ORIGINAL ARTICLE

DEPLETION OF ENDOGENOUS ZINC STORES INDUCES OXIDATIVE STRESS AND CELL DEATH IN HUMAN MELANOMA CELLS

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Summary: The role of intracellular free zinc and its chelation by TPEN (*N*,*N*,*N*',*N*'- tetrakis(2-pyridylmethyl)ethylenediamine) was studied in Bowes human melanoma cells. The content of free Zn pools was determined by fluorescent probe Zinquin. Depletion of zinc triggered apoptosis confirmed by cell blebbing, changes in mitochondrial transmembrane potential and GSH levels, caspase-3 activation and nuclear fragmentation. Apoptosis was only partially prevented by cyclosporin A or N-acetylcystein, suggesting various independent but likely interrelated mechanisms participating in this process.

Key words: *Human melanoma cells; Labile zinc; Cell death; Mitochondria; Oxidative stress*

Introduction

Zinc (Zn) is an essential component of all eukaryotic cells, where it is required for many biological activities including growth regulatory processes (10). In the cell, Zn ions may exist in a form of permanently bound protein complexes (metalloenzymes or Zn fingers) or be relatively free, constituting loosely bound labile Zn stores, which are participating in intracellular Zn fluxes. Under physiological conditions, cellular Zn content is strictly regulated, however, the presence of higher Zn concentrations in the surrounding environment results in an overall increase of intracellular Zn levels and is associated with documented Zn toxicity (4,17). Conversely, many studies have shown that Zn deficiency may lead to rapid depletion of endogenous Zn pools, thereby inducing oxidative stress and apoptosis in the exposed cells (5,11,15,23).

It is nowadays understood that the impaired regulation of normal cell death signaling most probably underlies cell malignant transformation. Furthermore, recent observations suggest that this process might occur or be enhanced in the presence of Zn ions since they are able to suppress apoptosis by interfering with several molecular targets in numerous cell types (4,14,22).

Thus the purpose of this work was to study the biological effects and significance of deprivation of labile endogenous Zn stores in human melanoma cells, which are known to be resistant to spontaneous or induced cell death (16,20). We report here that the depletion of intracellular Zn stores by permeable Zn chelator *N, N, N', N'*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) induces morphological changes and decreases mitochondrial activity of Bowes melanoma cells while generating oxidative stress and cell death – apoptosis.

Materials and Methods

Chemicals

Zinc sulfate; *N*,*N*,*N*',*N*'- tetrakis(2-pyridylmethyl)ethylenediamine (TPEN); N-acetylcystein (NAC); Zinquin E (ethyl ester), glutathione reductase (GR); β-NADPH; reduced glutathione (GSH); oxidized glutathione (GSSH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB);

4', 6-diamidino-2-phenylindole (DAPI); Triton-X and cyclosporin A were purchased from Sigma-Aldrich (Prague, Czech Republic).

4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate (WST-1) was from Boehringer Mannheim-Roche (Basel, Switzerland). JC-9 dye and secondary antibodies for caspase-3 detection were from Molecular Probes, Inc. (Eugene, U.S.A.) and from EXBIO (EXBIO-Institute of Molecular Genetics, Prague, Czech Republic). Monospecific antiserum for the detection of activated caspase-3 was obtained from New England Biolabs, Inc. (New England Biolabs, Beverly, U.S.A.). All other chemicals were of highest analytical grade.

Cell line

Bowes human melanoma cell line (ATCC, No. CRL – 9607, Manassas, United States) was grown as adherent cell culture in DMEM PAN (Aidenbach, Germany) supplemented with 10 % fetal bovine serum (Aidenbach, Ger-

many), 100 U/ml penicillin, and 100 g/ml streptomycin. Cells were maintained in an incubator at 37 °C and 5 % CO_2 atmosphere and were passaged two times a week using 0.05 % trypsin/EDTA. Only mycoplasma-free cells were used for experiments.

