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Original Article

Triethylene-glycol-dimethacrylate induces caspase-mediated apoptotic cell death in cementoblasts by the regulation of JNK and p38 pathways-an in vitro study

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Abstract *Background/Purpose:* Triethylene-glycol-dimethacrylate (TEGDMA) is one of the monomers used in composite resin matrix. Residual TEGDMA can be eluted from bulk fill composite resins and the amounts also increased with time. Composite resin used as root-end filling materials may invoke the critical biologic reactions in surrounding tissues. However, little is known about the effects of TEGDMA on cementoblasts. The aim of this study was to investigate the possible detrimental effects of murine cementoblast (OCCM.30) by TEGDMA in vitro.
Materials and methods: OCCM.30 cells were exposed to TEGDMA (0, 1, 2, 4 mM) for 24 h. Cell viability was determined by microculture tetrazolium assay. Flow cytometry was conducted to evaluate the cell cycle distribution and the type of cell death. Caspase-mediated apoptotic cascade and mitogen-activated protein kinase (MAPK) pathways were analyzed by Western blot.
Results: The concentrations of TEGDMA≧1 mM were found to significantly inhibit OCCM.30 cell viability in a dose-dependent manner ($P < 0.05$). TEGDMA dose-dependently induced apoptosis by the increase of sub-G1 population, early apoptotic cells, and later apoptotic cells. TEDGMA-induced apoptotic mechanisms were found to activate caspase 8, 9, and 3 in OCCM.30 cells, respectively ($P < 0.05$). In addition, both c-Jun N-terminal kinase (JNK) inhibitor JNK-in-8 and p38 inhibitor SB203580 dramatically reduced TEGDMA-induced caspase 8, 9, and 3 activations in OCCM.30 cells, respectively ($P < 0.05$).
Conclusion: Taken together, our results demonstrated that TEDGMA decreased cell viability and induced the apoptotic cell death in cementoblast. In addition, caspase-mediated cell apoptosis was found to be associated with JNK and p38 signal transduction pathways.

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Introduction

Composite resin is one of the most popular restorative materials for direct restorations in the world. They are widely used for decayed teeth restoration, root caries filling, endodontic perforation repair, and root-end retrograde filling. In addition to color, composite resins also demonstrate the most valuable wear resistance and improved adhesion to enamel and dentin.[1] In Taiwan, a significant increased trend of composite resin filling for teeth restoration was found from the nationwide registry-based study.[2]

Composite resins consist of resin matrix, inorganic filler, and coupling agent. Triethylene-glycol-dimethacrylate (TEGDMA) is a hydrophilic monomer in resin matrix which is used to reduce viscosity and enhance bonding strength to dentin. Unfortunately, the polymerization of composite resin is incomplete and the monomers could be eluted after polymerization including TEGDMA.[3–5] TEGDMA was reported as the main monomer eluted from polymerized composite resin especially in the aqueous environment.[3] TEDGMA is a small molecular weight monomer that exhibits higher mobility and is eluted more quickly than large molecules.[4] In addition, the eluted TEGDMA was found with higher concentrations than other monomer from bulk fill composite resins.[5] Therefore, the residual TEGDMA released from resin-based dental restorative materials might cause adverse effects to surrounding tissues. Previously, TEGDMA has been approved as a cytotoxic agent to interfere with various cellular functions of multiple target cells such as macrophages,[6–10] pulp cells, [6,7] apical papilla stem cells,[11] gingival fibroblasts,[12,13] and osteoblast-like cell lines.[14]

Cementoblasts play a critical role in the healing of periodontal ligament and cementum in periapical portions. Ideally, composite resin should be biocompatible and well tolerated with peri-radicular tissues when they are used for the repair of furcation as well as root perforation and for the retrograde root filling. However, little is known about the cytopathologic effects of residual monomers such as TEGDMA on cementoblasts. The purpose of this study was to investigate the cytotoxic effects of TEGDMA on murine immortalized cementoblast cell line (OCCM.30) by measuring cell viability, type of cell death, caspase-mediated apoptotic cascade, and mitogen-activated protein kinase (MAPK) signaling pathways in vitro.

Materials and methods

Chemicals and reagents

Cell culture materials including Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco-BRL (Gaithersburg, MD, USA) and Hyclone Laboratories, Inc (Logan, UT, USA), respectively. TEGDMA, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), dimethyl sulfoxide, and phosphate buffered saline (PBS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Antibodies specific for p38, phosphorylated p38, β-actin, pro-caspases-3, pro-caspases-8, and FITC (fluorescein isothiocyanate-labeled) Annexin V Apoptosis Detection Kit I were obtained from BD Biosciences (San Jose, CA, USA). Antibodies specific for extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), phosphorylated ERK, phosphorylated JNK, pro-caspases 9, cleaved caspases-3, cleaved caspases-8, and cleaved caspases-9 were purchased from Cell Signaling Technology (Danvers, MA, USA).

