Apoptotic and Necrotic Influence of Dental Resin Polymerization Initiators in Human Gingival Fibroblast Cultures

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The aim of this study was to examine the apoptotic and necrotic influence of four dental resin polymerization initiators — namely benzoyl peroxide (BPO), camphorquinone (CQ), dimethylaminoethyl methacrylate (DMAEMA), and dimethyl-para-toluidine (DMPT) — on human gingival fibroblast (HGF) cells. To this end, the growth inhibition of HGF cells with 1 mM BPO, CQ, and DMAEMA, and 500 μM DMPT was evaluated using Cell Counting Kit-8. Then, cell cycle analysis by flow cytometry was used to assess propidium iodide-stained cells (distribution of cells in G\textsubscript{0}/G\textsubscript{1}, S, G\textsubscript{2}/M phases). All four dental resin polymerization initiators induced G\textsubscript{0}/G\textsubscript{1} cell cycle arrest. As for the patterns of cell death (necrosis and/or apoptosis), they were analyzed using Annexin V-FITC/PI staining with flow cytometry. All four dental resin polymerization initiators most likely induced necrosis.

Keywords: Apoptotic and necrotic influence, Dental resin polymerization initiators, Human gingival fibroblast

INTRODUCTION

In dentistry, dental resins are widely used as restorative materials owing to their ease of handling and esthetic merits. However, problems such as water absorption and dissolution of residual monomers have been reported\textsuperscript{3,4}. It has been found that resin monomers from dental restorative materials are released into the saliva and diffuse into the tooth pulp, gingiva, mucosa, and circulating blood, where they have potentially toxic effects\textsuperscript{5,6}. Moreover, the polymerization initiators, which are present in smaller amounts than the monomers, are harmful because they generate radicals\textsuperscript{5,7}.

Polymerization initiators used for visible light (VL)-cured resins usually consist of a photosensitizer (primarily camphorquinone (CQ)) and a reducing agent (which is often a tertiary amine such as dimethyl-para-toluidine (DMPT) or dimethylaminomethyl methacrylate (DMAEMA)), as well as benzoyl peroxide (BPO) and DMPT for self-curing resins\textsuperscript{8,9}. Radicals are generated when CQ and tertiary amine in the VL resin are irradiated or when BPO is mixed with a tertiary amine. The resulting radicals attack not only the double bonds in the resin monomer but also the double bonds of polysaturated fatty acids and phospholipids, damaging the cell membranes in living systems\textsuperscript{10,11}. These radicals can also influence DNA, leading to genotoxic effects and cell death in living organisms\textsuperscript{12,13}.

Generally, two types of cell death can occur — namely apoptosis and necrosis\textsuperscript{14}. When cytotoxic stimuli are intense, cells may escape the cell cycle and undergo a programmed process of cell death called apoptosis. Apoptosis is an active physiological process characterized by various phenomena such as cell shrinkage. An injury to the cell caused by a toxic substance can quickly activate the apoptotic response, which rapidly causes cell death. The clearance of the remaining cell debris by phagocytes is also very quick, thereby avoiding an acute inflammatory reaction\textsuperscript{14}. By contrast, necrosis is “cell murder” — a passive process that follows the exposure of cells to a gross injury and which may trigger an inflammatory reaction in tissues\textsuperscript{15}.

Dental resin polymerization initiators have been shown to induce cytotoxicity in human gingival fibroblast (HGF) cells in vitro\textsuperscript{16,17}. However, the mechanism by which a dental resin polymerization initiator induces cell death and its effects on the cell cycle are not fully understood. Against this background, we sought to evaluate in vitro the effects, including apoptotic and necrotic effects, of dental resin polymerization initiators on HGF cells in terms of cell cycle and cytotoxicity.

MATERIALS AND METHODS

Chemicals

A solution of 75% BPO was purchased from Sigma—Aldrich (Tokyo, Japan). CQ, DMPT, and DMAEMA were from Tokyo Kasei (Tokyo, Japan). These solutions did not require further purification. For comparison purpose, a hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) solution
from Santoku Chemical Industries (Tokyo, Japan) was used in this study.

