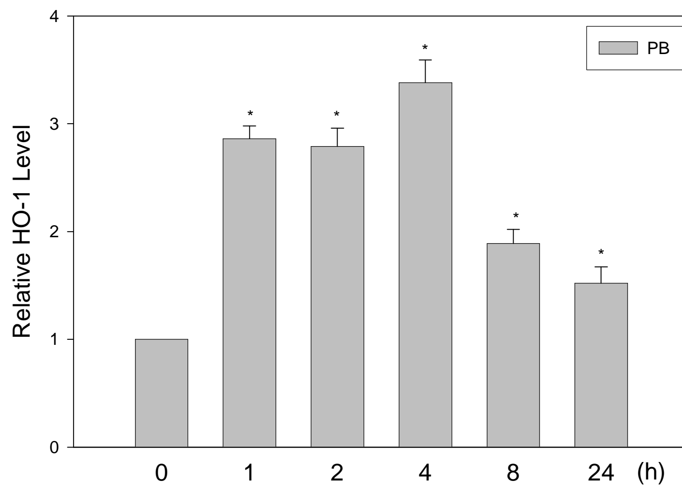

Fig. 3 (A) Kinetics of HO-1 mRNA gene expression in human pulp cells exposed to PB for 0, 1, 2, 4, 8, and 24 h, respectively. GAPDH was performed in order to monitor equal protein loading. (B) Levels of HO-1 mRNA treated with PB were measured by densitometer. The relative level of HO-1 mRNA expression was normalized against GAPDH signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p<0.05$.

Fig. 4 (A)

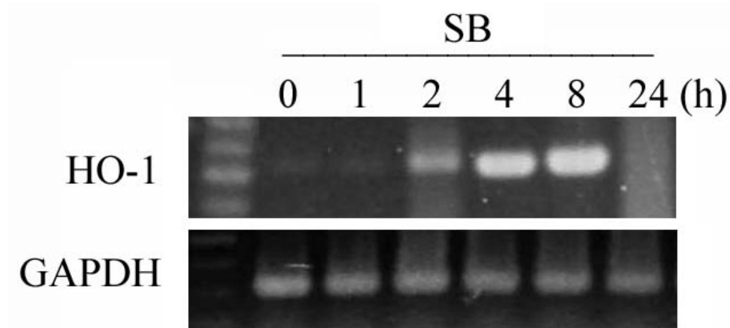

Fig. 4 (B)

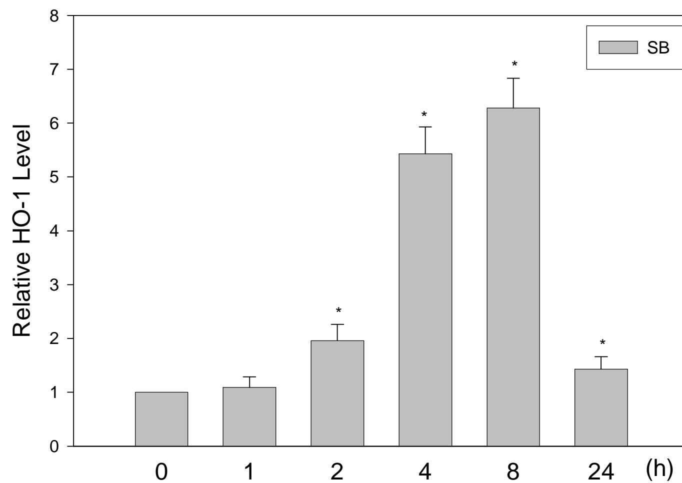

Fig. 4 (A) Kinetics of HO-1 mRNA gene expression in human pulp cells exposed to SB for 0, 1, 2, 4, 8, and 24 h, respectively. GAPDH was performed in order to monitor equal protein loading. (B) Levels of HO-1 mRNA treated with SB were measured by densitometer. The relative level of HO-1 mRNA expression was normalized against GAPDH signal and the control was set as 1.0. Optical density values represent the mean ± SD. * represents significant difference from control values with $p<0.05$.