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ORIGINAL ARTICLE

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The coinitiator DMABEE induces death by apoptosis and necrosis in human monoblastoid cells

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Abstract 4-*N*,*N*-Dimethyl amino benzoic acid ethylester (DMABEE), a leachable lipophilic component of polymer-based dental-filling materials, has been shown to interact with cell membrane phospholipids, such as phosphatidylcholine and phosphatidylserine (PS). One marker of cellular death by apoptosis is the change in architecture of the plasma membrane involving the translocation of the negatively charged PS from the inner to the outer leaflet of the cell membrane. We therefore hypothesized that DMABEE has the potential to induce apoptosis. The necrosis inducing potential was also investigated. To test our hypothesis human monoblastoid U-937 cells were exposed to 10, 20, 40, 80, 120, 160, and 200 μM of DMABEE for 24, 48, and 72 h. At the culture end-points apoptotic and necrotic cells were detected by flow cytometry. DMABEE enhanced cell death by apoptosis and necrosis in U-937 cells in a dose-dependent fashion. The data support our hypothesis that DMABEE triggers death-signaling pathways.

Keywords Dental filling · DMABEE · Cell death · Apoptosis · Necrosis

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Introduction

Polymer-based dental filling (PBDF) materials are widely used in contemporary general dental practices [1]. They consist of synthetic polymers, ceramic fillers, and molecules, which promote or modify their polymerization reaction. 4-*N*,*N*-Dimethyl amino benzoic acid ethylester (DMABEE) is a coinitiator in light-cured PBDF materials, and pit and fissure sealants. The compound is, among monomers, additives, contaminants, and degradation-products, eluted from PBDF materials [9]. DMABEE has been reported to have a moderate cytotoxic effect [7]. The log P_{ow} has been reported to be 3.75 [9], indicating a rather strong lipophilic compound. Lipophilic compounds may accumulate in cell membranes [16] and are likely to disrupt membrane integrity [17]. DMABEE has been found to interact with monolayers of saturated phosphatidylcolines (PC, i.e., markers of the outer membrane leaflet) and phosphatidylserines (PS., i.e., markers of the inner membrane leaflet) [10]. These observations were confirmed by studies on liposomes of PC and PS [10].

During apoptosis the architecture of the plasma membrane changes involving the redistribution of the various phospholipid species between the two leaflets of the membrane [20]. One early marker of cellular death by apoptosis is the translocation of the negatively charged PS from the inner to the outer leaflet of the cell membrane

[11]. PS exposure seems to be a universal phenomenon of apoptosis occurring in most if not all cell types independent of the initiating trigger [20]. Previous studies have shown that polymer-based dental materials induce apoptosis and necrosis [3, 4]. It was therefore of interest to investigate the potential of DMABEE to induce apoptosis and/or necrosis.

Apoptosis is a highly regulated physiological process characterized by shrinkage, condensation of nuclear chromatin followed by nuclear fragmentation, and preservation of the structural integrity and most of the functions of the cellular organelles and of the plasma membrane [19]. In vivo apoptotic cells are rapidly phagocytized without triggering an inflammatory reaction. In vitro in the absence of professional phagocytes apoptotic cells reach the stage of secondary necrosis during which they swell, the integrity of the plasma membrane is lost, and finally cell lysis occurs. A reliable method for quantification of apoptosis is based on the specific binding of annexin V to the externalized PS [20]. Necrosis is a passive process characterized by mitochondrial swelling, dissolution of the nucleus, and rupture of the plasma membrane. Necrosis occurs as a result of exposure to gross injury and triggers an inflammatory reaction in the tissue [19]. The usefulness of the consideration of the mode of cell death in the assessment of biocompatibility is twofold: a better understanding of pathomechanisms and the development of strategies for targeted intervention.

The aim of this study was to test our hypothesis that DMABEE can induce cellular death by apoptosis and/or necrosis.