For Annexin V-FITC/PI assay, stock solutions of four polymerization initiators in dimethylsulfoxide (DMSO) and \( \text{H}_2\text{O}_2 \) solution (for comparison purpose) were prepared. For CQ, DMPT, and DMAEMA stock solutions, they were prepared at 500 mM in DMSO. As BPO was not soluble at 500 mM in DMSO, its stock solution was prepared at 100 mM in DMSO. To achieve the required final concentrations, samples of each stock solution were diluted using Dulbecco’s modified Eagle medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal bovine serum (FBS; Daiichi Pure Chemicals, Tokyo, Japan) just before use.

**Cells and cell culture**

After obtaining informed consent from a patient, we obtained a fragment of gingiva attached to a tooth and from which we cultivated HGF cells. The gingival tissue was cut into pieces measuring 1–2 mm\(^3\), washed twice with phosphate buffered saline (PBS; Sigma–Aldrich) supplemented with 100 U/ml penicillin and 100 \( \mu \text{g/ml} \) streptomycin (Sigma–Aldrich), and then placed in tissue dishes. The explants were incubated in a culture medium — consisting of DMEM, 30% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin — at 37°C in a humidified atmosphere of 5% carbon dioxide (CO\(_2\)). When outgrowth was observed in the cultures, the medium was changed twice and the cells grown to confluence. Following which, the cells were treated briefly with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid (EDTA; Sigma–Aldrich) to detach them from the monolayer. The cells were recultured in DMEM containing 10% FBS until confluent monolayers were obtained.

**Growth assay**

To determine the inhibition of HGF cell growth caused by the polymerization initiators (BPO, CQ, DMPT, and DMAEMA), Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) was used in this study. An equal number of HGF cells (1×10\(^4\) cells/well) was seeded in 100 \( \mu \text{l} \) of DMEM with 10% FBS in 96-well microplates. These cells were incubated overnight at 37°C in a humidified atmosphere of 5% CO\(_2\).

For each stock solution of photoinitiator in DMSO, it was serially diluted in cell culture medium to obtain a final DMSO concentration of 1%. The cells were then treated with various concentrations of polymerization initiators diluted in 100 \( \mu \text{l} \) of DMEM, to obtain final concentrations of 50 \( \mu \text{M} \), 100 \( \mu \text{M} \), 500 \( \mu \text{M} \), 1 mM, and 5 mM for each initiator. After the cultures were incubated for 24 hours, 10 \( \mu \text{l} \) of Cell Counting Kit-8 solution was added to each well and the plates were incubated for two hours at 37°C. Absorbance at 450 nm was measured with an immunoreader (Microplate Reader Model 550, Bio-Rad, Tokyo, Japan), whereby cell number was calculated from the absorbance value relative to a standard curve.

**Cell cycle analysis**

A quantity of 5×10\(^5\) HGF cells was plated in 60-mm culture dishes. To synchronize the cells in G\(_0\)/G\(_1\) phase by serum deprivation\(^{22}\), HGF cells were incubated in DMEM with 0.5% FB+S for 48 hours. To induce the cells to re-enter the cell cycle, they were incubated in fresh medium containing 10% FBS with different concentrations of the four polymerization initiators (final concentrations of 500 \( \mu \text{M} \)–5 mM) for another 24 hours.

Cellular DNA content was determined by flow cytometry as described previously\(^{23}\). Floating and attached cells were collected using trypsin-EDTA and resuspended in DMEM (no FBS). The cells were then fixed for 30 minutes in a 70% ice-cold ethanol solution containing ribonuclease (RNase; 2 mg/ml). After washing with PBS, the cells were stained with PI for 10 minutes. PI-elicited fluorescence of individual cells was measured using a FACS Calibur Flow Cytometer (Becton Dickinson, Tokyo, Japan) with laser excitation at 488 nm. Emissions greater than 590 nm were collected in a linear/log scale fashion. A total of 1×10\(^4\) cells were analyzed for each sample, whereby the percentages of cells in G\(_0\)/G\(_1\), S, and G\(_2\)/M phases were determined using a standard ModiFit and Cell Quest software (Becton Dickinson).