Cell culture

OCCM.30 cells derived from root surface of the first mandibular molar of osteocalcin large T-antigen transgenic mice were the generous gift by Professor Chi-Cheng Tsai from Dr. Somerman Laboratory (University of Washington, Seattle, WA, USA).[15] The culture of OCCM.30 cells were described previously.[16,17]

Cytotoxicity assay

OCCM.30 cells were seeded at a density of 5×10^4 cells/well in 24-well plates, incubated overnight and exposed to different concentrations of TEDGMA (0, 1, 2, 4 mM) for 24 h. Then, cells were rinsed with PBS and incubated with a

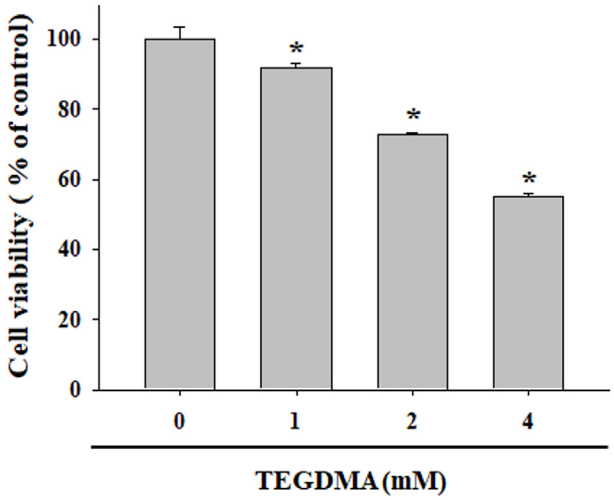

Figure 1 The inhibitory effects of triethylene-glycol-dimethacrylate (TEDGMA) on cell viability in OCCM.30 cells for 24 h. Data represent the mean ± standard deviation from three independent experiments. *$P < 0.05$ compared with the control.

