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Biochemical Pharmacology 67 (2004) 513–522
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Selenite-induced apoptosis in doxorubicin-resistant cells and effects on the thioredoxin system

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Received 18 June 2003; accepted 15 September 2003

Abstract

Selenium treatment of the doxorubicin-resistant cell line, U-1285dox, derived from human small cell carcinoma of the lung, resulted in massive apoptosis. This effect appeared maximal at 2 days after addition of selenite. The apoptosis was caspase-3 independent as revealed by Western blot analysis, activity measurement and by using caspase inhibitors. Induction of apoptosis was significantly more pronounced and occurred after addition of lower concentrations of selenite in the doxorubicin-resistant cells compared to the parental doxorubicin-sensitive cells. High levels of selenite caused necrosis in the doxorubicin-sensitive cells. Analysis of enzymatic activity (insulin reduction) of thioredoxin reductase (TrxR) and TrxR protein concentration, measured by ELISA, revealed increasing activity and protein levels after treatment with increasing concentrations of selenium. Maximum relative increase was induced up to 1 μM in both sublines and at this selenium level the concentrations of TrxR measured as insulin reducing activity or ELISA immunoreactivity were nearly identical. Increasing concentrations of selenite up to 10 μM resulted in increased activity and concentration of TrxR in the sensitive subline but decreasing levels in the resistant subline. The level of truncated Trx (tTrx) was higher in the resistant U-1285dox cells but the level did not change with increasing selenite concentrations. Our results demonstrate pronounced selective selenium-mediated apoptosis in therapy-resistant cells and suggest that redox regulation through the thioredoxin system is an important target for cancer therapy.

Keywords: Selenium; Apoptosis; Multi-drug resistance; Thioredoxin reductase; Thioredoxin

1. Introduction

Clones of cancer cells, which are more resistant to environmental factors such as free radicals, compared to normal cells, may arise during tumor promotion and progression. Furthermore, a secondary resistance may develop during chemotherapy and cause failure of an initially successful treatment.

In a recent study, we showed that two doxorubicin-resistant cell lines, GLC_4/ADR and U-1285dox, were more sensitive to the cytotoxic effects of selenite compared to the doxorubicin-sensitive cells and measurements of the viability revealed 4-fold lower IC_{50} in the doxorubicin-resistant cell lines. There were no synergisms between selenite and doxorubicin and the classical multi-drug resistance (MDR) [1] proteins were not affected, but the levels of the redox enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) were lower in the doxorubicin-resistant cells after treatment with selenite at a concentration corresponding to IC_{50} for 4 days [1].

Selenium is a well-known, strictly concentration-dependent, modulator of cell-growth. In lower concentrations,

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Abbreviations: MDR, multi-drug resistance; TrxR, thioredoxin reductase; GR, glutathione reductase; Trx, thioredoxin; tTrx, truncated thioredoxin.

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doi:10.1016/j.bcp.2003.09.021

selenium stimulates cell-growth and induces synthesis of selenoproteins. In this concentration-span selenium is mainly an antioxidant. In higher concentrations, selenium compounds turn from antioxidants to pro-oxidants with potent inhibitory effects on cell-growth. A generally accepted mechanism for this is the induction of oxidative stress [2].

Some selenium compounds are reduced by and modulate the activities of the thioredoxin system. This system, comprising thioredoxin (Trx), TrxR and NADPH, is a multifunctional general protein disulfide reductase system. Trx is a 12 kDa protein, consisting of 104 amino acids, functioning through cyclic oxidation–reduction of a single –S–S– group, in the active –C–X–X–C– site of the protein [3]. A variety of physiological functions of Trx have been described, e.g. regulation of receptors, enzymes and apoptosis [4,5]. Mammalian cytosolic TrxR is a homodimeric flavoenzyme containing a FAD, a catalytically active disulfide/dithiol and a penultimate C-terminal selenocysteine residue in each subunit of 58 kDa. A distinct mitochondrial TrxR (TrxR2) has recently been characterized [6,7]. Furthermore, a third mammalian TrxR has been described in mouse testis [8]. The two latter enzymes are, like the cytosolic enzyme, selenoproteins.

Truncated Trx (tTrx) is a shortened variant of Trx. It consists of the first 80 amino acids of the full-length 104 amino acids containing protein [9,10] and is suggested to be the result of an alternative splicing of the mRNA transcribed from the Trx gene [11]. This 10 kDa protein has an intact Trx active site [9,10], but has no thiol reductase activity and is not a substrate to TrxR [12]. The tTrx has a cytokine function, stimulating B cells [13], and has eosinophil cytotoxicity-enhancing factor (ECEF) activity [9].

In this study, we have investigated the morphological changes and apoptosis patterns in doxorubicin-sensitive U-1285 and doxorubicin-resistant U-1285dox cells derived from human small cell carcinoma of the lung after exposure to selenite. In addition, since selenite is a known potent modulator of the Trx-system the concomitant changes in TrxR, Trx and tTrx levels were investigated. The results suggest mechanisms for the differential toxicity to selenite in these two cell lines.

2. Material and methods

2.1. Chemicals and drugs

Sodium selenite and all chemicals employed for enzyme assays were procured from Sigma Chem. Co Trx from *E. coli* and the caspase inhibitor Z-VAD-fmk were purchased from Promega. Caspase-3/CPP32 inhibitor Z-DEVD-fmk was from Medical & Biological Laboratories Co Bovine TrxR was obtained from IMCO Corp. Doxorubicin (Adriamycin®) was purchased from Amersham Biosciences. RPMI1640, fetal calf serum and PBS Dulbecco's without calcium and magnesium, without sodium bicarbonate were from GIBCO BRL, InVitrogen.

