Superoxide anion inhibits drug-induced tumor cell death

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Abstract Intracellular superoxide ($O_2^{\bullet-}$) was manipulated in M14 melanoma cells by overexpression or repression of Cu/Zn SOD using a tetracycline-inducible expression system. Scavenging intracellular $O_2^{\bullet-}$ increased tumor cell sensitivity to daunorubicin, etoposide, and pMC540, whereas expression of the antisense SOD mRNA significantly decreased cell sensitivity to drug treatment. Whereas Cu/Zn SOD overexpressing cells exhibited higher activation of the executioner caspase 3 upon drug exposure, caspase 3 activation was significantly lower when Cu/Zn SOD was repressed by antisense expression. These data show that intracellular $O_2^{\bullet-}$ regulates tumor cell response to drug-induced cell death via a direct or indirect effect on the caspase activation pathway.

Key words: Superoxide anion; Apoptosis; Caspase; Chemotherapy

1. Introduction

Intracellular reactive oxygen species (ROS) are produced partly as byproducts of normal metabolism, and partly via activation of ROS-producing enzymes in response to exogenous stimuli [1–3]. Because intracellular accumulation of ROS is toxic [1,4], levels of ROS are tightly regulated by multiple defense mechanisms involving small anti-oxidant molecules which contain sulfhydryl groups and ROS-scavenging enzymes, like superoxide dismutase (SOD), catalase, and glutathione peroxidase [1,5]. Alterations of any of these components of the oxidant defense system modulate the fate of ROS in the cell by catalyzing reactions to remove $O_2^{\bullet-}$ and hydrogen peroxide (H_2O_2). The observation that tumor cells often have lower SOD activity [6] has resulted in the notion that an accumulation of intracellular ROS, or more generally a pro-oxidant state, provides tumor cells with a survival advantage over their normal counterparts. One possible explanation could be that $O_2^{\bullet-}$ or H_2O_2 might function as mitogenic stimuli [7]. However, our earlier findings suggest an important role for $O_2^{\bullet-}$ in tumor cell sensitivity to cell death induced by cell surface receptor CD95 [8,9].

Most anticancer agents kill tumor cells by an active process which involves key systems of the physiologic apoptosis program [10–13], characterized by a series of events culminating in specific morphological and biochemical changes leading to cell death [14–17]. The central executioners in the apoptotic pathway are members of a family of intracellular cysteine proteases, caspases [18]. Activation of one or other of the caspase members can trigger a cascade of events leading to cell death via proteolytic degradation of a number of cellular proteins, e.g. lamin, actin, and poly(ADP-ribose) polymerase [19–24].

Stimulated by these findings, we set out to investigate if the inhibition of tumor cell death by increased intracellular $O_2^{\bullet-}$ is restricted to the CD95 system or could also be a mechanism underlying tumor cell response to drug-induced cell death.

2. Materials and methods

2.1. Cells

Human melanoma cell line M14 was a generous gift from Dr. Armando Bartolazzi (Oncologica Clinica e Sperimentale, Rome, Italy) and was maintained in DMEM (Hyclone, Irvine, CA, USA) supplemented with 5% fetal bovine serum (FBS) (Hyclone), 1% glutamine and 0.5% gentamicin (Gibco-BRL, Gaithersburg, MD, USA).

2.2. Cloning of Cu/Zn SOD cDNA in the sense or antisense orientation

Cu/Zn SOD cDNA was amplified using the Quick clone human cDNA library from human leukocytes as template with the Advantage cDNA PCR Kit (Clontech Laboratories, Inc., Palo Alto, CA, USA). The following oligonucleotide primers were used to amplify the full length Cu/Zn SOD cDNA: forward with a *Mlu*I restriction site 5′-CAC GCG ACG CGT ATG GCG ACG AAG GCC GTG TGC GTG CTG AAG-3′ and reverse with a *Sal*I restriction site 5′-CAC GCG GTC GAC TTA TTG GGC GAT CCC AAT TAC ACC ACA AGC-3′, whereas for antisense cDNA amplification we used: forward with a *Sal*I restriction site 5′-CAC GCG GTC GAC ATG GCG ACG AAG GCC GTG TGC GTG CTG AAG-3′ and reverse with a *Mlu*I restriction enzyme site 5′-CAC GCG ACG CGT TTA TTG GGC GAT CCC AAT TAC ACC ACA AGC-3′. The PCR amplified SOD cDNA (464 bp) in the sense orientation was cloned into the bidirectional vector pBiL, whereas the bidirectional vector pBiG was used for antisense cloning. All vectors were purchased from Clontech.