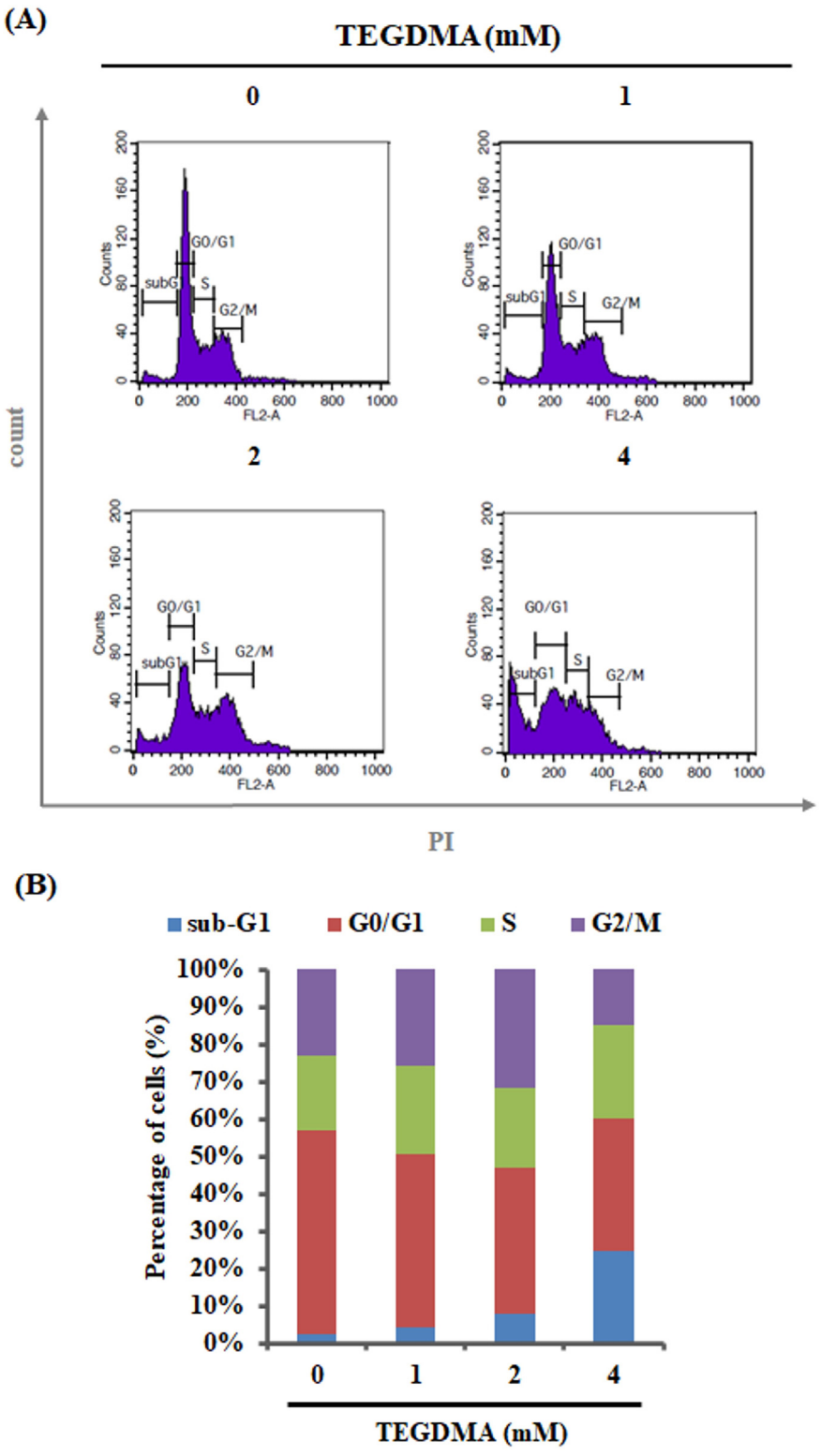

Figure 2 Effects of triethylene-glycol-dimethacrylate (TEDGMA) on the distribution of cell-cycle phase in OCCM.30 cells. (A) TEDGMA (0, 1, 2, 4 mM) increased the sub-G1 phase ratio in OCCM.30 cells for 24 h by FACS after propidium iodide staining. (B) The quantification results of the cell cycle population of OCCM.30 cells are shown in the bar graph for the quantification analysis of sub-G1, G0/G1, S, and G2M phases, respectively.

culture medium containing of 0.5 mg/mL MTT to evaluate cell viability according to previous studies.[18,19]

Flow cytometer for the distribution of cell cycle and the type of cell death

OCCM.30 cells were cultured with 0, 1, 2, and 4 mM TEGDMA for 24 h. Then the cells were step by step washed with PBS, incubated with 0.25 % trypsin–EDTA solution, collected, and fixed in cold 70 % ethanol for 24 h. The cells were suspended with propidium iodide (PI) buffer at room temperature for the distribution of cell cycle. Flow cytometer (BD Biosciences, San Jose, CA, USA) was used to analyze the distribution of cell cycle. Combined with PI staining, annexin V-FITC apoptosis staining was performed to differentiate apoptosis from necrosis. Cell Quest software (BD Biosciences) was used to quantitate and analyze the results. The details were as described previously.[20,21]

Western blot

To investigate the underlying mechanism of TEGDMA-induced apoptosis, the activations of caspase 8 (an extrinsic pathway), caspase 9 (the initiator of an intrinsic pathway), and caspase 3 (the final executioner) were detected in OCCM.30 cells. Nearly confluent monolayers of cells were washed with serum-free DMEM and immediately exposed with the different concentrations of TEGDMA (1, 2, 4 mM) for 24 h. Cultures without FCS were used as negative control. To further elucidate the relationships among TEDGMA-induced apoptosis, activation of caspases, and phosphorylation of MAPKs, OCCM.30 cells were pretreated with 10 μM ERK inhibitor U0126, 1 μM JNK inhibitor JNK-in-8, and 10 μM p38 inhibitor SB203580 for 1 h. Then, cells were treated with 2 mM TEDGMA for another 24 h. Cell lysates were collected and the extraction of proteins from cells and immunoblotting analysis were performed as described previously.[21,22]

Statistical analysis

Triplicate experiments were performed throughout this study. All assays were repeated three times to ensure reproducibility. Student's t-test was performed by using SigmaPlot (Sigma-Stat 2.0, San Jose, CA, USA). A P value < 0.05 was considered as statistical significance.

Results

The effects of TEGDMA (0, 1, 2, 4 mM) on OCCM.30 cells were measured by MTT assay for 24 h in Fig. 1. The concentrations of TEGDMA ≧ 1 mM revealed cytotoxicity to OCCM.30 cells in a dose-dependent manner ($P < 0.05$). At concentrations of 1 mM, 2 mM, and 4 mM TEGDMA were found to reduce cell viability about 91.67 %, 72.61 %, and 55.19 % as compared with control, respectively.

