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Critical role of superoxide anions and hydroxyl radicals in HEMA-induced apoptosis

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ABSTRACT

Objective. Resin monomers such as 2-hydroxyethyl methacrylate (HEMA) induce apoptosis because of the excess formation of reactive oxygen species (ROS). The portion of ROS including superoxide anions, hydrogen peroxide or hydroxyl radicals in monomer-induced apoptosis is unknown. Here, the effectiveness of superoxide anions or hydroxyl radicals was analyzed using tempol or sodium formate as radical scavengers.

Methods. RAW264.7 mouse macrophages were exposed to HEMA (0–6–8 mM) in the presence of tempol (0–0.05–0.5–5.0 mM) or sodium formate (0–1–5–10 mM). The formation of ROS using $DCFH_2$-DA or dihydrorhodamine 123 (DHR123) as fluorescent dyes and the induction of apoptosis was determined by flow cytometry after 1 h or 24 h exposure periods. Expression of enzymes related to ROS metabolism was detected by Western blotting.

Results. DCF fluorescence significantly increased after short exposure (1 h) while DHR123 fluorescence was enhanced after a long exposure period (24 h) in cells treated with HEMA. Although no influence was detected on the formation of ROS, tempol or sodium formate protected cells from HEMA-induced apoptosis. The number of cells in late apoptosis or necrosis induced with 6 or 8 mM HEMA was reduced in the presence of tempol or low concentrations of sodium formate. HEMA-induced expression of catalase, indicating oxidative stress, decreased in the presence of tempol.

Significance. Superoxide anions and hydroxyl radicals contribute to HEMA-induced apoptosis. The current findings support the development of strategies based on the pharmacological inhibition of enzymes producing superoxide anions finally converted to hydroxyl radicals to compensate for potential adverse tissue reactions associated with dental composites.

1. Introduction

Bioactive resin monomers like 2-hydroxyethyl methacrylate (HEMA) are released from non-polymerized or incompletely polymerized dental composite materials used to direct the course of dental therapy. These compounds may interfer with oral tissues or living pulp tissues after diffusion through dentin in a particular clinical situation in freshly cut deep dentinal cavity preparations [1,2]. Inflammation, disturbance of odontoblast function, or confined degeneration of pulp mesenchymal tissues may occur as responses towards unpolymerized dentin adhesives or composite filling materials depending on the remaining dentin thicknesses [3–5]. In addition, cell-specific responses have been reported

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after exposure of cell cultures to physiologically relevant levels of dental resin monomers. Matrix mineralizing capabilities or expression of gene products essential for tertiary dentin formation were disturbed in odontoblasts, and resin monomers also inhibited essential functions of cells of the innate immune system, such as the release of cytokines in cells stimulated with lipopolysaccharide (LPS) [6–9]. Moreover, resin monomers may cause oxidative DNA damage followed by programmed cell death or apoptosis [10–12].

This negative interference on vital functions observed in a variety of eukaryotic cells is associated with monomer-induced oxidative stress due to the formation of reactive oxygen species (ROS) beyond the capacities of the cellular redox homeostasis [1]. ROS, such as superoxide anions ($O_2^{\bullet -}$), may act as oxidizing agents per se, or convert into hydrogen peroxide (H_2O_2) and hydroxyl radicals ($HO^{\bullet}$) [13,14]. Although experimental evidence suggests that adaptive cell responses like the induction of apoptosis are causally related to the formation of hydrogen peroxide, the contribution of other ROS like superoxide anions or hydroxyl radicals is still unclear [15]. We hypothesize that identification of the function of superoxide anions and hydroxyl radicals in monomer-induced apoptosis will influence the development of therapeutic strategies to control adaptive cell responses associated with oxidative stress in tissues exposed to monomers in a clinical situation. For this purpose, we used the nitroxide tempol as a radical scavenger for trapping superoxide anions. Tempol has been described as a superoxide dismutase mimetic molecule because of its reaction with superoxide anions [16–19]. In addition, sodium formate was used as a scavenger of hydroxyl radicals. These reactive oxygen molecules are mostly generated from hydrogen peroxide by the Fenton reaction, and may cause oxidative damage in cellular macromolecules like proteins or DNA leading to apoptosis [20,21]. With respect to the analyses of apoptosis, we also considered the possible influence of tempol and sodium formate on the expression of enzymatic antioxidants and the formation of oxidative stress. Yet, it seems that the formation of ROS in cells exposed to resin monomers is not adequately depicted by the use of a single fluorescent dye currently available [22]. Since dihydroethidium (DHE) tested ineffective as a probe for the detection of superoxide anions in monomer-exposed cells in our previous investigation, $DCFH_2$-DA (2′-7′-dichlorodihydrofluorescein diacetate) was used to indicate general oxidative stress, and dihydrorhodamine 123 (DHR123) should allow for the analysis of hydrogen peroxide (H_2O_2) formation [14,23–25]. RAW264.7 mouse macrophages were used here as a suitable model cell line of the innate immune system as proven effective in numerous previous studies [6,9,25,26].

