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ORGAN TOXICITA AND MECHANISMS

**Franz-Xaver Reichl · Magali Esters · Sabine Simon
Mario Seiss · Kai Kehe · Norbert Kleinsasser
Matthias Folwaczny · Jürgen Glas · Reinhard Hickel**

Cell death effects of resin-based dental material compounds and mercurials in human gingival fibroblasts

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Abstract In order to test the hypothesis that released dental restorative materials can reach toxic levels in human oral tissues, the cytotoxicities of the resin-based dental (co)monomers hydroxyethylmethacrylate (HEMA), triethyleneglycoldimethacrylate (TEGDMA), urethanedimethacrylate (UDMA), and bisglycidylmethacrylate (BisGMA) compared with methyl mercury chloride (MeHgCl) and the amalgam component mercuric chloride ($HgCl_2$) were investigated on human gingival fibroblasts (HGF) using two different test systems: (1) the modified XTT-test and (2) the modified H 33342 staining assay. The HGF were exposed to various concentrations of the test-substances in all test systems for 24 h. All tested (co)monomers and mercury compounds significantly ($P < 0.05$) decreased the formazan formation in the XTT-test. EC_{50} values in the XTT assay were obtained as half-maximum-effect concentrations from fitted curves. Following EC_{50} values were found (mean [mmol/l]; s.e.m. in parentheses; $n = 12$; * significantly different to HEMA): HEMA 11.530 (0.600); TEGDMA* 3.460 (0.200); UDMA* 0.106 (0.005); BisGMA* 0.087 (0.001); $HgCl_2$* 0.013 (0.001); MeHgCl* 0.005 (0.001). Following relative toxicities were found: HEMA 1; TEGDMA 3; UDMA 109; BisGMA 133; $HgCl_2$ 887; MeHgCl 2306. A significant ($P < 0.05$) increase of the toxicity of (co)monomers and mercurials was found in the XTT-test in the following order: HEMA < TEGDMA < UDMA < BisGMA < $HgCl_2$ < MeHgCl. TEGDMA and MeHgCl induced mainly apoptotic cell death. HEMA, UDMA, BisGMA, and $HgCl_2$ induced mainly necrotic cell death. The results of this study indicate that resin composite components have a lower toxicity than mercury from amalgam in HGF. HEMA, BisGMA, UDMA, and $HgCl_2$ induced mainly necrosis, but it is rather unlikely that eluted substances (solely) can reach concentrations, which might induce necrotic cell death in the human physiological situation, indicating that other (additional) factors may be involved in the induction of tissue (pulp) inflammation effects after dental restauration.

Keywords (Co)monomers · Mercurials · Apoptosis · Necrosis · Human gingival fibroblasts

F.-X. Reichl (✉) · M. Esters · S. Simon · M. Seiss
Walther-Straub-Institute of Pharmacology and Toxicology,
Goethestr 33, 80336 Munich, Germany
E-mail: reichl@lmu.de
Tel.: +49-89-2180-75745
Fax: +49-89-2180-75746

K. Kehe
Bundeswehr Institute of Pharmacology and Toxicology,
Neuherbergstr 11, 80937 Munich, Germany

N. Kleinsasser
Head and Neck Surgery, Department of Otolaryngology,
University of Regensburg, Franz-Joseph-Strauß-Allee 11,
93053 Regensburg, Germany

M. Folwaczny · J. Glas · R. Hickel · F.-X. Reichl
Department of Operative Dentistry and Periodontology,
Ludwig-Maximilians-University, Goethestr. 70,
80336 Munich, Germany

Introduction

Cavities in the primary and in the permanent dentition are restored with tooth-colored materials, especially resin composites and glass ionomer cements, which in part have replaced amalgam (Geurtsen and Schoeler 1997). Resin composites are widely used in dental restorations because of their rapid polymerization, their final strength and adhesion to enamel and dentin. These resins are used as filling materials, dentine adhesives, cements or luting agents for inlays, crowns, veneers, and orthodontic brackets. Previous studies have documented that unpolymerized (co)monomers can be released from resin composites (Lee et al. 1998) into the oral cavity (Ferracane and Condon 1990; Ferracane 1994; Hamid et al. 1998; Mazzaoui et al. 2002), and/or can diffuse through the dentin into the pulp space (Gerzina and Hume 1994; Geurtsen 1998). The most common (co)monomers used are triethyleneglycoldimethacrylate