2.3. Development of a tetracycline-responsive transactivator protein expressing cell line

M14 cells were stably transfected with the pTet-off plasmid. This vector contains the gene for the tetracycline-responsive transactivator protein (tTA protein) and a gene for resistance to geneticin (G418). Stably transfected cells were selected using 1 mg/ml G418 (Gibco-BRL). After 4–6 weeks, positive clones were screened for tTA expression by transient transfection with the pBiGL reporter. The pBiGL reporter plasmid expresses both the *LacZ* and the luciferase genes, which provides two functional assays for tTA regulatory protein expression. Two cell lines, M14 Tet-off 1 and M14 Tet-off E, gave high β-galactosidase ('β-Gal staining set' from Boehringer Mannheim, Indianapolis, IN, USA) and luciferase (Luciferase Assay System from Promega Corporation, Madison, WI, USA) activities in the absence of tetracycline.

2.4. Development of Cu/Zn SOD and SOD antisense expressing M14 cell lines

M14 Tet-off E was transfected for a second time with pBiL re-

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sponse plasmid containing the Cu/Zn SOD cDNA or the pBiG response plasmid containing the antisense SOD cDNA, and the pTK-Hyg plasmid containing the hygromycin resistance gene. Stably transfected clones were screened for their ability to express β-galactosidase or luciferase under the regulation of tetracycline. Two clones were identified and amplified, the M14 SOD1 clone expressing Cu/Zn SOD and M14 AS-SOD3 antisense clone. Transfections of all cell lines were performed using the SuperFect Transfection Reagents from Qiagen Gmbh (Germany) according to the vendor's instructions.

2.5. Cell death triggers

Etoposide was obtained from Clontech, merocyanine 540 (MC540) from Sigma Chemical Co. (St. Louis, MO, USA), and daunorubicin (daunomycin hydrochloride) from ICN Biochemicals (Aurora, OH, USA). MC540 was photoactivated (pMC540) as described elsewhere [13].

2.6. Immunofluorescence for intracellular Cu/Zn SOD

M14 cells (1×10^6) were fixed in 1 ml of 4% paraformaldehyde for 15 min on ice. After a wash with phosphate buffered saline (PBS), cells were permeabilized in ethanol for 1 h on ice, washed with PBS and incubated with 1 μg of monoclonal anti-Cu/Zn SOD IgG2a (Pharmingen, San Diego, CA, USA) for 30 min on ice. Following another wash with PBS+1% FBS samples were exposed to FITC-conjugated goat anti-mouse IgG for 30 min, washed and resuspended in 0.5 ml of 2% paraformaldehyde, and analyzed by flow cytometry.

2.7. Chemiluminescence assay for detection of intracellular $O_2^{\bullet-}$

The lucigenin-based chemiluminescence assay was performed as previously described [8]. Chemiluminescence was monitored for 60 s in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Data are shown as relative light units per 60 s (RLU/60 s) ± S.D. from three independent measurements.

2.8. Cytotoxicity assays

To trigger drug-induced cell death, 2×10^4 cells/well were plated in 96-well microtiter plates in DME+5% FBS 24 h prior to the test. After 18 h of incubation with the anticancer drugs, the medium was aspirated and crystal violet staining was used to assess cell viability as described previously [8].

2.9. Annexin V staining for detection of apoptotic cells

Externalization of phosphatidylserine was assayed by the ApoAlert-Annexin V Apoptosis kit (Clontech). Briefly, M14 cells (1×10^6/ml) were exposed to 20 μM etoposide or 150 μg/ml of pMC540 or 0.4 μg/ml of daunorubicin for 12 h and cell surface staining and flow cytometry were performed as described elsewhere [13].

