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The intracellular antioxidant balance of HL-60 cells and its implication in the apoptosis induced by quinoidal compounds

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The quinoidal structure is the most widespread chemical structure in nature. Many of recently used anthracyclic neoplastic compounds have a quinoidal structure, which is a good substrate for the enzymes reducing them in one electron way with subsequent production of ROS (reactive oxygen species). It has been suggested that ROS may act as a second messenger regulating the activity of redox-sensitive enzymes and Ca^{2+} signaling involved in the induction of apoptosis. We found that 2-mercaptoethanol (2-ME) and N-acetyl-L-cysteine (NAC) were the best inhibitors of apoptosis: direct quenching of free radicals and protection of SH- group were most effective for the HL-60 cell line. Butylhydroxytoluene (BHT) and N, N'-diphenyl-*p*-phenylene diamine (DPPD), lipid soluble antioxidants show another critical point for apoptosis in HL-60 cells, which is lipid peroxidation. We also found that fetal bovine serum affected the induction of apoptosis by anthracyclic and anthracyclic antitumor compounds in HL-60 cells, probably through the penetration, efflux and accumulation of anthraquinones. The exact mechanism of this phenomenon needs more experimental data. Our findings contribute to the understanding of the intracellular conditions of apoptosis induction in the HL-60 cell line.

Key words: leukemic cells, antioxidants, ROS, apoptosis

INTRODUCTION

Oxidative stress is defined as a disturbance in the pro-oxidant/antioxidant systems in favor of the former [1]. The amount of ROS produced in the mitochondria, endoplasmic reticulum, nucleus and cytoplasm under normal physiological conditions is relatively low and regulates cell differentiation, proliferation and apoptosis [2]. Production of ROS can result from the extracellular exposure to inflammatory cytokines, chemical carcinogens, chemotherapeutic agents and irradiation or from a decreased antioxidant capacity of the organism [3]. The quinoidal structure is one of the most widespread chemical structures in nature [4]. Many of recently used chemotherapeutic agents such as antibiotics or anthracycline antineoplastic drugs have a quinoidal structure. The biological activity of quinones is substantiated by induction of ROS and electrophilicity that enables them to form adducts with intracellular constituents. Which of them is leading in the apoptosis depends on the qualities of a biological object and the origin of a chemical. The induction of irreversible injury or necrosis requires high concentrations of ROS or a long exposure to ROS [5]. However, a lower level of ROS could be more specific in oxidizing particular targets and inducing apoptosis [1]. An increasing amount of literature shows that redox status of the cells plays an important role in cellular signaling [1, 5]. Of particular interest are tyrosine phosphatases because of a critical thiol group in the active site of the enzyme [6]. In addition, the loss of protein kinase C (PKC) is an essential element in the death of a cell exposed to oxidative stress [7]. Moreover, distinct mitogen activated kinase (MAPK) cascades with extracellular signal regulated kinase (ERK), p-38 and JNK/SAPK pathways can be involved in the apoptosis [8]. As a result of environmental stress, a class of immediate-early response genes or transcription factors are constitutively activated as a response to ROS [9]. The intensity and duration of stress as well as a balance with the intracellular antioxidant system can influence the signaling systems, leading to cell death

or surviving [2]. The goal of our investigation was to estimate the origin of ROS and intracellular antioxidant balance involved in the induction of apoptosis in the promyelocytic leukemia HL-60 cell line. Our result could be valuable for the further discovery of potent antileukemic medicaments.

MATERIALS AND METHODS

Cell culture and chemicals. RPMI medium, FBS, penicillin-streptomycin, Doxorubicin were purchased from GIBCO BRL Life Sciences Technologies. Dr. K. Ollinger, Linkoping University, kindly provided rhein and naphthazarin (Nz). All other chemicals were purchased from Sigma Chemical Co. Cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C in a humidified 5% CO_2 atmosphere and passaged twice a week. Stock solutions of chemicals were prepared in DMSO. 2-mercaptoethanol (2-ME), N-acetyl-L-cysteine (NAC), butylhydroxytoluene (BHT), dithiotreitol (DTT), catalase, o-phenanthroline, pyrrolidine dithiocarbamate (PDTC), N,N'-diphenyl-*p*-phenylene diamine (DPPD) were added immediately before naphthazarin. Buthionine sulfoximine (BSO) and diethylmaleate (DMA) were added 22 h and 15 min in advance, respectively. The concentration of DMSO or ethanol never exceeded 0.3%. Apoptotic response was examined within 24 h. The viability of control cells was always no less than 97%.