2.2. Cell lines

The doxorubicin-sensitive human U-1285 small cell carcinoma of the lung cell line and its doxorubicin-resistant subline U-1285dox, previously described [14,15], were kindly provided by Prof. J. Bergh (Radiumhemmet, Karolinska Hospital). The U-1285dox subline is 24 times more resistant to doxorubicin than the parental doxorubicin-sensitive U-1285 cell line, and has an overexpression of the MDR-protein MRP1.

All cell lines were cultured in suspension in RPMI1640 medium with Glutamax-I, 25 mM HEPES and supplemented with 10% fetal calf serum at 37° in a humidified atmosphere with 5% CO_2. All cultures were free of mycoplasma.

To eliminate revertants, the doxorubicin-resistant subline was cultured in the presence of doxorubicin every fourth week, and cultured at least two passages without selecting doxorubicin prior to the experiments.

2.3. Preparation of slides

Cells were seeded at a density of 0.55×10^5 cells/mL in standard cell culture flasks and incubated continuously with selenite for 0–4 days. The concentrations chosen were 0, 1, 2, 5, and 10 μM for both cell sublines. In addition, the doxorubicin-sensitive cells were exposed to 30 μM, a concentration too toxic for the doxorubicin-resistant cells [1].Within each experiment, determinations were performed in duplicate and compared to unexposed cells in duplicate. The entire experiment was set in four duplicates, one duplicate harvested every day. Fifty thousand cells per slide were selected for apoptosis detection and morphological investigations, respectively. For morphological investigations cells were cyto-centrifuged for 5 min at 18.06*g* (Cytospin 3) and stained by Giemsa staining.

2.4. Apoptosis determination (TUNEL)

The commercial *in situ* apoptosis detection kit (ApopTag, NeoMarkers) was used for determination of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) positivity. Cells were fixed in buffered 4% formaldehyde for 10 min and cyto-centrifuged for 5 min at 18.06*g*, 50,000 cells per slide. The cells were stained according to the instructions of the manufacturer. The cells were counterstained with propidium iodide (PI) 1 μg/mL in DABCO anti-fade (Sigma Chem. Co). The percentage of cells with FITC-positive nuclei (TUNEL-positive cells) was based on differential counts of at least 200 nucleated cells. As a negative control, cells from day 0 without TdT enzyme were analyzed. Slides from three independent experiments were examined in fluorescence microscope and counted twice.

2.5. Morphological investigation

Duplicate slides with cells from three different preparations were made and examined by light microscopy and counted twice. Slides were coded and examined in a double blind manner. Four hundred randomized cells were enumerated on each slide. (1) Enlargement/swelling (necrotic), (2) apoptotic or (3) mitotic cells were recorded and expressed as percentage of total cell number. The enlarged/swollen cells were classified as necrotic due to their morphological appearance and since they were stained blue by trypan blue.

2.6. Apoptosis by Annexin V staining

U-1285 and U-1285dox cells were cultured for 0–4 days with and without 10 μM selenite. For determination of caspase dependency, the selenite exposed cells were cultured with or without the presence of a caspase-3 inhibitor (Z-DEVD-fmk) and a general caspase inhibitor (Z-VAD-fmk) in concentrations recommended by the manufacturers. Cells were stained with FITC-conjugated Annexin V, and PI using the Apoptosis detection kit (Nexins Research Apoptest-FITC kit^{TM}) as described by the manufacturer's protocol. Twenty thousand events were collected on a FACS Calibur flow cytometer (Becton Dickinson) and analyzed using the CellQuest software (Becton Dickinson). Low-fluorescence debris was gated out prior to analysis.

2.7. Analysis of caspase-3 activation by SDS–PAGE and immunoblot analysis

U-1285 and U-1285dox cells were cultured in the presence and absence of 10 μM selenite for 2 days. As a positive control of caspase-3 cleavage Jurkat cells (obtained from American Type Culture Collection) were incubated continuously with doxorubicin, 0.2 μM for 2 days or 1.0 μM for 16 hr, respectively. Cells were lysed for 30 min on ice in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 25 mM EDTA, 0.01% sodium azide, 1 mM sodium fluoride, 0.5 mM PMSF, leupeptid 10 μg/mL, aprotinin 10 μg/mL, pepstatin 2 μg/mL, 1 mM DTT, 1 mM sodium orthovanadate, 1% Igepal) and stored at −70°. Cell lysates were thawed and mixed with double concentrated Laemmli buffer. Electrophoresis of 25 μg protein aliquots were carried out in a 12% SDS–PAGE at 100 V for 2 hr and the proteins were transferred to PVDF membranes (Bio-Rad). The PVDF membranes were blocked in Tris-saline buffer containing 10% non-fat dry milk. Caspase-3 protein was detected by staining with polyclonal antibody (1:1000; BD-Pharmingen International). After washing, a peroxidase-conjugated secondary antibody (1:2500; Amersham Biosciences) together with the enhanced chemiluminiscence (ECL + Plus; NEN, Perkin-Elmer) were used for visualization by exposure to Hyperfilm ECL (Amersham Biosciences).

2.8. Caspase-3 activity assay

For determination of caspase-3 activity a caspase-3/CPP32 Fluorometric Assay Kit (Medical & Biological Laboratories Co) was used. U-1285 and U-1285dox cells were exposed to 1 and 20 μM doxorubicin, respectively. U-1285dox cells were exposed to 10 μM selenite and Jurkat cells were exposed to 0.2 μM doxorubicin or 10 μM selenite. All cell lines were incubated for 2 days and analyzed according to the protocol provided by the manufacturer. The samples were analyzed in a fluorimeter equipped with a 400-nm excitation filter and 505-nm emission filter. The caspase-3 activity was expressed as relative fluorescence.

