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Production of Intracellular Superoxide Mediates Dithiothreitol-Dependent Inhibition of Apoptotic Cell Death

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ABSTRACT

Apoptotic cell death proceeds from the activation of cysteine proteinases called caspases. As full enzymatic activity of caspases requires reduction of cysteine residues in and around the catalytic site of the proteases, cysteine-reducing agents such as dithiothreitol (DTT) are expected to facilitate caspase activity upon induction of apoptosis. However, DTT has been shown to efficiently protect cells from apoptosis. The mechanism involved in DTT-mediated inhibition of apoptosis has been attributed to its antioxidant activity. Interestingly, under physiological conditions, thiol–mediated antioxidant reaction has also been shown to result in intracellular generation of superoxide ($O_2^{\bullet -}$). In line with our earlier findings implicating a slight prooxidant state in resistance to apoptosis, we set out to investigate if the death-inhibitory activity of DTT could be mediated by intracellular $O_2^{\bullet -}$. Our results show that incubation of human melanoma cell line M14TF or human bladder carcinoma cell line T24 with DTT induced an increase in intracellular $O_2^{\bullet -}$ with concomitant inhibition of apoptosis triggered by CD95 signaling, staurosporine, or hydrogen peroxide. Moreover, preincubation of either cells with Tiron, a specific $O_2^{\bullet -}$ scavenger, reverted DTT-induced inhibition of apoptosis. These results show that the apoptosis-inhibitory activity of DTT may not be due to its antioxidant property, but instead linked to its ability to induce an increase in intracellular $O_2^{\bullet -}$ level. *Antioxid. Redox Signal.* 7, 456–464.

INTRODUCTION

APOPTOSIS is an important physiological cell death process triggered by a family of cysteine proteases called caspases. Caspases are normally present in the cells as proenzymes that require proteolysis for enzymatic activity. The activated caspases require reduction of a catalytic site cysteine residue, as well as other cysteine residues around the catalytic site for enzymatic activity (13, 14). Thiol alkylating agents and spontaneous thiol oxidation inhibit caspase activity. Hence, by their ability to reduce cysteine thiols, compounds such as dithiothreitol (DTT) would be expected to favor caspase activation and apoptotic cell death. However, DTT is a well-known inhibitor of apoptosis, specifically during radiation-induced apoptosis (3, 16). In agreement with the opinion that reactive oxygen species (ROS) participate in the induction of apoptosis by a variety of triggers (4), inhibition of apoptotic cell death by thiols such as DTT has been attributed to their efficient antioxidant capacity (19). However, the observation that hypoxia can induce apoptosis supports the argument that ROS are not always necessary for mediating apoptotic cell death (15, 28). In addition, we and others have shown that a slight increase in intracellular ROS, in particular superoxide ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2), inhibits apoptotic signaling in tumor cells, irrespective of the trigger (5–8, 11, 12, 23–26).

Interestingly, during thiol–mediated antioxidant reaction, thiols undergo one-electron oxidation with the formation of thiyl radicals. In addition, the protective and repairing efficacy of thiols depends not only on their capacity to detoxify free radical, but also on the chemical character and reactivity of the formed thiyl radicals. Under physiological conditions, thiyl radicals can react with thiolate anion yielding disulfide radical anion $(RSSR)^-$ as an intermediate and finally disulfides and $O_2^{\bullet -}$ (30). Hence, the main goal of this study was to

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assess if the antiapoptotic activity of the model thiol compound DTT could be a function of an increase in intracellular $O_2^{\bullet-}$ rather than its ROS-scavenging capacity. Here we show that DTT treatment of M14 human melanoma cells expressing a fusion protein consisting of the extracellular domain of the tumor necrosis factor (TNF) receptor p55 (TNF-R p55) and the transmembrane and intracellular domains of CD95 (M14TF) (7) and T24 human bladder carcinoma cells resulted in a significant inhibition of apoptosis triggered by CD95, staurosporine, or H_2O_2, which was accompanied by a significant increase in intracellular $O_2^{\bullet-}$. Moreover, the presence of Tiron, a selective scavenger of $O_2^{\bullet-}$, prevented DTT-dependent production of intracellular $O_2^{\bullet-}$ and reverted the inhibitory effect of DTT on apopotosis. Taken together, these results support the critical role of intracellular $O_2^{\bullet-}$ production in DTT-mediated inhibition of apoptotic cell death.

MATERIALS AND METHODS

Reagents

DTT, Tiron (4,5-dihydrooxy-1,3-benzene disulfonic acid), crystal violet, lucigenin, and diethyldithiocarbamate (DDC) were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The pan-caspase inhibitor ZVAD and the caspase-specific peptides were purchased from Bio-Rad Laboratories, (Hercules, CA, U.S.A.). Lucigenin stock solution (3.5 m*M*) was diluted in distilled water.

Cells

The human melanoma M14TF cell line was developed in the human melanoma cell line M14 as described previously (7). The human bladder carcinoma T24 cell line was purchased from ATCC (Rockville, MD, U.S.A.). M14TF cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum (both from HyClone, Logan, UT, U.S.A.) and 1 mg/ml G418 (GibcoBRL, Gaithersburg, MD, U.S.A.). T24 cells were maintained in McCoy's media (GibcoBRL) supplemented with 5% fetal bovine serum.