Cell viability and morphology. Cell viability was assessed by their ability to exclude trypan blue. Apoptotic cells were identified using AO/EB staining as described in [10]. Data were expressed as a mean ± S. E. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Our previous studies showed that quinoidal compounds of different origin are substrates for many intracellular flavo enzymes reducing them in one electron way with subsequent production of ROS [11]. For that reason we compared induction of apoptosis in HL-60 cells by different quinones. The results (Figure 1) show that naphthoquinone naphthazarin (Nz) was the most potent inducer of apoptosis. The threshold concentration of Nz inducing apoptosis was 0.5 μM and the highest amount of apoptotic cells was reached in 12 h (data not shown). The maximum of ROS production was reached during the first 5 min of incubation (data not shown). Prolongation of incubation or increasing concentrations of the chemical initiated the necrotic process. Apoptosis was determined as described in Materials and Methods. Since Nz was the most effective inducer of ROS, we used it as an apoptotic model in our study. The estimation of optimal conditions of apoptosis induction is very important, at least for the anticancer agents such as daunorubicin, doxorubicin, cisplatin, 1-β-D-arabinofuranosyl-cytosine and others. The level of side effects of these compounds on patients could strongly depend on the dose and on the amount of induced ROS. There still exists a problem in identifying ROS, detection of its exact amount and the way of its detoxification. One of the possibilities to investigate the origin of ROS in the process of apoptosis is through antioxidants. We found that N-acetyl-L-cysteine (NAC) or 2-mercaptoethanol (2-ME) were the most effective antioxidants and inhibitors of apoptosis induced by Nz (Figure, 2). NAC is well known as a quencher of free radicals ($O_2^{\bullet -}$, $OH^{\bullet}$) and precursor of glutathione, a very important intracellular molecular antioxidant [12]. N- or S-acidic derivatives of cystein penetrate the cell membrane easier and are quicker transformed to cystein. 2-ME also has a similar mode of activity. These results show that intracellular GSH or enzymes with an SH-group in the active center could be strongly involved in the induction of apoptosis by Nz. We have tried lipid soluble antioxidants such as butylhydroxy toluene (BHT) and a synthetic analog of vitamin E N'N-diphenyl-*p*-phenylenediamine (DPPD). Their antioxidant mechanism is based on the protection of cell membranes from peroxidation of lipids. For this model of apoptosis we used a higher concentration of DPPD and BHT, compared to hepatocytes [13]. Probably, the higher amount of ROS is involved in the apoptosis or/and the cells are less sensitive. Dithiotreitol, a reducer of protein SH- groups, did not protect from apoptosis, neither did the metal chelators pyrrolidine dithiocarbamate (PDTC) and o-phenantroline. Another component of oxidative stress, hydrogen peroxide, did not dominate in the apoptosis of HL-60 cells since catalase did not protect the cells (Figure, 2a). Another step of our study was artificial abolishment of the antioxidant system. We challenged HL-60 cells with buthionine sulfoximine, a specific inhibitor of γ-glutamylcysteine sinthetase and diethylmaleate, a depleter of intracellular GSH [14] (Figure, 2b). Our results show that both compounds increased apoptosis by more than 50% and proved that intracellular GSH is a very important antioxidant in HL-60 cells.