Flow cytometry was used to determine the effect of TEGDMA on the distribution of cell cycle (Fig. 2A). The quantitative analysis of sub-G1 apoptotic fraction was about 2.37 %, 3.97 %, 8.00 %, and 24.59 % in the concentrations of 0, 1, 2, and 4 mM TEGDMA, respectively (Fig. 2B). Apoptosis induced by TEGDMA was further confirmed by Annexin V-FITC/PI double-staining in Fig. 3A. As illustrated in Fig. 3B, the percentage of early apoptotic cells (LR) and late apoptotic cells (UR) was about 8.66 %, 11.25 %, 24.92 %, and 33.63 % after treating OCCM.30 cells in the concentrations of 0, 1, 2, and 4 mM TEGDMA, respectively. In addition, cell necrosis (UL) was also noted about 2.73 %, 5.90 %, 6.03 %, and 10.93 % in the treatment of concentrations of 0, 1, 2, and 4 mM TEGDMA in OCCM.30 cells, respectively (Fig. 3B).

Caspase cascades are the proteolytic enzymes regarding the control of cell death. The results showed that TEGDMA decreased the expression of pro-caspase-8, -9, and -3 in OCCM.30 cell in Fig. 4A. The quantitative results of these protein levels were illustrated in Fig. 4B. The concentration of 4 mM TEGDMA significantly inhibited pro-caspases-8, -9, and -3 expression about 0.25, 0.82, and 0.72 fold as compared with control ($P < 0.05$), respectively. Fig. 4C demonstrated that TEGDMA induced the activation of cleaved caspase-8, -9, and -3 in OCCM.30 cells. As shown in Fig. 4D, 4 mM TEGDMA significantly stimulated the levels of cleaved caspase-8, -9, and -3 about 2.45, 3.13, and 139.74 fold as compared with control ($P < 0.05$), respectively. Taken together, TEGDMA-induced cell apoptosis was found to activate extrinsic caspase 8, intrinsic caspase 9, and their downstream effector caspase 3.

MAPKs play a central role in the regulation of cell apoptosis. As expectedly in Fig. 5A, 2 mM TEGDMA significantly increased the phosphorylation of ERK, JNK, and p38 expression as compared with control ($P < 0.05$). Furthermore, 10 μM ERK inhibitor U0126, 1 μM JNK inhibitor JNK-in-8, and 10 μM p38 inhibitor SB203580 with or without treatment with 2 mM TEGDMA were used to identify the regulation of caspases 8, 9, and 3 in OCCM.30 cells (Fig. 5A). Both JNK in 8 and SB203580 significantly inhibited the TEDGMA-induced the increase of protein levels of cleaved caspase-8, -9, and -3 as compared with TEGDMA alone ($P < 0.05$). However, they could not be affected by the co-treatment with ERK inhibitor U0126 (Fig. 5B). Taken together, these findings suggested that activation of the p-JNK as well as p38 signaling pathways may involve in the caspases-mediated apoptotic cell death by TEGDMA in OCCM.30 cells.

Discussion

Cementoblasts play an important role during periodontal regeneration procedures. Composite resin perforation repair materials may impair the periodontium by the direct contact or by releasing monomers. Thus, the selection of cementoblasts to evaluate the cellular interaction of TEGDMA-released form composite resin perforation repair materials may have clinical relevance in vivo. To the best of our knowledge, this is the first study shown that TEGDMA is a cytotoxic agent to OCCM.30 cells. Similar findings have reported that TEGDMA exhibited cytotoxicity in HeLa cells,[7] human pulp derived cells,[6,7] murine macrophage RAW264.7 cells,[7–10] apical papilla stem cells,[11] human gingival fibroblasts,[12,13] and human osteoblast-like cell lines (MG-63, Saos-2, and hFOB 1.19).[14] In summary, the cytotoxicity of TEGDMA is non-cell type specific.

