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An *in vitro* evaluation of two resin-based sealers on proliferation and differentiation of human periodontal ligament cells

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Abstract

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Aim To evaluate the effects of a polymethyl methacrylate resin–based sealer [Superbond sealer (SB)] on the proliferation and osteogenic differentiation of human periodontal ligament cells (HPDLCs) *in vitro*, compared with a methacrylate resin–based sealer [Epiphany SE sealer (EP)].

Methodology Human periodontal ligament cells were obtained from of healthy third molar teeth of two participants with informed consent. To determine the effects of the eluent from set resin sealers on HPDLCs, the 7-day-washed (washed) or non-washed freshly prepared (fresh) set SB or EP discs were prepared. Cells cultured on these discs were evaluated by the WST-1 proliferation assay and scanning electron microscopy (SEM). The osteogenic differentiation of HPDLCs on washed SB discs was then evaluated by gene expression analysis of osteopontin (OPN) and osteocalcin (OCN) by using quantitative RT-PCR.

Results Human periodontal ligament cells exhibited growth on washed SB discs, whereas fresh SB and EP discs and washed EP discs inhibited proliferation of HPDLCs. SEM observation revealed that HPDLCs tightly attached and spread on the surface of washed SB discs, whilst no HPDLCs were observed on the surface of fresh and washed EP discs. Furthermore, HPDLCs significantly upregulated gene expressions of OPN and OCN when cultured on washed SB discs in osteogenic differentiation medium for 2 weeks.

Conclusions Although Superbond sealer initially exerted cytotoxic effects on HPDLCs, these effects were reduced during washing for 7 days compared to EP, which continued to be cytotoxic even though the specimens were washed for the same period of time. Washed Superbond allowed HPDLCs to differentiate into osteogenic cells.

Keywords: biocompatibility, Epiphany SE sealer, human periodontal ligament cells, Superbond sealer.

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Introduction

The major requirement for endodontic sealers is to fill the gaps between the core materials and the root canal wall (Limkangwalmongkol *et al.* 1991). Biocompatibility and insolubility of sealers are important, because they come in contact with host tissues apically, might affect wound healing and help to maintain the effectiveness of the root filling (Peters 1986). Low solubility of endodontic sealers is a requirement of the ISO 6876 standard (Geurtsen 2001). The recent demand for resin-based sealers has increased because of their potential adhesive properties and penetration into root canal walls (Shipper & Trope 2004, Ishimura *et al.*

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2007). Recent adhesive resin sealers have been improved with regard to their adhesive capacity to dentine. However, concerns associated with their cytotoxicity remain (Lodiene *et al.* 2008).

It is well known that methyl methacrylate (MMA)-based resins demonstrate strong adhesion to dentine (Kataoka *et al.* 2000, Ari *et al.* 2003). Previously, it has been demonstrated that Superbond, an MMA-based resin, exhibited biocompatible properties when transplanted into a rat mandible (Maeda *et al.* 1999). However, this resin has limitations, including low radiopacity and difficulty in removal. Therefore, Superbond sealer (SB; Sun Medical Co. Ltd., Shiga, Japan) was developed to overcome these challenges. SB, also known as RC sealer, is composed of polymethyl methacrylate (PMMA), zirconium oxide and 4-methacryloxyethyl trimellitate anhydride/methyl methacrylate (4-META/MMA) as a monomer and partially oxidized tri-n-butylborane (TBBO) as a catalyst. SB applied to root dentine has been reported to form substantial resin tags extending over 100 μm into the dentine matrix (Hayashi *et al.* 2008) and have similar sealing potential of AH Plus and Rocanal 2 (Cobankara *et al.* 2006).

Epiphany SE sealer (EP; Pentron Clinical Technologies LLC, Wallingford, CT, USA) is a self-etch, dual-cure, hydrophilic resin sealer and is composed of ethoxylated bisphenol A dimethacrylate, bisphenol A glycidyl dimethacrylate, 2-hydroxyethyl methacrylate (HEMA) and acid methacrylate resins. Its development was based on the original Epiphany sealer. Because methacrylate resin–based sealers are hydrophilic, they can be applied to wet root canals (Zmener *et al.* 2008). Furthermore, the sealing ability of EP is almost the same as that of AH Plus (Shokouhinejad *et al.* 2010). However, some studies have reported cytotoxicity of the original Epiphany sealer (Lodiene *et al.* 2008, Camargo *et al.* 2009, Al-Hiyasat *et al.* 2010). A recent report also demonstrated that EP exhibited a similar level of cytotoxicity as the Epiphany sealer (Gambarini *et al.* 2009).