(TEGDMA), 2-hydroxyethylmethacrylate (HEMA), urethanedimethacrylate (UDMA), and bisglycidylmethacrylate (BisGMA) (Geurtsen 1998; Kaga 2001). Released substances from composites can induce adverse effects (Hanks et al. 1992; Stanley 1993; Gerzina and Hume 1996; Bouillaguet et al. 1998; Soderholm and Mariotti 1999; Sadoh et al. 1999), for example, tissue (pulp) inflammation effects (Stanley 1993; Gerzina and Hume 1996; Bouillaguet et al. 1998). In vivo, necrosis generally sets off a tissue inflammation associated with clinical symptoms because dying cells typically release intracellular substances into the circulating fluids (Majno and Joris 1995).

In the case of amalgam it has been demonstrated that mercury vapour (Hg^0) and mercury ions (Hg^{2+}) have been released from amalgam in measurable quantities and transferred via the oral and/or other tissues into the blood (Halbach 1994; Wild et al. 1997; Malich et al. 1998; Denny and Atchison 1996; Lipfert et al. 1996; Belletti et al. 2002), leading also to tissue inflammation effects (Kehe et al. 2001; Leite et al. 2004).

Scarce information is available about the cell death effects of dental composite components on human oral cells directly compared with mercurials (e.g., from amalgam).

It has been shown that determination of viability by the tetrazolium colorimetric assay (e.g., XTT-assay) is an useful in vitro screening method to compare the cytotoxic potentials of chemical compounds (Bean et al. 1995). The H 33342 staining assay has been performed for histochemical and biochemicals evaluation of cell apoptosis or necrosis.

In order to test the hypothesis that released dental restorative materials can reach toxic levels in human oral tissues and can induce necrotic (inflammation) effects, the cell death effects of (co)monomers directly compared with mercurials were investigated on human gingival cells using the XTT- and the H 33342 staining assays.

Materials and methods

Chemicals

The (co)monomers HEMA, TEGDMA, BisGMA, and UDMA were purchased from Degussa (Düsseldorf, Germany). Triton-X 100, $HgCl_2$, and MeHgCl were obtained from Merck (Darmstadt, Germany).

HEMA and $HgCl_2$ were directly dissolved in the medium. TEGDMA, UDMA, BisGMA, and MeHgCl were dissolved in DMSO and diluted with the medium (final DMSO concentration $<1\%$).

Cell culture

In the human physiological situation gingival fibroblasts are exposed to high concentrations of (co)monomers (eluted from composites) and/or mercury (released from amalgam) from human beings wearing composites and/or amalgam (Wan et al. 2001). Therefore, in this study human gingival fibroblasts (HGF) were used.

Two sets of primary HGFs obtained from different individuals (Oligene, Berlin, Germany, Cat.-No.: 1110412) were cultured from biopsies of the attached gingival of healthy premolar and molar teeth. The HGF were cultured in 75 cm^2 or 175 cm^2 cell culture flasks with culture medium, Quantum 333 with L-glutamate, supplemented with 50 U/ml penicillin and 50 µg/ml streptomycin (all chemicals were obtained from PAA Laboratories GmbH [Cölbe, Germany]). They were grown at 37°C in a humidified atmosphere of 5% CO_2 and the medium was replaced twice weekly until the cells reached a confluence of approximately 70%.

After reaching confluence of 70%, the cells were washed with phosphate buffer saline (PBS), detached from the flasks by a brief treatment with trypsin/EDTA, seeded into 96-well microtiter plate at a density of 20,000 cells/well in 200 µl growth media and incubated for 24 h.

