Influence of polymerization properties of 4-META/MMA-based resin on the activity of fibroblast growth factor-2

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Dental adhesive resins based on 4-methacryloxyethyl trimellitate anhydride (4-META)/methyl methacrylate (MMA) have been utilized for root-end filling and the bonding of fractured roots. To increase the success rate of these treatments, it would be beneficial to promote the healing of surrounding tissue by applying growth factors. In this study, the influences of the polymerization properties of 4-META/MMA-based resins on the activity of fibroblast growth factor-2 (FGF-2) were evaluated *in vitro*. The temperature increase caused by the heat generation during polymerization of the 4-META/MMA-based resin was insufficient to change the structure and function of FGF-2. Unpolymerized monomers released from the cured 4-META/MMA-based resin had no negative influences on the ability of FGF-2 to promote the proliferation of osteoblast-like cells. These findings suggest that it is possible to use FGF-2 in combination with 4-META/MMA-based resins.

Keywords: 4-META/MMA-based resin, Fibroblast growth factor-2, Osteoblast-like cells

INTRODUCTION

The application of dental adhesive resins is expanding to include various treatments such as the restoration of fractured roots^{1–4}, root-end fillings^{5,6}, and perforation sealing⁷. Among a plethora of adhesive resins, those based on 4-methacryloxyethyl trimellitate anhydride (4-META)/methyl methacrylate (MMA) exhibit high biocompatibility with the periodontal tissues and excellent bonding and sealing capabilities in wet conditions^{3,4,8–11}. Imazato *et al.*^{12,13} reported that cured 4-META/MMA-based resin produces minimal deleterious effects on cell proliferation and differentiation in osteoblast-like cells and pluripotent mesenchymal precursor cells, suggesting a potential advantage for regeneration of periapical and/or periodontal tissues. However, 4-META/MMA-based resins have no intrinsic ability to promote regeneration, and in some cases severe damage of the surrounding tissue can be an unintended consequence of its use². Furthermore, when used for bonding of fractured roots, there have been reports of incomplete healing (*e.g.*, residual periodontal pockets) due to insufficient regeneration of periodontal tissue¹⁴.

In order to increase the success rate of treatments using such adhesive materials, it would be beneficial to co-administer growth factors to promote healing in the surrounding tissue. Fibroblast growth factor-2 (FGF-2) stimulates growth and the development of angiogenesis and tissue development¹⁵ and is widely used to accelerate wound healing^{15,16}. In addition, several studies reported the efficacy, safety, and clinical

significance of FGF-2 for periodontal regeneration¹⁷. It promotes the proliferation of various types of cells such as vascular endothelial cells, chondrocytes, osteoblasts, and mesenchymal stem cells^{18–21}. Therefore, for treatments such as root-end filling or perforation repair—where severe damage has been caused to surrounding tissues—the co-application of FGF-2 with 4-META/MMA-based resin could enhance results. However, it has not yet been clarified if FGF-2 and 4-META/MMA-based resins are compatible, or whether the resin affects the activity of FGF-2. In this study, the effects of the heat generation during polymerization and monomer release from 4-META/MMA-based resin on FGF-2 activity were evaluated in osteoblast-like cells *in vitro*.

MATERIALS AND METHODS

Heat generation upon curing of 4-META/MMA-based resin

The monomer, powder and catalyst of commercial 4-META/MMA-based adhesive resin (Super Bond C&B, clear type; Sun Medical, Moriyama, Japan; SB) (Table 1) were mixed according to the manufacturer's instructions. Briefly, to a mixture of four drops of liquid monomer and one drop of catalyst, 0.08 g of the polymer powder was added and mixed for 30 s. The mixed paste was placed in a mold (10 mm diameter and 1 mm thickness), and a thermocouple (Handy Thermo DP-41; Eiwa Denshikeiki, Kyoto, Japan) was immediately inserted at its center (5 mm radius and 0.5 mm depth; Fig. 1). Temperature changes in the specimens were recorded every 30 s at ambient temperatures of 25 and 37°C. These experiments were repeated on five

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Table 1 Material composition

Material	Manufacturer	Components	Compositions
Super Bond C&B	Sun Medical, Moriyama, Japan	Monomer	MMA, 4-META
		Powder	PMMA
		Catalyst	TBB

MMA: methyl methacrylate, 4-META: 4-methacryloxyethyl trimellitate anhydride, PMMA: polymethyl methacrylate, TBB: tri-n-butyl borane.