Cell viability assay

Cell viability was assessed using the crystal violet assay, performed in 96-well microtiter plates as described previously (7). Cell viability was determined by dye absorbance at 595 nm on an automated ELISA reader and calculated as the mean of triplicate optical density (OD) values of cells incubated with the specific apoptotic inducer divided by the mean of triplicate OD values of cells incubated in culture medium, and expressed as a percentage. The standard error of the mean of triplicate OD values never exceeded 5%. The data presented show the means ± SE of two experiments done in triplicate.

$O_2^{\bullet-}$ measurement

The intracellular level of $O_2^{\bullet-}$ was assessed using a lucigenin-based chemiluminescence assay as previously described (7). Chemiluminescence was monitored for 60 s in a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, U.S.A.). Data are shown as relative light units (RLU) in DTT-treated cells over RLU in cells left in normal medium (control cells) expressed as the percentage of untreated control cells (% of control cells).

Caspase activity

Activities of caspases 3, 8, and 9 were detected using the tetrapeptide inhibitors Ac-DEVD-AFC (for caspase 3), Ac-LETD-AFC (for caspase 8), and Ac-LEHD-AFC (for caspase 9) peptides. Cells were incubated with or without DTT for 1 h prior to the induction of apoptosis. Caspase activity was determined as described previously (9).

RESULTS

DTT inhibits CD95-mediated apoptosis in M14TF cells via an increase in intracellular $O_2^{\bullet-}$

The effect of DTT on cell sensitivity to apoptosis was first assessed using the human melanoma cell line M14TF. M14TF cells express a fusion protein consisting of the extracellular domain of TNF-R p55 and the transmembrane and intracellular domains of CD95. Hence, incubation of M14TF cells with recombinant TNF (rTNF) triggers CD95-mediated apoptosis (7). Cell viability was assessed using the crystal violet assay. As shown in Fig. 1A, incubation of M14TF cells with 30 ng/ml rTNF induced a time-dependent loss of cell viability indicative of efficient signaling through the intracellular domain of the CD95 death receptor. In addition, incubation of cells with the pan-caspase tetrapeptide inhibitor ZVAD-fmk (50 μ*M*) prior to receptor activation completely blocked death signaling (Fig. 1A), thus confirming a critical role for caspase activation in death signaling in this system. To assess the effect of DTT on CD95-mediated signaling, M14TF4 cells were preincubated for 1 h with increasing concentrations of DTT followed by the addition of 30 ng/ml rTNF. Cells pretreated with DTT were significantly resistant to apoptosis triggered by CD95 stimulation, with a maximum inhibition of cell death obtained with 1 m*M* DTT (Fig. 1). Interestingly, the level of cell death inhibition obtained with 1 m*M* DTT was comparable to that seen with the caspase inhibitor ZVAD-fmk, suggesting inhibition of caspase activation by DTT.

To provide further evidence that death inhibition by DTT was mediated via its ability to block caspase activation, we set out to investigate the effect of DTT pretreatment on caspases 3, 8, and 9 activities triggered in response to CD95 activation. Results of kinetics of caspase activation clearly indicate that all three caspases were activated in a time-dependent manner with the peak of activation at ~2 h after triggering of apoptosis (Fig. 2A–C). Interestingly, preincubation of M14TF cells with 1 m*M* DTT significantly blocked caspase 3, 8, and 9 activities induced upon exposure to rTNF (Fig. 2A–C). Moreover, in agreement with a caspase-dependent regulation of apoptosis in M14TF4 cells by DTT, a dose-dependent inhibition of caspase 3 activity, induced upon 2-h incubation with rTNF, was observed with increasing concentrations of DTT (Fig. 2D).

Although DTT is a reducing agent and caspase activation is facilitated in a reducing environment, the inhibition of death signaling by DTT stimulated us to investigate the mechanism of DTT-mediated inhibition of apoptosis in this system. In

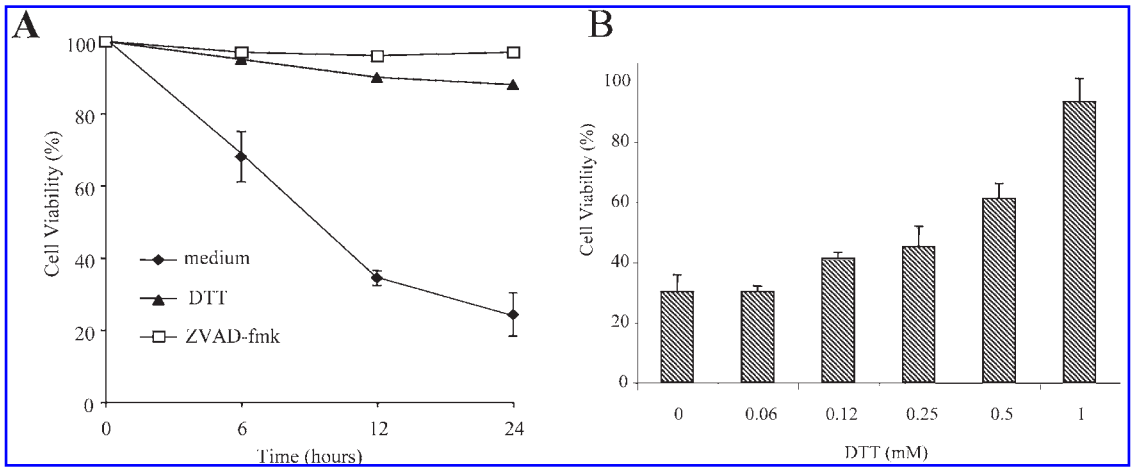

FIG. 1. Preincubation of M14TF cells with DTT inhibits CD95-mediated apoptosis. M14TF cells were incubated for 1 h in medium containing **(A)** 1 m*M* DTT, 50 μ*M* ZVAD-fmk, or control medium or **(B)** increasing concentration of DTT prior to the addition of 30 ng/ml rTNF to trigger CD95-mediated cell death. Cell viability was assessed using the crystal violet assay after 6, 12, and 24 h of incubation with rTNF in A and 12 h in B. Data are shown as means ± SE of three experiments done in triplicate.