2.9. Preparation of cell extracts

For biochemical determinations cells were cultured in the presence of selenite in increasing concentrations 0, 1, 2, 5, and 10 μM (and 30 μM). The cells were harvested after 2 days, washed twice with PBS and resuspended in 1 mL PBS. In the presence of protease inhibitors (Complete Mini, EDTA-free, 1836170, Roche) cells were lysed by freezing and thawing three times in liquid nitrogen and in 37° water-bath, respectively. The cells were then homogenized by a standard knife-homogenizer for 30 s at 4°. The homogenates thus obtained were subsequently centrifuged at 25,200 rcf for 7 min and the resulting supernatants were used for biochemical measurements.

2.10. Enzyme assay

The activities of TrxR in the cell extracts were determined as described elsewhere [16]. Protein concentration was quantified employing the Biuret procedure. A volume corresponding to 50 μg protein from each cell extract was incubated with 80 mM HEPES (pH 7.5), 0.9 mg/mL NADPH, 6 mM EDTA, 2 mg/mL insulin and 10 μM *E. coli* Trx at 37° for 20 min in a final volume of 120 μL. By the addition of 500 μL DTNB (0.4 mg/mL) in 6 M guanidine hydrochloride/0.2 M Tris–Cl (pH 8.0) the reaction was terminated. A blank sample, containing everything except Trx, was incubated and treated in the same manner as each unknown sample. The absorbance at 412 nm was measured and the blank value subtracted from the corresponding absorbance value of the sample. A standard curve was prepared by using purified calf thymus TrxR, with a defined specific activity.

2.11. Enzyme-linked immunosorbent assay (ELISA) for TrxR, Trx and tTrx

ELISA for human TrxR was performed as previously described [17]. Briefly, 96-well ELISA plates (Nunc Immuno-Plate MaxiSorp) were coated with 100 μL per

well of anti-TrxR-clone 3 mAbs (1 μg/mL) in carbonate buffer, pH 9.6 for 16 hr at 4°. The plates were rinsed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 200 μL PBS containing 3% BSA (PBS-BSA) for 1 hr. The wells were rinsed four times with PBS-T and incubated with 100 μL samples or standard TrxR serially diluted in PBS containing 0.1% BSA, 0.05% Tween 20 (PBS-BSA-T), and 0.5 mM AEBSF, for 2 hr at 4°. The plates were covered with aluminum foil to minimize photo-damage. The wells were rinsed four times with PBS-T and then incubated with 100 μL biotinylated anti-TrxR-clone 2 mAbs (75 ng/mL) for 1 hr at room temperature on a rocker platform. This mAb has different epitope specificity compared to the coating mAb. The wells were then rinsed four times with PBS-T and incubated with 100 μL alkaline phosphatase-conjugated streptavidine (AX02-0402X; diluted 1:4000) (Amersham Biosciences) in PBS-BSA-T on a rocker platform. The plates were washed four times in PBS-T and incubated with *p*-nitrophenyl phosphate (Sigma Chem. Co) in diethanolamine, pH 9.0, containing 0.5 mM $MgCl_2$ and 0.02% NaN_3 for 40 min. The absorbance was measured at 405 nm in a Multiskan RC photometer Labsystem. Human placental TrxR, kindly provided by Dr. A. Holmgren, Stockholm or human recombinant TrxR kindly provided by Dr. G. Spyrou, were used in the range 437–1.8 ng/mL for the standard curve. The method showed linearity from 1.8 to 400 ng TrxR/mL when plotted in a log-log diagram.

ELISA for human Trx was performed as described above for TrxR except for using the monoclonal anti-Trx clone 2G11 Ab as a catcher Ab (5 μg/mL; BD Pharmingen) and biotinylated IgG of goat anti-human Trx (IMCO Co) as an indicator Ab. The unknown samples and the standards were diluted in PBS-TB containing 1 mM DTT. Human recombinant Trx (IMCO Co) was used as a standard in the range 100–0.41 ng/mL.

ELISA for truncated Trx was performed as described above for TrxR and Trx except for using anti-truncated Trx (1–80) clone 7D11 mAb as a catcher (15 μg/mL) and biotinylated goat anti-human Trx as an indicator Ab. Standard human truncated Trx (used in the range 100–0.41 ng/mL) was kindly provided by Dr. G. Spyrou, Stockholm.

2.12. Statistical analysis

Statistical analysis was performed using Student's *t*-test.

3. Results

3.1. Apoptosis and necrosis

Selenium-induced apoptosis was determined in a concentration- and time-dependent manner detected by TUNEL-assay, morphologic investigation and FACS-analysis. After a continuous incubation of 10 μM selenite the U-1285 cells showed maximum of apoptosis at day 4 (Figs. 1A and 2A). The doxorubicin-resistant U-1285dox cells, however, showed a maximum of apoptosis already day 2 (Figs. 1B and 2B). The proportion of cells in apoptosis was significantly ($P < 0.01$) higher in the U-1285dox cells compared to the parental U-1285 cells (Fig. 1A and B). These data obtained by TUNEL-assay and morphological investigation were confirmed by Annexin V/FACS-analysis (Fig. 3A and B), i.e. a larger fraction of apoptotic cells in the U-1285dox cells compared to U-1285 after incubation with selenite (10 μM).