Measurement of intracellular labile Zn

Cultures pretreated with 25–100 μM TPEN (2h, 37 °C) were washed with phosphate buffered saline (PBS) and incubated with 25 μM Zinquin E in DMEM for 30 min at 37 °C. After 30 min, the slides were washed with PBS and Zinquin fluorescence was recorded under a fluorescence microscope Nikon Eclipse E400 (Nikon Corporation, Kanagawa, Japan) using an excitation filter 330–380 nm and emission filter 420 nm. The images were collected with a digital color matrix camera COOL 1300 (VDS, Vosskűhler, Germany) and LUCIA DI Image Analysis System (Laboratory Imaging Ltd., Prague, Czech Republic). In all experiments, the results were compared with those originating from untreated cultures.

Bowes human melanoma cell toxicity

Effects of labile Zn deprivation on human melanoma cells were assessed by morphological analysis as well as by measurement of mitochondrial activity. Cultures were exposed to 25–100 μM TPEN (2h, 37 °C), rinsed with phosphate buffered saline (PBS) and resuspended in DMEM PAN without serum. Their morphological appearance was photographed at regular intervals under an inverted microscope Olympus IX-70 (Olympus Optical Co, Ltd., Tokyo, Japan) by a digital camera Olympus C-4040 (Olympus Optical CO, Ltd., Tokyo, Japan). Mitochondrial activity was quantified by WST-1 colorimetric assay, which is based on the cleavage of the tetrazolium salt to colored formazan by mitochondrial dehydrogenases in viable cells. Melanoma cells (6,000 cells/well) in 200 μl of DMEM PAN with 10% fetal bovine serum were seeded into 96–well microtiter plates, with the first two columns of wells without cells (blank) and left overnight at 37 °C and in 5 % CO_2. Next, the cells were treated with varying concentrations of TPEN for up to 2h. After 2h, the cells were rinsed with PBS and 100 μl of WST-1 was added. The cells were further incubated for 2h upon same conditions. The absorbance of samples was recorded at 450 nm with 650 nm of reference wavelength by a scanning multiwell spectrophotometer Titertek Multiscan MCC/340 (ICN Biochemicals, Frankfurt, Germany). In all cases, the absorbance of TPEN in medium alone was recorded to determine whether it interfered with the assay.

GSH assay

Control and treated cells were detached by a cell scraper and collected by centrifugation at 1,000 x g for 5 min at 4 °C (JOUAN, Nantes, France). The obtained pellet was rinsed with PBS and mixed with distilled water to the final volume of 2 ml. Aliquots of this lyzate were either used for measurement of protein content by bicinchoninic acid assay – BCA assay (Bicinchoninic acid kit for protein determination, Sigma-Aldrich, Prague, Czech Republic) or diluted with EDTA-phosphate buffer and used for GSH assay. For this assay, sample aliquots were mixed with NADPH, GR and DTNB. After adding NADPH, the change in absorbance during 6 min was followed at 412 nm using a Shimadzu UV – Visible Spectrophotometer UV – 1601 (SHIMADZU DEUTSCHLAND GmbH, Duisburg, Germany). Data were expressed as nM GSH/mg protein on the basis of a GSH calibration curve.

Mitochondrial transmembrane potential ($\Delta\Phi m$) analysis

Bowes melanoma cells were seeded into cultivation flasks and allowed to grow overnight. After treatment with TPEN, cells were rinsed in PBS and stained with JC-9 dye for 15 min at 37 °C. Mitochondrial membrane potential was assessed by flow cytometry analysis using a flow cytometer equipped with cell sorter (COULTER EPICS Elite *ESP*, Coulter, U.S.A.). Mitochondrial transmembrane potential changes were indicated as an increase in fluorescence intensity at 528 nm.