Although resin-based sealers directly contact periapical tissues and are consistently subjected to tissue fluids, these sealers are known to initially leach cytotoxic components. Therefore, in this study, the cytotoxic effects of SB and EP have evaluated using 7-day-washed (washed) or non-washed freshly prepared (fresh) set sealers on human periodontal ligament cells (HPDLCs) that were cultured in direct contact with the materials. Additionally, the effects of these sealers on osteogenic differentiation of HPDLCs were investigated.

Materials and methods

Materials

Superbond sealer and EP were mixed according to the respective manufacturer's instructions. The discs of each sealer were then prepared following a modification of methods reported previously (Maeda *et al.* 2010). Briefly, mixed sealers were dispensed into a mould inset 9 mm diameter and 1 mm thickness. SB was placed in a humidified chamber at 37 °C for 24 h. EP was light-cured for 20 s at a distance 1 cm away from an LED light-curing device (Pencure; J. Morita MFG. Corp., Kyoto, Japan) and placed in a humidified chamber at 37 °C for 24 h. For washed groups (washed), set discs were rinsed every day for 7 days with 1.25 mL per disc of fresh alpha-minimum essential medium (α-MEM; Gibco-BRL, Grand Island, NY, USA), supplemented with 50 μg mL^{-1} streptomycin and 50 U mL^{-1} penicillin (Gibco-BRL). For non-washed freshly prepared groups (fresh), sealers were mixed 24 h prior to the beginning of co-culture, and set discs were prepared as described earlier. The washed or fresh discs were then placed in a 48-well or a 24-well tissue culture plate (one disc per well; Becton Dickinson Labware, Lincoln Park, NJ, USA), and cells were co-cultured on the discs.

Cell culture

Human periodontal ligament cells (denoted as HPDLC-DD and HPDLC-TD) were isolated as previously reported (Wada *et al.* 2001, Fujii *et al.* 2006) from the healthy third molars of two participants who visited the Dental Hospital of Kyushu University for orthodontic extractions. The PDL tissues removed from the middle portion of the root surface were incubated at 37 °C for 20 min in α-MEM containing 0.2% collagenase and 0.25% trypsin. The cells were initially cultured on 35-mm Primaria dishes (Becton Dickinson Labware) in α-MEM containing 10% foetal bovine serum (FBS; Gibco-BRL). Cells cultured from passages four through seven were used. HPDLCs were maintained in 10% FBS/α-MEM throughout the culture systems. The procedures were performed in compliance with the Research Ethics Committee, Faculty of Dentistry, Kyushu University, and informed consent was obtained from each patient.

For osteogenic differentiation assays, both HPDLC populations were cultured with washed SB or EP discs in control medium (CM) composed of 10% FBS/α-MEM, or in osteogenic differentiation medium (DM) composed

of CM including 50 mg mL^{-1} ascorbic acid (Nacalai Tesque, Kyoto, Japan) and 2 mmol L^{-1} β-glycerophosphate (Sigma-Aldrich Co., St Louis, MO, USA) (Maeda *et al.* 2004). All cultures were maintained in 100% humidity at 37 °C in a CO_2 incubator.

WST-1 proliferation assay

Human periodontal ligament cells (5×10^3 cells per well) were cultured in direct contact with washed or fresh discs of SB or EP in a 48-well plate in 250 μL of 10% FBS α-MEM per well for 3 days. For each group, four discs were prepared. As a positive control, HPDLCs were cultured on the plastic dish with no discs. On days 0, 1 and 3 of co-culture, viable cells were measured using a Cell Proliferation Assay kit (Millipore Corp., Billerica, MA, USA). Briefly, at indicated time-points, 25 μL of kit reagent, WST-1, was added to the culture medium of each well. After 90 min, 110 μL of supernatant was collected from each well, and the density was measured using an ImmunoMini NJ-2300 (System Instruments Co., Ltd., Tokyo, Japan) at absorbance of 450 nm. Experiments were performed in duplicate. Statistical analysis was performed by Student's *t*-test.