Fig. 1 Measurement of temperature changes in SB resin during polymerization. A: thermocouple (Handy Thermo DP-41), B: diagram shows position of the thermocouple probe during temperature measurement.

independent occasions.

Release of unpolymerized monomers from cured 4-META/MMA-based resin

Monomer, powder, and catalyst were mixed as described above. Disc-shaped specimens (9 mm diameter and 1 mm thickness) were prepared by placing the mixture in a mold, sealing with a celluloid strip and a glass slide, and curing for 30 min at 25°C.

Cured specimens were immersed in 500 μ L of distilled water. After storage for 24 h, the concentration of unpolymerized monomer released was measured by high performance liquid chromatography (HPLC). The HPLC system (Prominence; Shimadzu, Kyoto, Japan) used a reverse-phase column (Inertsil ODS-3 column; GL Science, Tokyo, Japan). Acetonitrile and 5 mM phosphate buffer solution including 100 mM sodium perchlorate mixed at 70/30 (v/v) were used

for the mobile phase at a flow rate of 1.0 mL/min, and readings were performed at 240 nm. Since 4-META is quickly hydrolyzed to 4-methacryloxyethoxy carbonylphthalic acid (4-MET) in aqueous solutions, 4-MET (Sun Medical) and MMA (Tokyo Kasei Kogyo, Tokyo, Japan) were used for standard solutions. These experiments were repeated three times independently.

Influences of unpolymerized monomers on the activities of FGF-2

1. Cell culture

Mouse calvaria-derived osteoblast-like cells (MC3T3-E1; RIKEN Cell Bank, Tsukuba Science City, Japan) were maintained in humidified 5% CO₂ at 37°C with a growth medium containing α -minimum essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (SAFC Biosciences, Lenexa, KS, USA), 100 U/mL penicillin (Invitrogen), and 100 μ g/mL streptomycin (Invitrogen). Differentiation into osteoblasts was induced by adding ascorbic acid (Invitrogen) and β -glycerophosphoric acid (Wako Pure Chemical Industries, Osaka, Japan) to the growth media.

2. MTT assay

The MC3T3-E1 cells were seeded into the wells of a 96-well tissue culture plate at 2.0×10^4 cells/well and cultured for 12 h in a humidified 5% CO₂ incubator at 37°C. The media was replaced with fresh growth media containing 4-MET or MMA. The concentrations of each monomer tested (5 or 10 μ g/mL of 4-MET, and 5, 10, or 25 μ g/mL of MMA) were decided based on the results of a previously-conducted HPLC analysis of monomer release. FGF-2 (Fiblast; Kaken Pharmaceutical, Tokyo, Japan) was added to each well at a final concentration of 5 ng/mL and the culture was continued for another 24 h. Cells cultured without FGF-2 were used as controls.

Cell proliferation was determined by MTT assay. Briefly, 20 μ L of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl-tetrazolium bromide solution (MTT; Sigma Chemical, St. Louis, MO, USA) was added to each well and the plates were incubated for 4 h at 37°C. After removing the media, 110 μ L of 2% SDS/0.01 N HCl (Nacalai Tesque, Kyoto, Japan) was added to each well. The absorbance at 570 nm was measured using a microplate reader (Wallac 1420 ARVO Mx/Light; PerkinElmer, Waltham, MA, USA). The experiments were repeated three times independently.