Detection of apoptosis

The slides with TPEN-treated and control cells were fixed with 4 % formaldehyde solution. The specimens were rinsed with phosphate saline buffer with Triton X (PBS-T) and a primary antibody against caspase-3 was added. After overnight cultivation a secondary antibody (anti-mouse conjugated with Alexa 488) was added, and the cells were incubated for 90 min at RT. The specimens were post-labeled with DAPI, mounted into SlowFade® medium (Molecular Probes, Inc. Eugene, U.S.A.) and examined under a fluorescence microscope Nikon Eclipse E400 (Nikon Corporation, Kanagawa) equipped with a digital color matrix camera COOL 1300 (VDS, Vosskűhler, Germany), using an excitation filter 510–560 nm and emission filter 590 nm. Photographs were taken using the software LUCIA DI Image Analysis System LIM (Laboratory Imaging Ltd., Prague, Czech Republic) and analyzed. All the experiments were done in triplicate.

Statistics

Statistical analysis was carried out with a statistical program GraphPad Prism (GraphPad Software, Inc. San Diego, U.S.A.). We used one-way Anova test with Dunnet's post test for multiple comparisons. Results were compared with control samples, and means were considered significant if $P<0.01$.

Results

Intracellular labile Zn fluorescence. Endogenous labile Zn stores appear to localize mostly in the cytoplasm of Bowes human melanoma cells (Fig. 1A). Membrane per-

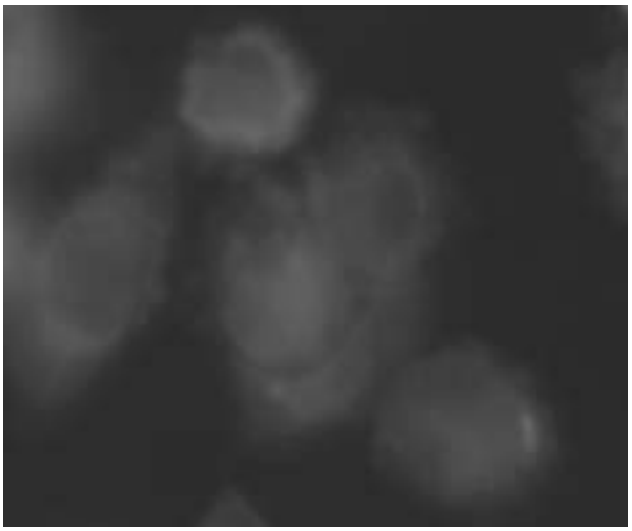

Fig. 1A: Intracellular labile zinc stores in Bowes human melanoma cells after incubation with 25 μM Zinquin. Zinc localizes predominantly to the cytoplasm of cells. Fluorescence 600x.

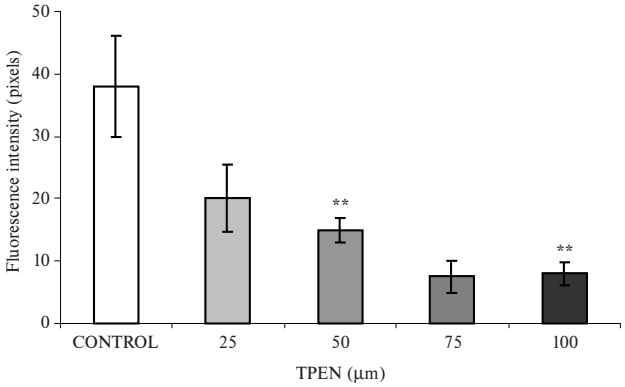

Fig. 1B: Time and concentration dependent reduction of intensity of Zinquin fluorescence after exposure to various of TPEN concentrations. Cultures of Bowes human melanoma cells were exposed for 2h to this membrane permeable zinc chelator and changes in intracellular zinc content were measured using an image analysis system. Values represent the mean ± SD of three different experiments. **P<0.01 with one way-Anova test and Dunnet's post test for multiple comparisons.