Scanning electron microscopy (SEM) observation

Human periodontal ligament cells (1×10^4 cells per well) cultured on washed SB and EP discs for 3 days were fixed at 4 °C for 3 h with 2.5% glutaraldehyde in phosphate-buffered saline (PBS) and post-fixed with 1% OsO_4 in 0.1 mol L^{-1} cacodylate buffer (pH 7.4) for 1 h. The cells were then dehydrated with an increasing ethanol series and finally treated with hexamethyldisilazane (Sigma-Aldrich Co.). After the specimens were dried completely and gold-sputter-coated, they were observed using FE-SEM (Hitachi Co. Ltd., Tokyo, Japan).

Quantitative RT-PCR

Human periodontal ligament cells (1×10^4 cells per well) were cultured in direct contact with washed SB and EP discs in CM or DM. For each group, four discs were prepared. After culturing for 2 weeks, first-strand cDNA was synthesized from 1 μg of total cellular RNA using an ExScript™ RT Reagent kit (Takara Bio Inc., Shiga, Japan), and PCR was performed using SYBR Green I (Takara Bio Inc.) in a Thermal Cycler Dice™ Real Time System (Takara Bio Inc.) according to previous description (Tomokiyo *et al.* 2008, Maeda *et al.* 2010). After initial denaturation at 95 °C for 10 s, subsequent amplification was followed by 40 cycles of 95 °C for 5 s, the appropriate annealing temperature for 30 s, 72 °C for 30 s (amplification) and a dissociation programme at 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. Specific primer sequences were as follows: osteopontin (OPN) (115 bp), forward 5′-ACACATATGATGGCCGAGGTGA-3′, reverse 5′-TGTGAGGTGATGTCCTCGTCTGTAG-3′; osteocalcin (OCN) (112 bp), forward 5′-CCCAGGCGCTACCTGTATCAA-3′, reverse 5′-GGTCAGCCAACTCGTCACCAGTC-3′; β-Actin (β-Act) (89 bp), forward 5′-ATTGCCGACAGGATGCAGA-3′, reverse 5′-GAGTACTTGCGCTCAGGAGGA-3′. A β-Act primer was used as an internal control. Expressions of the target genes were calculated from the delta–delta C_t ($\Delta\Delta C_t$) values. Experiments were performed in duplicate. Statistical analysis was performed by Student's *t*-test.

Results

Proliferation of HPDLCs on SB and EP discs

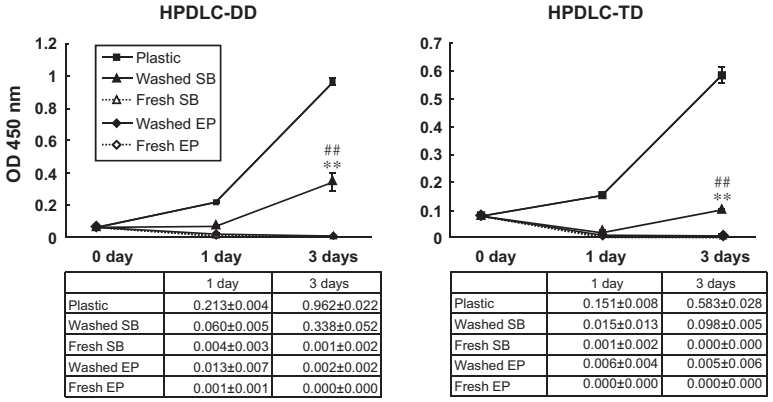
Although the growth of HPDLC-DDs and HPDLC-TDs cultured on washed SB discs for 1 day was inhibited, both HPDLCs exhibited significant cell growth after 3 days of culture compared to the growth on the other discs; however, the growth was slower than that of control cells cultured on plastic dishes (Fig. 1). The growth of HPDLCs cultured on fresh SB discs or washed and fresh EP discs was almost completely inhibited during 3 days of culture (Fig. 1).

SEM observation

Human periodontal ligament cells cultured on washed SB and EP were examined. The surface of SB discs was rough, and two kinds of particles were observed under SEM (Fig. 2a). HPDLCs were tightly attached onto zirconium oxide as well as PMMA particles of washed SB discs and spread on these discs after 3 days of co-culture (Fig. 2b,c). Contrarily, no cells were observed on the surface of washed EP discs (Fig. 2d). We also found no HPDLCs on fresh SB or EP discs (data not shown).