**Annexin V-FITC/PI assay**

To identify cells undergoing apoptosis, the cells were stained with PI and fluorescein isothiocyanate (FITC)-conjugated Annexin V, using an Annexin V-FITC Apoptosis Detection Kit I (Becton Dickinson). PI detects cells that have lost their plasma membrane integrity (i.e., necrotic and secondary necrotic cells), while Annexin V detects early apoptotic cells based on externalized phosphatidylserine.

Polymerization initiators of various concentrations (500 \( \mu \text{M} \)–5 mM) were added to HGF cultures, and the cells incubated for 24 hours at 37°C. After incubation, the cells were harvested using trypsin-EDTA treatment and washed with DMEM containing 10% FBS. The cells were resuspended in 50 \( \mu \text{L} \) of 1× binding buffer supplemented with 5 \( \mu \text{L} \) of Annexin V-FITC and 10 \( \mu \text{L} \) of PI solutions, and then the cells kept at room temperature in the dark for 15 minutes according to manufacturer’s instructions. After the addition of 450 \( \mu \text{L} \) of 1× binding buffer, the stained cells were kept on ice and subjected to fluorescence-activated cell sorter (FACS) analysis using a FACS Calibur Flow Cytometer. FITC fluorescence (green)
between 515 and 545 nm was collected, as well as PI (red) fluorescence between 564 and 606 nm.

**Data analysis**

Quantitative data are expressed as mean±standard error (SE) of at least three independent experiments and were assessed using Student’s *t*-test. Significance was accepted at *p*<0.05.

**RESULTS**

**Growth assay**

Cell proliferation was expressed as the percentage of cells proliferating in the presence of an initiator relative to the cells proliferating in the absence of initiators. BPO, CQ, and DMAEMA at 1 mM and DMPT at 500 μM inhibited the growth of HGF cells (*p*<0.05; Fig. 1). In contrast, low concentrations of BPO (100 and 500 μM) and DMAEMA (500 μM) promoted cell growth, suggesting that these two polymerization initiators could stimulate cell growth at low concentrations.

**Cell cycle analysis**

Serum-deprived cells accumulated in G0/G1 phase (Fig. 2B; control 0 h). When these cells were returned to culture medium containing 10% FBS, cell proliferation was stimulated after 24 hours with or without a polymerization initiator. After 24 hours

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**Fig. 1** Effects of dental polymerization initiators (BPO, CQ, DMPT, and DMAEMA) on the growth of HGF cells. Cells (1×10⁶ cells) in 96-well culture plates were exposed to different concentrations of dental polymerization initiators for 24 h, and the cells were counted with Cell Counting Kit-8. *: Significantly different (*p*<0.05) from control (*n*=6).
Fig. 2  A: Cell cycle of HGF cells. B: Histogram representing the percentage of cells in each cell cycle phase, determined by DNA content and PI staining (n=3). Control 0 h (0.5% FBS): cells synchronized in G0/G1 phase by serum deprivation in 0.5% FBS for 48 h. Control 24 h (10% FBS): cells re-entering cell cycle after addition of DMEM containing 10% FBS and 24-h incubation. C: Representative flow cytometry profiles of HGF cells. The tall peak (left) represents cells in G0/G1 phase, the small peak (right) represents cells in G2/M phase, and the area between the peaks represents cells in S phase.