2.10. Determination of caspase 3 activity

Caspase 3 activity was assayed by the ApoAlert CPP32 Fluorescent Assay kit (Clontech). M14 SOD1 and AS-SOD3 cells (1×10^6 cells/ml) were exposed to etoposide (20 or 40 μM), pMC540 (150 μg/ml) or daunorubicin (0.4 μg/ml) for 4–12 h. Cells were then resuspended in 50 μl of chilled cell lysis buffer (provided by the supplier) and incubated on ice for 10 min. 50 μl of 2× reaction buffer containing 10 mM dithiothreitol and 5 μl of the substrate DEVD-AFC were added to each sample and incubated at 37°C for 30 min. Detection of protease activity was performed by measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a spectrofluorimeter (Luminescence Spectrometer LS50B, Perkin Elmer, Buckinghamshire, UK).

2.11. Western blot analysis

Lysates from the Cu/Zn SOD clone or the M14 AS-SOD3 clone (5×10^6 cells) were prepared following incubation in the presence and absence of 2 μg/ml tetracycline using 0.5 ml of RIPA/NP-40 lysis buffer (5 mM Tris pH 7.4, 30 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, and 0.5% NP-40). 30 μg of total protein

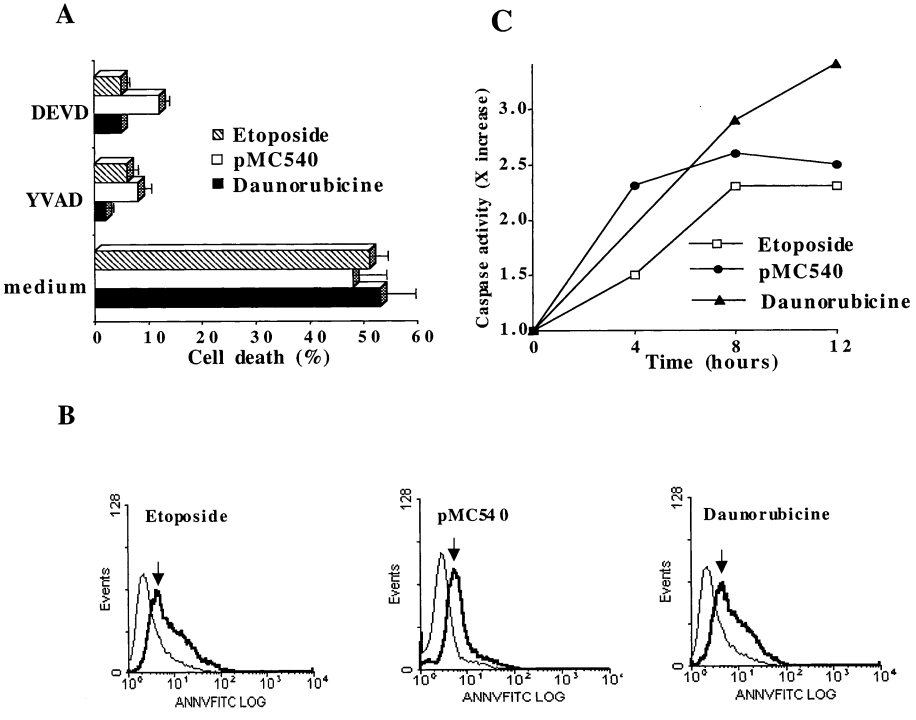

Fig. 1. Etoposide, pMC540, and daunorubicin induce apoptotic cell death in M14 cells. M14 cells (1×10^6 cells/ml) were exposed to 20 μM etoposide or 150 μg/ml of pMC540 or 0.4 μg/ml of daunorubicin for 18 h and cell death was determined by the crystal violet assay as described in Section 2. To assess the effect of caspase inhibitors on drug-induced cell death, M14 cells were treated with 100 μM DEVD-CHO or YVAD-CHO prior to the addition of the three triggers. Data shown are mean ± S.D. of three independent experiments performed in triplicate. B: M14 cells were treated as above for 12 h and externalization of PS was detected by annexin V-FITC (1 μg/ml) staining and analyzed by flow cytometry. At least 10 000 events were analyzed by WINMDI software. Arrows indicate log increase in fluorescence over untreated control cells. C: Caspase 3 activity was assayed in lysates of drug-exposed M14 cells (4–12 h) as described in Section 2. Data shown are representative of at least three independent observations and expressed as fold increase in activity over the untreated M14 cells (1×).

was subjected to 10% PAGE, transferred to nitrocellulose, blocked overnight with 5% dried milk in Tris buffered saline/0.1% Tween 20 (TBST), blotted with 2 μg/ml of a polyclonal sheep anti-human Cu/Zn SOD (UBI, Lake Placid, NY, USA) for 1 h at room temperature. After three washes with TBST, the Cu/Zn SOD protein was detected using 1:5000 dilution of an anti-sheep HRP-conjugated IgG (Pierce, Rockford, IL, USA) and visualized by the SuperSignal Substrate Western Blotting kit (Pierce). The autoradiogram was scanned using the Scan Jet 4C/T scanner from Hewlett Packard and analyzed with Desk Scan software. Results are shown in density level per square inch (level × sq.in).