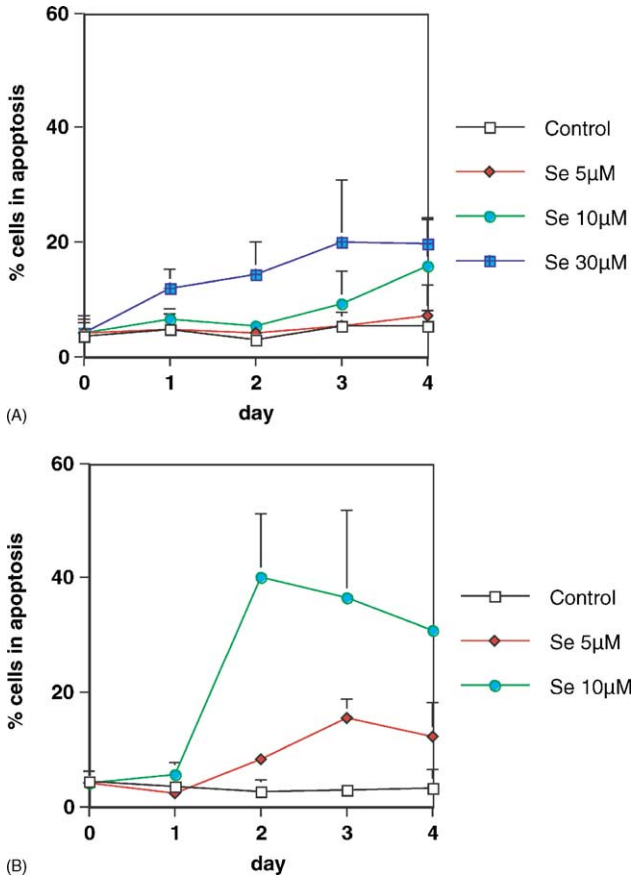

Fig. 1. Cells in apoptosis determined by the TUNEL-assay in doxorubicin-sensitive U-1285 (A) and doxorubicin-resistant U-1285dox (B) in a concentration- and time-dependent manner. Values are mean of three independent preparations. Bars show +SD.

To analyze the caspase-3 dependency, Western blot analyses and caspase-3 activity assays were performed. The ability of U-1285 and U-1285dox to cleave procaspase-3 was investigated by treatment of the cells with doxorubicin in concentrations corresponding to IC_{50}. After 2 days incubation with doxorubicin, the relative fluorescence increased 3- and 4-fold, respectively indicating procaspase cleavage. Furthermore, 3 days incubation of Jurkat cells with selenite (10 μM) resulted in 6-fold increase in relative fluorescence clearly indicating caspase activation. The active subunit p17 and the intermediate p19

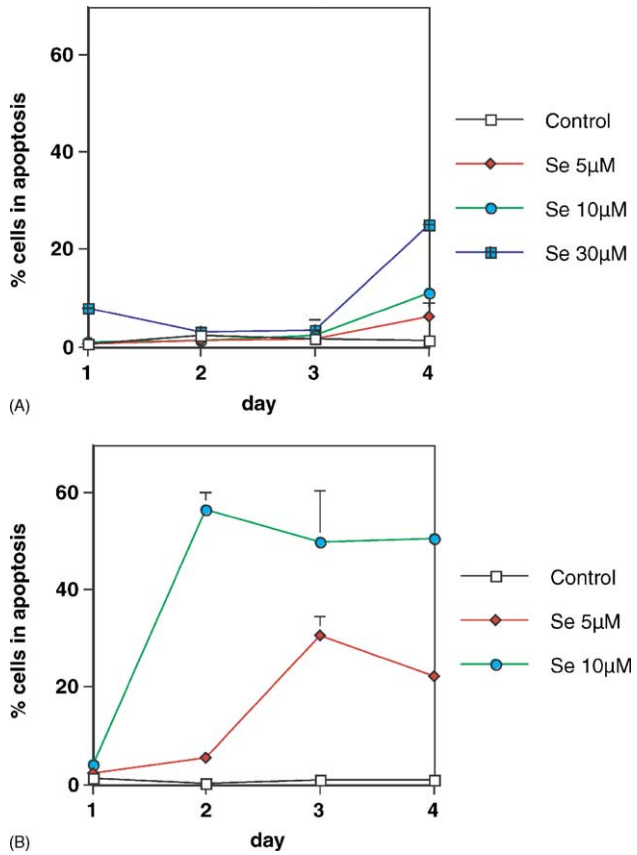

Fig. 2. Cells in apoptosis determined by morphological investigations in doxorubicin-sensitive U-1285 (A) and doxorubicin-resistant U-1285dox (B) in a concentration- and time-dependent manner. Values are mean of three independent preparations. Bars show +SD.

of caspase-3 could only be detected by immunoblot analysis in positive apoptosis control (Jurkat) cells after incubation with doxorubicin, while selenite exposure after 48 hr did not significantly activate procaspase-3 cleavage in U-1285 or U-1285dox cells (Fig. 4A). Caspase-3 activity in the selenite exposed U-1285dox cells was not increased compared to unexposed cells (Fig. 4B). Furthermore, addition of the caspase-3 inhibitor Z-DEVD-fmk or the general caspase inhibitor Z-VAD-fmk to the selenite exposed U-1285dox cells did not alter the number of cells in apoptosis determined by Annexin V/FACS-analysis described above (data not shown).

In the presence of 10 μM selenite 7% of doxorubicin-sensitive and 4% of the resistant cells were enlarged (necrotic) (Fig. 5A and B). After incubation with 30 μM selenite in the doxorubicin-sensitive cells (i.e. the concentration corresponding to IC_{50} for these cells [1]) the percentage of necrotic cells increased to 15% (Fig. 5A).