Exposition procedure

After removal of the culture medium, the adherent and confluent cells were treated in the microtiter plate (XTT test) or directly in the flask (H 33342 assay) with various concentrations of HEMA (0.1–30 mM), TEGDMA (0.03–10 mM), BisGMA (0.01–0.3 mM), UDMA (0.01–1 mM), $HgCl_2$ (0.003–1 mM), or MeHgCl (0.001–0.03 mM). Cells were incubated in all test systems for 24 h.

XTT assay

A modified XTT-test was used for the quantification of cells and their viability. The biochemical procedure of this test is based on the activity of mitochondrial enzymes, which are only active in living cells. Metabolic active cells are able to reduce the tetrazolium salt XTT-(sodium 3'[1-phenyl-aminocarbonyl]-3,4-tetrazolium bis[4-methoxy-6-nitro] benzene sulphonic acid hydrate) labeling reagent to an orange colored soluble formazan dye (Scudiero et al. 1988). The dye intensity is analyzed spectrophotometrically at 450 nm (reference wavelength 670 nm) using a microtiter plate reader. The intensity of the dye is directly proportional to the number of metabolic active cells.

20,000 cells/well were seeded from the culture flasks into a 96-well microtiter plate and precultured for 24 h in 200 µl medium before the assay. The proliferating cells were then incubated in the absence or presence of (co)monomers and mercury compounds in various concentrations as described in the section 'Exposition procedure' for 24 h. After incubation for 20 h, a mixture of XTT-labeling reagent and electron coupling reagent was added 4 h before photometrical analysis. Each

experiment was repeated six times for each set of primary gingival cells. For this experiment two control groups were added. Controls received either medium only, or medium + DMSO (with maximum of formazan formation [= 100%]; final DMSO concentration < 1%). Cells treated with 2% Triton-X were used as negative control.

H 33342 staining assay

The modified Hoechst H 33342 staining assay was used for qualitative and quantitative morphological analysis of cell death by fluorescence microscopy.

In general, two main types of cell death must be distinguished, apoptosis and necrosis (Majno and Joris 1995). Necrosis can cause tissue (pulp) inflammation effects (Rathburn et al. 1991; Hanks et al. 1992; Stanley 1993; Bouillaguet et al. 1998).

The fluorescent dye H 33342, a fluorescent DNA-binding, membrane-permeable dye was used to define nuclear chromatin morphology. After exposure of cells to various concentrations of the test substances, and in consideration of the specific morphologic alterations of apoptosis or necrosis (Thompson 1995), cell death was evaluated qualitatively and quantitatively by fluorescence microscopy. Individual cells were visualized at 400-fold magnification in order to distinguish the viable mitotic cells with uniform nuclear pattern, the apoptotic cells with randomly coalesced pattern of chromatin condensation and apoptotic bodies (Savill et al. 1993), and also the necrotic cells, characterized by swelling and organelle spread. For the test, 10^6 cells were first placed in 75 cm^2 tissue flasks and precultured for 1 week to reach confluence. HGFs were then incubated with culture medium containing (co)monomers or the mercurials in various concentrations for 24 h as described in the section 'Exposition procedure'. Two control groups were added. Controls received either medium only, or medium + DMSO (final DMSO concentration < 1%).

After incubation for 24 h, the cells were rinsed with PBS, detached from the flasks with a brief treatment of trypsin and the media, and all detached cells were transferred into falcon tubes and centrifuged at 250*g* for 5 min at 4°C. The cell pellets were resuspended in 1 ml PBS and prepared for the glass slides: 100 µl from the cells were seeded into Eppendorf cups and treated with 100 µl formaldehyde (after dilution with distilled water, dilution factor = 5). About 20 µl of the treated cells were then transferred on a glass slide. Then 100 µl H 33342 (0.3 mg/ml) was added and the slide was incubated at 37°C for further 30 min. The glass slides were washed with distilled water and mounted in 20 µl 'Antifade solution' (Prolong Antifade, Molecular Probes), subsequently covered with a glass cover slip. They were analyzed under a fluorescence microscope. From each glass slide, 100 cells were counted, analyzed for their morphology and classified as apoptotic, necrotic, or viable cells. The apoptotic cells were identified by increased fluorescence of nuclei because of chromatin condensation (Sanwal et al. 2001), and the formation of apoptotic bodies, which are membrane-bound vesicles, containing condensed chromatin (Savill et al. 1993). Necrotic cells, also called "abnormal or ghost" cells (Abend et al. 2000), were swelled and took on the appearance of a flocculent mass or a nuclear ghost, with weakly stained cytoplasm containing three- to sixfold enlarged nuclei (Abend et al. 2000). Cells that showed an uniform nuclear pattern with uniform DNA staining throughout the nuclear interior were counted as viable cells as described by Liu et al. (1998). Apoptotic, necrotic, or viable cells were calculated as percentages of the total cell number. Each experiment was repeated three times.