3. Results

3.1. Etoposide, pMC540, and daunorubicin trigger cell death via caspase 3 activation in M14 cells

Exposure of M14 cells to pMC540 (up to 150 μg/ml) induces cell death by apoptosis [13]. Similar results were obtained following exposure of M14 cells to 20 μM etoposide or 0.4 μg/ml of daunorubicin as shown in Fig. 1. We used annexin V staining and caspase 3 activity as markers of apoptosis. All three drugs induced cell death in M14 cells (Fig. 1A) with characteristic apoptotic morphology (Fig. 1B), and induced activation of caspase 3 (Fig. 1C). Caspase 3 activation was detected as early as 4 h after treatment and peaked by 12 h (Fig. 1C). The role of caspases in the death process induced by etoposide, pMC540, and daunorubicin in M14 cells was confirmed by the ability of YVAD-CHO (100 μM) and DEVD-CHO (100 μM) to inhibit cell death (Fig. 1A). Hence, through this set of experiments we demonstrate that all three anticancer agents used in our study induced cell death in M14 melanoma cells via activation of the apoptotic machinery.

3.2. Effects of modulation of intracellular $O_2^{\bullet-}$ on tumor cell sensitivity to anticancer drugs via the caspase 3 activation pathway

We used a tetracycline-inducible system whereby the expression of Cu/Zn SOD could be manipulated in vivo. Selective gene expression is induced upon removal of tetracycline. Stable clones of M14 melanoma cell line were generated where the level of Cu/Zn SOD protein could be increased by induction of transcription of Cu/Zn SOD cDNA (M14 SOD), or decreased by transcription of an antisense SOD mRNA (M14 AS-SOD). In the absence of tetracycline for 48 h, the M14 SOD1 clone showed an increase of 1.78-fold (89%) in the expression of Cu/Zn SOD protein compared to the same clone where overexpression of Cu/Zn SOD was inhibited by the presence of 2 μg/ml tetracycline (Fig. 2). In contrast, the M14 AS-SOD3 clone showed a 30% decrease in the Cu/Zn SOD protein level when cultured in the absence of tetracycline as shown in Fig. 2. It should be pointed out that in the presence of tetracycline the level of SOD expression was not significantly different in the two clones. Hence, the level of SOD expression was 2.6-fold higher in the M14 SOD1 clone than in M14 AS-SOD3 in the absence of tetracycline. In a separate set of experiments, we confirmed our Western blot data by immunofluorescence staining for Cu/Zn SOD in the clones (Table 1). In addition, to ascertain the effect of overexpression or repression of Cu/Zn SOD on the intracellular $O_2^{\bullet-}$ level, we directly measured intracellular $O_2^{\bullet-}$ using a lucigenin-based chemiluminescence assay as described in Section 2. As shown in Table 1, M14 cells overexpressing Cu/Zn SOD (M14 SOD 'ON') showed a drop in intracellular $O_2^{\bullet-}$ concentration, whereas antisense expression (M14 SOD antisense 'ON') resulted in an increase in $O_2^{\bullet-}$ levels compared to the clone cultured in the presence of tetracycline (M14 SOD antisense 'OFF'). An increase or decrease in Cu/Zn SOD level did not affect tumor cell survival in culture.