3.2. Mitosis

Investigation of the mitosis index in the doxorubicin-sensitive cells showed significant decrease in the interval 5–10 μM, day 2. In the doxorubicin-resistant subline, however, the decrease occurred already day 1 and in the lower concentration interval, 2–5 μM selenite.

3.3. Levels of TrxR

The levels of TrxR were measured by enzyme activity assay (insulin reduction) and ELISA.

3.3.1. Activity assay

In the doxorubicin-sensitive U-1285 cells the TrxR activity increased from 300 ng active TrxR/mg protein in absence of selenium to 620 ng/mg protein in the presence of 1 μM selenite ($P < 0.001$) (Fig. 6A). In the presence of 10 μM selenite the level of active TrxR/mg protein was 1041 ng/mg protein in these cells. The activity of TrxR increased even further in the sensitive subline up to 30 μM selenite.

In the case of the doxorubicin-resistant cells the level of active TrxR increased from the initial level of 315 ng/mg protein to 595 ng/mg protein in the presence of 1 μM selenite ($P < 0.001$) (Fig. 6B). However, at increasing concentrations of selenite the TrxR activity decreased and was only 426 ng/mg protein when the cells were exposed to 10 μM selenite.

3.3.2. ELISA

In the doxorubicin-sensitive subline the amount of TrxR increased significantly from 420 to 990 ng/mg protein upon exposure to selenite ($P = 0.01$) (Fig. 6A). In contrast, the doxorubicin-resistant subline was more refractory to selenite addition, with values of 460, 656, 640, 435, and 280 ng/mg protein after selenite exposure (Fig. 6B).

3.3.3. Comparison of the two methods

The two assays showed a statistical significant correspondence, $r^2 = 0.78$, $P = 0.0001$. However, there were some interesting discrepancies: (1) In cells not treated with selenium the amount of TrxR measured by ELISA was around 40% higher compared to the activity. (2) At a selenite concentration of 1 μM the amount measured by the two methods were nearly identical. (3) At the highest concentration of selenite the amounts measured by activity were somewhat higher compared to the level measured by ELISA.

3.4. Concentration of Trx and tTrx in cells

The resistant and sensitive sublines of U-1285 were investigated for their cellular content of Trx and tTrx. Figure 7A shows that there was no statistical significant difference in the Trx levels between the two sublines and the levels did not significantly change with selenite exposure up to 10 μM. Truncated Trx showed a profile of expression similar to Trx but at an expected lower level (Fig. 7B). However, the difference between the sublines was statistically significant ($P < 0.01$) with about double level of tTrx in the resistant subline.

