Activation of stress-regulated transcription factors by triethylene glycol dimethacrylate monomer

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Abstract
Triethylene glycol dimethacrylate (TEGDMA) is a resin monomer available for short exposure scenarios of oral tissues due to incomplete polymerization processes of dental composite materials. The generation of reactive oxygen species (ROS) in the presence of resin monomers is discussed as a common mechanism underlying cellular reactions as diverse as disturbed responses of the innate immune system, inhibition of dentin mineralization processes, genotoxicity and a delayed cell cycle. Yet, the signaling pathway through a network of proteins that finally initiates the execution of monomer-induced specific cell responses is unknown so far. The aim of the present study was to extend the knowledge of molecular mechanisms of monomer-induced cell death as a basis for reasonable therapy strategies. Thus, the monomer-induced expression and phosphorylation of stress-related transcription factors was analyzed in various cell lines. The time-related induction of apoptosis was investigated as well. The expression of p53 increased in HeLa cell cultures treated with camptothecin (positive control) for 24h, and the formation of p53Ser15 and p53Ser46 was detected in cell nuclei by Western blotting. TEGDMA (3 mM) appeared to stimulate p53 expression only slightly, but increased p21 expression was found in cell nuclei and cytoplasm. Both camptothecin and TEGDMA increased p53 expression to some extent in the nuclear fraction in human transformed pulp-derived cells (tHPC), and similar effects were detected in RAW264.7 macrophages. No clear induction of c-Jun and phospho-c-Jun by TEGDMA was detected in HeLa cell nuclei, and the expression of ATF-2 and phospho-ATF-2 was inhibited in the presence of the monomer. ATF-3 expression was found only in the nuclear fraction of camptothecin-treated HeLa cultures. TEGDMA seemed to inhibit the formation of phospho-c-Jun and phospho-ATF-2 in tHPC, and the monomer acted negatively on the expression of c-Jun, ATF-2 and ATF-3 in RAW264.7 macrophages. These changes in the expression and activation of stress-related transcription factors were time-related to the induction of apoptosis by TEGDMA in all cell lines. The present study provides experimental evidence that TEGDMA interferes with the regulation of cellular pathways through transcription factors as a consequence of DNA damage like p53 or initiated downstream of MAPK (mitogen-activated protein kinases) like c-Jun, ATF-2 and ATF-3. The direct causal correlation between DNA damage, activation or inhibition of MAPKs and transcription factors, and apoptosis is under current investigation. However, the induction of apoptosis in different cell lines in the presence of monomers like TEGDMA may be subject to a higher level of complexity than currently suggested by simple linear models.

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1. Introduction

Tissues of the oral cavity are impacted by a variety of biological stressors including compounds released from dental restorative biomaterials. Numerous investigations have shown that dental composite materials are not chemically inert in the human oral cavity. Residual unreacted monomers are available for short exposure scenarios due to incomplete polymerization processes of the organic matrix of dental composites. Triethylene glycol dimethacrylate (TEGDMA) was found to be a major comonomer eluted even from polymerized resin composites in an aqueous environment [1]. In addition, polymerized composites are also susceptible to biodegradation through hydrolytic enzymes in saliva, making the
polymeric network most likely a source of continuous release of compounds to which cells and tissues are then exposed for a long period of time [2].

Among others, the resin monomers TEGDMA and HEMA caused specific stress responses in a wide variety of eukaryotic cells in vitro [3]. These substances induced cell death via apoptosis in various cell types including pulp and gingiva cells and it appears as if cell death was mediated, at least in part, by the generation of reactive oxygen species (ROS) disturbing the redox balance [4,5]. Furthermore, genotoxic and mutagenic effects caused by TEGDMA are probably to some extent a consequence of ROS-induced DNA damage. A significant increase in the levels of 8-oxoguanine as a marker for oxidative DNA damage was found in cell cultures after long exposure to TEGDMA [6]. As a result of DNA damage, mammalian cells activate functional cell cycle checkpoints through the coordinated activities of regulatory proteins. A corresponding monomer-induced delay of the cell cycle was, however, overridden in the presence of the antioxidant N-acetylcysteine (NAC) [7,8]. Furthermore, low TEGDMA concentrations and chemically related substances like PMMA (poly-methyl methacrylate) even inhibited specific odontoblast functions including alkaline phosphatase activity, the matrix mineralizing capability, calcium deposition, and gene expression, such as dentin sialoprotein [9,10]. Oxidative stress is most likely the cause of disturbed mineralization because NAC dramatically reduced cytotoxicity of bone substitutes to maintain osteoblastic viability and function, implying enhanced bone regeneration around NAC-treated inorganic biomaterials [11]. The monomer TEGDMA also influenced specific cell responses of the innate immune system. The monomer instantaneously downregulated the LPS-induced cytokine production in macrophages and inhibited the expression of surface antigens like CD14 and other surface markers essential for the controlled interaction of immune cells [12].