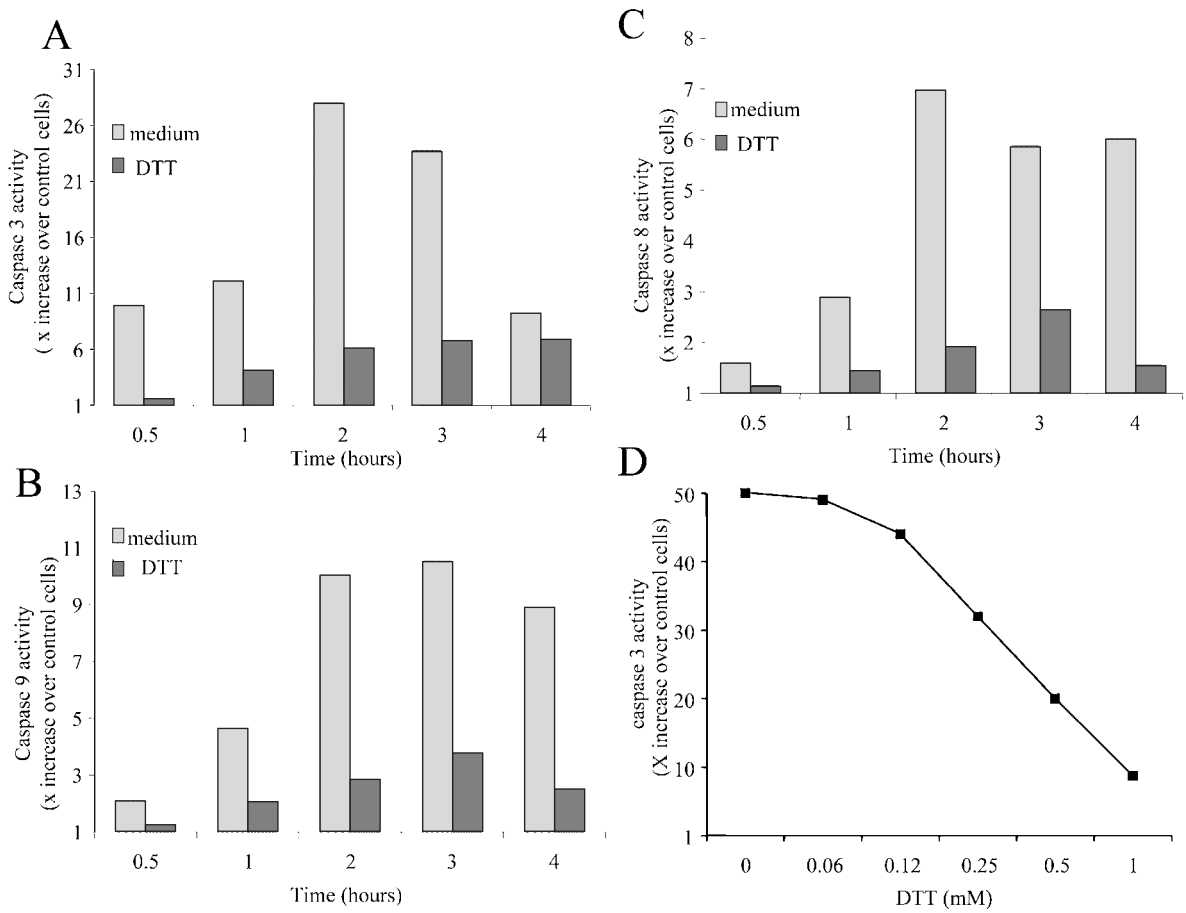

FIG. 2. Preincubation of M14TF cells with DTT inhibits caspase 3, 8, and 9 activities. M14TF cells were incubated for 1 h in medium containing 1 m*M* DTT or control medium prior to the addition of 30 ng/ml rTNF to trigger CD95-mediated cell death, and cells were harvested to determine **(A)** caspase 3, **(B)** caspase 9, and **(C)** caspase 8 activities at different time points. Caspase activities were assessed as described in Materials and Methods. **(D)** M14TF cells were incubated for 1 h in medium containing increasing concentrations of DTT or control medium prior to the addition of 30 ng/ml rTNF for 2 h. Caspase 3 activity was assessed as described in Materials and Methods.