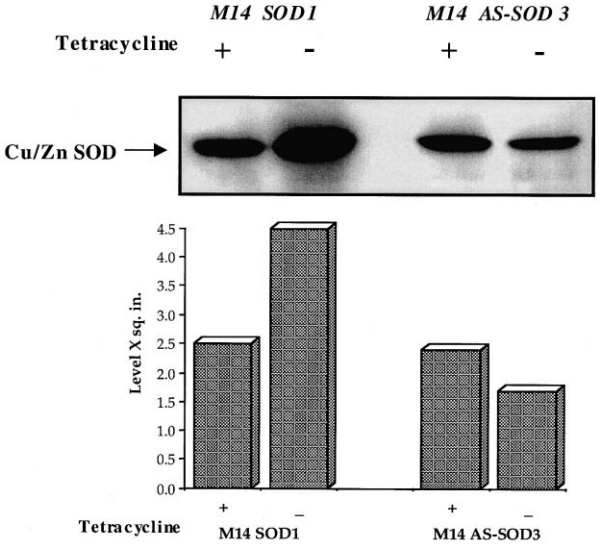

Fig. 2. Western blot analysis of Cu/Zn SOD expression in lysates of M14 SOD1 and M14 AS-SOD3 clones. 5×10^6 of M14 SOD1 and M14 AS-SOD3 clones were cultured in the presence of 2 μg/ml of tetracycline (+) or 48 h after the removal of tetracycline (−). Western blot analysis of Cu/Zn SOD was performed as described in Section 2.

Table 1
Analysis of intracellular Cu/Zn SOD and determination of intracellular O_2^- concentration in SOD1 and AS-SOD3 clones

Clone	Tet	SOD-FITC (MFI)	O_2^- (RLU/60 s) ± S.D.
SOD1	+	214	0.540 ± 0.059
	−	386	0.262 ± 0.095
AS-SOD3	+	256	0.498 ± 0.06
	−	180	0.588 ± 0.084

Intracellular Cu/Zn SOD was determined by flow cytometry using an anti-human Cu/Zn SOD IgG and secondary anti-mouse FITC-conjugated IgG. 1×10^6 cells were stained and 10 000 events were analyzed. Data are shown as mean fluorescence intensity (MFI). For detection of intracellular O_2^-, 2×10^6 cells were lysed as described in Section 2, and O_2^- was immediately measured by a lucigenin-based chemiluminescence assay. Data are shown as relative light units/60 s (RLU/60 s), and indicate mean ± S.D. of three independent measurements.

M14 SOD1 and M14 AS-SOD3 clones were subsequently tested for their sensitivity to etoposide-, pMC540-, and daunorubicin-induced cell death in the presence or absence of tetracycline. Overexpression of intracellular Cu/Zn SOD in the M14 SOD1 clone, by removal of tetracycline for 48 h (SOD 'ON'), increased cell death in response to all anticancer agents (Fig. 3A–C). In contrast, a decrease in Cu/Zn SOD protein level in the M14 AS-SOD3 clone upon culturing without tetracycline (SOD antisense 'ON') decreased sensitivity to anticancer agents as compared to the sensitivity in the presence of tetracycline (SOD antisense 'OFF') (Fig. 3A–C). Furthermore, whereas the percentage of cell death to all triggers obtained with both M14 SOD1 and AS-SOD3 clones in the 'OFF' state was comparable, the percentage of cell death in the SOD 'ON' state (higher sensitivity) was consistently 2–4-fold higher as compared to that obtained with the cells in the

SOD antisense 'ON' state (lower sensitivity). These data indicate that intracellular level of Cu/Zn SOD can regulate sensitivity of tumor cells to a variety of anticancer drugs. To confirm that this effect was mediated via $O_2^{\bullet-}$, we tested the effect of tiron, an $O_2^{\bullet-}$ scavenger [25]. Our results (Fig. 4A,B) demonstrate that preincubation of M14 cells in the SOD antisense 'ON' state with 1 mM tiron (SOD antisense 'ON'/tiron) enhanced tumor cell death sensitivity to daunorubicin and etoposide compared to that obtained when the SOD antisense was not expressed (SOD antisense 'OFF'). Moreover, the presence of tiron in the SOD antisense 'OFF' (SOD antisense 'OFF'/tiron) state further increased tumor cell sensitivity to both cell death triggers to a level similar to that observed when M14 cells were overexpressing SOD (SOD 'ON') (Figs. 3A,C and 4A,B). We addressed the possibility that the increase in tumor cell sensitivity to anticancer agents in response to overexpression of SOD may be due to increased production of H_2O_2. However, co-culturing cells with catalase did not inhibit the increase in tumor cell death in response to anticancer agents in cells tailored to overexpress Cu/Zn SOD (data not shown).