These findings clearly indicate that the adverse effects of resin monomers involve interference with a complex regulatory network of specific cell functions. However, except for the well-established effects regarding the induction of oxidative stress by resin monomers, there is only one additional minor insight available in the research thus far into the specific mechanisms behind these phenomena, and the inhibition of specific functions in cells of various origins.

A more detailed understanding will provide a better estimation of the consequences associated with dental therapies. Thus, the analysis of the mechanisms of the cytotoxicity of dental composite materials is highly attractive because it leads the way to the development of useful strategies and materials for the protection of tissues of the oral cavity [13].

Research related to this goal has primarily focused on signal transduction pathways through the mitogen-activated protein kinase (MAPK) cascade. These proteins regulate basic cell functions modified by resin monomers like cell viability and apoptosis, cell proliferation and the expression of cytokines and cell surface antigens [14]. It has been reported that ERK1/2 was activated by HEMA and TEGDMA in a salivary gland cell line after long exposure periods, and a role of p38 in the apoptotic cell response to HEMA has been suggested [15]. Moreover, the sustained activation of both ERK1/2 and p38 kinases by TEGDMA in a human monocyte cell line (THP-1) was associated with monomer-induced cytotoxicity, and a pro-survival role of ERK1/2 was indicated in primary human pulp cells after short exposure periods to HEMA [6,16]. ERK1/2, p38 and JNK (c-Jun N-terminal kinase) were differentially activated by phosphorylation in the presence of lipopolysaccharide (LPS), a known inducer of MAPK activity, and the resin monomer TEGDMA in RAW264.7 mouse macrophages. In contrast to the immediate inhibition of cytokine release, apoptosis and necrosis caused by LPS and TEGDMA was a late response associated with a strong increase in MAPK activation [17].

Besides the suggested role of MAPKs in the regulation of cell death and survival, the function of the tumor suppressor p53 as a key regulator of apoptosis is firmly established [18]. P53 is activated through the ATM signaling pathway when DNA damage occurs and, in addition to many other functions, acting as a transcription factor controls cell cycle progression and apoptosis-related gene expression or interacts with proteins after posttranslational modifications including phosphorylation at Ser15 and Ser46 residues. Among the p53 target genes, p21Cip1 expression is activated, which in turn acts as an inhibitor of cell proliferation or apoptosis depending on its cellular localization [19].

Although not experimentally proven so far, it has been proposed that an increase in ROS caused by resin monomers is the main origin of the upstream activation of MAPK [17]. In contrast, the related downstream activation of MAPK-related gene expression of specific transcription factors that finally initiate the execution of monomer-induced apoptosis is unknown to date. The activation of cascades of phosphorylation, in many cases increases expression of immediate early genes as an early stress response. These immediate early genes code for transcription factors, which then regulate downstream genes of the cell survival network [20]. Therefore, it was the aim of the present study to broaden our understanding of the molecular mechanisms of dental resin monomer-induced cell death. To this end, we analyzed the monomer-induced expression and phosphorylation of transcription factors that were associated with the induction of apoptosis.

First, we hypothesized that the induction of apoptosis may depend on a differential activation of various transcription factors which are targets of the MAPK signaling pathway. For instance, the activity of the transcription factor AP-1 is rapidly elevated due to the induction of fos and jun gene transcription by many stimuli including genotoxic agents [21,22]. In addition to the activation at the transcriptional level, the activity of c-Jun is greatly enhanced in response to phosphorylation by the MAPK JNK [23]. Activating transcription factor 2 (ATF-2), as a member of the ATF/CREB family of transcription factors, is activated via phosphorylation by MAPKs in the cellular response to stress [24]. It has been reported that activation of ATF-2 by p38/JNK is necessary for the induction of apoptosis, and ATF-2 has also been shown to coordinate the cellular response to DNA damage upon activation of ATM [25,26]. ATF-3 has been discussed as a stress-inducible gene or an ‘adaptive response’ gene. Its biological role is currently not well defined but probably related to the regulation of cell proliferation and apoptosis [27]. Second, because of the demonstrated genotoxicity of dental resin monomers, monomer-induced apoptosis may also be initiated by the cellular response to DNA damage followed by the activation of a signaling cascade through ATM, p53, and p21 [18].