Material and methods

Chemicals

The study employed DMABEE (CAS#10287-53-3) purchased from Merck-Schuchardt (Hochenbrunn, Germany) and acetone purchased from Reinheit-Merck (Darmstadt, Germany).

Cell culture

U-937 human histiocytic lymphoma promonocytic cells (CRL-1593.2, American Type Culture Collection, Manassas, Va., USA) were cultured in suspension in 25 cm^2 Costar polystyrene flasks (Cambridge, Mass., USA) in RPMI-1640 medium (BioWhittaker, Walkersville, Md., USA) supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum, 1% L-glutamine, and 2% mix of penicilline/streptomycine/fungisone (BioWhittaker, Verviers, Belgium) and kept in a fully humidified atmosphere of 5% CO_2 and 95% air at 37°C. The density of the cells was maintained between 5×10^5 cells/ml and 1×10^6 cells/ml. The doubling time of the cells at these densities was 22–24 h. The cells were routinely subcultured every 2–3 days.

Exposure of cells to DMABEE

U-937 cells with viability higher than 95% (tested by exclusion of 0.2% trypan blue) were cultured at a density of 5×10^5/ml in 24-well Costar plates in RPMI-1640 enriched with the supplements mentioned above. The cells were exposed to DMABEE for 24, 48, and 72 h. The following concentrations of DMABEE were added to the cells: 10, 20, 40, 80, 120, 160, and 200 μM. Acetone served as solvent for DMABEE. The following concentrations of acetone were used in the samples (v/v): 0.1%, 0.2%, 0.4%, 0.8%, 1.2%, 1.6% and 3.2% respectively. For control U-937 cells were also exposed to volumes of acetone equal to those used for dissolving DMABEE. Cells treated with 100 UI/m human recombinant tumor necrosis factor α (R&D Systems, Abingdon, UK) and 1 μg/ml cycloheximide (Sigma Aldrich, Oslo, Norway) served as positive controls [6]. U-937 cells exposed only to RPMI-1640 plus supplements provided the negative control.

Annexin V–FITC/propidium iodide assay

The assay used has been described in detail elsewhere [3]. Redistribution of PS to the outer leaflet of the plasma membrane was detected by incubating cells with fluorescein isothiocyanate (FITC) conjugated annexin V (Apoptest V-FITC Kit A-700, NeXins Research, Kattendijke, The Netherlands). Cells that lost the integrity of the plasma membrane (i.e., necrotic and secondary necrotic) were detected by propidium iodide (PI). At the culture endpoints cells were stained with annexin V–FITC and PI according to the manufacturer's instructions. During the assay the cells were kept on ice to arrest further progress through the stages of life towards death. Stained cells were analyzed by a Coulter Epics XL-MCL (Coulter, Harpender, UK) flow cytometer, equipped with a single argon ion laser. Excitation was at 488 nm. Green (FITC) fluorescence was collected between 505 and 545 nm and the red (PI) fluorescence between 605 and 635 nm. According to the manufacturer's instructions, at least 2,500 cells were analyzed per sample. Since the assay was performed on live cells, it was very important to do the analysis in a shorter amount of time and thus, a lower then usual number of cells was run through the flow cytometer.

Quadrant settings were based on the negative control. Each experiment was run in duplicate and repeated at least three times. Data analysis was carried out with the Coulter Epics XL software version 1.5 and with WinMDI 2.7 [18]. To avoid ambiguities regarding the mode of cell death cells were also viewed by light microscopy.

Statistical analysis

Statistical analysis of data obtained by flow cytometry was performed using the one-way analysis of variance test considering P values less than 5% as significant. All statistical analyses were carried out with SPSS 11.0 [12].

Results

Quadrants were set to define the maximum fluorescence intensity for background staining by annexin V–FITC and PI. Test samples with higher levels of staining were considered positive. Typical dot plots of green fluorescence (annexin V–FITC) vs. red fluorescence (PI) are shown in Fig. 1. Three separate clusters are displayed: viable cells contained neither stain (lower left quadrant), apoptotic cells with intact plasma membrane integrity were stained only by annexin V–FITC (lower right quadrant), whereas cells in secondary necrosis (i.e., the phase consecutive to apoptosis in vitro) and necrotic cells contained both stains (upper right quadrant).