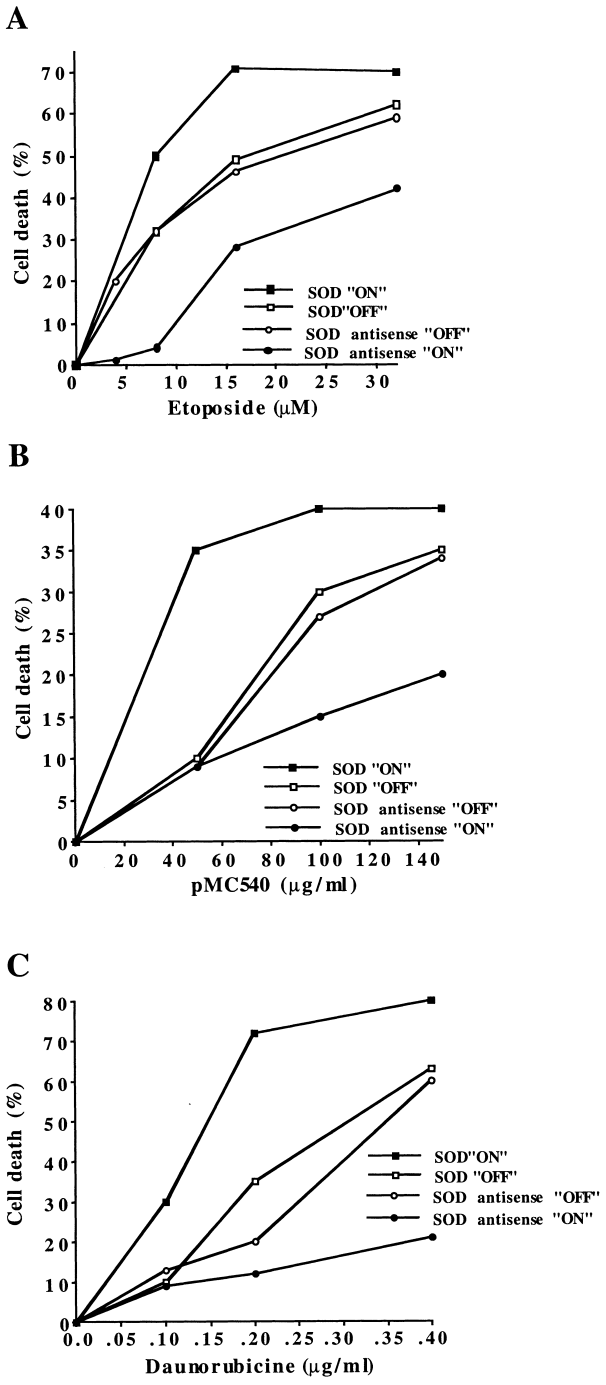

Fig. 3. Sensitivity of the M14 SOD1 clone and the M14 AS-SOD3 clone to cell death triggered by increasing concentrations of etoposide or pMC540 or daunorubicin. Cell death of the M14 SOD1 clone and the M14 AS-SOD3 clone in the presence of tetracycline (SOD 'OFF' or SOD antisense 'OFF') or following removal of tetracycline for 48 h (SOD 'ON' or SOD antisense 'ON') was determined following 18 h treatment with increasing concentrations of (A) etoposide, (B) pMC540 or (C) daunorubicin.

The caspase 3 activities correlated with the cytotoxicity data. Exposure to 20 μM etoposide resulted in a 2.4-fold increase in caspase 3 activity in the presence of tetracycline (SOD 'OFF' and AS-SOD 'OFF'), but caspase 3 activity was higher in the SOD 'ON' (3.4-fold increase in activity over the control cells) than in the SOD antisense 'ON' state (1.8-fold increase in activity over the control cells) as shown in Fig. 5A. Similar results were obtained with 0.4 μg/ml of daunorubicin and 150 μg/ml of pMC540 (Fig. 5B and data not shown).

4. Discussion

Evidence for the involvement of ROS in tumor promotion derives from the earlier observations that free radical scavengers could inhibit numerous actions of tumor promoters [26]. Thus a pro-oxidant state might provide tumor cells with a survival advantage over their normal counterparts [9,26]. Here we provide evidence that the intracellular $O_2^{\bullet-}$ level is a central regulator of tumor cell response to apoptosis induced by a variety of anticancer drugs. Since our previous report showed inhibition of CD95-induced apoptosis by intracellular $O_2^{\bullet-}$ [8], one possible explanation for our results could have been an indirect activation of the CD95 pathway in M14 cells following incubation with the different drugs [27–30]. However, M14 melanoma cells are deficient in both the death receptors CD95 and CD120a [8], and none of the apoptotic triggers used in our study induced expression of either of the receptors (S. Pervaiz and M.-V. Clément, unpublished data).

