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# Function of MAPK and downstream transcription factors in monomer-induced apoptosis

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ABSTRACT

The resin monomer triethylene glycol dimethacrylate (TEGDMA) disrupts vital cell functions, and the production of oxidative stress is considered a common underlying mechanism. The precise signaling pathways, however, that initiate monomer-induced effects, which disturb responses of the innate immune system, inhibit dentin mineralization processes, or induce apoptosis in target cells *in vitro* are still unknown. The present study provides insight into the causal relationship between TEGDMA-induced apoptosis and the activation of MAPK and transcription factors downstream using pharmacological inhibitors of the ERK1/2, p38 and JNK pathways. The endotoxin lipopolysaccharide (LPS; 0.1 μg/ml) was included as an inducer of MAPK activity in RAW264.7 mouse macrophages. Cell viability was decreased from 95% in untreated cultures to about 43% after a 24 h exposure to 3 mM TEGDMA. Inhibition of the ERK1/2 pathway by the MEK1/2 inhibitor PD98059 reduced cell viability to 84%. While apoptosis induced by TEGDMA remained unchanged, Western blot analyses revealed that the activation of ERK1/2 in the presence of TEGDMA was inhibited by PD98059. LPS-induced expression of activated transcription factors c-Jun, ATF-2, ATF-3 and phospho-Elk1 was decreased in cells co-treated with TEGDMA. This inhibition was more intense in the presence of PD98059, indicating that the MEK/ERK pathway is involved in the inhibition of the LPS-induced activation of transcription factors by TEGDMA. No clear effects of the p38 inhibitor SB203580 and the JNK inhibitor SP600125 on TEGDMA-induced apoptosis were detected. The antioxidant N-acetylcysteine (NAC) protected cells from TEGDMA-induced cell death, and inhibited the activation of ERK1/2, p38 and JNK by TEGDMA. Moreover, the TEGDMA-induced downregulation of the expression of the transcription factors c-Jun and ATF-2 was prevented as well. In conclusion, physiologically relevant concentrations of inhibitors differentially modified the expression of MAPK and transcription factors in cell cultures exposed to LPS and the monomer TEGDMA. The absence of a drastic effect of the MAPK pathway inhibitors on TEGDMA-induced apoptosis on the one hand, and the protective effect of NAC and PD98059 in particular on TEGDMA-induced MAPK activation and apoptosis on the other hand, leads to a new model for the role of MAPK in the regulation of cell homeostasis in monomer-exposed cells and tissues.



## 1. Introduction

Dental restorative materials are a lasting source of bioactive compounds in various tissues of the oral cavity in short and long-term scenarios. Unreacted resin monomers are environmental stressors released from the organic matrix of dental composites after incomplete polymerization. Uncured hydrophilic monomers like 2-hydroxyethyl methacrylate (HEMA) or triethylene glycol dimethacrylate (TEGDMA) were identified along with a wide variety of monomers and additives in an aqueous environment [1,2]. Moreover, clinically polymerized composites are predisposed to biodegradation through enzyme activities in saliva [3]. The monomers released the most, HEMA followed by TEGDMA, cause specific stress responses in various eukaryotic cells *in vitro* [4]. It appears that the production of reactive oxygen species (ROS) is a first major response of cells to exposure to resin monomers. Most

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relevant, the extent of monomer-induced ROS production clearly exceeds the capacities of the cells' enzymatic and nonenzymatic antioxidant system, including the availability of reduced glutathione (GSH) [5]. As a consequence, resin monomers disturb the cellular redox balance and lead to the disruption of specific cell functions *in vitro* [4].