Gene expressions of OPN and OCN in HPDLCs cultured with washed SB

After 14 days of culture with washed SB discs, both HPDLCs incubated in DM significantly upregulated



	1 day	3 days
Plastic	0.213±0.004	0.962±0.022
Washed SB	0.060±0.005	0.338±0.052
Fresh SB	0.004±0.003	0.001±0.002
Washed EP	0.013±0.007	0.002±0.002
Fresh EP	0.001±0.001	0.000±0.000

	1 day	3 days
Plastic	0.151±0.008	0.583±0.028
Washed SB	0.015±0.013	0.098±0.005
Fresh SB	0.001±0.002	0.000±0.000
Washed EP	0.006±0.004	0.005±0.006
Fresh EP	0.000±0.000	0.000±0.000

Figure 1 Proliferation assay of human periodontal ligament cell (HPDLC)-DDs and -TDs cultured on Superbond sealer (SB) or Epiphany SE sealer (EP) discs. After 1 day of co-culture, the proliferation of HPDLCs was inhibited compared to the cells cultured on the plastic dish. However, after 3 days, proliferation of HPDLCs cultured on washed SB discs significantly increased in comparison with the culture on fresh SB discs or washed or fresh EP discs. The growth of HPDLCs cultured on fresh SB discs or washed and fresh EP discs was almost completely inhibited. Control culture was performed on plastic dishes. **< 0.01 versus fresh SB, ##< 0.01 versus washed and fresh EP.

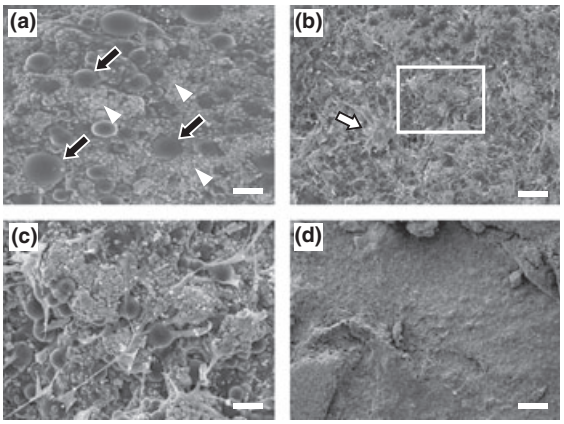


Figure 2 Scanning electron microscopy images. Superbond sealer (SB) discs exhibited rough surfaces, and two kinds of particles in different sizes were observed (a). The larger particles indicated by black arrows are zirconium oxide, and smaller particles indicated by white arrowheads are polymethyl methacrylate (PMMA). The growth of human periodontal ligament cells (HPDLCs) cultured for 3 days on washed SB discs was observed (b, c). HPDLCs were tightly attached and spread on PMMA as well as zirconium oxide particles and were aggregated (arrow) on the surface of washed SB discs (b). A higher magnification of the square in (b) is shown in (c). No cells were observed on the surface of Epiphany SE sealer discs after 3 days of culture (d). Bars in a and c: 50 μm. Bars in b and d: 150 μm.

gene expression of OPN when compared with HPDLCs incubated in CM, even though this upregulation was lower compared with its expression in cells cultured on the plastic dishes in DM (Fig. 3a). The upregulated expression of OCN in both HPDLCs cultured in DM was gradual compared with that of OPN. The expression level of OCN in HPDLCs cultured with washed SB discs in DM was close to that of cells cultured on plastic dishes in DM (Fig. 3b). Although HPDLCs cultured with washed EP discs were also subjected to the same analysis, no transcripts were detected because no significant mRNA was collected (data not shown).

Discussion

This study demonstrated by culturing HPDLCs in direct contact with two resin-based sealers that washed SB displayed biocompatibility for HPDLCs, whereas EP exerted cytotoxic effects on HPDLCs in the current culture system and furthermore that HPDLCs cultured on washed SB discs in DM showed osteogenic differentiation. The significant finding of this study is that the cytotoxic eluents from set SB are reduced in 7 days and then that SB allowed the osteogenic differentiation of HPDLCs.