this regard, we questioned whether this inhibitory activity was a function of a slight prooxidant state induced by DTT in M14TF4 cells. Using a lucigenin-based chemiluminescence assay, we specifically investigated the effect of DTT treatment on intracellular levels of $O_2^{\bullet-}$. This assay is widely used to detect $O_2^{\bullet-}$ in biological systems, and despite reports questioning its validity, Li *et al.* (17) and Myhre *et al.* (20) have elegantly demonstrated that lucigenin is a reliable assay for detecting $O_2^{\bullet-}$ production by enzymatic and cellular sources. Exposure of M14TF cells to 1 m*M* DTT resulted in a steady time-dependent increase in intracellular $O_2^{\bullet-}$ levels as shown in Fig. 3A. Interestingly, intracellular production of $O_2^{\bullet-}$ could be detected at concentrations of DTT as low as 0.12 m*M*, a concentration that can still induce inhibition of M14TF cell death upon triggering with rTNF (Fig. 1B and data not shown). However, it should be pointed out that the kinetics of intracellular $O_2^{\bullet-}$ production was much slower with 0.12 m*M* than that obtained with 1 m*M* DTT. These data suggest that a longer preincubation with lower concentrations of DTT (0.12 m*M*) could potentially have the same inhibitory effect on apoptosis as observed with relatively higher concentrations (1 m*M*) that trigger a rapid increase in intracellular $O_2^{\bullet-}$ generation.

Intrigued by these results, and to provide a link between DTT-mediated inhibition of apoptosis and its ability to trigger an increase in intracellular $O_2^{\bullet-}$, we exploited the $O_2^{\bullet-}$ scavenging property of the commonly used compound Tiron (22). Cells were coincubated with 1 m*M* Tiron and DTT prior to the addition of rTNF. Not only did Tiron revert the DTT-induced increase in intracellular $O_2^{\bullet-}$ (Fig. 3B), but also more importantly caspase 3 activity and sensitivity to CD95-mediated apoptosis were restored in M14TF4 cells (Fig. 3C and D). These data provide evidence for a prooxidant effect of DTT and implicate intracellular $O_2^{\bullet-}$ in its apoptosis inhibitory activity.

$O_2^{\bullet-}$-mediated inhibition of death signaling by DTT is neither cell type- nor stimulus-specific

We have previously demonstrated that a slight increase in intracellular $O_2^{\bullet-}$ inhibits death signaling irrespective of the trigger (5–8, 23–25). Against the backdrop of our findings