HeLa were included in the current investigation as an established human cancer cell line and a control for the expression of the p53 tumor suppressor protein. In addition, cell responses were analyzed in cells important for clinical applications and tissue-specific reactions. RAW264.7 mouse macrophages were used as a model cell line of the innate immune system, and human pulp-derived cells were utilized as a model of pulp tissue responses [17]. The dental resin TEGDMA served as a model monomer to study the expression and posttranslational modification of transcription factors because of the demonstrated relationship between monomer structure and cytotoxicity [3,28].

2. Materials and Methods

2.1. Chemicals and reagents

Triethylene glycol dimethacrylate (TEGDMA; CAS-No. 109–16–0) and Nonidet P-40 substitute (NP-40; 74385) were purchased from Sigma–Aldrich (Taufkirchen, Germany). Camptothecin was obtained from BioVision (Mountain View, CA, USA). All other chemicals were purchased from standard suppliers.
necrosis (annexin V−; PI+). Early apoptosis was determined with Annexin V−; PI− cells in the lower left quadrant (Q3) of density.

2.4. Analysis of the expression of transcription factors and protein extraction

The cells (1.0×10^6) were seeded in six-well plates for 24 h at 37°C and 5% CO2. Then, cell cultures were treated with cell culture medium containing TEGDMA (1 and 3 mM) or 1 μm camptothecin for 24 h. The exposure of Hela cells was not detected compared to untreated cultures.

2.5. Western blot analysis

The amount of viable human transformed pulp cells (tHPC) only slightly from 85% to 73%, and 8% (3 mM TEGDMA) compared to 1% found in untreated cell cultures. The percentage of cells in late apoptosis increased to 16% in cell cultures treated with 3 mM TEGDMA, but the proportion of necrotic cells induced by camptothecin and TEGDMA was below 1% (Fig. 1A).

3. Results

3.1. Induction of apoptosis in Hela, human pulp cells and mouse macrophages

The induction of apoptosis by the resin monomer TEGDMA was determined in various cell lines to analyze for responses of cells with tissue-specific functions. In Hela cells, a significant decrease in the number of viable cells to about 82% caused by camptothecin was related to an increase in the percentage of cells in early apoptosis (14%) after a 24 h exposure period (Fig. 1A). Likewise, the resin monomer TEGDMA induced a concentration-related increase in the number of cells in early apoptosis to 3% (1 mM TEGDMA) and 8% (3 mM TEGDMA) compared to 1% found in untreated cell cultures. The percentage of cells in late apoptosis increased to 16% in cell cultures treated with 3 mM TEGDMA, but the proportion of necrotic cells induced by camptothecin and TEGDMA was below 1% (Fig. 1A).

The amount of viable human transformed pulp cells (tHPC) was reduced to 55% and 38% by 1 and 3 mM TEGDMA, respectively compared to 85% in untreated controls. The proportion of cells in late apoptosis increased in parallel from 19% to 36% in cultures treated with 1 and 3 mM TEGDMA compared to 6% in control cultures, and a significant increase in cells in necrosis was detected in TEGDMA-treated cultures. Camptothecin, however, reduced the number of viable tHPC only slightly from 85% to 73%, and a significant increase in the number of cells in the various stages of cell death was not detected compared to untreated cultures (Fig. 1B).

RAW264.7 mouse macrophages (ATCC TIB71) were cultivated in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and penicillin-streptomycin. Human pulp-derived cells (tHPC) were routinely kept in MEM supplemented with 10% FBS, penicillin (100 μg/ml), and streptomycin (100 μg/ml).

2.3. Determination of apoptosis

The cells (5.0–1.0×10^6)well) were cultivated in six-well plates for 24 h at 37°C and 5% CO2. Then, cell cultures were treated with cell culture medium containing TEGDMA (1 and 3 mM) or 1 μm camptothecin for 24 h. The exposure of Hela cells was not detected compared to untreated cultures.

2.2. Cell culture and exposure of cells

The cells were stained with propidium iodide (PI) to differentiate between cells in apoptosis (annexin V− and necrosis (PI−) [17,30]. Finally, the stained cell cultures were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). FITC fluorescence (FL-1) was collected through a 530/30 band pass filter, and PI fluorescence (FL-3) through a 650 nm long pass filter. Data acquisition (2×10^6 events for each sample) was performed with the FACSDiva software. The same software was used to count the numbers of viable (annexin V−; PI−) cells in the lower left quadrant (Q3) of density plots, and the percentages of cells in apoptosis (annexin V−; PI−; lower right quadrant Q4), late apoptosis (annexin V−; PI−; upper right quadrant Q2), and necrosis (annexin V−; PI−; upper left quadrant Q1) were determined accordingly [17,30].