Statistics

XTT assay

The calculations were performed using Graph Pad Prism 4 (Graph Pad Software Inc., San Diego, USA). Values from exposed cells were calculated as percentage of the 100% controls. The values were plotted on a concentration log-scale and range of the maximum slope was detected. Half maximum-effect substance concentration at the maximum slope was assessed as EC_{50}. The EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves and calculated from repeated experiments (n = 12). Data are presented as means ± s.e.m.

The statistical significance of the differences between the experimental groups was checked using the t-test, corrected according to Bonferroni-Holm (Forst 1985).

H 33342 staining assay

Data are presented as means ± s.e.m; $n = 3$.

Results

Similar results were obtained for both sets of primary HGFs from different individuals for each test system. Similar results were obtained, when control cells received either medium only, or medium + DMSO (data not shown). The formazan formation and the graphs have been made in relation to the control cells, which received medium + DMSO.

XTT assay

All (co)monomers and mercurials induced a dose-dependent loss of viability in HGF (Fig. 1).

The EC_{50} values and the relative toxicities of (co)monomers and mercurials are shown in Table 1.

A significant ($P < 0.05$) increase of the toxicity of (co)monomers and mercurials was found in the following order: HEMA < TEGDMA < UDMA < BisGMA < $HgCl_2$ < MeHgCl.

TEGDMA and MeHgCl induced mainly apoptotic cell death, involving more than 70% of HGF at the highest concentrations of these substances.

Photographs were taken (not shown).

H 33342 assay

All substances induced a dose-dependant increase of apoptotic or necrotic effects (Fig. 2).

HEMA, BisGMA, UDMA, and $HgCl_2$ induced mainly necrosis, involving more than 90% of HGF at the highest concentrations of these substances.