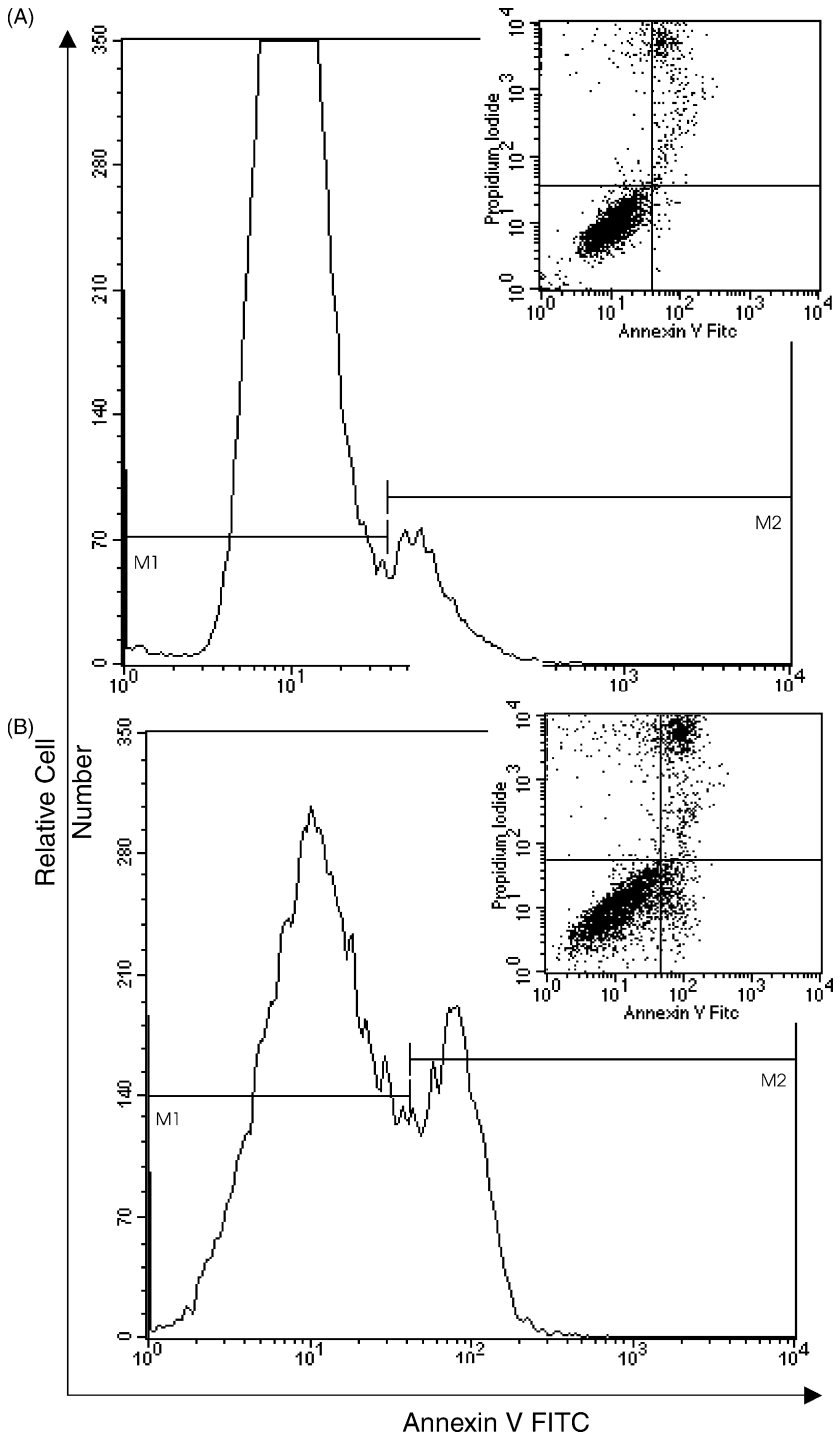

Fig. 3. Annexin V–FITC/PI-staining and FACS-analysis of apoptotic cells. U-1285 (A) and U-1285dox (B) cells in early and late apoptosis after incubation with 10 μM selenite for 2 days.

4. Discussion

This study demonstrates increased selenium-mediated apoptosis in doxorubicin-resistant human lung cancer cells compared to doxorubicin-sensitive parental cells. The difference was accompanied by decreased mitotic index and differential induction of TrxR.

Drug-resistant cells are less inclined to undergo apoptosis compared to drug-sensitive cells, as previously reported [18]. Several reports indicate that selenium can induce apoptosis [19–21], but differences between drug-resistant and -sensitive cells have not been described. In a recent study, we showed that the doxorubicin-resistant U-1285dox and GLC4/ADR cell lines were about four

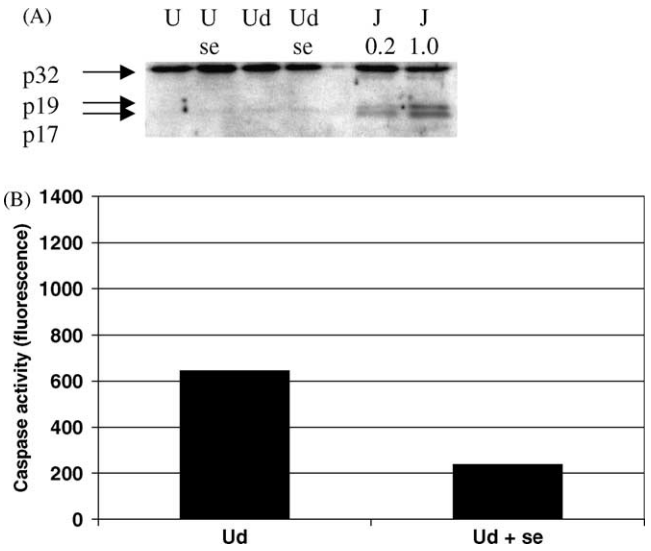

Fig. 4. (A) Immunoblot analysis of caspase-3 cleavage products were detected in cell lysate preparations from U-1285 (U) and U-1285dox (Ud) cells incubated with and without 10 μM selenite (se). As a positive control for apoptotic proteolysis Jurkat cells were exposed to doxorubicin for 48 hr at 0.2 μM (J 0.2) and for 16 hr at 1.0 μM (J 1.0). Unprocessed 32 kDa procaspase-3 and 19 and 17 kDa subunits of active caspase-3 are indicated. (B) Caspase-3 activity in U-1285dox cells. Unexposed control cells (Ud) and cells exposed to 10 μM selenite (Ud + se). Bars show relative fluorescence. The fluorescence of the positive control (Jurkat cells) was 9700 (data not shown).

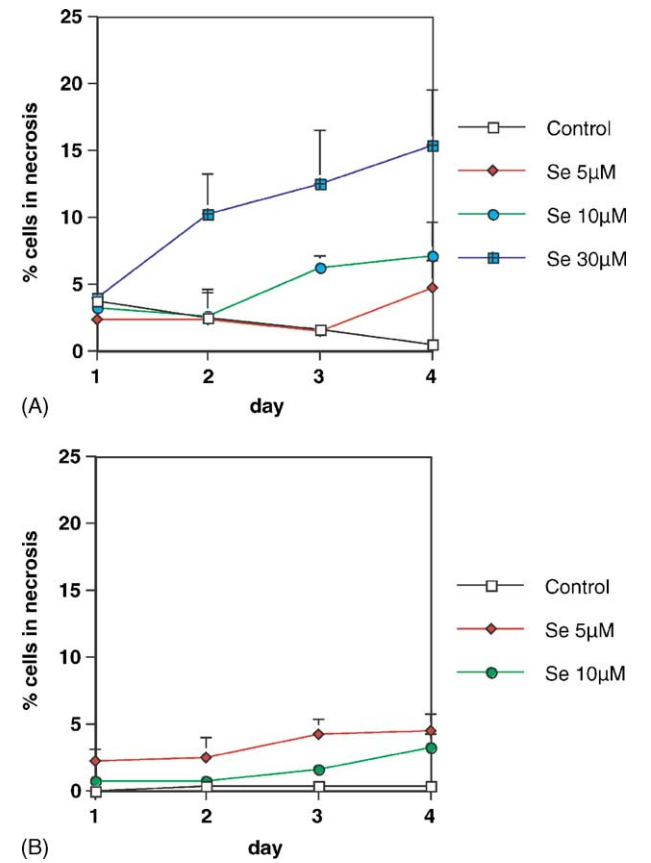

Fig. 5. Necrotic cells determined by morphological investigations in doxorubicin-sensitive U-1285 (A) and doxorubicin-resistant U-1285dox (B) in a concentration- and time-dependent manner. Bars show +SD.