In order to investigate the inhibitory effect of increased intracellular $O_2^{\bullet-}$ concentration on apoptotic cell death, we manipulated the intracellular $O_2^{\bullet-}$ level by overexpressing or decreasing the cytoplasmic Cu/Zn SOD protein level in vivo using a tetracycline-inducible expression system. This allows the same clone to be analyzed in the presence or absence of tetracycline. M14 cells tailored to overexpress or suppress cytoplasmic Cu/Zn SOD showed a direct relationship between intracellular Cu/Zn SOD, $O_2^{\bullet-}$ concentrations, and tumor cell sensitivity to apoptotic triggers. Overexpression of Cu/Zn SOD enhanced tumor cell sensitivity to drug treatment, in agreement with a recent finding that overexpression of Cu/Zn SOD increased sensitivity of mouse neurons transgenic for Cu/Zn SOD to apoptosis induced by kainic acid [31]. Conversely, our results with the M14 antisense SOD clone demonstrated that a decrease in intracellular Cu/Zn SOD in tumor cells protected them from apoptosis induced by anticancer drugs. The role of intracellular $O_2^{\bullet-}$ in regulating tu-

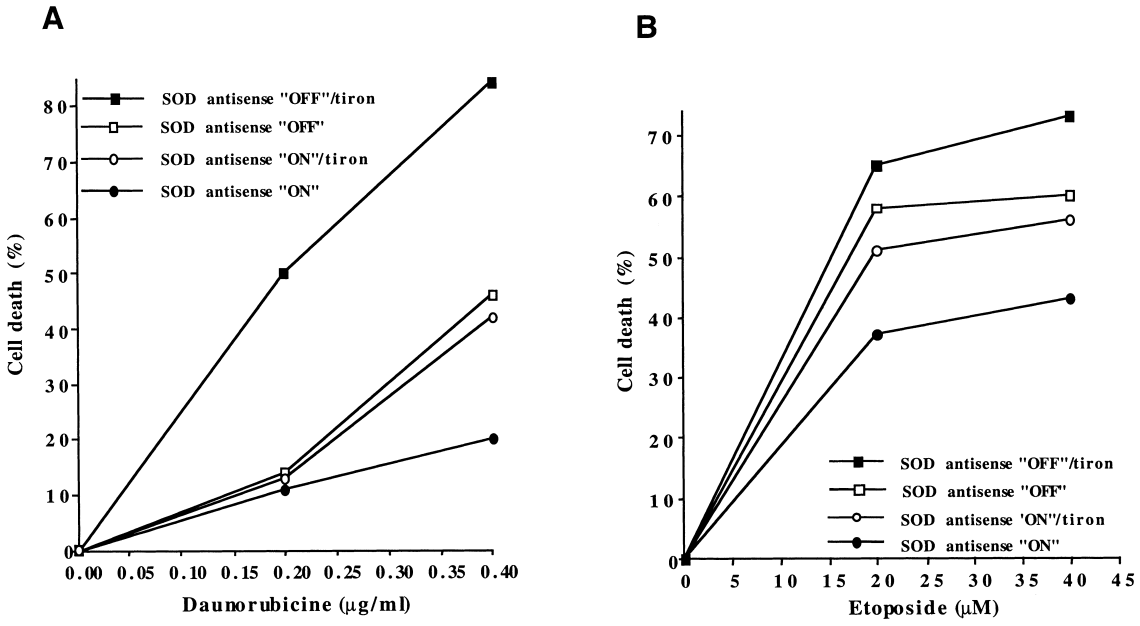

Fig. 4. Effect of tiron on the sensitivity of the M14 AS-SOD3 clone to cell death induced by daunorubicin or etoposide. M14 AS-SOD3 cultured with (SOD antisense 'OFF') or without (SOD antisense 'ON') 2 μg/ml tetracycline was tested for sensitivity to (A) daunorubicin or (B) etoposide in the presence of 1 mM tiron (SOD antisense 'OFF'/tiron and SOD antisense 'ON'/tiron). Cell death was determined by the crystal violet assay.