Figure 2 gives an overview of the levels of apoptosis (A–C) and necrosis (D–F) at 24, 48, and 72 h. We found that the percentage of dead cells increased with the concentration of DMABEE. DMABEE induced statistically significant higher levels of cell death ($P<0.05$) than the control represented by corresponding volumes of solvent (i.e., acetone) except for the following: apoptosis at 10 μM (24, 48 h) and 40 μM (72 h) and necrosis at 10 μM (72 h), 20 μM (24 h), and 40 μM (24, 48, 72 h). When compared to cells grown in RPMI, DMABEE enhanced necrosis and apoptosis ($P<0.05$) at 80, 120, 160, and 200 μM at all time points. For the higher concentrations of DMABEE (120, 160, and 200) necrosis surpassed apoptosis. There was a decrease in the level of apoptosis and an increase in the level of necrosis at 72 h compared to the previous time points. There was no statistically significant difference between the percentages of apoptosis and necrosis induced by the different volumes of acetone.

Examination by light microscopy confirmed the results obtained by flow cytometry (results not shown). U937 apoptotic cells displayed typical morphological signs of apoptosis such as decreased cell volume, more regular shape of the nuclei, and chromatin condensation. Necrotic cells were recognized by cell swelling, decondensation of chromatin, and disruption of cell membranes.

Discussion

Recent reports have shown that PBDF dental materials activate death signaling pathways. For example, resin-based dental materials polymerized by using blue light caused cell death by apoptosis and necrosis in rat submandibular acinar cells [14] and in human endothelial cells [13]. The resin monomer BisGMA and the comonomer TEGDMA have been confirmed to augment apoptosis in a dose-and time-dependent manner in gingival human fibroblasts [5, 8]. Still, more needs to be known about the mechanisms by wich PBDF materials and their degradation products activate cell death.

The present findings demonstrate that a coinitiator that elutes from light-cured PBDF materials (i.e., DMABEE) augments cell death by apoptosis and necrosis in U-937 cells in a concentration-dependent manner. These findings are in accord with a recent report by Lygre et al. [10], which showed that DMABEE interacted with membrane phospholipids (i.e., PS and PC). Changes in the phospholipid composition may result in altered membrane fluidity and metabolite uptake and changes in the receptors. Ultimately cell death may occur. During the early stages of apoptosis