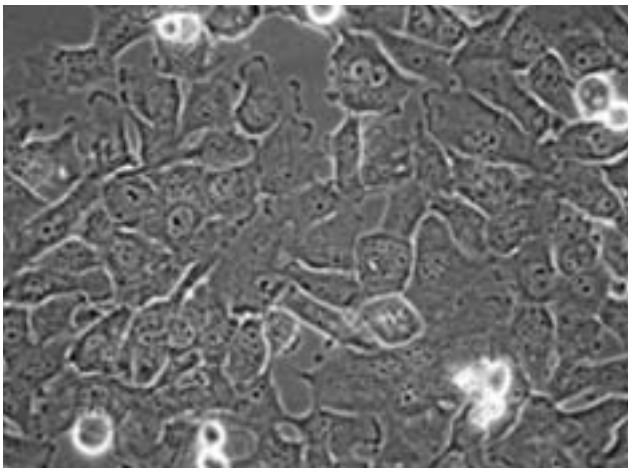

Fig. 2A: Bowes human melanoma cells in control cultures. Phase contrast 400x.

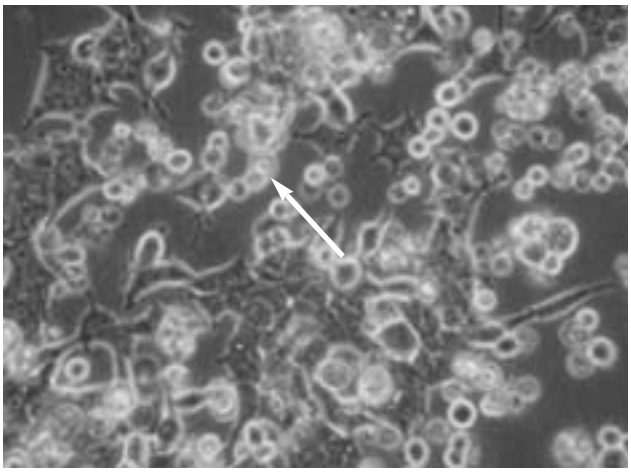

Fig. 2B: Bowes human melanoma cells treated with membrane permeable chelator of zinc TPEN. Following 2h exposure, many cells lost adherence and became rounded. Phase contrast 400x.

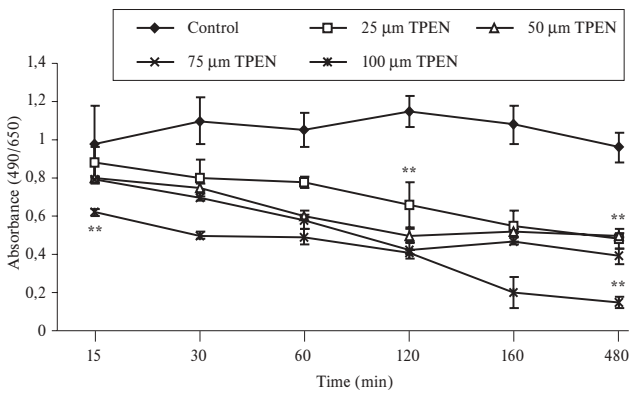

Fig. 2C: The activity of mitochondrial enzymes as measured by WST-1 tetrazolium cleavage assay in Bowes human melanoma cells after treatment with various concentrations of membrane permeable zinc chelator TPEN. All the employed TPEN concentrations suppressed mitochondrial enzymes, in case of the highest TPEN concentration as early as 15 min after the beginning of treatment. Values represent the mean ± SD of three independent experiments. **P<0.01 significantly different from control with one way-Anova test and Dunnet's post test for multiple comparisons.

meable Zn chelator TPEN was capable of reducing labile Zn content in the same cells in a concentration-dependent manner (Fig. 1B).

Effect of Zn deprivation on Bowes cell morphology and mitochondrial enzymes. Exposure to <25 μM TPEN had no effect on appearance of studied cells as well as on performance of mitochondrial enzymes during 8h (data not shown). TPEN at concentrations of 25–100 μM caused vacuolization and subsequent cell rounding followed in some cases by moderate blebbing (Fig. 2A,B). Concurrently, activity of mitochondrial enzymes was suppressed. These changes were more pronounced and occurred earlier with increasing concentrations of employed TPEN (Fig. 2C), with the highest concentration producing the first changes as early as 15 min after the beginning of the treatment.