We analyzed the effect of FBS on the induction of apoptosis by anthra- and naphthoquinones and H_2O_2 (Figure, 3). There is a possibility that the induction of apoptosis by quinoidal compounds depends on their penetration and accumulation in the cell regulated by FBS [15]. The total amount of apoptotic cells (Figure, 3) was almost the same for

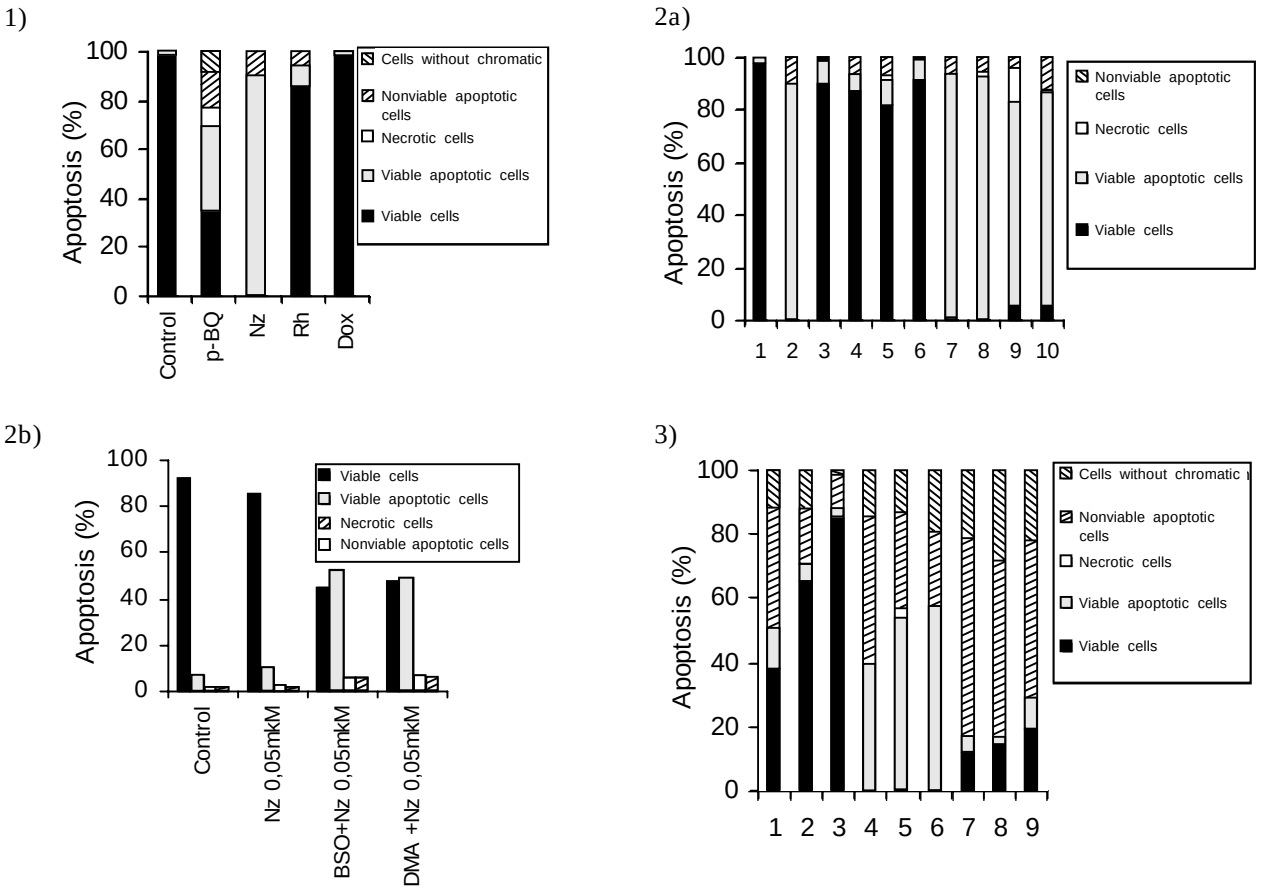

Figure. 1) Induction of apoptosis by different quinoidal compounds. *p*-benzoquinone (*p*-BQ) 10 μM, naphthazarin (Nz) 0.5 μM, rhein (Rh) 0.5 μM, doxorubicin (Dox) 2 μM. Cells were incubated in RPMI 1640 with 10% FBS. Apoptosis was determined in 24 h

