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Dequalinium induces cell death in human leukemia cells by early mitochondrial alterations which enhance ROS production

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Abstract

Dequalinium (DQA) has been proposed as a selective antitumoral agent due to its preferential accumulation in mitochondria of cancer cells. Our aim was a better understanding of DQA cytotoxicity. DQA-induced NB4 and K562 cell alterations are initiated within the first 30 min of treatment at a high DQA concentration with a mitochondrial membrane depolarization. Cytochrome *c* release to cytoplasm, superoxide anion overproduction and ATP depletion in NB4 cells induce, 16 h later, apoptosis by a typical caspase-9/caspase-3-dependent intrinsic pathway. K562 cells were more resistant to the DQA effect than NB4 cells, remaining viable for longer time periods.

Keywords: Dequalinium; Antitumoral; Leukemia; Apoptosis; Necrosis; Mitochondria; Reactive oxygen species

1. Introduction

Mitochondria have raised a large interest due to their implication in many pathways related to both cell life and death by integrating pro- and antiapoptotic stimuli [1,2]. The majority of conventional anticancer drugs exert their cytotoxic action on mitochondria indirectly via different signalling pathways. A direct targeting on mitochondrial function can be of therapeutic relevance to trigger cancer cell death [3–7]. In addition to the essential role in apoptosis, mitochondria play a critical role in redox metabolism and ATP synthesis. One important event is the production of superoxide during respiration, which can be converted to other reactive oxygen species (ROS) potentially toxic to the cells. Since evidence indicates that cancer cells exhibit increased intrinsic oxidative stress, new therapeutic strategies that take advantage of increased ROS in cancer cells to enhance therapeutic activity and selectivity have been proposed [8].

There are different approaches to target small molecules to mitochondria [9]. Delocalized lipophylic cations (DLCs) comprise a potential new class of antitumor small membrane-permeable agents which accumulate in mitochondria, driven by the negative electric potential across the mitochondrial membrane. They have proved to be selectively more toxic in tumor than in normal cells [10,11]. This behaviour provides an attractive basis for the use of DLCs in selective tumor cell eradication. Among the wide variety of DLCs, dequalinium (DQA), initially used as an anti-microbial agent, has been reported to display a potent in vitro and in vivo antitumor activity in cells from different malignancies by a mechanism that is not yet well-understood [12–15]. Several studies relate the antitumor DQA effect to an impairment of mitochondrial function [16–19].

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Our aim is to analyse the mechanism by which DQA exerts its effects on two human leukemia cell lines: NB4, derived from acute promyelocytic leukemia [20], and K562, derived from chronic myeloid leukemia in blastic crisis. K562 cells are known to express the fusion protein Bcr-Abl that provides continuous cell survival signalling [21]. Previous studies from our group have demonstrated that DQA displays differential cytotoxicity in both cell lines. NB4 cell death occurs mainly through apoptosis in response to relatively low DQA concentrations and involves the activation of caspase-3, whereas high DQA concentrations induce necrotic cell death. The K562 cells were resistant to apoptosis and died by necrosis after DQA treatment. DQA-induced mitochondrial alterations including loss of mitochondrial transmembrane potential, $O_2^{\bullet -}$ overproduction and ATP depletion, as notable intracellular events, have been observed in both NB4 and K562 cells following long DQA treatments. The present study was aimed at understanding the earliest mitochondrial DQA effect that leads NB4 and K562 cells to death. A better knowledge of the DQA effects would allow an improvement of DQA application for therapeutic purpose.