Original data from at least four independent experiments were summarized as medians (25–75% quartiles), and differences between median values were statistically analyzed using the Mann–Whitney U test (SPSS 18.0, SPSS, Chicago, IL, USA) for pairwise comparisons among groups at the 0.05 level of significance.

2.4. Analysis of the expression of transcription factors and protein extraction

The cells (1.0–2.0×10^6) were seeded into cell culture plates (15 cm in diameter) and left untreated for 24 h at 37°C and 5% CO2. Then the medium was replaced with fresh cell culture medium containing TEGDMA (1 and 3 mM) or 1 μm camptothecin for 24 h at 37°C and 5% CO2. Exposure was stopped by collecting the exposure media (DMEM) containing 5% FBS, 4.5 g/l glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml).

The amount of viable tHPC only slightly from 85% to 73%, and a significant increase in the number of cells in the various stages of cell death was not detected compared to untreated cultures (Fig. 1B).
3.2. Expression of transcription factors associated with apoptosis

3.2.1. Expression of transcription factors in HeLa

The expression of transcription factors associated with the induction of apoptosis in the presence of TEGDMA was analyzed in the same cell lines. The tumor suppressor protein p53 was not detected in either the cell nuclei or cytoplasmic fraction of untreated HeLa cultures. However, p53 expression was greatly enhanced in cultures treated with camptothecin, which was used as a positive control after a 24 h exposure period. A very strong single band was detected with a specific antibody in the nuclear fraction (Fig. 2A). In contrast, TEGDMA appeared to increase p53 expression only slightly in cell nuclei at a high concentration (Fig. 2A). The formation of p53 Ser15 and Ser46 phosphorylation was also clearly detectable in nuclei isolated from cell cultures exposed to camptothecin. However, this posttranslational modification of p53 was not unequivocally detected in TEGDMA-treated HeLa cells under the current experimental conditions. The p53-induced cyclin-dependent kinase (CDK) inhibitor p21 was found in the cytoplasm as well as in the nuclear fraction of untreated HeLa cells. Moreover, the expression of p21 was slightly enhanced in nuclei of cells treated with camptothecin, and TEGDMA appeared to increase p21 expression in both cell nuclei and cytoplasm (Fig. 2A). Lamin A/C and GAPDH were used as markers for the nuclear and the cytoplasmic cell fraction. Typical results from one of at least two independent experiments are shown.
cultures as expected, while GAPDH was present only in the cytoplasmic fraction. Noteworthy is that a faint band was present in the nuclear fraction of camptothecin-treated HeLa cells representing the cleavage product of lamin A (Fig. 2A).

Transcription factors downstream of the MAPK cascade were differentially expressed after exposure to the resin monomer and camptothecin. Exposure of HeLa to camptothecin increased the expression of c-Jun in cell nuclei compared to untreated controls, and phosphorylation of c-Jun was detected as well. Only a very faint band indicating c-Jun was visible in the cytoplasmic fraction (Fig. 2B). Most importantly, a clear induction of phospho-c-Jun and c-Jun by TEGDMA was not detected in HeLa cells despite a faint band in extracts of cells exposed to 3 mM TEGDMA under the current experimental conditions. In contrast to this pattern, phosphorylated ATF-2 and total ATF-2 were clearly detectable in the nuclear fraction of cell extracts of untreated cultures. However, the expression of phospho-ATF-2 and ATF-2 was not activated but appeared to be inhibited by camptothecin and TEGDMA. None of the protein species were detectable after the same exposure periods in the cytoplasmic fractions. ATF-3 expression was found only in the nuclear fraction of camptothecin-treated HeLa cell cultures (Fig. 2B).

3.2.2. Expression of transcription factors in tHPC

Transformed human pulp-derived cells (tHPC) expressed the p53 protein in both cell nuclei and the cytoplasmic fraction of untreated cells (Fig. 3A). Camptothecin increased p53 expression to some extent in the nuclear fraction, and it appeared as if 1 mM TEGDMA was not detected in HeLa cells despite a faint band in extracts of cells exposed to 3 mM TEGDMA under the current experimental conditions. In contrast to this pattern, phosphorylated c-Jun was visible in the cytoplasmic fraction as well. Camptothecin increased the amounts of phosphorylated c-Jun in cell nuclei while TEGDMA appeared to inhibit the formation of phospho-c-Jun (Fig. 3B). A very similar pattern of expression was detected with ATF-2 and phospho-ATF-2. As with HeLa, expression of ATF-3 was detected only in the nuclear fraction of camptothecin-treated tHPC cultures (Fig. 3B).