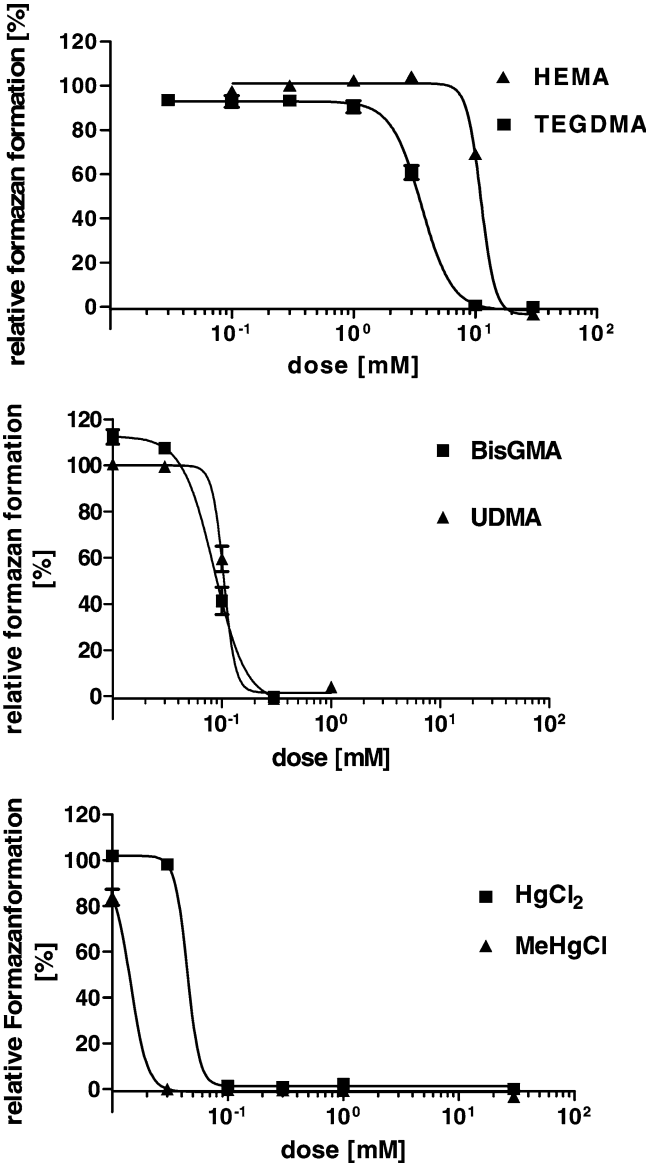

Fig. 1 Effect of HEMA, TEGDMA, BisGMA, UDMA, $HgCl_2$, and MeHgCl on the viability of HGFs. Cell viabilities were assessed by the XTT-test. Curves are drawn from parameters obtained by fitting a sigmoid function to the data points. Data points represent mean values ± s.e.m ($n = 12$)

Discussion

It has been demonstrated that ionized inorganic mercury (released from amalgam fillings) and/or (co)monomers (released from composites) can enter the organism via oral and/or other tissues (Geurtsen et al. 1998; Reichl 2003) and can induce adverse effects, for example, tissue (pulp) inflammation effects (Martin et al. 1997; Kehe et al. 2001; Van Miller et al. 2003).

The aim of this study was to examine the cell death effects of dental composite components directly compared with mercurials. Furthermore, in order to test the hypothesis that released dental restorative materials can reach levels, which can induce cell death effects in human oral tissues, these results were estimated to the human physiological situation (see next).

For a convenient risk assessment of mercury from amalgam, the uptake of mercury from food (~90% MeHgCl, mainly from fish) must be taken into account (Kehe et al. 2001; Mahaffey et al. 2004). Therefore, the organic mercury compound MeHgCl was included in this study.

The XTT test was assessed for measuring the cell viability. Only viable cells with intact mitochondria and sufficient oxygen supply are able to reduce the colorless tetrazolium salt to a formazan dye. The advantage of the XTT assay, compared with other cytotoxic assays is that the measurement of the generated, soluble, colorimetric formazan salt is directly correlated to the viability of tested cells. Therefore, several authors consider this assay preferable to other alternatives (Landbeck and Paulsen 1995; Witte et al. 1995).

Table 1 EC_{50} values and relative toxicities of (co)monomers and mercurials in the XTT-test

Substance	EC_{50}	Relative toxicity
HEMA	11.530 (0.600)	1
TEGDMA	3.460 (0.200)[a]	3
UDMA	0.106 (0.005)[a,b]	109
BisGMA	0.087 (0.001)[a–c]	133
$HgCl_2$	0.013 (0.001)[a–d]	887
MeHgCl	0.005 (0,001)[a–e]	2306

Values were calculated as percentage of the maximum formazan formation of the control HGFs (100%). Values were plotted on a concentration log-scale and the range of maximum slope was detected. EC_{50} values were obtained as half-maximum-effect concentrations from the fitted curves and calculated from repeated experiments ($n = 12$). Data are presented as means (mmol/l) ± s.e.m in parentheses

[a]significantly ($P < 0.05$) different to HEMA
[b]significantly ($P < 0.05$) different to TEGDMA
[c]significantly ($P < 0.05$) different to UDMA
[d]significantly ($P < 0.05$) different to BisGMA
[e]significantly ($P < 0.05$) different to $HgCl_2$