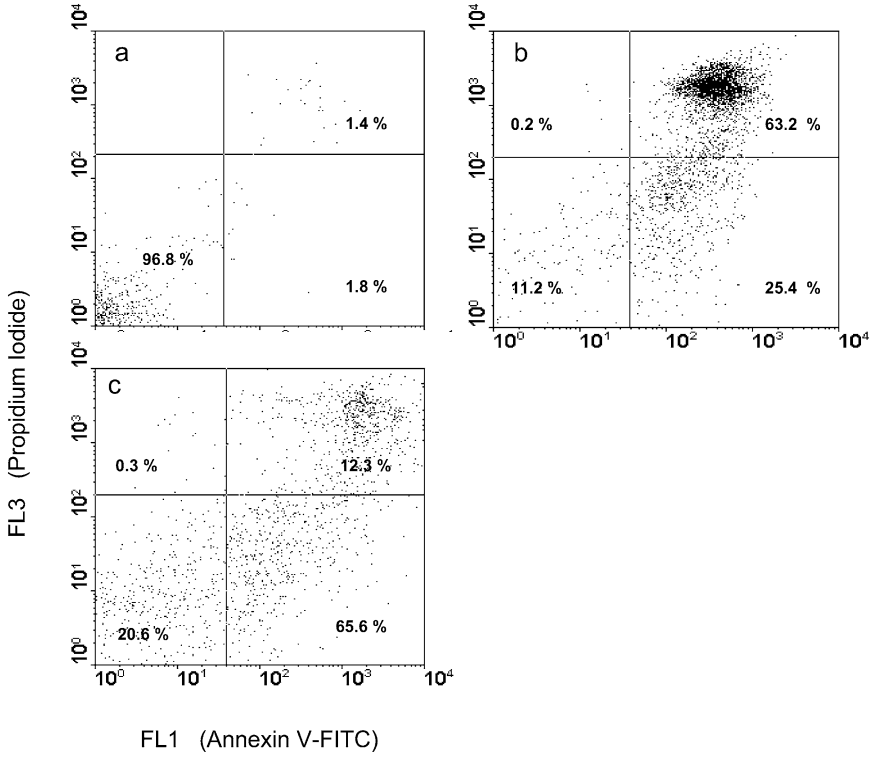

Fig. 1 Dotplots of annexin V–FITC/PI staining of U-937 cells. Three phenotypes can be observed: viable (*lower left quadrant*), apoptotic (*lower right quadrant*), and necrotic and/or secondary necrotic (*upper right quadrant*). **a** Untreated cells. **b** Cells exposed to 160 μM DMABEE for 72 h. **c** Cells exposed to 100 UI/ml tumor necrosis factor α and 1 μg/ml cycloheximide