3.2.3. Expression of transcription factors in RAW264.7 mouse macrophage cells

In RAW264.7 macrophages, p53 was detectable in cell nuclei of untreated cell cultures (Fig. 4A). Moreover, camptothecin and 1 mM TEGDMA caused an increase in the expression of p53, and a single band was even detected in the cytoplasmic fraction of camptothecin-treated cells. Furthermore, p53 was phosphorylated at Ser15 in the nuclear cell fraction after exposure of cells to camptothecin and TEGDMA. Again, a distinct single band indicating p53Ser15 was present in the cytosol of cells treated with camptothecin. Unfortunately, an antibody specific for the detection of p53Ser46 in mouse cells is currently not available. High amounts of p21 protein were detected in the cytoplasmic fraction of RAW264.7 cells compared with the faint bands present in some nuclear fractions. It seemed as if p21 was upregulated in cells exposed to the higher TEGDMA concentration (Fig. 4A). Lamin A/C and GAPDH were detected in nuclear and cytoplasmic fractions of all cell cultures. A cleavage product of lamin A was present in the nuclear fraction of RAW264.7 cells exposed to 3 mM TEGDMA (Fig. 4A).

Similarly to the observations found with HeLa and tHPC, camptothecin and TEGDMA acted negatively on the expression of transcription factors downstream from MAPK as suggested in a linear signaling model. The expression of c-Jun was observed in cell nuclei of untreated controls and a weak expression was detected in the cytoplasmic fraction as well (Fig. 4B). However, c-Jun expression was lower in cell cultures exposed to camptothecin and TEGDMA. The amounts of phospho-c-Jun were reduced in the nuclear fraction, and no phospho-c-Jun was detected in the cytoplasmic fraction except for a very faint band in extracts of untreated cells. The expression of ATF-2 and phosphorylated ATF-2 was inhibited by camptothecin and a high TEGDMA concentration in cell nuclei, and neither ATF-2 nor phospho-ATF-2 were detected in the cytosol. ATF-3 expression was also inhibited after treatment of cells with camptothecin and TEGDMA (Fig. 4B).

The inhibitory effects of camptothecin and TEGDMA on the expression of ATF-2 and ATF-3 in RAW264.7 mouse macrophages were unexpected and contrary to those observed in human HeLa and tHPC. Therefore, we questioned whether these transcription factors could be activated by a stimulus different than camptothecin or TEGDMA. In fact, exposure of RAW264.7 cells to the bacterial...
with a complex redox-sensitive regulation of specific oxygen species (ROS) indicating the interference of resin monomers triggered at least in part by the established production of reactive species. Hypothesis that these diverse effects on vital cell responses are genotoxicity, inhibition of the mineralization process and disturbance of cell functions.

Endotoxin lipopolysaccharide (LPS) strongly increased the expression of ATF-3 in cell nuclei compared to untreated cell cultures, and the expression of ATF-2 was activated as well (Fig. 5). A decrease in ATF-2 and ATF-3 expression in cultures exposed to camptothecin and TEGDMA in the same experiment was again observed, while camptothecin activated ATF-2 and ATF-3 in HeLa control cultures (Fig. 5).

4. Discussion

4.1. TEGDMA-induced apoptosis in mouse and human cell lines

Dental resin monomers like TEGDMA and HEMA released from dental composite materials elicit a wide variety of responses in eukaryotic cells in vitro including cytotoxicity through apoptosis, genotoxicity, inhibition of the mineralization process and disturbance of functions of the innate immune system. We adhere to the hypothesis that these diverse effects on vital cell responses are triggered at least in part by the established production of reactive oxygen species (ROS) indicating the interference of resin monomers with a complex redox-sensitive regulation of specific cell functions [3]. However, thus far, only the role of mitogen-activated protein kinases in the induction of apoptosis has been studied in some detail [6,15–17]. It was recently found that extracellular signal-regulated kinases (ERK1/2) and the stress-activated kinases p38 and JNK were differentially phosphorylated in mouse macrophages depending on the exposure period. Long exposure of RAW264.7 cell cultures to TEGDMA resulted in a substantial increase in the phosphorylation of both ERK1/2 and p38 kinases. It was assumed that this activation was related to the induction of apoptosis as observed after the same exposure period [17]. Cell death via apoptosis was detected in the presence of TEGDMA in mouse macrophages and human pulp-derived cells similar to the observations in the present investigation. Here, increasing concentrations of TEGDMA reduced the number of viable mouse macrophages, and the percentage of cells in early and late apoptosis increased in parallel. TEGDMA was less effective in transformed human pulp-derived cells (tHPC), and HeLa were most resistant to TEGDMA and camptothecin as well. It is suggested that the greater susceptibility of RAW264.7 macrophages to TEGDMA might be related to the downregulation of stress-activated transcription factors like ATF-2 and ATF-3 as discussed below.