3. Alkaline phosphatase (ALP) activity measurement

Cell differentiation was evaluated by determining ALP activity. MC3T3-E1 cells were seeded at 2.0×10^4 cells/well and cultured for 12 h, after which the culture medium was replaced with fresh differentiation medium containing 4-MET or MMA at the same concentrations as for the MTT assay. FGF-2 (5 ng/mL final concentration) was added to each well and the culture was continued for another 3 days. The medium was then replaced with fresh differentiation medium free of both FGF-2 and monomer, and the culture was continued for another 7 or 14 days.

After lysis of the cells by the addition of PBS containing 0.02% Triton-X (Nacalai Tesque), ALP activity was determined with the ALP Substrate Kit (Bio-Rad Laboratories, Hercules, CA, USA). To each 20 μ L sample was added 80 μ L of *p*-nitrophenyl phosphate (Sigma-Aldrich) and the mixture was incubated for 30 min at 37°C. The reaction was terminated by the addition of 100 μ L of 0.4 N NaOH (Wako Pure Chemical Industries), and the optical density at 405 nm was measured and compared with a series of *p*-nitrophenol standard values. Total cell protein was measured using the BCA Protein Assay Kit (Invitrogen), and ALP activity was calculated in micromoles of *p*-nitrophenol produced per min per mg of protein. Cells cultured without the addition of FGF-2 acted as controls. The experiments were repeated three times independently.

Statistical analysis

Statistical differences in the data from the MTT and ALP activity assays between cultures with and without FGF-2 were analyzed using Student's *t*-tests, with *p*-values less than 0.05 ($p < 0.05$) considered statistically significant. The rates of cell proliferation of FGF-2-stimulated cells in the presence or absence of unpolymerized monomers were statistically analyzed using analysis of variance (ANOVA) and the Tukey's HSD test with a significance level of $p < 0.05$.

RESULTS

Temperature increase induced by polymerization of 4-META/MMA-based resin

The temperature changes of the resin during polymerization are shown in Fig. 2. At an ambient temperature of 25°C (Fig. 2A), the mixture of the monomer, powder and catalyst became stringy after 1.5 min and cured after 5.5 min. The temperature of the cured specimens thereafter increased moderately, peaking at 17–24 min. The peak temperature increase was $1.30 \pm 0.75^\circ\text{C}$.

At an ambient temperature of 37°C (Fig. 2B), the setting time was shortened to approximately 1 min. The temperature of the specimens reached a peak of $2.54 \pm 0.18^\circ\text{C}$ at 7.5–10 min.

Release of unpolymerized monomers from cured 4-META/MMA-based resin

By HPLC analysis, MMA and 4-MET released from

Fig. 2 Temperature changes during polymerization of 4-META/MMA-based resin.

Temperature changes in the specimens were recorded at ambient temperatures of 25°C (A) and 37°C (B). The bar represents the standard deviation of three replicates.

cured SB resin were detected separately, showing peaks at 15 and 17 min, respectively. The released concentrations of 4-MET and MMA were 2.2 ± 1.0 and 9.8 ± 3.3 $\mu\text{g/mL}$, respectively.

Influence of unpolymerized monomer on the activity of FGF-2

Data showing the proliferation of MC3T3-E1 cells in the presence and absence of resin monomer and FGF-2 are presented in Fig. 3. As for the culture free of any monomers, cell proliferation was significantly increased by culture with FGF-2 compared with the control ($p < 0.05$, Student's *t*-tests). In the presence of 4-MET or MMA at all concentrations, cell proliferation was significantly increased by culture with FGF-2 compared with those without FGF-2 ($p < 0.05$, Student's *t*-tests). The presence of 4-MET had no significant effect on either unstimulated or FGF-2-stimulated cell proliferation ($p > 0.05$, ANOVA and Tukey's HSD test). While MMA appeared to increase in both unstimulated and FGF-2-stimulated cell proliferation (Fig. 3B), no significant differences in the rate of FGF-2-stimulated cell proliferation were found between the presence and absence of MMA ($p > 0.05$, ANOVA and Tukey's HSD test). These indicated that