mor cell sensitivity thus appears not to be exclusive for CD95 signaling-dependent apoptosis, but may be common to a variety of anticancer drugs known to induce cell death by activation of the apoptotic machinery. We addressed the possibility that the increase in tumor cell sensitivity to anticancer agents, in response to overexpression of SOD, may be due to increased production of H_2O_2. However, co-culturing cells with catalase did not inhibit the increase in tumor cell death in response to anticancer agents in cells tailored to overexpress Cu/Zn SOD (data not shown). These data are further supported by a recent report which demonstrates that Cu/Zn SOD overexpressing cells have reduced steady-state level of $O_2^{\bullet-}$ as well as H_2O_2 and an increased reducing environment [32]. In light of our findings, the survival advantage that a pro-oxidant environment provides the tumor cells may well be a function of not only increased proliferation of cells [7,33], but also resistance to apoptotic signal.

Through these series of experiments we have established that intracellular $O_2^{\bullet-}$ regulates tumor cell sensitivity to drug-induced apoptosis. However, the intracellular target of $O_2^{\bullet-}$ remains undefined. One logical candidate could be the caspases, the central executioner of the apoptotic pathway [34]. Indeed, an increase in Cu/Zn SOD level facilitated caspase 3 activation, whereas a decrease of Cu/Zn SOD protein

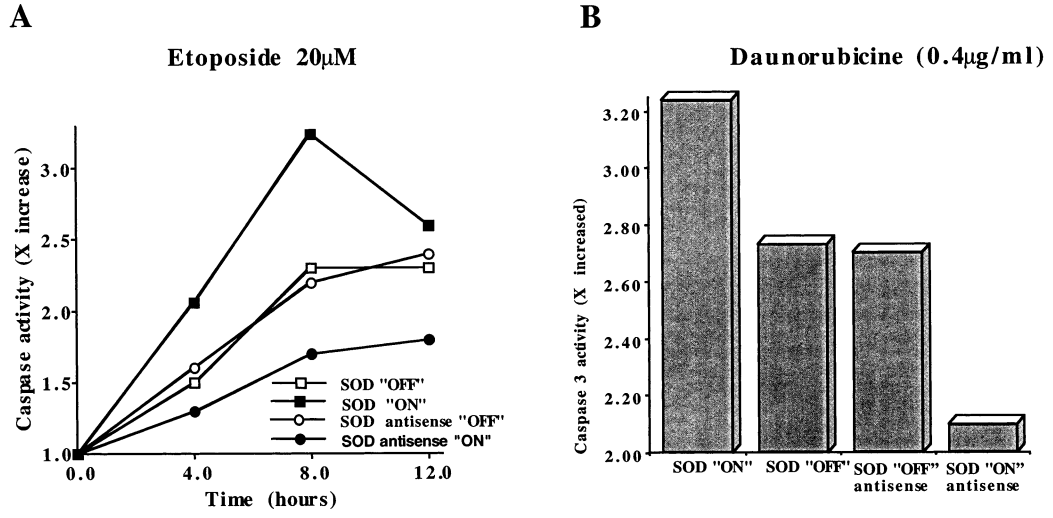

Fig. 5. Caspase 3 activation following induction of cell death with daunorubicin and etoposide. The M14 SOD1 and M14 AS-SOD3 clones were incubated with 20 μM (A) etoposide for 4–12 h, or (B) daunorubicin (0.4 μg/ml) for 12 h in the presence of 2 μg/ml tetracycline (SOD 'OFF' and SOD antisense 'OFF') or 48 h after the removal of tetracycline (SOD 'ON' and SOD antisense 'ON'). Caspase activity is represented as fold increase over the activity obtained for cells without the apoptotic trigger (1×).

level lowered caspase 3 activation, thus suggesting that regulation of tumor cell sensitivity to apoptotic triggers by intracellular $O_2^{\bullet-}$ may be via its effect on intracellular caspase activation. Is the effect of scavenging intracellular $O_2^{\bullet-}$ directly on caspase activation or on events upstream of the caspase cascade? The targets of $O_2^{\bullet-}$ in the regulation of tumor cell response to apoptosis are the subjects of our ongoing investigations.

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