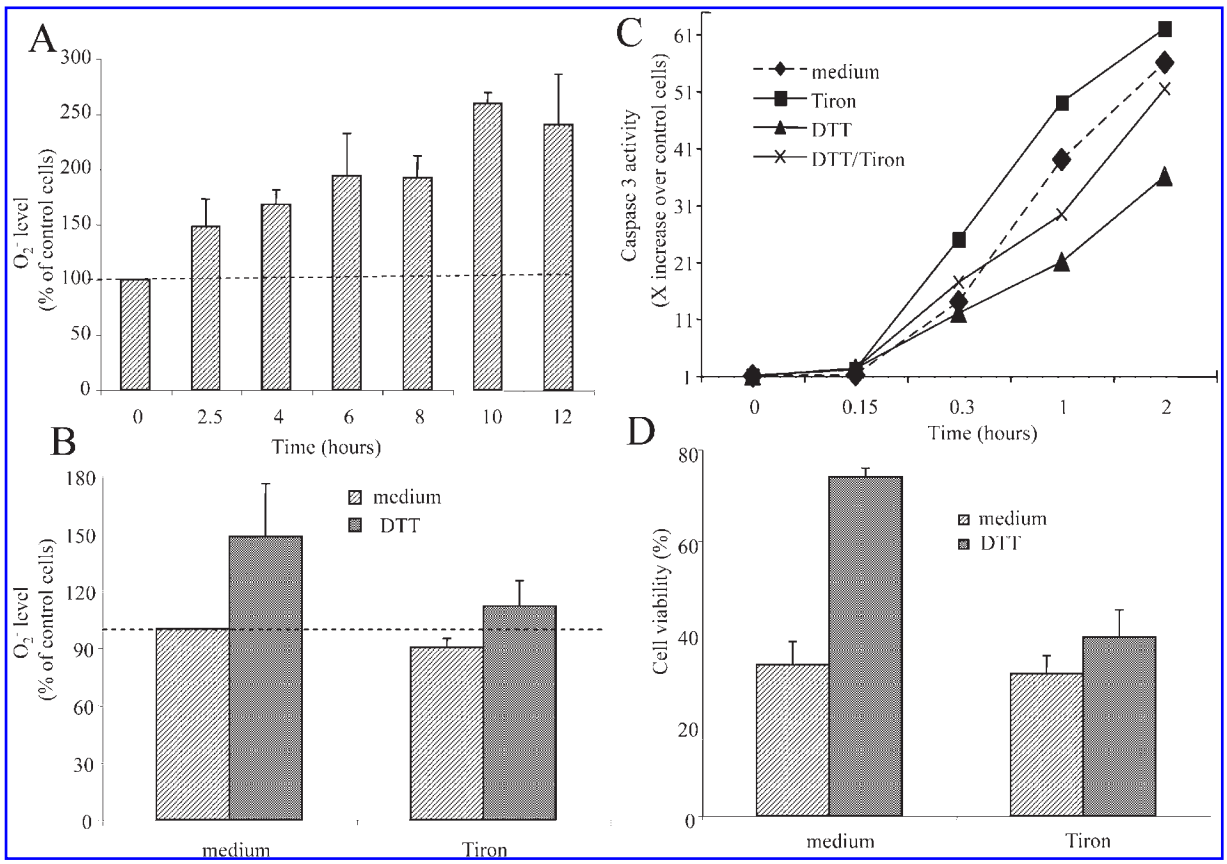

FIG. 3. Inhibition of CD-95-mediated apoptosis in M14TF cells is associated with production of $O_2^{\bullet-}$. **(A)** M14TF cells were incubated for 2.5–12 h in medium with or without 1 m*M* DTT. Intracellular level of $O_2^{\bullet-}$ was assessed using a lucigenin-based assay as described in Materials and Methods. Data are shown as % of intracellular $O_2^{\bullet-}$ increase compared with cells left in control medium for the same period of time and represent means ± SE of two experiments done in duplicate. **(B)** M14TF cells were incubated for 1 h in medium with or without 1 m*M* Tiron and with or without 1 m*M* DTT before assessment of intracellular levels of $O_2^{\bullet-}$. **(C)** Caspase 3 activity following 2 h of incubation with 30 ng/ml rTNF. **(D)** Cell viability following 14 h of incubation with 30 ng/ml rTNF. Intracellular $O_2^{\bullet-}$ was assessed as described in Materials and Methods. Cell viability was assessed using the crystal violet assay. Data are shown as means ± SE of three experiments done in triplicate.

with CD95-mediated apoptosis, we next investigated if the mechanism of DTT-mediated death inhibition was exclusive to either M14TF cells or CD95-directed apoptosis. Therefore, we used two widely utilized triggers of apoptosis, namely, staurosporine and H_2O_2, and an unrelated bladder carcinoma cell line T24. M14TF4 or T24 cells were exposed to either 1 μ*M* staurosporine (Fig. 4A) or 500 μ*M* H_2O_2 (Fig. 4B) with or without prior incubation for 1 h with 0.5 m*M* DTT or 50 μ*M* ZVAD-fmk. Exposure of M14TF4 and T24 cells to staurosporine and H_2O_2 resulted in a significant decrease in cell viability, which was blocked by the pan-caspase inhibitor ZVAD-fmk (Fig. 4A and B). More importantly, and consistent with the results obtained with CD95-mediated apoptosis, preincubation with DTT blocked staurosporine- or H_2O_2-induced apoptosis in both tumor cell lines (Fig. 4A and B).

To ascertain if the death inhibitory activity of DTT in T24 cells was also related to its prooxidant activity, we next assessed the effect of DTT on intracellular $O_2^{\bullet-}$ levels in T24 cells. Indeed, exposure of T24 cells to 0.5 m*M* DTT resulted in a steady increase in intracellular $O_2^{\bullet-}$ (Fig. 4C). Furthermore, simultaneous exposure to the $O_2^{\bullet-}$ scavenger Tiron not only inhibited the increase in intracellular $O_2^{\bullet-}$, but also reversed DTT-induced inhibition of apoptosis (data not shown). These data clearly indicate that the inhibitory effect of DTT on apoptotic signaling is not exclusive to a particular trigger or cell line, but is a function of an intracellular milieu nonpermissive for death execution, *i.e.,* a slight increase in intracellular $O_2^{\bullet-}$.

Manipulating intracellular $O_2^{\bullet-}$ by inhibiting superoxide dismutase (SOD) activity blocks death signaling in M14TF4 and T24 cells

To provide a more direct evidence to demonstrate the ability of intracellular $O_2^{\bullet-}$ to inhibit apoptotic signaling in the two cell lines used in this study, we manipulated the intracellular levels of $O_2^{\bullet-}$ by pharmacologically inhibiting the activity of SOD, the major enzyme responsible for the dismutation of $O_2^{\bullet-}$. Incubation of M14TF4 with 1 m*M* DDC, a specific inhibitor of SOD activity, resulted in a significant increase in