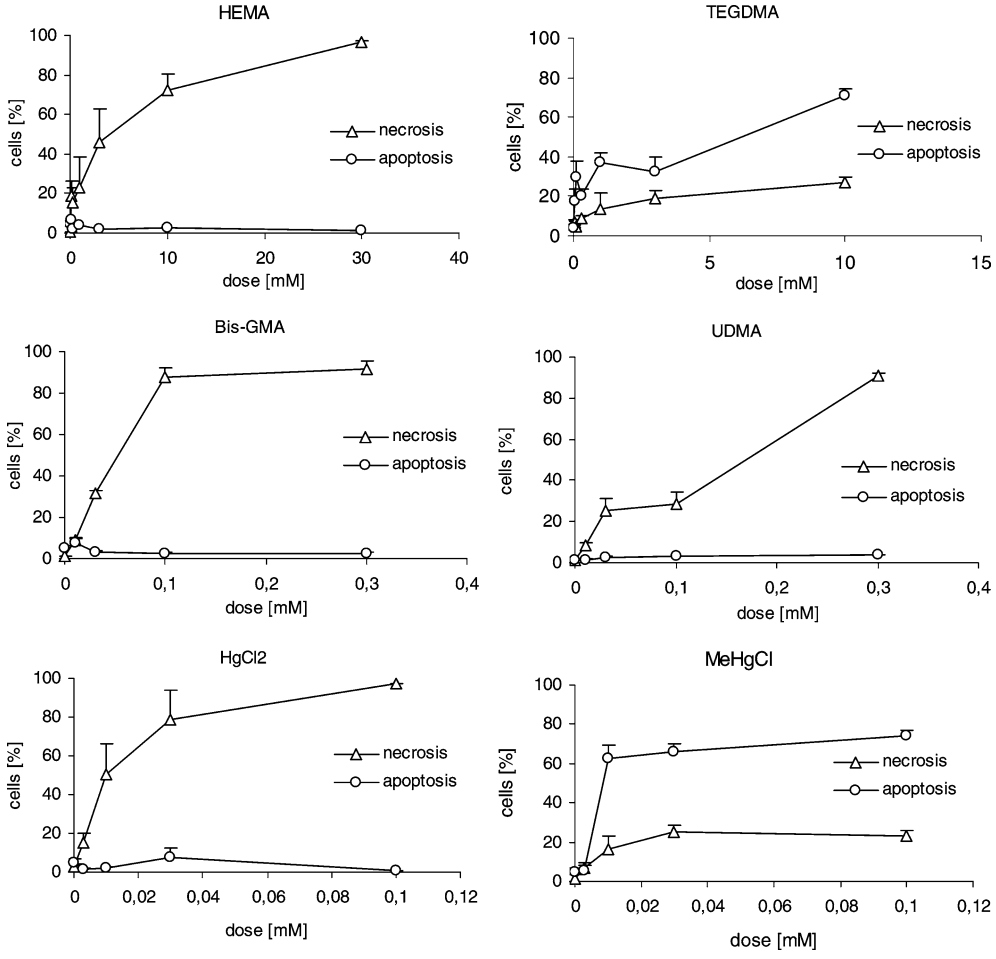

Fig. 2 Effect of HEMA, TEGDMA, BisGMA, UDMA, $HgCl_2$, and MeHgCl on the mode of cell death of HGFs. Nuclear morphology was assessed by the H 33342 assay. Data points represent mean values ± s.e.m. ($n = 3$)

The H 33342 staining assay was chosen to examine the mode of cell death, apoptosis, or necrosis induced by dental materials.

In the XTT assay a dose-dependent decrease in the viability of cells was found for all substances tested in HGF. A higher toxicity of mercury compounds was found compared with the composite components (see Table 1). This may be explained by the interaction of mercury with critical proteins in the cells (Kehe et al. 2001; Zalups et al. 2004). Mercury compounds are capable of inactivating sulfhydryl enzymes even in low concentrations, and therefore, can interfere with cellular metabolism and function as it has been already described by Viarengo and Nicotera (1991) and Boot (1996).