2. Materials and methods

2.1. Preparation of DQA stock solution

The preparation of DQA stock solutions has been detailed previously [12,22,23]. Briefly, a 10 mM dequalinium chloride (Sigma Chemical Co., St. Louis, MO, MW 527.6) stock solution was prepared by dissolving an adequate amount of DQA in methanol in a round bottom flask. The organic solvent was removed with a rotary evaporator. The DQA-film obtained was resuspended in NaCl-free 5 mM HEPES, pH 7.4 and sonicated for 1 h. Finally, the sample was centrifuged ($1000 \times g$, 5 min) to remove metal particles from the probe as well as larger DQA aggregates. This procedure yielded an opaque solution of liposome-like DQA vesicles, which was then filtered using a 0.2 μM filter. The DQA concentration was determined by fluorimetry (Perkin-Elmer LS-50 B Spectrofluorimeter, excitation $\lambda = 335$ nm, emission $\lambda = 360$ nm). The DQA standard curve was found to be linear between 0.001 and 0.01 mM DQA ($r^2 = 0.998$). Dilutions of this stock solution to a final concentration of 0.5–20 μM DQA were used for cell incubations.

2.2. Cell cultures

NB4 and K562 leukemia cell lines were obtained from the American Type Culture Collection. Cells were grown in RPMI 1640 medium (Gibco-Life Technologies, Scotland, UK) supplemented with 8% heat-inactivated foetal calf serum (FCS, Gibco-Life Technologies) and gentamycin (80 μg/ml). Cells were seeded at a density of 2 to 3×10^5 cells/ml. Cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

2.3. Viability assays

Exponentially growing NB4 or K562 cell cultures (2 to 3×10^5 cells/ml) were treated with increasing (0.5–20 μM) DQA concentrations for the indicated time periods (1–16 h). Metabolic activity of the cells was assessed with an MTT kit (Roche Mannheim, Germany) to detect mitochondrial dehydrogenase activity. Viable cells, with functional mitochondria, were able to reduce the tetrazolium ring to a blue formazan product whereas dead cells remained uncoloured. The IC_{50} is defined as the drug concentration that induced a 50% loss of metabolic activity.

2.4. Necrotic or apoptotic cell death evaluation

The characteristic feature of the necrotic process, such as the loss of cell membrane integrity, was analysed by propidium iodide (PI) free influx in cells [24]. Cells were incubated with 50 μg/ml of PI and the emitted fluorescence was analyzed by flow cytometry in a FACScan (Becton Dickinson, San Jose, CA) with an FL2 detector (620 nm band pass filter). Under these conditions, necrotic cells are brightly stained by PI and appear as a peak at very high fluorescence values.

The characteristic loss of DNA in the apoptotic process was analyzed by flow cytometry of permeabilized PI-stained cells [24]. Samples containing 3 to 5×10^5 cells were incubated with 0.5 mg/ml of RNase A for 30 min. Cells were then permeabilized with 0.1% nonidet P-40 and incubated with 50 μg/ml of PI. DNA content analysis was carried out by flow cytometry (FL-2 detector in a linear mode) using the WinMDI and the cylchred programs. Apoptotic cells are identified as a hypodiploid peak (sub-G_0/G_1 content) whereas non-apoptotic, primary necrotic, cells are generally found among the healthy ones.

To study phosphatidyl serine (PS) exposure on the cell surface, cells were double-stained with annexin V-FITC and PI (Annexin V-FITC Apoptosis Detection Kit, Calbiochem, US) and analyzed by flow cytometry (FACScan, Becton Dickinson, San José, CA).

2.5. Caspase activities

Caspase-8 and -9 activities were determined with the corresponding colorimetric commercial assay kit (Calbiochem, USA and Canada). Caspase-3 activity was determined in vitro by measuring the capacity of cell extracts to cleave the caspase-3-specific substrate Ac-DEVD-pNA [22]. Samples containing 10×10^6 cells were washed in PBS and resuspended in 100 μl of ice-cold lysis buffer (50 mM Tris–HCl, pH 7.5; 0.03% (v/v) nonidet P-40, 1 mM dithiothreitol) for 30 min at 4 °C. Cell extracts were centrifuged ($13{,}000 \times g$ for 15 min at 4 °C) and aliquots of the supernatants (20 μg of proteins) were incubated for 30 min at 37 °C with 200 μM of Ac-DEVD-pNA (Calbiochem, Germany) in a final volume of 200 μl. Enzymatic caspase-3 activity was calculated from the increase of absorbance at 405 nm during 2 h. The caspase