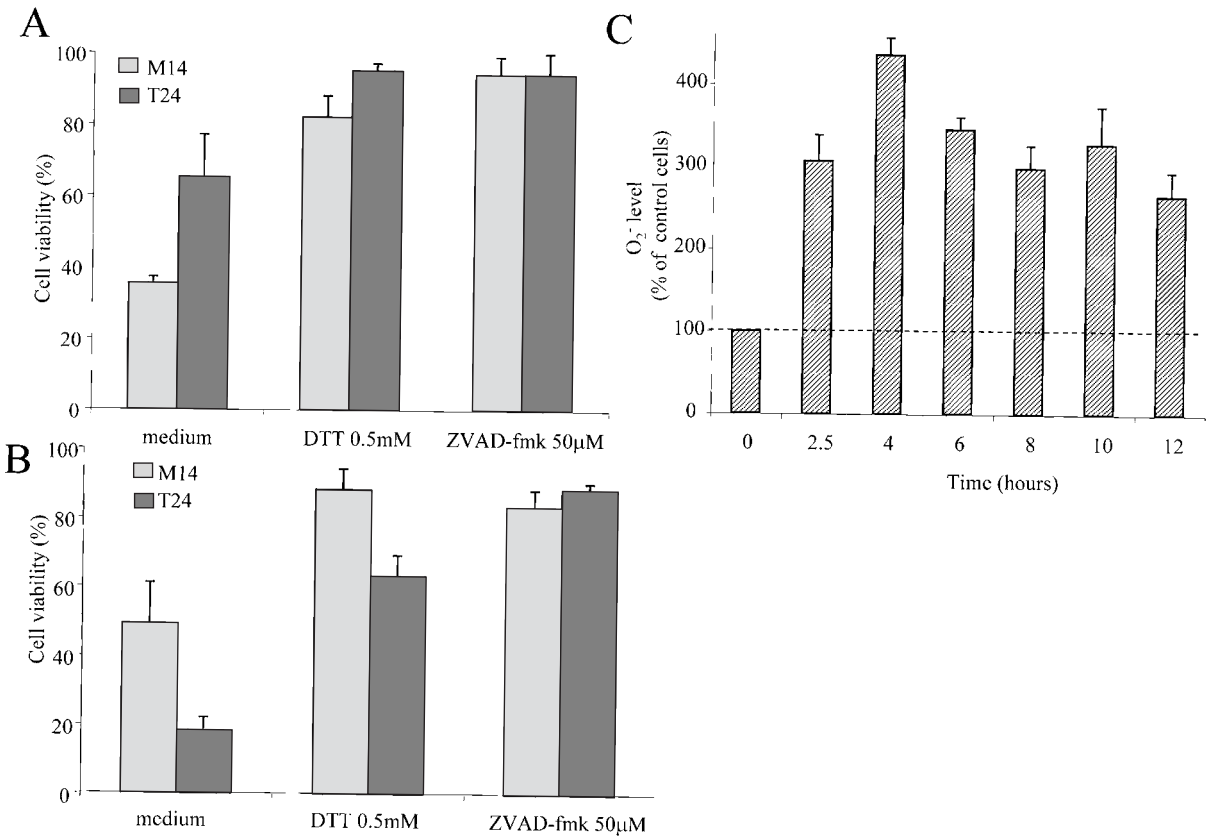

FIG. 4. Preincubation of M14TF and T24 cells with DTT inhibits staurosporine- and H_2O_2-mediated apoptosis. M14TF and T24 cells were incubated for 1 h in normal medium or medium containing 0.5 m*M* DTT or 50 μ*M* ZVAD-fmk prior to the addition of **(A)** 500 μ*M* H_2O_2 or **(B)** 1 μ*M* staurosporine for 18 h. Cell viability was assessed using the crystal violet assay. Data are shown as means ± SE of three experiments done in triplicate. **(C)** T24 cells were incubated in the presence of 0.5 m*M* DTT for 2.5–12 h. Intracellular level of $O_2^{\bullet-}$ was measured using a lucigenin-based assay as described in Materials and Methods. Data are shown as % of increase compared with cells left in control medium for the same period of time, and represent the average ± SE of two experiments done in duplicate.