in the presence of 10% FBS without polymerization initiators, the number of cells in S and G2/M phases increased and the number in G0/G1 phase decreased, compared with control 0 h (Fig. 2B; control 24 h). A low concentration (500 μM) of the polymerization initiators BPO, CQ, and DMAEMA (excluding DMPT) had no effect on the cell cycle phase at 24 hours compared with the control (Fig. 3). By contrast, high concentrations of initiators (1 mM and 5 mM) caused a large percentage of the cells to remain in G0/G1 phase, with a small percentage in S and G2/M phases.
Annexin V-FITC/PI assay
Compared with control cells at 24 hours (Fig. 4a), cells exposed to 2 mM H$_2$O$_2$ for 24 hours exhibited significantly more apoptosis (Fig. 4b; lower right quadrant). Cells exposed to 5 mM CQ, 1 and 5 mM DMPT, 1 and 5 mM DMAEMA (Figs. 4d-3, 4e-2, 3, 4f-2, 3) showed increased numbers of necrotic cells (upper left and right quadrant), resulting in cell death without entering apoptosis. These polymerization initiators (5 mM CQ, 1 and 5 mM DMPT, 1 and 5 mM DMAEMA) resulted in increased numbers of necrotic/apoptotic cells (upper left quadrant, uptake of annexin V and PI) but no increase in the number of apoptotic cells alone (lower right quadrant, uptake of annexin V but not PI). Thus, treatment with 2 mM H$_2$O$_2$ induced apoptosis, whereas a high con-
Annexin V-FITC

Fig. 4  FACS analysis after staining with annexin V-FITC and PI, detecting the induction of apoptosis and necrosis in HGF cells by dental polymerization initiators after 24 h. In each plot, the lower left quadrant represents viable cells, the upper left quadrant represents partial loss of membrane integrity (uptake of PI but not annexin V), the lower right quadrant represents apoptotic cells, and the upper right quadrant represents necrotic or apoptotic/necrotic cells. (a) Non-treated cells (negative control); (b) Cells incubated with 2 mM H$_2$O$_2$ (positive control). Cells treated with (c) BPO, (d) CQ, (e) DMPT, and (f) DMAEMA. (a) through (f) show diagrams for one representative experiment; –1, –2, and –3 indicate 500 μM, 1 mM, and 5 mM respectively.
centration of dental resin polymerization initiators might induce toxicity via a necrotic pathway.

**DISCUSSION**

Materials used in dentistry can have harmful effects, manifested mainly as pulp damage and contact dermatitis or in the form of allergic reactions\(^4\). Numerous *in vitro* studies addressing cellular responses to dental monomers and polymerization initiators have shown that some monomers, including bisphenol A diglycidylether methacrylate ( Bis-GMA), triethylene glycol dimethacrylate (TEGDMA), 2-hydroxyethyl methacrylate (HEMA), and methyl methacrylate (MMA), can be cytotoxic\(^24,25\). Moreover, some polymerization initiators can potentially cause problems via estrogenicity and genotoxicity. For example, BPO is found to be estrogenic and genotoxic in *vitro*\(^6\). Although DMPT and CQ are not genotoxic, the metabolic products of DMPT are estrogenic\(^15,26\).

Dental resins usually contain about 0.2–1.5 wt% of polymerization initiator relative to the monomer\(^6\). Unpolymerized initiators will diffuse into the mouth. Moin *et al.*\(^2\) reported that a dental polymerization initiator (0.05–0.15 wt% of dental resin) diffused from the cured resin into an organic solvent (methanol) during a 24-hour period. Therefore, it is important to evaluate the safety of polymerization initiators with regard to leaching over time.

Exposure time may significantly influence the biocompatibility of dental resins\(^27\). In the present study, HGF cultures were exposed to polymerization initiators at various concentrations for 24 hours. In laboratory conditions, leaching is completed within 24 hours\(^29\). Polymerization initiators that leak from dental resins first leach into the oral environment and influence oral tissues, including HGF cells. Various reports have indicated that dental polymerization initiators induce toxicity in HGF cells\(^30,31\).