Currently, it appears as if oxidative stress caused by monomer exposure modulates tightly regulated cellular signaling networks of vital importance. For instance, it was reported that long-term exposure of deciduous teeth stem cells to low concentrations of HEMA and TEGDMA delayed the differentiation and mineralization processes [6]. In addition, it has been shown that resin-induced oxidative stress is the most likely cause of inhibited mineralization because the antioxidant N-acetylcysteine (NAC) considerably reduced cytotoxicity of methacrylates to maintain osteoblastic viability and function [7,8]. Furthermore, resin monomers also interfere with signaling pathways triggered by the bacterial endotoxin lipopolysaccharide (LPS). In cells of the innate immune system, LPS leads to the activation of a CD14 and TLR4 complex, and a cascade of signal proteins finally activates mitogen-activated protein kinases (MAPK) or the transcription factor NF-κB. As a result, the pro- and anti-inflammatory mediators such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), or interleukin-10 (IL-10) are released [9]. It has been shown recently that the release of TNF-α, IL-6, and IL-10 from LPS-stimulated mouse macrophages was significantly reduced by TEGDMA even after very short exposure periods. Thus, the ability of macrophages to induce an appropriate immune response may be inhibited by this resin compound [10]. Furthermore, genotoxic and mutagenic effects observed in cells exposed to HEMA or TEGDMA are probably, at least to some extent, caused by ROS-induced DNA damage. The activation of functional cell cycle checkpoints due to oxidative DNA damage may not only lead to a delay of the mammalian cell cycle, but also to the induction of apoptosis through the coordinated activities of regulatory proteins [11].

The induction of cytotoxicity via apoptosis is a major stress response of cells targeted by resin monomers. This phenomenon has been described on many occasions and underlying mechanisms have been deciphered in part so far [4]. Besides monomer-induced oxidative DNA damage, the redox-sensitive activation of MAPK has been considered to be another major pathway possibly leading to apoptosis. The ERK1/2, JNK, and p38 serine–threonine kinase modules of the MAPK pathways are regulated by various stress-related stimuli including ROS. While the function of ERK1/2 has been linked to the regulation of cell survival, JNK and p38 MAPK were considered stress kinases related to the induction of apoptosis [12]. It has been shown recently that the onset of apoptosis and necrosis in RAW264.7 mouse macrophages was associated with the induction of activated ERK1/2 and stress kinases p38 and JNK after exposure to TEGDMA [10]. Since signaling through the MAPK cascade is linked to transcription factors downstream, it was hypothesized that apoptosis caused by TEGDMA was related to the monomer-induced downregulation of the transcription factors c-Jun, ATF-2 or ATF-3 [13]. It is well established that the expression of the transcription factor AP-1 is elevated due to the induction of *fos* and *jun* gene transcription by stressors including genotoxic agents [14,15]. Moreover, the activity of c-Jun is increased in response to the phosphorylation by JNK [16]. Similarly, activating transcription factor 2 (ATF-2) responded to p38/JNK, and it is necessary for the induction of apoptosis as well as for the coordination of cell responses to DNA damage [17,18]. Likewise, ATF-3 is considered a stress-inducible 'adaptive response' gene whose biological role is related to the regulation of cell proliferation and apoptosis [19]. Besides a possible role of MAPK, monomer-induced apoptosis may also be initiated by response to DNA damage followed by the activation of a signaling cascade through ATM, transcription factor p53, and p21 [13,20].

In spite of this progress in the elucidation of the mechanism of monomer-induced apoptosis, the activation of MAPK and related transcription factors downstream has not yet been critically linked to monomer-induced apoptosis thus far. The characterization of a particular role of MAPK and transcription factors downstream in monomer-induced apoptosis, however, is a topic of considerable importance for the clinical use of dental composite materials as well as for therapeutic purposes in dental practice. Here, we provide information on the causal correlation between apoptosis, MAPK and downstream transcription factors using specific pharmacological inhibitors of MAPK. To this end, signal transduction through MAPK ERK1/2 was inhibited by the use of PD98059 (2′-amino-3′-methoxyflavone). It binds to the inactive form of MEK1/2, thus preventing the phosphorylation of ERK1/2 and the generation of phospho-c-Jun downstream [21–23]. The pyridinyl imidazole compound SB203580 was employed as a specific inhibitor of the catalytic unit of MAPK p38 through competition with ATP. The phosphorylation of the stress-activated transcription factor ATF-2 is thus inhibited [24,25]. Finally, SP600125 was utilized as a specific inhibitor of JNK function preventing downstream phosphorylation of c-Jun [26]. Two different stressors were used here to characterize effects in RAW264.7 mouse macrophages as a model of the innate immune system. Cell responses towards the resin monomer TEGDMA were related to the influence of lipopolysaccharide (LPS), a known inducer of MAPK activity and related transcription factors [10,13].