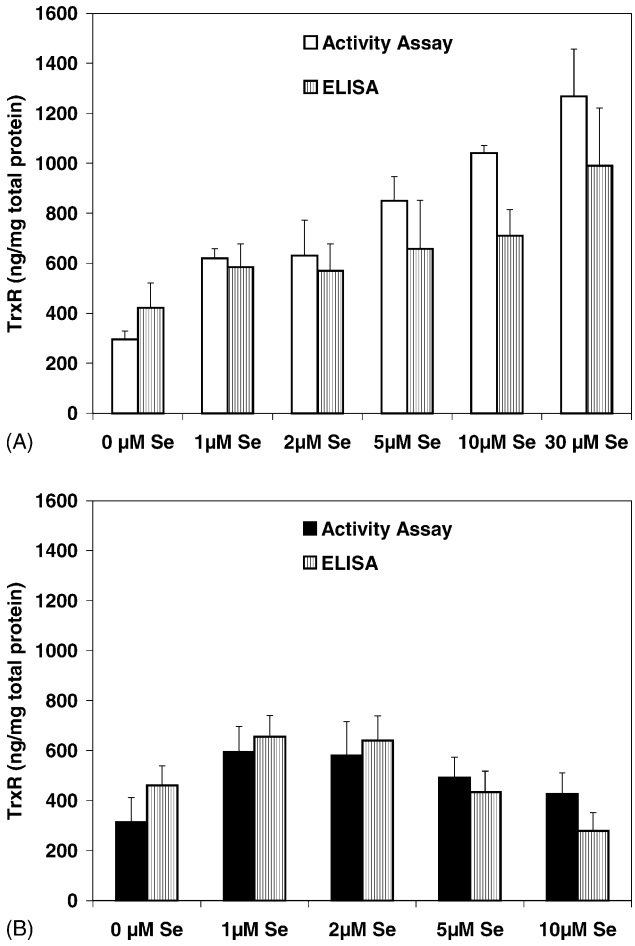

Fig. 6. TrxR measured by activity assay (insulin reduction) and by ELISA, in doxorubicin-sensitive U-1285 (A) and doxorubicin-resistant U-1285dox (B) cells cultured in the presence of 0, 1, 2, 5, and 10 μM (and 30 μM) selenite. The values are mean + SD from three repeat assays performed on extracts from three independent preparations.

times more sensitive to the cytotoxic effects of selenite compared to the parental, doxorubicin-sensitive U-1285 and GLC4 cell lines. The IC_{50} values determined by an ATP-luminescence assay for cell viability were 7 and 30 μM for U-1285dox and U-1285, 4 and 14 μM for GLC4/ADR and GLC4, respectively [1]. There was no synergism between doxorubicin and selenite and the expressions of MDR proteins (MRP-1, LRP and topoisomerase) were not affected by the selenite treatment.

In this study, we show that selenite-induced apoptosis in a significantly larger proportion of the doxorubicin-resistant cells compared to the doxorubicin-sensitive cells. The maximum apoptosis was achieved at day 2, indicating that the selenite exposure did not have an instant toxic effect. The major mechanism in the doxorubicin-sensitive cells, however, was necrosis at the higher selenite concentration (30 μM) corresponding to the 4-fold higher IC_{50} of these cells. The results indicate a specific cytotoxicity in the doxorubicin-resistant cells since the major mechanism was apoptosis. The differences in cytotoxic mechanisms between U-1285dox and U-1285 upon

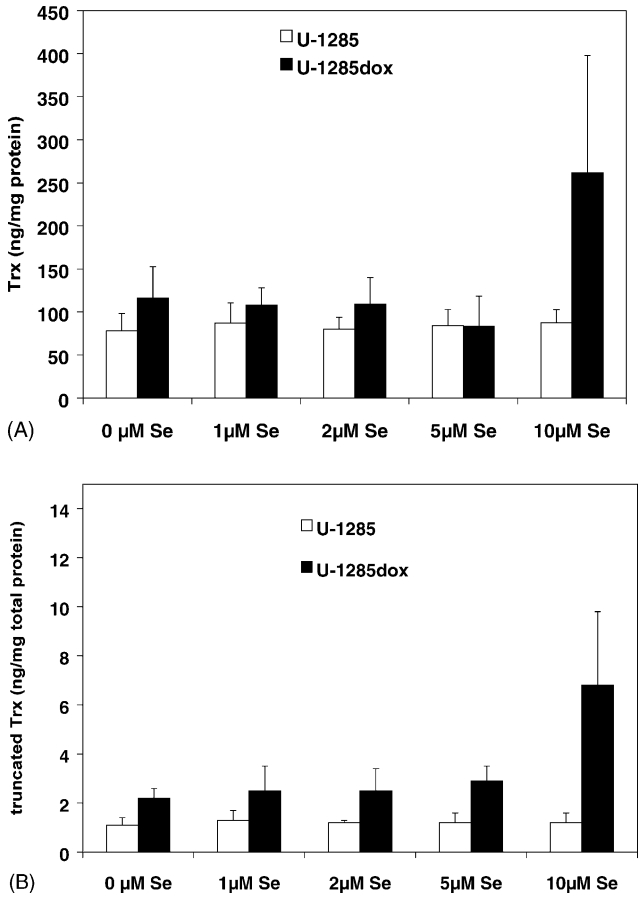

Fig. 7. Trx values (A) and tTrx (B), measured by ELISA, in U-1285 and U-1285dox cells in the presence of 0, 1, 2, 5, and 10 μM (and 30 μM) selenite. The values are mean + SD from three repeat assays performed on extracts from three independent preparations.

selenium exposure were confirmed by FACS-analysis. Although, as expected, the absolute values differed between the methods, all three experimental approaches showed several fold higher selenium-mediated apoptosis in U-1285dox cells.