In the present study, growth assays provided the first evidence that elevated concentrations of dental resin polymerization initiators have severe chemical and biological effects on HGF cells *in vitro*. The effective inhibitory concentrations were 1 mM for BPO, CQ, and DMAEMA, and 500 \(\mu\)M for DMPT (Fig. 1), which concurred with published data\(^31\). Results of cell cycle analysis suggested that these concentrations of initiators induced a significant and sustained accumulation of cells in \(G_0/G_1\) phase of the cell cycle, compared with the control at 24 hours (Fig. 3). This implied that the growth inhibitory effect of the polymerization initiators stemmed from the arrest of DNA replication in the cell cycle.

The effects of dental polymerization initiators on cell death were also examined using an Annexin V-FITC/PI assay. Cell death occurs via necrosis and apoptosis, involving reproductive cell death and programmed cell death respectively\(^39\). The morphological changes associated with necrosis are swelling, followed by cell membrane rupture and lysis of organized cell structure. By contrast, during the process of apoptosis, condensation and fragmentation of the cytoplasm and nucleus occur in the target cells while normal organelle structure is maintained. Several studies have indicated that exposing cells to exogenous oxidants can induce apoptosis\(^39\).

During the early stages of apoptosis, a loss of phospholipid asymmetry occurs, which results in the translocation of negatively charged aminophospholipid phosphatidylserine (PS) from the inner leaflet of the cell membrane to the outer leaflet\(^30\). In the present study, the Annexin V-FITC/PI assay used to identify apoptotic cells detected this modification as a marker of apoptotic cells. In the presence of calcium ions, annexin V binds specifically to PS\(^31\). The externalization of PS is an important cell surface signal for the macrophage clearance of apoptotic cells\(^32\).

Recent reports have shown that resin-based dental materials caused cell death by apoptosis and necrosis in rat submandibular acinar cells and human endothelial cells\(^32,33\). The resin monomer Bis-GMA and comonomer TEGDMA augment apoptosis in dose- and time-dependent manners in HGF cells\(^34,35\). 4-N,N-dimethyl amino benzoic acid ethylester (DMAE), which is a polymerization initiator, augments cell death via apoptosis and necrosis in human histiocyctic lymphoma promonocytic cells in a concentration-dependent manner\(^36\).

In this study, the Annexin V-FITC/PI assay showed that 5 mM CQ, 1 and 5 mM DMPT, and 1 and 5 mM DMAEMA induced cell death without producing an apoptotic signal (Fig. 4). Lee *et al.*\(^39\) demonstrated that 2 mM \(H_2O_2\), which was used as a positive control for apoptosis in the present study, induced a characteristic DNA fragmentation pattern of giant DNA fragments together with small fragments. In the present study, the dental polymerization initiators did not produce such a characteristic DNA ladder pattern specific for apoptosis (data not shown). Therefore, dental polymerization initiators might cause necrosis of HGF but not apoptosis. Although the mechanisms of cell cycle arrest and cell death caused by polymerization initiators have not been elucidated, we demonstrated that four dental polymerization initiators consistently induced \(G_0/G_1\) cell cycle arrest and necrosis. In other words, dental polymerization initiators may induce toxicity owing to growth arrest, followed by necrosis.

We showed that, by differentiating necrosis and apoptosis based on their significantly different sequelae *in vivo*, the toxic potential of oral biomaterials could be more accurately assessed *in vitro*. It has been recommended that permanent cell lines be used for initial toxicity screening because of their
metabolic and genetic stability and good reproducibility. However, in the present study, primary human oral cell lines derived from potential target tissues were used instead to determine the cytotoxicity of dental resin polymerization initiators. At this juncture, it must be mentioned that even primary cell culture systems differ from the in vivo conditions for the use of initiators. On this ground, only limited conclusions about the possible toxicity of dental initiators in vivo should be drawn.

Thus far, investigations that use controlled cell cultures under identical and interlaboratory-repeatable conditions have contributed invaluablely to the evaluation or screening of isolated components. Nonetheless, to clarify the mechanisms leading to apoptosis and necrosis and to determine the clinical relevance of our findings, further in vitro and in vivo studies are required.

REFERENCES


25) Samuelson J, Dahl J, Karlsson S, Morshak E, Becher R. Apoptosis induced by the monomers