An important reaction hereby is the enzyme catalyzed reaction with reduced glutathione. After binding of metal ions (e.g., Hg^{2+}) to glutathione, inorganic mercury can be detoxificated, leading however, to a decrease of intracellular glutathione level. Furthermore, the cellular energy metabolism can be impaired rapidly and can lead to cell swelling and finally necrotic cell death (Kehe et al. 2001). In the present study necrotic cell death was also found for the amalgam component $HgCl_2$ in HGF.

The organic MeHgCl showed a higher toxicity compared with the inorganic $HgCl_2$ in the XTT-test. These results are in agreement with the results of Sanfeliu et al. (2003), who found that short-chain alkyl mercury compounds are more toxic than inorganic mercury compounds. This can be explained by the higher liposolubility of MeHgCl compared with $HgCl_2$. The higher lipophilic MeHgCl can cross more easily the lipid bilayer of cell membranes leading to a faster cell death than $HgCl_2$ (Lipfert et al. 1996; Belletti et al. 2002).

The increased cytotoxicity of the (co)monomers in the following order (XTT-test): HEMA < TEGDMA < UDMA < BisGMA can be explained by several factors: HEMA and TEGDMA were upto 133-fold less toxic than BisGMA, whereas HEMA was less toxic than TEGDMA. This can be explained by the more hydrophilic character of HEMA, which has an additional hydroxyl group compared with TEGDMA. The higher cytotoxicity of TEGDMA compared with HEMA could further be explained by its ability to interact with the lipid bilayer of cell membranes in a surfactant-like manner (Schuster et al. 1996) and/or by its ability to cause lipid peroxidation in vitro as it was already described by Fujisawa et al. (1984) and by Terakado et al. (1984). Both membrane effects can cause cell death (Raffray and Cohen 1997). It has been demonstrated that the (co)monomer TEGDMA may also significantly interfere with intracellular glutathione (Freidig et al.

2001; Engelmann et al. 2002) and that early and extensive depletion of the intracellular GSH level in tissues may significantly contribute to cell death (Engelmann et al. 2002). Furthermore, the higher cytotoxicity of BisGMA compared with other (co)monomers might depend on its higher liposolubility compared with for example, HEMA (the octanol/water partition of BisGMA is about tenfold higher compared with the octanol/water partition of HEMA) (Issa et al. 2004).

The increased (co)monomer toxicity in the following order: HEMA < TEGDMA < UDMA < BisGMA may also be explained by the increased molecular weights of these (co)monomers (e.g., HEMA = 130 Da, TEGDMA = 286 Da, UDMA = 471 Da, BisGMA = 512 Da). These results are in agreement with the results of Geurtsen et al. (1998), who listed in their paper 35 different components with their respective molecular weights. They found that there is a direct correlation between molecular weight and degree of cytotoxicity of substances. The higher the molecular weight, the higher is the cytotoxicity (Geurtsen et al. 1998).

All substances tested induced a dose-dependent increase of apoptotic or necrotic processes. Within the mercurials $HgCl_2$ induced more cell necrosis (see also the earlier discussion), whereas MeHgCl induced more apoptosis. Within the composite components HEMA, BisGMA, and UDMA induced more necrotic cell death, whereas TEGDMA induced more apoptotic cell death.

Apoptosis is an important active and physiological mechanism of cell depletion characterized by various phenomena (e.g., cell shrinkage) and apoptotic cell death can be caused by many physiological cues (Heil et al. 1996; Mac Lellan and Schneider 1997; Ashkenazi and Dixit 1998). A detrimental injury of the cell, for example, due to a toxic substance, can quickly activate the apoptotic response, which rapidly causes cell death. Necrosis is a pathologic process generated by a massive, lethal injury of the cells. Generally, apoptotic cells are efficiently and rapidly recognized and phagocytosed by macrophages and surrounding cells without the induction of inflammation (Savill et al. 1993) in contrast to necrosis. In vivo, necrosis generally sets off a tissue inflammation associated with clinical symptoms because dying cells typically release intracellular substances into the circulating fluids (Majno and Joris 1995).