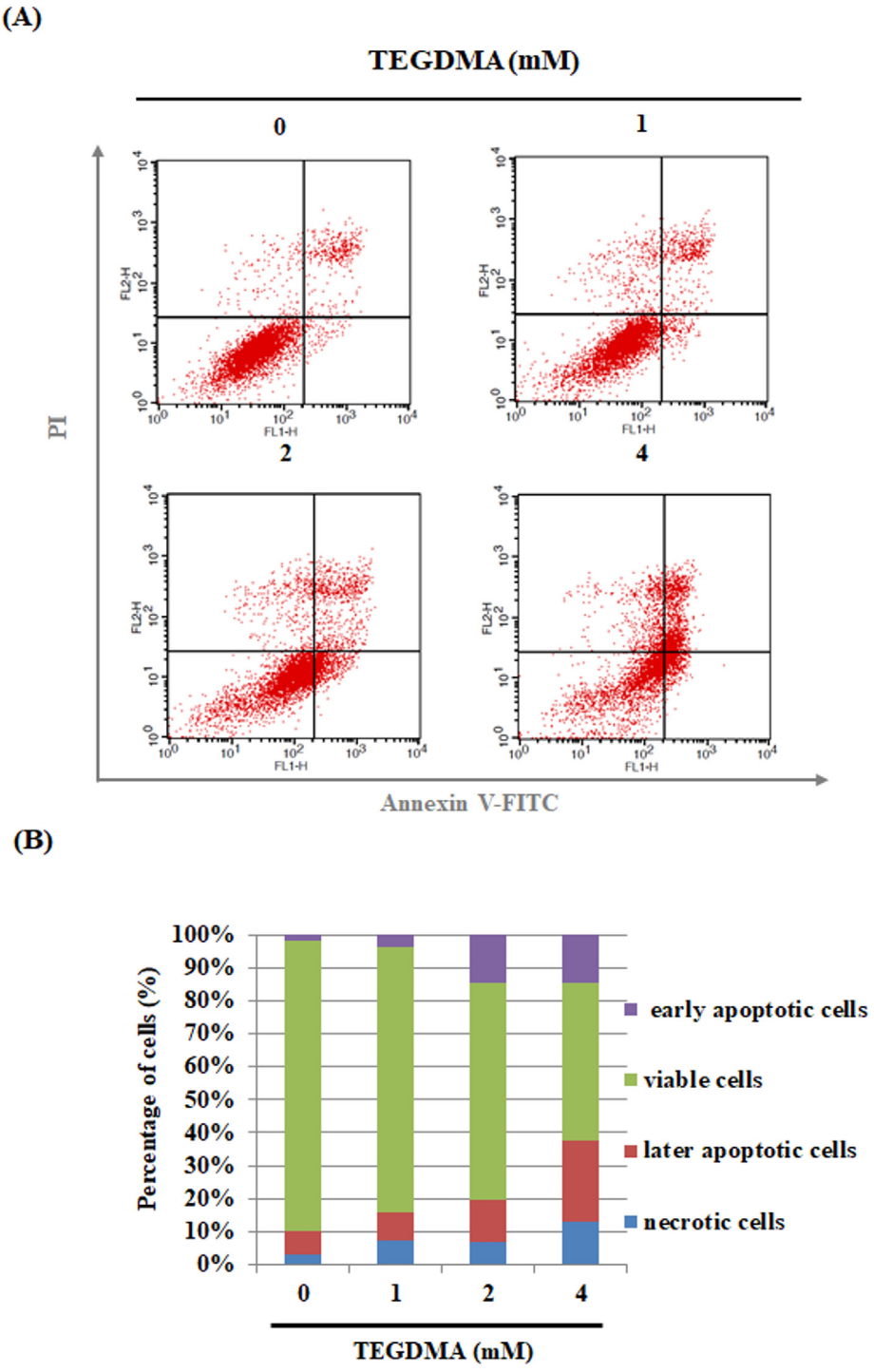

Figure 3 Effects of triethylene-glycol-dimethacrylate (TEDGMA) on the type of cell death in OCCM.30 cells. (A) Annexin-V/propidium iodide double-staining was used to quantify the type of cell death in OCCM.30 cells treated with TEDGMA (0, 1, 2, 4 mM) for 24 h. In this dot plot, lower right quadrant (LR) represents early apoptosis. Upper right quadrant (UR) represents late apoptosis cells. Upper left (UL) quarter represents the necrotic cells. Lower left quarter (LL) represents the viable cells. (B) The quantitative analysis of OCCM.30 cells in each quarter was summarized to differentiate apoptosis or necrosis as illustrated by bar graph.