Fig. 3 Cell proliferation in MC3T3-E1 cells cultured in the presence of unpolymerized monomer. Cells were grown in the presence and absence of FGF-2 (light gray and dark gray columns, respectively) and either 4-MET (A) or MMA (B), and their proliferation rates measured using an MTT assay. The bar represents the standard deviation of three replicates. The asterisk indicates a significant ($p < 0.05$ by Student's *t*-test) difference in proliferation between cultures with and without FGF-2. The number above the asterisk indicates the rate of cell proliferation stimulated by FGF-2 (Mean $\pm$ S.D.) calculated as: (Absorbance value in presence of FGF-2 / Mean absorbance value in absence of FGF-2) $\times 100\%$.

the presence of 4-MET or MMA at any concentrations did not affect the cell proliferation of FGF-2.

The results of ALP activities are shown in Fig. 4. When cells were cultured in the absence of any monomer (Fig. 4A), their ALP activity was significantly suppressed (compared with control) by FGF-2 at 3 and 7 days, but not at 14 days. This activity was not significantly affected by the presence of 4-MET or MMA at any of the tested concentrations (Fig. 4B–F).

DISCUSSION

Most of the proteins that maintain stability at temperature in a living body are denatured by heat. Above a certain temperature, the protein loses its three-dimensional structure and, with it, its function. For FGF-2, this structural change occurs at temperatures above 41°C, with complete denaturation at approximately 55°C²³. Since the procedure of radical polymerization generates heat, the temperature increase associated with the curing of resin may be sufficient to breach these thresholds and affect the function of FGF-2. Therefore, the temperature change caused after mixing of SB was measured under two environment condition at 25 and 37°C. Results in this study showed that the temperature increase on curing the SB resin was less than 3°C in the center of the specimen, even at an ambient temperature of 37°C. Thus, even at body temperature, the cured resin does not reach the 41°C threshold that would start to affect FGF-2 structure and function.

Tri-*n*-butyl borane (TBB), a polymerization initiator of SB, is known to promote radical formation in the presence of water, permitting high polymerization of SB under conditions of poor moisture control^{6,23} and greater bonding effectiveness than other adhesives in wet environments¹¹. Additionally, SB provides a favorable surface for cell attachment¹², meaning it can be directly applied to soft tissues in procedures such as direct pulp capping^{8–10}. However, Nezu *et al.*²⁴ reported that unpolymerized hydrophilic N-methacryloyl glycine monomer binds to bovine tendon collagen and alters its structure. In many cases, such structural alteration of proteins leads to changes in their function(s)²⁵. Thus, it is of importance to elucidate whether unpolymerized monomers released from cured 4-META/MMA-based resin influence FGF-2 activities. Therefore, the release concentration of unpolymerized monomers from SB was first analyzed by HPLC by placing the cured specimen into distilled water. 4-MET and MMA were found to be released at 2.2 ± 1.0 and 9.8 ± 3.3 µg/mL, respectively. Based on these release concentrations, the activities of FGF-2 in the presence of unpolymerized 4-MET or MMA were assessed. However, the ability of FGF-2 to promote cell proliferation and differentiation was not diminished by the presence of 4-MET or MMA at any of the tested concentrations, suggesting that the release of unpolymerized monomers from SB after curing has no negative influence on FGF-2 activity.

Vemuri *et al.* investigated the denaturation of FGF-2 at pH values ranging from 2–9. By measuring the fluorescence intensity of FGF-2 solutions, they found that FGF-2 was not denatured in the pH range 5–8²⁶. Although 4-MET is an acidic monomer, the pH of the 4-MET aqueous solution used in this study was between 5.24 and 5.65, which would be insufficient to produce any denaturation of FGF-2. Thus, we conclude that the release of low concentrations of unpolymerized 4-MET from cured SB does not influence the activity of FGF-2 *via* pH-mediated denaturation.

Fig. 4 Differentiation of MC3T3-E1 cells cultured in the presence of unpolymerized monomer.