## 2. Materials and methods

### 2.1. Chemicals and reagents

Triethylene glycol dimethacrylate (TEGDMA; CAS-No. 109-16-0), lipopolysaccharide (LPS; E. coli, serotype 055:B5), N-acetylcysteine (NAC) (CAS-No 616-91-1), and nonidet P-40 substitute (NP-40; 74385) were purchased from Sigma-Aldrich (Taufkirchen, Germany). PD98059, 2′-amino-3′-methoxyflavone), SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4- pyridyl)imidazole), and SP600125 (anthra[1,9-cd]pyrazole-6 (2H)-one) were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). RPMI 1640 medium containing L-glutamine and 2.0 g/l $NaHCO_3$ was purchased from PAN Biotech (Aidenbach, Germany). Fetal bovine serum (FBS), penicillin/streptomycin, and phosphate-buffered saline supplemented with 5 mM EDTA (PBS-EDTA) came from Life Technologies, Gibco BRL (Eggenstein, Germany). Antiphospho-specific p44/42 (ERK1/2)(Thr202/Tyr204), anti-p44/42 (ERK1/2), antiphospho-ATF-2(Thr69/71) (no. 9225), antiphospho - Elk-1, anti-Elk-1, anti-lamin A/C (no. 2032), and anti-rabbit IgG HRP-linked antibody (no. 7074) were obtained from Cell Signaling (NEB Frankfurt, Germany). Antiphospho-specific p38(pT180/pY182) (clone 30/p38 MAPK), and anti-p38α (clone 27/p38α/SAPK2a) were purchased from BD Biosciences (Heidelberg, Germany), and phospho-specific JNK (anti-ACTIVE JNK, pTPpY) was obtained from Promega (Madison, WI, USA). Anti-c-Jun (sc-1694), anti-ATF-2 (C-19) (sc-187), and anti-ATF-3 (C-19) (sc-188) came from Santa Cruz Biotech (Heidelberg, Germany). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (clone 6C5) was purchased from Millipore GmbH (Schwalbach, Germany), and goat anti-mouse IgG (H + L)-HRP conjugate came from Bio-Rad Laboratories (Munich, Germany). Amersham hyperfilm ECL came from GE Healthcare (Munich, Germany). A protease inhibitor cocktail (complete mini) was obtained from Roche Diagnostics (Mannheim, Germany), and a FACS Annexin V-FITC apoptosis detection kit came from R&D Systems (Minneapolis, MN, USA). All other chemicals used in the present study were at least chemical grade.

### 2.2. Cell culture, exposure of cells, and determination of apoptosis

RAW264.7 mouse macrophages (ATCC TIB71) were cultivated in RPMI 1640 medium containing L-glutamine, sodium-pyruvate and 2.0 g/l $NaHCO_3$ supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. The cells ($0.5–1.0 \times 10^5$/well) were cultivated in six-well plates for 24 h at 37 °C. Then, the cell cultures were treated with 0.0, 0.1, 1.0 or 3.0 mM TEGDMA, or 0.1 μg/ml LPS, or a combination of LPS/TEGDMA for 24 h. The cells were exposed in the presence or absence of 10 or 30 μM PD98059 (MEK1/2 inhibitor), 10 μM or 30 μM SB203580 (p38 inhibitor) and 3 or 10 μM SP600125 (JNK inhibitor). Cell cultures were preincubated with the inhibitors for 1 h at 37 °C. Only results obtained with 30 μM PD98059 or SB203580 and 10 μM SP600125 are presented in the Results section for clarity