Apoptosis is a key regulator of physiological growth control and regulation of tissue homeostasis. In this study, TEDGMA induced apoptotic effect in OCCM.30 cells by the elevation of sub-G1 cell population. In addition, both early and later apoptotic cells were noted in TEDGMA-induced cell death by Annexin V-FITC apoptosis assay. Our results were in agreement with previous studies that TEDGMA could induce cell apoptosis in HeLa cells,[6] human pulp

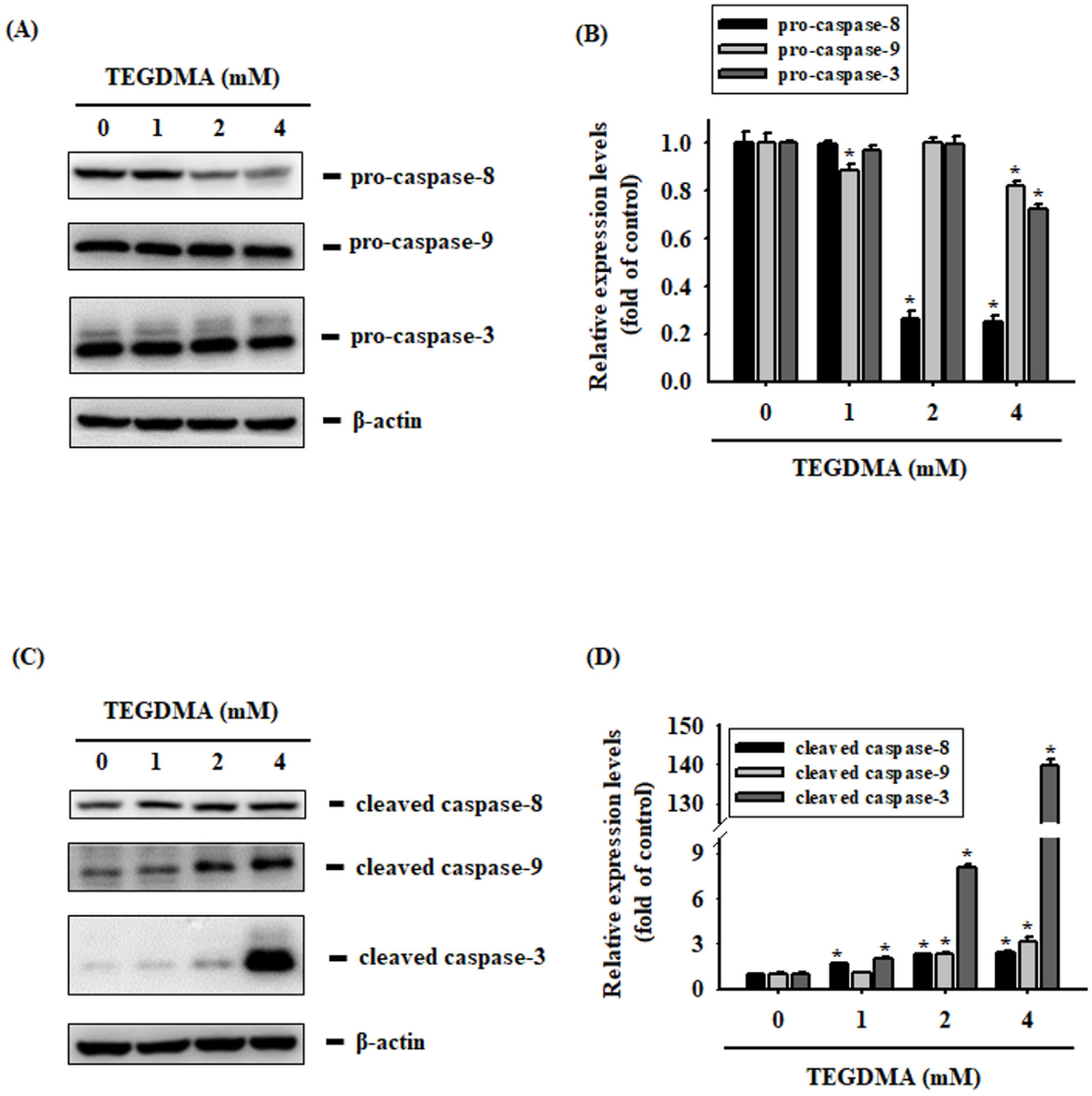

Figure 4 Triethylene-glycol-dimethacrylate (TEDGMA)-induced caspases-mediated apoptosis in OCCM.30 cells. (A) The effects of TEDGMA (0, 1, 2, 4 mM) on the inhibition of pro-caspase-8, -9, and -3 in OCCM.30 cells for 24 h were assayed by Western blot. (B) The quantitative results of these protein levels were adjusted by β-actin levels. Values represent the mean ± standard deviation from three independent experiments. *$P < 0.05$ compared with the control. (C) The effects of TEDGMA (0, 1, 2, 4 mM) on the activation of cleaved caspase-8, -9, and -3 in OCCM.30 cells for 24 h were evaluated by Western blot. (D) The quantitative results of these protein levels were adjusted by β-actin levels. Values represent the mean ± standard deviation from three independent experiments. *$P < 0.05$ compared with the control.

derived cells,[6] mouse macrophages RAW264.7 cells,[6–9] and human gingival fibroblasts.[12] Moreover, TEDGMA-induced cell necrosis was also revealed in OCCM.30 cells. Similar results were found in HeLa cells,[7] human pulp derived cells,[6,7] and mouse macrophages RAW264.7 cells.[6–10] Taken together, the type of TEDGMA-induced cell death is varied in different cells.