In the present study TEGDMA induced mainly apoptosis. These apoptotic processes were observed at concentrations > 3 mM. These results are in agreement with the results of Janke et al. (2003), who found in their study that TEGDMA generated mainly apoptosis in tissues depending on treatment time and concentration. TEGDMA had no significant toxic effect at concentrations lower than 2.5 mM after 24 h exposition, but HGF incubated with 5 mM or 7.5 mM TEGDMA showed an increase of apoptotic cells to ~28 and ~49%, respectively. The apoptotic cell death induced by TEGDMA may be explained by its conjugation with intracellular glutathione because Engelmann et al. (2001) have found that TEGDMA is conjugated to intracellular glutathion leading to decreased glutathione content due to a detoxification process.

Ratanasathien et al. (1995) have determined the biocompatibility of UDMA in mouse odontoblasts and they concluded that UDMA might cause odontoblastic death primarly by necrosis. These results are in agreement with the results in the present study where UDMA also induced mainly necrotic cell death in HGF.

Human pulp and gingival inflammation effects induced by resin composites after tooth restoration were described in some studies (Rathburn et al. 1991; Hanks et al. 1992; Stanley 1993; Bouillaguet et al. 1998). But can (co)monomers and/or mercurials released from dental materials reach concentrations, which might induce necrotic (inflammation) effects in oral tissues in the human physiological situation?

For a complete and convenient risk assessment of xenobiotics for human beings, the results of the xenobiotics from many test batteries in vitro and in vivo are necessary. Nevertheless, reached physiological concentrations (in saliva or blood) of free (co)monomers from composites and/or of mercury released from amalgam from people are much lower than those required to induce toxic effects as shown in this study. In the case of amalgam the release of mercury in the saliva (and in the blood) have been measured (median of people without amalgam fillings < 2.5 nmol Hg/l saliva, < 0.5 nmol Hg/l blood, and people with many [> 12] amalgam fillings < 25 nmol Hg/l saliva, < 10 nmol Hg/l blood) (Ott 1984). These concentrations are far below what is considered to be toxic in man. The physiological mercury concentrations in the saliva (and/or in the blood) in people with many amalgam fillings (including the uptake of organic mercury from food), as described previously, are far below the EC_{50} values of MeHgCl or $HgCl_2$ and far below those values, which induced apoptotic or necrotic effects found in the present study in HGF.

Hamid et al. (1998) have found in vitro (in the experimental human physiological situation) that the total accumulated release of HEMA or TEGDMA into water at 30 days after several resin-based material applications ranged from 0.01 to 1 μmol/l. Gerzina and Hume (1996) and Spahl et al. (1998) have found that the release of the lipophilic BisGMA from composites into water is much lower compared with the release of the hydrophilic HEMA. In the present study BisGMA was the most toxic composite component. The minimal EC_{50} value of BisGMA was about 100-fold higher, and for HEMA even about 11.000-fold higher, compared with the total accumulated release of HEMA described in the study by Hamid et al. (1998). Therefore, the results in the present study indicate that even with the most toxic BisGMA (and consequently with the other tested [co]monomers released from composites), for people wearing composites and/or amalgam (in the physiological situation), it is rather unlikely that eluted (co)monomers and mercurials can reach concentrations, which might induce necrotic or apoptotic cell death in HGF. However, it is noted that necrotic and tissue

(pulp) inflammation effects have been described after tooth restorations with amalgam and/or composites (Stanley 1993; Majno and Joris 1995; Bouillaguet et al. 1998). Therefore, the results in the present study indicate that other (additional) factors (e.g., synergistic effects, bacteria) may be involved in the induction of the mentioned pulp inflammation effects after tooth restoration.

Conclusion

The results of this study indicate that resin composite components have a lower toxicity than mercury from amalgam. HEMA, BisGMA, UDMA, and $HgCl_2$ induced mainly necrosis, but it is rather unlikely that eluted substances can reach concentrations, which might induce necrotic cell death in the physiological situation, indicating that other (additional) factors may be involved in the induction of those tissue (pulp) inflammation effects.

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