Biocompatibility of root canal sealers is important, particularly because the sealers maintain contact with host periapical tissues. Notably, resin-based sealers are known to release cytotoxic components. Although the biocompatibility of Superbond cement *in vivo* has been reported (Maeda *et al.* 1999), it was not suitable as a root canal sealer because of its short working time, low

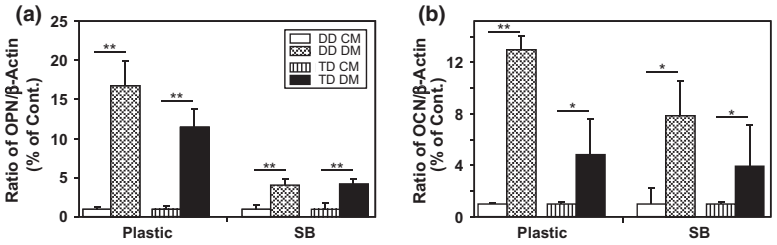


Figure 3 Gene expressions of osteopontin (OPN) (a) and osteocalcin (OCN) (b) in human periodontal ligament cell (HPDLC)-DDs and -TDs. HPDLCs were cultured alone on a plastic dish or cultured with washed Superbond sealer discs for 14 days in control medium (CM) or osteogenic differentiation medium (DM). Total RNAs were then subjected to real-time PCR analysis. Four wells per group were subjected to the analysis. Experiments were performed in duplicate. **< 0.02, *< 0.05.

radiopacity and difficulty in removal from the root canal (Imai & Komabayashi 2003). In this context, to resolve these problems, SB was developed for use as a root canal sealer (Imai & Komabayashi 2003). Att *et al.* (2009) reported the cytotoxicity of PMMA. However, SEM analysis revealed that HPDLCs attached and spread on the PMMA particles in washed SB discs. This suggested that the cytotoxicity of PMMA might be reduced by washing. Furthermore, SB includes partially oxidized TBBO as a catalyst and 4-META/MMA as a monomer. Eldeniz *et al.* (2007) reported that extracts from fresh SB (RC sealer) exhibited cytotoxic effects on human gingival fibroblasts and a mouse fibroblastic cell line. The authors suggested that such cytotoxicity might be caused by the TBBO component in SB, based on a previous study that indicated that the cytotoxicity of a 4-META/MMA-TBBO resin might be preferably associated with TBBO (Fujisawa & Atsumi 2004). Therefore, it is speculated that PMMA and TBBO in SB would exert cytotoxic effects in fresh SB on HPDLCs, whereas the cytotoxic effects of these components could be reduced by washing within 7 days. Thus, once such cytotoxic constitutes are removed from SB, HPDLCs might be able to easily attach and grow on the SB surfaces, and even show osteogenic differentiation. Therefore, when SB is used to fill the root canal, there is a possibility that periodontal ligament cells or cementoblasts could fill the apical foramen following the formation of cementum-like tissues.

Heitman *et al.* (2008) suggested that the degree of cytotoxicity of the original Epiphany sealer increased with increasing material concentration or exposure time. A recent report indicated that the cytotoxicity of EP might be attributable to partial degradation associated with the solubility of the EP sealer (Donnelly *et al.* 2007), such as leaching of filler particles (calcium hydroxide, barium sulphate, barium glass and silica (Versiani *et al.* 2006)) contained in the sealer. Brackett *et al.* (2009) reported that Epiphany sealer did not activate the secretion of inflammatory cytokines in THP1 monocytes by itself *in vitro*, but the degradation products from Epiphany might exert cytotoxic effects on these cells even though the sealer was immersed in PBS for 12 weeks. These degraded products might cause the detection of no cells and no significant mRNA in washed or fresh EP cultures in our current experiment. HEMA also exhibits cytotoxicity and inhibits the growth of a variety of cells (Ratanasthien *et al.* 1995, Chang *et al.* 2005). Additional reports suggested that the cytotoxicity was a result of the residual monomers in EP, which are the main components released from cured composite resins (Eldeniz *et al.* 2007). The latter report also indicated low cytotoxicity of SB (RC sealer). These results could support the current data. However, Sousa *et al.* (2006) reported that Epiphany sealer implanted in bone did not induce inflammation, but demonstrated intraosseous biocompatibility. Thus, more studies are needed to discern the cytotoxicity and biocompatibility of EP and its efficiency *in vivo*.

Conclusions

The cytotoxicity of washed and fresh EP observed in the current study might be related to the continuous elution of the uncured monomer or some soluble components from set EP. The results suggest that the cytotoxic components in SB could be eliminated in a comparatively short time after the set by washing, and thus, SB might be a more biocompatible material compared to EP.

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