Effect of Zn deficiency on oxidative stress. Various thiols are known to participate in intracellular redox balance mechanisms that protect against oxidative stress responsible for cell injury and cell death. In order to verify the relationship between Zn status, oxidative stress and spontaneous cell death, the effect of endogenous labile Zn deficiency on intracellular GSH was examined. TPEN-in-

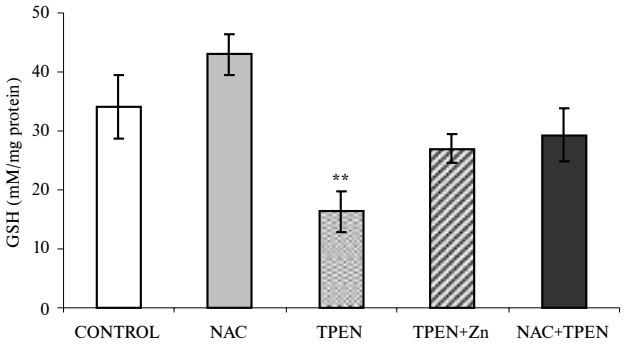

Fig. 3: The levels of reduced glutahione (GSH) in Bowes human melanoma cells. Zinc chelation induced reduction in GSH content, which was partially preventable by addition of zinc or pretreatment of cells with NAC. Values represent the mean ± SD of three independent experiments. **P<0.01 significantly different from control with one way-Anova test and Dunnet's post test for multiple comparisons.

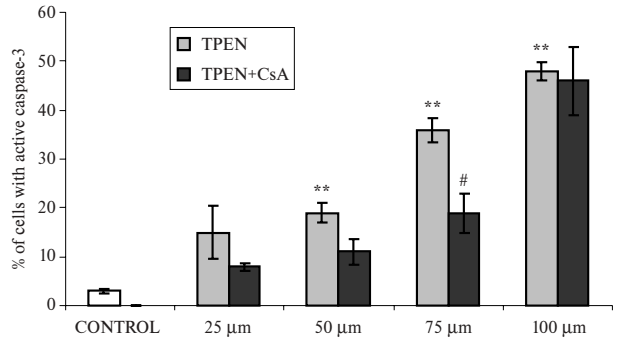

Fig. 4B: Apoptosis in Bowes human melanoma cells treated with various TPEN concentrations with or without 5 μM cyclosporin A. In all cases, zinc chelation resulted in activation of caspase-3, which was partially reduced by cyclosporin A. Values represent the mean ± SD of three independent experiments. **P<0.01 significantly different from control, # significantly different from TPEN treated cultures with one way-Anova test and Dunnet's post test for multiple comparisons.

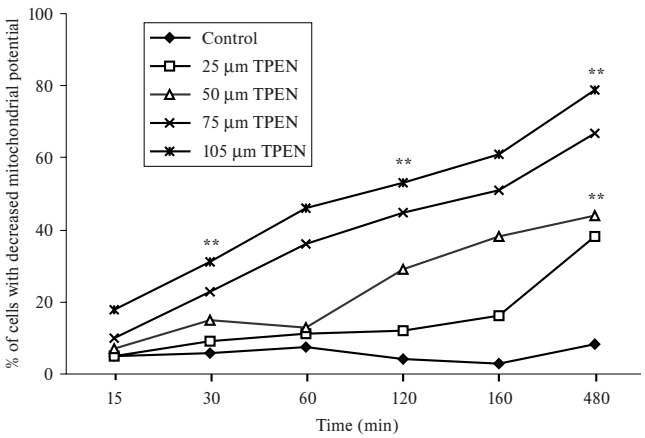

Fig. 4A: Mitochondrial membrane potential in Bowes human melanoma cells treated with various TPEN concentrations. In all cases, zinc chelation promoted a decrease in mitochondrial potential – an early sign of apoptosis. Values represent the mean ± SD of three independent experiments. **P<0.01 significantly different from control with one way-Anova test and Dunnet's post test for multiple comparisons.