2. Materials and methods

2.1. Chemicals and reagents

2-Hydroxyethyl methacrylate (HEMA; CAS-No. 868-779) was obtained from Merck (Darmstadt, Germany). Dihydrorhodamin123 (DHR123; CAS-No. 109244-58-8), phorbol myristate acetate (PMA; CAS-No. 16561-29-8), and a bicinchoninic acid (BCA) assay kit came from Sigma (Taufkirchen, Germany). RPMI 1640 medium containing L-glutamine and 2.0 g/l $NaHCO_3$ was from PAN Biotech (Aidenbach, Germany). Fetal bovine serum (FBS), penicillin/streptomycin, and phosphate-buffered saline supplemented with 5 mM EDTA (PBS-EDTA) were purchased from Life Technologies, Gibco BRL (Eggenstein, Germany). The FACS Annexin V-FITC apoptosis detection kit was obtained from R&D Systems (Minneapolis, MN, USA). 2′7′-Dichlorodihydrofluorescin diacetate (H_2DCF-DA; CAS-No. 4091-99-0) was purchased from MoBiTec (Göttingen, Germany).

Anti-catalase (H-300, sc-50508), and anti-Cu-Zn superoxide dismutase (SOD-1, sc-271014), anti-glutathione peroxidase 1/2 (GPx1/2, sc-133152) and anti-heme oxygenase 1 (HO-1, sc-1797), antibodies came from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit IgG HRP-linked antibodies were obtained from Cell Signaling (NEB Frankfurt, Germany). Goat anti-mouse IgG (H + L)-HRP conjugate came from Bio-Rad Laboratories (Munich, Germany), and Amersham hyperfilm ECL was obtained from GE Healthcare (Munich, Germany). The protease inhibitor cocktail (complete mini) was purchased from Roche Diagnostics (Mannheim, Germany), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody (clone 6C5) came from Millipore (Schwalbach, Germany). All other chemicals used in the present study were at least chemical grade.

2.2. Intracellular detection of reactive oxygen species (ROS) by flow cytometry

RAW264.7 mouse macrophages (ATCC TIB71) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. For the analysis of ROS formation, cells were cultured in 6-well plates (5×10^4/well) for 48 h at 37 °C as described in Ref. [25]. Briefly, the cells were preincubated in 2 ml cell culture medium supplemented with tempol (0–0.05–0.5–5.0 mM) or sodium formate (0–1–5–10 mM) for 3 h, and then exposed to HEMA (0–8 mM) or phorbol myristate acetate (0.1 μM PMA) for 1 h or 24 h in the presence or absence of tempol or sodium formate. Concentrations of tempol or sodium formate were optimized in preliminary experiments (not shown). PMA was used as a positive control substance to show induction of reactive oxygen species [27]. Exposure periods for all experiments done to analyze ROS production were selected following the positive analysis of ROS in monomer-exposed RAW264.7 mouse macrophages observed in our recent investigation [25]. Cells were incubated in culture medium with 10 μM H_2DCF-DA or 5 μM DHR123 prior to harvesting in PBS/5 mM EDTA. Finally, DCF fluorescence intensity was measured by flow cytometry (FACSCanto, Becton Dickinson, San Jose, CA, USA) at an excitation wavelength of 488 nm and an emission wavelength of 519 nm (Fl-1). DHR123 fluorescence was analyzed at an excitation wavelength of 488 and an emission wavelength of 578 nm (Fl-2, DHR123). Mean fluorescence intensities were obtained by histogram statistics (FACSDiva™ 5.0.2 software). Individual values of fluorescence intensities were normalized to fluorescence detected in untreated control cultures (0 mM HEMA = 1.0) as described in Ref. [25].