The differences between the effectiveness of TEGDMA and camptothecin in RAW264.7 macrophages compared to tHPC and HeLa indicate different mechanisms in the activity of both substances depending on the cell line. It was reported that camptothecin acts during DNA synthesis via strand scission, thus causing cell death via apoptosis during the S-phase of the cell cycle [31]. In contrast, the current hypothesis for the induction of cell death by resin monomers like TEGDMA favors an indirect mechanism through the generation of ROS [3]. The observations of TEGDMA-induced apoptosis in the various cell lines in the present investigation correspond with results obtained from primary human pulp-derived cells and gingival fibroblasts after long exposure [17,32,33]. Moreover, the concentration-dependent decrease in the number of vital cells after long exposure periods precisely correlated with the activation of MAPK in the presence of TEGDMA in both mouse macrophages and human pulp-derived cells [17].

It has been previously suggested that TEGDMA-induced apoptosis in primary pulp cells might not be mediated through ERK1/2 activation since the percentage of cells in apoptosis only slightly changed after pharmacological inhibition of this pathway [33]. In addition, there is some evidence that ERK1/2 might be
activated as a pro-survival factor in cells exposed to high TEGDMA concentrations, and sustained activation of the stress kinases p38 and perhaps JNK is related to TEGDMA-induced apoptosis in various cell lines [17,33]. We observed in a recent study that ERK1/2 and p38 were activated in THP-1 cells after exposure to TEGDMA for 24 h and 48 h. This activation was reduced in the presence of the ROS scavenger NAC [6]. Thus, it appears as if activation of MAPK by TEGDMA is possible through the generation of oxidative stress in cells of the innate immune system.

Here we hypothesized that, in case differential activation of cell responses to monomer-induced stress was mediated through different MAPKs, appropriate downstream target genes would be activated as well. Therefore, the monomer-induced expression and phosphorylation of transcription factors associated with the induction of apoptosis was investigated in the present study to broaden our understanding of the molecular mechanisms of dental resin monomer-induced cell death in various cell lines. In addition, monomer-induced apoptosis may originate from DNA damage and a subsequent activation of a signaling cascade including p52 and p21 [18]. The favorite model of activation of the tumor suppressor protein p53, a transcription factor activated upon DNA damage, involves signaling via ATM/ATR and checkpoint kinases Chk1/Chk2. As the guardian of the genome, p53 is a key regulatory protein of the cell cycle, DNA repair, and the induction of apoptosis due to DNA damage [34,35]. While p53 activity is regulated upstream by external stress factors, both at the transcriptional and posttranslational level, it in turn regulates a wide variety of factors downstream. Phosphorylation of p53 at Ser15 is linked to the induction of the expression of the cyclin-dependent kinase (CDK) inhibitor p21 which inhibits cell proliferation. Phosphorylation at Ser46, on the other hand, is thought to be related to the regulation of p53-dependent apoptosis [18,36].