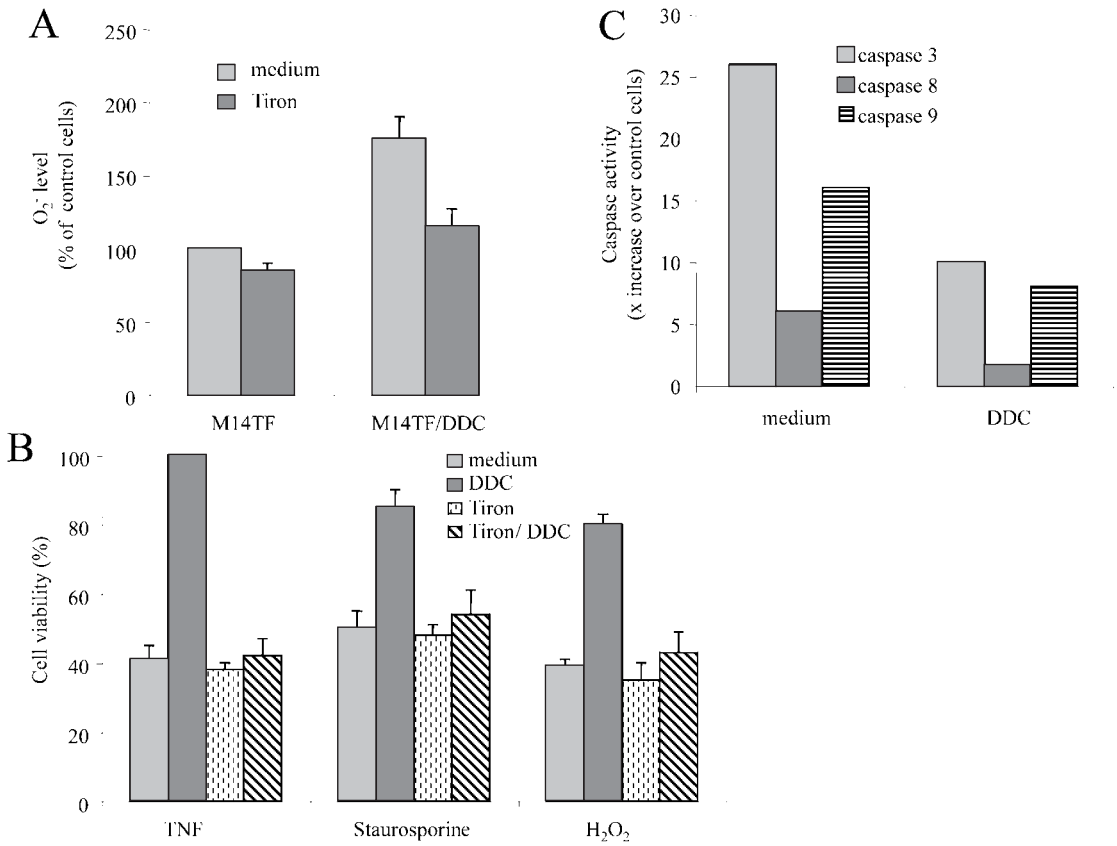

FIG. 5. Tiron prevents DDC-dependent production of $O_2^{\bullet-}$ and reverts DDC-induced inhibition of CD95-, staurosporine-, and H_2O_2-mediated apoptosis. M14TF cells were incubated in normal medium or medium containing 1 m*M* DDC, 1 m*M* Tiron, or a combination of 1 m*M* Tiron and 1 m*M* DDC for 1 h prior to **(A)** the measurement of intracellular level of $O_2^{\bullet-}$ or **(B)** addition of 30 ng/ml rTNF, 1 μ*M* staurosporine, or 500 μ*M* H_2O_2 to trigger apoptosis. Intracellular level of $O_2^{\bullet-}$ was measured by a lucigenin-based assay as described in Materials and Methods. Results are shown as means ± SE of three experiments done in triplicate. Cell viability was assessed using the crystal violet assay after 14 h of incubation time. **(C)** M14TF cells were incubated in normal medium or 1 m*M* DDC for 1 h prior to addition of 30 ng/ml rTNF, and caspase 3, 8, and 9 activities were determined 3 h following triggering of apoptosis as described in Materials and Methods.

intracellular $O_2^{\bullet-}$ levels (Fig. 5A) (7). In addition, prior incubation of M14TF cells for 1 h with 1 m*M* DDC followed by exposure to rTNF, staurosporine, or H_2O_2 resulted in significant inhibition of death signaling (Fig. 5B) (7). That this inhibition was, indeed, a function of intracellular increase in $O_2^{\bullet-}$ was further corroborated by the ability of Tiron to reverse DDC-dependent increase in intracellular $O_2^{\bullet-}$ and restore the sensitivity of M14TF4 cells to all apoptotic triggers (Fig. 5B) (7). Moreover, as shown with DTT, preincubation of M14TF cells with DDC inhibited rTNF-mediated activation of caspases 3, 8, and 9. Similar results were obtained with T24 cells using staurosporine or H_2O_2 as apoptotic triggers (data not shown).

DISCUSSION

Using two different tumor cell lines and three different apoptotic tiggers, the data presented in this report provide evidence to support the notion that production of intracellular $O_2^{\bullet-}$ contributes to the apoptosis-inhibitory activity of DTT. Although the inhibitory effect of DTT and other thiol compounds on death signaling has recently been reported, the commonly held view seems to favor the antioxidant property of thiols as the mechanism for their antiapoptotic activity (19). The data presented in this communication challenge that notion and provide an alternative explanation for the apoptosis-inhibitory activity of DTT. Incubation of M14TF and T24 cells with DTT induced a rapid increase in intracellular $O_2^{\bullet-}$ rather than what would have been expected of an antioxidant, *i.e.,* scavenging or inhibiting the production of ROS. Moreover, unlike TNF-induced apoptosis, where the role of ROS is well documented, CD95-mediated apoptosis does not depend on the generation of ROS. CD95-mediated cell death in M14TF cells is not inhibited by catalase (Clément, unpublished observations), and scavenging intracellular $O_2^{\bullet-}$ increases rather than decreases cell death (7). Corroborating these observations, DTT-induced increase in intracellular $O_2^{\bullet-}$ resulted in a significant inhibition of apoptotic signaling through the CD95 death receptor in M14TF cells. We also demonstrate that this

effect of DTT was not exclusively observed in M14TF cells or during CD95-mediated apoptosis, but also similarly impeded apoptotic execution triggered by staurosporine or H_2O_2 in M14TF as well as T24 tumor cells. More interestingly, these data point to a common mechanism that could provide an explanation for the inhibitory effect of DTT or other thiol compounds on a variety of death stimuli. Inhibition of apoptosis by all three triggers could be reverted by preventing intracellular $O_2^{\bullet-}$ accumulation with Tiron, a selective scavenger of $O_2^{\bullet-}$, thus implicating the slight prooxidant state induced by DTT in tumor cells as the inhibiting factor. Indeed, these findings are in agreement with our current hypothesis that elevated intracellular $O_2^{\bullet-}$ is an efficient inhibitor of apoptotic cell death (5, 6, 23, 24). To that end, we have previously demonstrated that an increase in intracellular $O_2^{\bullet-}$ concentration, achieved either by its overproduction (direct or drug-induced) or by inhibition of the $O_2^{\bullet-}$ scavenger Cu/Zn SOD, inhibits tumor cell apoptosis triggered via ligation of the CD95 receptor or by anticancer drugs (7, 25, 26). Similar results have been reported by Lin *et al.* with virus-induced apoptosis; elevated intracellular $O_2^{\bullet-}$ served as a survival signal, and strategies to lower $O_2^{\bullet-}$ favored death execution (18).

How does $O_2^{\bullet-}$ inhibit apoptotic signaling?