2a) Effect of antioxidants on the induction of apoptosis by Nz. 1) Control; 2) Nz 0.5 μM, 3) 2-ME 1 mM + Nz 0.5 μM; 4) NAC 1 mM + Nz 0.5 μM; 5) DPPD 2a) 10 μM + Nz 0.5 μM; 6) BHT 0.5 mM + Nz 0.5 μM; 7) DTT 0.1 mM + Nz 0.5 μM; 8) Catalase 572 U/ml + Nz 0.5 μM; 9) PDTC 40 μM + Nz 0.5 μM; 10) *o*-phenanthroline 0.5 mM + Nz 0.5 μM. 2b) Cells were preincubated with 0.5 mM BSO and 100 μM DMA for 22 h and 15 min respectively and challenged with 0.05 μM Nz for 24 h

3) Eeffect of FBS in the induction of apoptosis by quinones. 1–3 – 50 μM of rhein; 4–6 – Nz 0.5 μM; 7–9 – H_2O_2 1 μM, 1, 4, 7 – without serum, 2, 5, 8 – 1% of FBS, 3, 6, 9 – 10% of FBS. Control cells with FBS alone did not induce apoptosis

all inducers, but the amount of viable or viable apoptotic cells strongly varied depending on the concentration of serum, especially for rhein. This phenomenon could be frequently associated with the expression of P-glycoprotein (P-gp) and multidrug resistance protein (MRP), as well as with the size of special penetration pores in the membrane regulated by FBS [15]. Therefore, it is necessary to investigate which component of FBS and how affects apoptosis, incorporation, promotion or efflux of a chemical. Finally, we can conclude that the main mechanism of apoptosis induction in HL-60 cells by the quinones used in our study is induction of ROS, particularly $O_2^{\cdot -}$ and $OH^{\cdot}$. Antioxidants with a combined mode of action (direct quenching of ROS and protecting of -SH) were the best in the HL-60 cell line. Lipid peroxidation is another possible way of apoptosis induction by the compounds used in our study. The apoptosis induced by anthraquinones or anthracyclic antineoplastic compounds depends on the concentration of FBS that could regulate the penetration of compounds. This fact should not be ignored.

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VIDINIO ANTIOKSIDACINIO BALANSO REIKÐMË CHINOIDINIAIS JUNGINIAIS INDUKUOTAI HL-60 LÀSTELIØ APOPTOZEI

S a n t r a u k a

Chinoniniai dariniai yra viena ið labiausiai gamtoje paplitusiø cheminiø junginiø grupiø, kuriai priklauso dauguma ðiuo metu naudojamø prieðvëþiniø vaistø. Ðias medþiagas, vienelektroniniu bûdu redukuojant iki semichinonø, susidaro reaktyvûs deguonies dariniai (RDD). Pastaruoju metu literatûroje pasirodo vis daugiau duomenø apie RDD kaip antrinio apoptotinio signalo svarbà. Šio darbo tikslas buvo išanalizuoti vidulàsteliniø prooksidantinës/antioksidantinës sistemø pusiausvyros reikðmæ HL-60 lasteliø apoptozei. Nustatyta, kad 2-merkaptoetanolis (2-ME) ir N-acetil-L-cisteinas (NAC), t. y. antioksidantai, tiesiogiai gaudantys laisvuosius radikalus ir apsaugantys vidulàstelines tiolines grupes, yra efektyviausi. Kitas svarbus momentas HL-60 làsteliø apoptozës mechanizme yra lipidø peroksidacija: lipiduose tirpûs antioksidantai (N,N'-difenil-*p*-fenilendiaminas ir butilhidroksitoluenas) stabdë apoptozæ. Taip pat buvo nustatyta, kad embrioninis jauèio serumas veikia antrachinonø bei antracikliniø junginiø sukeltà apoptozæ, labiausiai tikëtina, moduliuodamas jø patekimà, kaupimàsi ir iðsiskyrimà ið làstelës. Tikslesnis veikimo mechanizmas tiriamas. Ðie duomenys atskleidþia RDD susidarymo bei vidulàstelinës antioksidantinës sistemos reikðmæ chinoniniø dariniø sukeltai apoptozei HL-60 làstelëse.