4.2. TEGDMA-induced activation of transcription factors related to DNA damage

While the expression of the p53 protein was greatly increased in the nuclear fraction of HeLa cells after exposure to camptothecin, the resin monomer TEGDMA enhanced p53 expression only slightly at a high concentration. Interestingly, p53 was not detected in the cytoplasmic fraction of exposed HeLa cells. Camptothecin also enhanced the expression of p53 in cell nuclei of HeLa and RAW264.7 macrophages, and TEGDMA was slightly effective. Camptothecin also stimulated the formation of p53 phosphorylation at Ser15 and Ser46 in cell nuclei, while this activation of p53 was apparently very weak in TEGDMA-treated HeLa cells under the current experimental conditions. These results indicate that p53 activation by the resin monomer is probably lower than the effect of camptothecin, although TEGDMA was active in treated cultures of the various cell lines as indicated by the reduction in viable cells. Our observations with HeLa and RAW264.7 macrophages are consistent with the reported low levels of p21, and possibly a slight increase in TEGDMA. The functional consequence of phosphorylation of Ser46 has been reported to be related to the onset of p53-dependent apoptosis after DNA damage through the activation of apoptotic target genes like PUMA and Noxa [37]. This pathway might also be the molecular basis behind the effects of monomers like TEGDMA and is under current investigation. Here, the expression of p53Ser15 was clearly detected in HeLa cells, and present in THP and RAW264.7 macrophages indicating phosphorylation of p53 by ATM as a response to DNA damage [18]. Recently, the expression of ATM and p53Ser15 has also been reported in a salivary gland cell line after long exposure to HEMA [38]. Besides the p53-independent regulation of p21 through, for instance, Sp1 or transforming growth factor β (TGF-β), phosphorylation of p53 at Ser15 upon DNA damage is discussed as a modification which activates the transcription of the cyclin-dependent kinase inhibitor 1 (p21) gene [18]. The p21 protein was detected here in the cytoplasmic as well as the nuclear fraction of untreated cells. In most fractions, the expression of p21 was enhanced in nuclei and the cytoplasm of cells treated with camptothecin or TEGDMA. The p21 protein plays a dual role in cell cycle progression by regulating both DNA synthesis and CDK activity, but p21 has also been discussed as an enhancer of cell survival. For example, it was reported that differentiated cells like macrophages contain high amounts of p21, and low levels were associated with apoptosis. The cellular localization of p21 is part of the control of its various functions. While nuclear p21 functions are related to cell cycle progression, the cytoplasmic p21 pool may be involved in the maintenance of cell survival. p21 probably forms a complex with the apoptosis signal-regulating kinase 1 (ASK1) in the cytoplasm to inhibit stress-related activation of MAPK. It has been shown that p21 may protect cells from death after exposure to cytotoxic agents and ionizing radiation [39,40]. Thus, it is possible that the relative high levels of p21 detected in the cytoplasmic fraction in the present study act as a defense mechanism against the cell damage caused by TEGDMA. This finding might be associated with the detection of the cleavage product of lamin A in RAW264.7 cell cultures exposed to TEGDMA or HeLa exposed to camptothecin. Lamin A/C and GAPDH were used as markers to identify the expression of proteins analyzed here in the nuclear and cytoplasmic cell fractions. Lamin cleavage, however, as detected here on some occasions, is also considered to play a role in the nuclear apoptotic process [41].

4.3. TEGDMA-induced activation of transcription factors related to the activation of MAPK

Cellular stress response is mediated through the activation of signal transduction pathways that may also modify the expression of immediate early genes. These genes encode transcription factors which consecutively regulate the activation of downstream genes, finally leading to apoptosis or supporting cell survival. It has been reported that the induction of immediate early genes like c-Jun and c-Fos, and activating transcription factors ATF-2 and ATF-3 was inducible by a wide variety of external signals including serum, growth factors, active phorbol esters, ionizing radiation, pharmacological agents, stress and cytokines. The MAP kinases ERK1/2, the stress-activated protein kinases JNK (c-Jun N-terminal kinase) and p38 may function as second messenger pathways [20]. The transcription factor complex AP-1, which is a homo- or heterodimer composed of Fos/Jun and ATF family members, is activated through phosphorylation of Jun by JNK. The JNK/Jun pathway regulates a vast number of target genes containing AP1-binding sites, including genes that control the cell cycle, as well as survival and apoptosis [20,42]. In the present study, camptothecin increased the expression of the proto-oncoprotein c-Jun in HeLa
cell nuclei while phospho-c-Jun and c-Jun were not noticeably increased by the monomer TEGDMA. This observation is in line with a previous report on camptothecin as an inhibitor of DNA topoisomerase that suggested a link between topoisomerase-mediated DNA damage and intra-cellular signaling events through a JNK pathway in HeLa [43]. In contrast, the expression of c-Jun in tHPC was detected in the nucleic and cytoplasmic fraction, and TEGDMA probably inhibited the phosphorylation of c-Jun. Other investigations demonstrated that phosphorylation of c-Jun through JNK could stabilize the protein and in that way contribute to its transcriptional activity. On the other hand, phosphorylation of c-Jun was also discussed as the signal for degradation through the ubiquitin pathway [20]. We have no plausible hypothesis at present regarding the reduced expression of both c-Jun and phospho-c-Jun in RAW264.7 macrophages exposed to the genotoxic agents camptothecin and TEGDMA. However, it was speculated that the major apoptotic pathway in neurons through JNK-c-Jun may be inhibited by degradation of c-Jun, thereby allowing neurons to tolerate apoptotic levels of JNK activation [20]. Since c-Jun is an ATF-2 target gene, it is also possible that the reduced expression of c-Jun is the consequence of a downregulation of ATF-2 [24].