Caspases are initially synthesized within cells as inactive procaspases and must become active cleaved caspases during apoptosis. Apoptosis is triggered by two different signaling pathways. Effector and initiator caspases (caspase 3) is activated by extrinsic stimuli (caspase 8) through death receptors or intrinsic stimuli (caspase 9) that lead to mitochondrial depolarization.[23] To the best of our current knowledge, TEDGMA was first found to decrease pro-caspase-3, -8, and -9 as well as stimulate cleaved caspase-3, -8, and -9 expression in OCCM.30 cells. Beside to the effector caspase 3, TEDGMA-induced cell apoptosis was found to activate both extrinsic and intrinsic apoptotic processes in OCCM.30 cells. The protein expression of cleaved caspase-3 was the most inducible amount. This finding suggests that caspase 3 may play a major cell

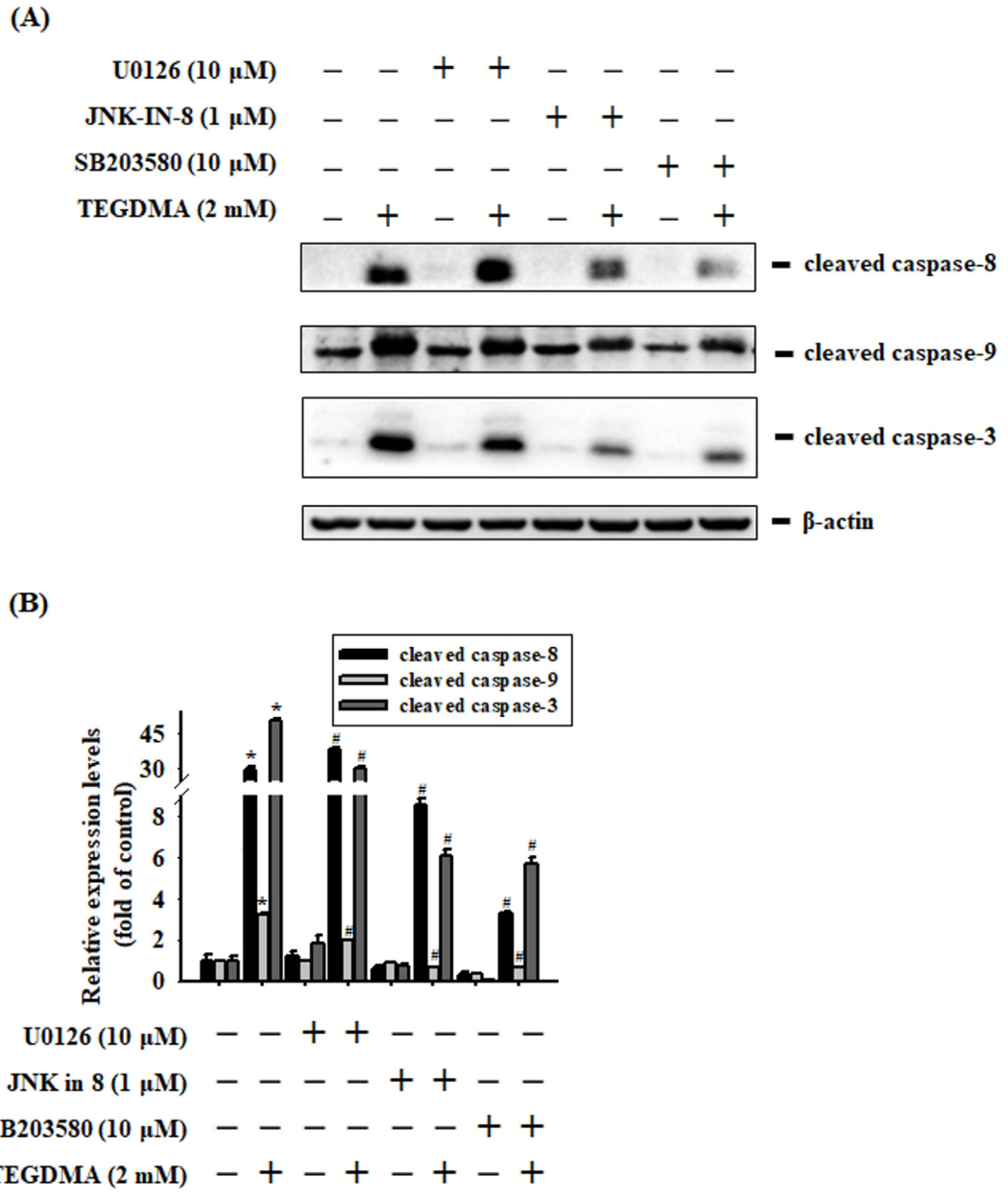

Figure 5 Relationship among the activation of mitogen-activated protein kinases in 2 mM triethylene-glycol-dimethacrylate (TEDGMA)-triggered apoptosis of OCCM.30 cells. (A) OCCM.30 cells were pre-treated with UO126, JNK in 8, and SB203580 for 1 h before 2 mM TEDGMA treatment and untreated cells for 24 h by Western blot. (B) The quantification data are shown in the bar graph adjusted by β-actin levels. Data represent the mean ± standard deviation from three independent experiments. *: $P < 0.05$ compared with the control. #: $P < 0.05$ compared with the 2 mM TEDGMA treatment group.

apoptotic pathway in OCCM.30 cells. Similar results were found in macrophages that the activation of caspase 3, 8, and 9 were generated by TEGDMA in a dose-dependent manner assessed by fluorometric assay.[9,10] In addition, Teti et al.[13] who also reported that a light band corresponding to the cleaved caspase-3 expression in human gingival fibroblasts by the treatment of TEGDMA.[13] Taken together, caspases-mediated cell apoptotic pathway may play an important signaling pathway in the TEGDMA-induced cell apoptotic death.

MAPKs, threonine and serine protein kinases, include ERK, JNK, and p38 which have been reported in the regulation of apoptotic cell death including the caspase-mediated apoptotosis.[24] Many reports have demonstrated that TEGDMA could activate MAPKs in human pulp cells[5] and RAW264.7 cells[5,7–9] with different expression pattern. In this study, the activity of ERK, JNK, and p38 were dominantly increased after 2 mM TEDGMA treatment. Therefore, the effects of TEDGMA on MAPK activities appear to be dependent on the cell types and the concentrations used. Moreover, our study directly found that the induction of caspase-mediated apoptosis by TEDGMA was retarded by JNK and p38 inhibitors, but not in ERK signaling pathway. Therefore, factors that inhibit JNK and p38 phosphorylation