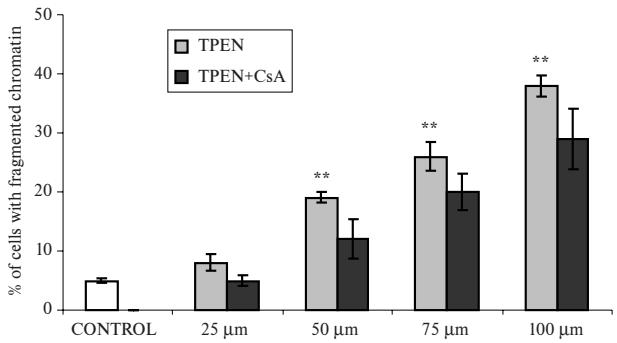

Fig. 4C: Apoptosis in Bowes human melanoma cells treated with various TPEN concentrations with or without 5 μM cyclosporin A. In all cases, zinc chelation resulted in nuclear fragmentation, which was not significantly reduced by cyclosporin A. Values represent the mean ± SD of three independent experiments. **P<0.01 significantly different from control with one way-Anova test and Dunnet's post test for multiple comparisons

duced labile Zn depletion caused a decrease in intracellular GSH which was partially prevented by addition of $ZnSO_4$ or pretreatment with NAC (Fig. 3).

Role of mitochondria in Zn depletion-induced apoptosis. Incubation of Bowes human melanoma cells with <25 μM TPEN had a very little effect on mitochondrial membrane potential and other observed markers of apoptosis; i.e. activation of caspase-3 and nuclear fragmentation (data not shown). When higher TPEN concentrations were added to cultures, mitochondrial membrane potential decreased in a time and concentration dependent manner, suggesting an impaired functioning of mitochondrial respiratory chain (Fig. 4A). In addition, mitochondrial alteration was paralleled by caspase-3 activation and nuclear fragmentation – hallmarks of apoptotic cascade. To determine whether caspase-3 activation was mediated by cytochrome c released from mitochondria, cultures were pretreated or co-treated with 5 μM cyclosporin A which is known as a suppressor of cytochrome c release. In all but the highest TPEN concentration (100 μM), caspase-3 activation and nuclear fragmentation were reduced or at least delayed in time (Fig. 4B,C).

Discussion

Malignant melanoma belongs among the most aggressive forms of skin cancer, which is often resistant to chemotherapeutically induced cell death. Although it has been shown that melanoma cells are capable of undergoing apoptosis via several distinct mechanisms, there is also compelling evidence suggesting that the same cells may employ a variety of mechanisms for regulating apoptosis and generating apoptosis deficiency. Thus one of the aims of current research concerning malignant melanoma is to elucidate how melanoma cells circumvent proapoptotic signals, and which apoptotic pathways are possibly altered in them (6,18,20).

It is nowadays known that Zn is a critical element in management of many cellular processes. Recent studies have shown that besides its physiological role, Zn appears to be involved in regulation of cell proliferation and cell death – apoptosis or necrosis. In the past decade, research into Zn status and mechanisms of cell death has helped identify the endogenous labile Zn pools whose altered homeostasis have been reported to accompany numerous pathological states including cancer (1,5).

In our work we have focused on the role of labile Zn pools in regulation of apoptosis in Bowes human melanoma cells. Incubation of Bowes cells with a specific fluorophore Zinquin showed that these cells, indeed, contain labile Zn ions distributed in the cytoplasm (Fig. 1A). The fact that these endogenous Zn stores may be reduced or depleted by addition of a membrane permeable Zn chelator TPEN concurs with observations made on different cellular and tissue models (21,22,23).