Caspase-3 remained uncleaved, although the morphological investigation, the TUNEL-assay and the Annexin staining/FACS-analysis revealed a substantial apoptosis. Furthermore, there was no increase in caspase-3 activity in the selenite exposed cells and there was no alteration of the apoptosis pattern when a caspase-3 inhibitor or a general caspase inhibitor was added. Taken together, this strongly indicates a caspase-independent mechanism. Procaspase-3 cleavage was induced by treatment of U-1285 and U-1285dox by doxorubicin demonstrating that these cells may undergo caspase-3 dependent apoptosis after exposure to factors other than selenite. Furthermore, caspase-3 activation was monitored after exposure of Jurkat cells to selenite. These data clearly indicate that selenite-mediated caspase-3 activation is highly dependent on cell type. Induction of caspase-3 independent apoptosis is also supported by a recent study showing that selenite-induced apoptosis was instead associated with phosphorylation of JNK and p38 MAPK [22]. These proteins are phosphorylated and activated by apoptosis signaling kinase 1 (ASK-1), which in turn is regulated by Trx. The reduced form of Trx binds to and inactivates ASK-1, thereby inhibiting apoptosis [5]. Alternatively, selenite-induced apoptosis could operate via the caspase-independent apoptosis inducing factor pathway [23].

Selenium compounds affect several proteins of importance for cell-growth, cell-defence and regulation of apoptosis, e.g. the thioredoxin system [24], AP-1 [24], cdk2 kinase [25] and p53 [26,27]. Furthermore, selenite treatment was previously shown to accumulate cells in S-phase where also the highest activity of TrxR occur [28]. Impairment of the DNA-synthesis in the S-phase is explained by the fact that selenite and selenodiglutathione are efficient inhibitors of ribonucleotide reduction [28] and the function of several of these proteins important for cell-growth are regulated by the Trx-system.

The basal activity of TrxR was similar in the two cell lines after 2 days of incubation. After 4 days of incubation, the basal activity was higher in the doxorubicin-resistant cells [1]. This discrepancy is likely explained by differences in cell-cycle distribution since the expression of TrxR varies in the cell cycle with a peak expression during the S-phase [28]. The activity and concentration of TrxR was investigated after incubation with increasing concentrations of selenite for 2 days. Without addition of selenite the concentration (measured by ELISA) of TrxR was 40% higher compared to the activity suggesting that the enzyme was not selenium saturated. This observation is supported by previously published data of a 60–70% selenium saturation in preparations of the mammalian enzyme [29,30]. The highest relative increase in both cell types was up to 1 μM. Conditions that are close to saturation are supported by the observation that the activity and the concentration were nearly identical at 1 μM concentration. However, above this level the activity decreased and the concentrations reached a plateau in the resistant subline. In the case of the doxorubicin-sensitive cells both the activity and the protein increased with increasing selenite concentrations. The level of TrxR activity, as measured by the insulin assay, and the level of TrxR as determined by ELISA (based on the monoclonal antibody reactivity), do not discriminate between the cytosolic TrxR1 and the mitochondrial TrxR2. The TrxR2 is highly homologous to TrxR1, including the conserved active site and has in addition a 33 amino acid N-terminal extension characteristic of a mitochondrial translocation signal. The anti-TrxR antibodies used in this study recognize a common epitope of TrxR1 and TrxR2, thus both species are measured in the ELISA, as well as in the insulin activity assay.

The efficient induction of both activity and concentration of TrxR, upon exposure to increasing concentrations of selenite, in the doxorubicin-sensitive subline may explain the relatively low cytotoxicity of selenite in this

subline, since TrxR could protect the cell from selenium mediated oxidative stress. Furthermore, a high activity of TrxR ensures a high functional level of reduced Trx and may prevent apoptosis by inhibition of ASK-1, JNK and p38.

The Trx and tTrx levels were not inducible by selenite, indicating a separate regulatory mechanism for these proteins compared to TrxR. The higher level of tTrx in the doxorubicin-resistant cells is an interesting observation, since this protein is previously not associated with resistance and cellular defense. The main known function for tTrx has so far been in the immune system as a cytokine and chemokine in the activation of B cells. However, our results indicate a role also in resistant tumor disease, a role that deserves further investigation.

Our results show that selenite-induced massive, casapse-3 independent, apoptosis in doxorubicin-resistant U-1285dox cells at concentrations that to a limited extent only affected the parental doxorubicin-sensitive U-1285 cells. Selenium-mediated selective induction of apoptosis in therapy-resistant cells is an interesting observation especially since drug-resistant cells are known to be less inclined to undergo apoptosis by other known agents. Redox regulation by selenium offers novel possibilities for future treatment of resistant tumor diseases and we are currently investigating effects of selenium on clinical material.

Acknowledgments

The authors are grateful to Prof. Jonas Bergh at Radiumhemmet, Karolinska Hospital, Sweden, for kindly providing the U-1285 and U-1285dox cell lines. We are also grateful to Hernán Concha Quezada for valuable help with the FACS-analysis, Sofia Bengtzén and Christina Nyström for excellent technical assistance and to Jan Videsäter for valuable help in the preparation of illustrations.

This investigation was supported by grants from The Swedish Cancer Society no. 4238-B00-02XBB (M.B.), 016772, (C.P.), 1682-B01-15XAB (L.C.E.), 3171 B00-12xAG. GSD (A. R.), Magnus Bergvall's Stiftelse and Karolinska Institutet.

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