may appear useful in preventing apoptosis mediated by TEGDMA. The activities of caspases suppressed by JNK in 8 and SB203580 might be the valuable therapeutics in TEGDMA-induced cytotoxic effects.

The interference of monomers with cementum could occur clinically which may lead to periodontal and apical bone destruction. However, it is difficult to determine the exact concentrations of TEGDMA that would act on cementoblasts in vivo. Cebe et al.[5] who have reported that the residual monomers eluted from bulk fill composite resins were detected in all time periods and the amounts were also increased with time by high-performance liquid chromatography. The highest concentrations of eluted monomers detected were reviewed that could cause the cytotoxic reactions in vitro. Therefore, long term accumulation of TEDGMA may be expected to reach the cytotoxic concentrations used in this experiment. The continued biocompatibility assessment of resin-based materials is necessary until the fulfillment of all properties as the ideal dental restorative materials.

Within the limitations of this in vitro experiments, the following conclusions can be addressed. TEDGMA-induced cytotoxicity was related to the inhibition of cell viability, the impairment of cell cycle, and the cell death with necrosis as well apoptosis. TEDGMA up-regulated sub-G0 cell fraction, early apoptotic cells, and late phase of apoptotic cells by flow cytometry. TEDGMA significantly increased the protein levels of pro-caspase-8, -9, and -3, whereas the expression of cleaved-caspase-8, -9, and -3 were elevated in OCCM.30 cells. The stimulation of caspase-8, -9, and -3 activation by TEDGMA were retarded by JNK inhibitor JNK-in-8 and p38 inhibitor SB203580. These results suggested that the activation of JNK and p38 signaling cascades may regulate caspases-dependent cell apoptotic death induced by TEGDMA in OCCM.30 cells. Further animal studies may be required to investigate the detailed effects and possible mechanism of TEGDMA in vivo.

Declaration of competing interest

The authors have no conflicts of interest relevant to this article.

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