In order to confirm the nature of changes promoted by TPEN in Bowes human melanoma cells, we followed several apoptosis-specific markers. Chelation of endogenous labile Zn brought a loss of cellular adherence, cell rounding and shrinkage, which was followed by moderate and rather random membrane blebbing (Fig. 2B). It has been noted that morphological features of apoptosis are preceded by other, more discreet changes such as a shift in redox balance. Since Zn is known as an antioxidant factor, being most active in maintenance of CuZn-SOD stability as well as in protection of thiol groups (8), we examined the effects of labile Zn deprivation on cellular GSH levels and subsequent apoptosis. We found that upon TPEN treatment, GSH concentration in Bowes cells decreased and this reduction was partially abolished by concurrent addition of NAC, a precursor in GSH synthesis, or external Zn. Still, apoptosis induced by Zn depletion was not suppressed entirely by either of the above-mentioned chemicals (i.e. Zn or NAC). This finding is in contrast with the results of the study conducted on rat hepatocytes where TPEN induced apoptosis was blocked by NAC (13). One possible explanation of this discrepancy might reside in the type of the employed model. Unlike hepatocytes, which are metabolically very active and harbor high concentrations of GSH, mesenchymal cells from which melanoma arises show lower activity and, perhaps, a varying sensitivity to redox changes. Furthermore, as stated before melanoma cells may possess unpredictably altered signaling pathways and thus it is possible that Zn depletion started other mechanisms, GSH independent, which eventually triggered an executory apoptosis cascade.

Mitochondria represent the cellular compartment responsible for generation of energy in the form of ATP. In addition, they may activate apoptosis by releasing cytochrome c along with other proapoptotic proteins, thereby activating caspase-9 and caspase-3, the major apoptosis execution enzymes (12). Release of mitochondrial cytochrome c have been reported to occur via permeability transition pore (PTP) whose opening is triggered by various signals such as a mechanical insult to the mitochondrial membrane, decreased expression of antiapoptotic proteins or suppression of mitochondrial function (7,9,12). In addition, recent studies indicate that mobilization of endogenous labile Zn pools might interfere with normal functioning of mitochondria too and be responsible for activation of apoptosis (19). Consistent with these reports we found that chelation of intracellular labile Zn pools corresponds well with a detected impaired function of mitochondrial dehydrogenases as well as with loss of mitochondrial transmembrane potential. The later parameter, in particular, is known to be an early feature of apoptosis and provides indirect evidence on possible activation of PTP. To determine whether labile Zn depletion induced release of cytochrome c from mitochondria in Bowes human melanoma cells, we pretreated or co-treated cultures with PTP-blocking agent cyclosporin A. We found that in all cultures treated with various concentrations of TPEN cyclosporin A was able to decrease basic markers of apoptosis – nuclear fragmentation and

activation of caspase-3 although this decrease was found significant only at 75 μM TPEN. This observation clearly suggests that some intracellular Zn localizes to mitochondria and its sequestration from this compartment likely induces various changes in mitochondria culminating in release of apoptosis-activating proteins. The fact that apoptosis was not entirely prevented by cyclosporin A implies that depletion of endogenous Zn interferes with other apoptosis-relevant targets.

One of them is most possibly procaspase-3, whose conversion to the active form – caspase-3 has been shown to be inhibited in the presence of Zn ions (2,3). Our data prove that Zn-deprived Bowes cells are caspase-3 positive in comparison with control cells. Addition of external Zn to such cells then suppressed a number of caspase-3 positive cells or at least delayed this process (data not shown).

Conclusion

Bowes human melanoma cells contain labile Zn stores which localize mostly to the cytoplasm and possibly to mitochondria too. TPEN induced Zn deprivation leads to a concentration and time dependent activation of apoptosis by at least three interrelated mechanisms; i.e. suppression of mitochondrial function and release of cytochrome c, triggering redox changes exemplified by reduction of GSH, and direct activation of caspase-3. Temporal and spatial organization of these mechanisms as well as determination of the exact relationship between endogenous Zn pools and mitochondria in the process of apoptosis remain obscure, thus calling for further studies.

Acknowledgment

This work was supported by Ministry of Education Czech Republic Research Project MSM 111500001 Serious organ failure, experimental and clinical aspects, possibilities for prevention and therapeutic management.

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Submitted January 2004.
Accepted April 2004.

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