ATF-2 (activating transcription factor 2) is a member of the basic region leucine zipper (bZIP) family of proteins and part of activating protein-1 (AP-1) [26]. It is phosphorylated through stress-activated protein kinases JNK or p38 in the presence of stressors like pro-inflammatory cytokines, UV irradiation, DNA damage or changes in the level of reactive oxygen species (ROS). Nonetheless, ATF-2 is also activated via the Ras-ERK pathway. Diverse signaling in the activation of ATF-2 is thought to result in a diversity of ATF-2 heterodimeric partners activated in a stimulus-specific manner. Among other functions, ATF-2 is required for cellular stress and DNA damage response including the protection of cells from ionizing irradiation [24]. It appears as if ATF-2 expression solely in cell nuclei was downregulated to some extent in tHPC after exposure to camptothecin and TEGDMA, and clearly in HeLa and RAW264.7 macrophages. As a target of JNK/p38 signaling, phosphorylation of ATF-2 was increased in RAW264.7 macrophages after stimulation with lipopolysaccharide (LPS). Consequently, it was suggested that ATF-2 might be involved in the transcriptional control mediated by Toll-like receptors (TLRs) in mouse macrophages [44]. We have recently shown that LPS-stimulated cytokine production was instantaneously downregulated in the presence of TEGDMA [17]. Since an inhibition of ATF-2 expression was also found in the present study, we speculate that ATF-2 might be involved in TEGDMA-induced inhibition of cytokine production in macrophages. This finding is underscored by the fact that RAW264.7 macrophages responded to LPS by enhanced ATF-2 and ATF-3 expression in the present investigation. These observations appear to be in line with those reported by other investigators as well [44,45].

ATF-3 is another member of the ATF/CREB family of transcription factors among the immediate early genes without any obvious tissue-specific or stimulus-specific activation. However, it has been suggested that ATF-3 may be an ‘adaptive response’ gene responsible for the coordination of cell reactions to extra- and/or intra-cellular changes. To date, the biological role of ATF-3 remains obscure, although it has been reported to be involved with the cell cycle control, apoptosis and the stress response [46]. The activation of ATF-3 in HeLa and tHPC in the nuclear fraction of camptothecintreated HeLa and tHPC cultures suggests that ATF-3 plays a functional role in the stress response, while the consequences of its downregulation in RAW264.7 macrophages remain unclear at present. We are currently pursuing the possibility that TEGDMA might interfere with the suggested role of ATF-3 to regulate TLR-stimulated inflammatory responses [47].

5. Conclusion

The present study provides experimental evidence that TEGDMA interferes with the regulation of cellular pathways through transcription factors activated as a consequence of DNA damage, or initiated downstream from MAPK as suggested in linear signaling models. Differential activation of these transcription factors was detected in cells derived from organs as diverse as the innate immune system or human pulp tissue after a long exposure period. The activation of the tumor suppressor protein p53 by TEGDMA in the various cell lines and its negative effect on the expression of transcription factors linked to MAPK activity was unknown thus far. The time-related induction of apoptosis and the modification of the various transcription factors in the presence of TEGDMA is also a novel finding. Yet, the direct causal correlation between DNA damage, activation or inhibition of MAPKs and transcription factors, and apoptosis is still under current investigation using pharmacological and genetic inhibitors. However, the present study indicates that induction of apoptosis in different cell lines in the presence of monomers like TEGDMA may be subject to a higher level of complexity than currently suggested by simple linear models.

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Appendix

Figures with essential color discrimination. Fig. 1 in this article is difficult to interpret in black and white. The full color images can be found in the on-line version, at doi:10.1016/j.biomaterials.2010.11.031.

References

[2] Santerre JP, Shaji I, Leung BW. Relation of dental composite formulations to DNA damage response mediated by Toll-like receptors (TLRs) in mouse macrophages. [44]. We have recently shown that LPS-stimulated cytokine production in RAW264.7 macrophages exposed to the genotoxic agents camptothecin and TEGDMA. However, it was speculated that the major apoptotic pathway in neurons through JNK-c-Jun may be inhibited by degradation of c-Jun, thereby allowing neurons to tolerate apoptotic levels of JNK activation [20]. Since c-Jun is an ATF-2 target gene, it is also possible that the reduced expression of c-Jun is the consequence of a downregulation of ATF-2 [24].

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