Cells were grown in growth medium without (dark gray columns) or with (light gray columns) FGF-2 for 3, 7 or 14 days. The growth medium included no monomer (A), 4-MET at 5 $\mu\text{g/mL}$ (B) or 10 $\mu\text{g/mL}$ (C), or MMA at 5 $\mu\text{g/mL}$ (D), 10 $\mu\text{g/mL}$ (E) or 25 $\mu\text{g/mL}$ (F). Error bars represent the standard deviation of three independent replicates. The asterisk indicates a significant ($p < 0.05$ by Student's t -test) difference in ALP content between cultures grown with and without FGF-2. The number above the asterisk indicates the inhibition in ALP activity (Mean $\pm$ S.D.) (%) calculated as: (ALP activity in presence of FGF-2/Mean ALP activity in absence of FGF-2) $\times 100$.

It has been reported that FGF-2 induced the expression of Chicken ovalbumin upstream promoter-transcription factor II (COUP-TFII) in mesenchymal lineage cells by MEK1/2 pathway activation²⁷. Since COUP-TFII acts downstream of this signaling pathway, the exposure of FGF-2 had a negative effect on osteogenic differentiation owing to greater COUP-TFII

expression. Also in MC3T3-E1 cells, FGF-2 induces COUP-TFII expression²⁷. As evidenced by decreases in ALP activity and osteogenic marker gene expression, FGF-2-stimulated cells showed a low potential for osteoblast differentiation^{27, 28}. As for the absence of any monomer in this study, the ALP activity of MC3T3-E1 cells was suppressed by FGF-2 until 7 days in accordance

with the results of previous reports above, suggesting that the differentiation of MC3T3-E1 cells would be suppressed by FGF-2. At 14 days, since the inhibitory effect could not continue owing to short-term (*i.e.* only 3 days) exposure of FGF-2, the ALP activity of FGF-2-stimulated cells was statistically same with that of FGF-2-unstimulated cells. At least, these facts suggested that the presence of 4-MET or MMA had no influence on the activity of FGF-2 to suppress the ALP activity of MC3T3-E1 cells. To assess the detailed mechanism of osteoblast differentiation of FGF-2-stimulated MC3T3-E1 cells, further experiments such as the gene expression of osteogenic differentiation markers are required.

As a non-biodegradable carrier suitable for delivery of proteins or antimicrobials^{29,30}, we have developed poly(2-hydroxyethylmethacrylate)/trimethylolpropane trimethacrylate (polyHEMA/TMPT) particles. When FGF-2 was loaded into such particles, a sustained release of FGF-2 was maintained for up to 14 days²⁸. In addition, the released FGF-2 promoted the proliferation of osteoblast-like cells, suggesting that FGF-2 within the particles preserved its activity for at least two weeks. These non-biodegradable FGF-2-loaded polymer particles could be applied to the 4-META/MMA-based resin as a releasing vehicle for FGF-2. From the results of present study, we believe that FGF-2 released from these particles should be able to demonstrate its ability to promote healing around the 4-META/MMA-based resin. However, control of moisture in the operating field is difficult *in vivo*. Although TBB can promote radical formation even in the presence of water, excessive wetness may hinder polymerization of SB, increasing the release of unpolymerized monomer to levels above those detected in this *in vitro* study. Therefore, further study to assess the influence on the conversion rate of the 4-META/MMA-based resin under wet condition, which is combined with the FGF-2 loaded polymer particles, is needed. Moreover, to assess the clinical effectiveness of FGF-2 applied in the vicinity of 4-META/MMA-based resin, further *in vivo* tests are required with appropriate simulation of clinical procedures.

CONCLUSION

Temperature increases caused by heat generation during polymerization of 4-META/MMA-based resin was within a range whereby the structure and function of FGF-2 would not be changed. *In vitro* tests indicated that release of unpolymerized 4-MET and MMA from cured 4-META/MMA-based resin had no influence on FGF-2 activities in terms of the proliferation and differentiation of osteoblast-like cells. These findings suggest that it is possible to apply FGF-2 around cured 4-META/MMA-based resin to promote tissue regeneration.

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