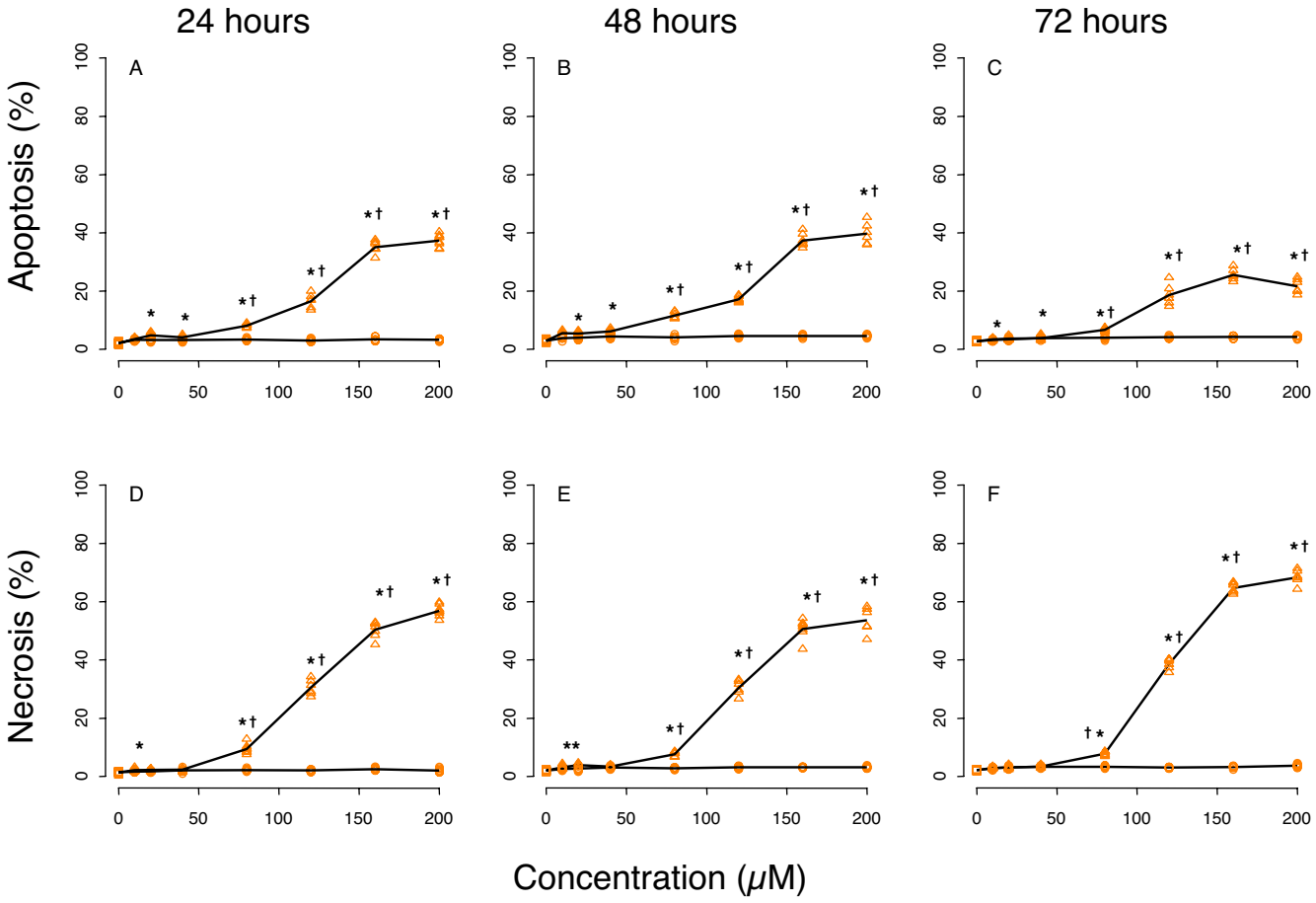

Fig. 2 Percentages of apoptosis (**A**–**C**) and necrosis (**D**–**F**) induced by 10, 20, 40, 80, 120, 160, and 200 μM DMABEE (*triangles*) and by volumes of acetone equal to those used as solvent for DMABEE (*circles*). The point of origin (*square*) represents the negative control (untreated cells). Each experiment was run in duplicate at least three times and each symbol (i.e., *triangles* and *circles*) represents one observation. $P<0.05$ *DMABEE vs. acetone, $P<0.05$ †DMABEE vs. RPMI, one-way analysis of variance

loss of phospholipid asymmetry occurs, which results in the translocation of the negatively charged aminophospholipid PS from the inner to the outer leaflet of the cell membrane [11]. The flow cytometry assay employed in this study for identifying apoptotic cells used this modification as a marker. Annexin V binds, in the presence of calcium ions, specifically to PS [20]. It is essential to determine whether cell death is apoptotic or necrotic because the biological significance of the two processes is different. Tissues more likely adapt when a biomaterial induces apoptosis since this results in little or no inflammatory consequence. If the biomaterial induces mainly necrosis, the subsequent inflammatory phenomena can result in severe tissue reactions.

Applied concentrations of DMABEE were chosen in accordance with previous findings [9]. DMABEE augmented necrosis more than apoptosis at 120, 160, and 200 μM, indicating that at higher concentrations DMABEE is more likely to induce strong inflammatory tissue reactions. However, it is most probable that the concentrations needed for such strong reactions are not reached in vivo. The level of necrosis also increased with the duration of cell exposure to DMABEE. However, for apoptosis this trend was opposite. This was to be expected as in vitro in the absence of phagocytes apoptotic cells undergo post-mortem changes similar to necrosis [19]. Hence the number of necrotic cells increases with time whereas that of apoptotic cells decreases.

We chose the permanent U-937 cell line in our study because of its human origin and ability to perform many of the monocytes' functions. Permanent cell lines are recommended for toxicity screening as a first approach because of their metabolic and genetic stability and good reproducibility [15]. U-937 cells are of monocytic lineage and thus alterations in their functions caused by DMABEE may mimic an altered local immune response. Moreover, this cell line was proposed as a good candidate for in vitro evaluation of biocompatibility [2].

Our investigation provides evidence and validates our hypothesis that DMABEE can elicit cellular death by apoptosis and necrosis. In general the deleterious effects became stronger in a dose- and time-dependent fashion. Further in vitro and in vivo studies are required to decipher the mechanisms leading to apoptosis and necrosis and to determine the clinical relevance of our findings.

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