The evidence is definitely in support of a critical role of intracellular $O_2^{\bullet-}$ in the regulation of cellular response to death signaling; however, the intracellular target(s) of $O_2^{\bullet-}$ are not clearly understood. At least in the tetracycline-inducible system to overexpress Cu/Zn SOD, we were able to correlate the death inhibitory activity of $O_2^{\bullet-}$ with a direct or indirect effect on the activation/activity of the main executioner caspase, caspase 3. A decrease in intracellular $O_2^{\bullet-}$ facilitated activation/activity of caspase 3, whereas an increase in intracellular $O_2^{\bullet-}$ significantly blocked caspase 3 activity (25). A more physiological example of this is the NADPH oxidase–derived oxidants generated by stimulated neutrophils that prevent caspase activation in these cells (10). Human neutrophils have a short half-life and are believed to die by apoptosis both *in vivo* and *in vitro*; caspases are activated in a time-dependent manner in neutrophils undergoing spontaneous apoptosis. However, in cells treated with the potent neutrophil activator phorbol 12-myristate 13-acetate, caspase activity was only evident after pharmacological inhibition of $O_2^{\bullet-}$ production by the NADPH oxidase, thereby strongly suggesting an inhibitory effect of intracellular $O_2^{\bullet-}$ on caspase activation. Similarly, inhibition of the NADPH oxidase in constitutive as well as CD95/Fas-triggered apoptosis resulted in an increased rather than suppressed level of caspase activity, suggesting that ROS may prevent caspases from functioning optimally in these cells. These redox-sensitive death-executing enzymes are suppressed in activated neutrophils, and an alternative oxidant-dependent pathway is used to mediate neutrophil clearance under these conditions (10). Finally, the data presented in the present report clearly demonstrate that preincubation with either DTT or DDC significantly inhibited the activation of caspases 3, 8, and 9. Taken together, these observations support the hypothesis that manipulation of cellular redox state serves as a productive strategy to modulate caspase-mediated death. Interestingly, a recent report from Smith *et al.* demonstrated that caspase 8 is susceptible to reactive oxygen-based inactivation even in the presence of DTT (29). This result could explain why the reducing activity of DTT is not able to overcome the inactivation of the initiator caspase 8 by DTT-mediated production of $O_2^{\bullet-}$, preventing various apoptotic triggers such as CD95 signaling, staurosporine, and even H_2O_2 to initiate the caspase cascade and kill the cells by apoptosis (29). Alternatively, intracellular $O_2^{\bullet-}$ production due to preincubation of M14TF or T24 cells with DTT or DDC could have induced a stress response leading to the expression of heat shock proteins (Hsps). Indeed, at least three Hsps, namely, Hsp70, Hsp90, and Hsp27, have shown antiapoptotic activity that could be linked to the inhibition of caspase 3 and 9 activities (for review, see 2).

The thioredoxin paradox

In vivo the cellular reducing environment is maintained by thioredoxins. Supporting the contention that reductants may facilitate caspase activity, Baker *et al.* recently showed that various recombinant human thioredoxins directly activate caspase 3 (1). Paradoxically though, similar to the presence of DTT, thioredoxin-1 expression seems to protect rather than increase apoptosis as one would have expected due to its thiol-reducing activity. This antiapoptotic effect of thioredoxin-1 has been reported with diverse apoptotic triggers such as TNF-α, anti-CD95/fas antibody, H_2O_2, activated neutrophils, ischemia–reperfusion injury, and anticancer drugs (27). Although the mechanism of this protective effect of thioredoxin-1 is unknown, in the light of our findings on the role of ROS in the regulation of apoptosis by DTT, one could put forward a new hypotheses vis-à-vis the apoptosis-inhibitory effect of thioredoxin-1. Interestingly, upon screening a cDNA library from a B-cell population of Epstein–Barr virus-transformed human peripheral blood lymphocytes for thioredoxin-binding proteins by the yeast two-hybrid system, a plasmid containing an insert sharing homology with the human p40phox, a cytosolic component of phagocyte oxidase, was found. In contrast, no interaction was observed with substituted mutant thioredoxin (C32S/C35S), which lacks reducing activity (21). These results showed that p40phox interacts with thioredoxin and indicate the possibility of thioredoxin-dependent regulation of oxidase activity. These data strongly suggest that thioredoxin, in addition to scavenging H_2O_2, may also regulate intracellular production of $O_2^{\bullet-}$. It is then tempting to propose that the antiapoptotic activity of thioredoxin may be associated with the induction of an intracellular prooxidant state. Thus, the combination of an H_2O_2-scavenging activity and induction of intracellular $O_2^{\bullet-}$ production may overcome the thiol-reducing activity of thioredoxin and result in inhibition rather than induction of the apoptotic pathway.

Conclusion

The surprising mechanism involved in DTT-mediated inhibition of apoptosis presented in this report adds to the list of conditions involving increases in intracellular level of $O_2^{\bullet-}$ as the principal mechanism for the resistance of cells to apoptotic triggers, thereby supporting a general inhibitory role for

intracellular $O_2^{\bullet-}$ in apoptotic signaling. In the light of these findings, we propose that $O_2^{\bullet-}$ be called an "antiapoptotic anion" in contrast to H_2O_2, which could be considered as a "proapoptotic ROS" as evidenced in numerous examples of oxidative stress-induced apoptosis (4). More importantly, our data suggest that we may want to reassess if the antioxidant activity of other thiol-containing compounds is the only explanation for their apoptosis-inhibiting activity.

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ABBREVIATIONS

DDC, diethyldithiocarbamate; DTT, dithiothreitol; H_2O_2, hydrogen peroxide; Hsp, heat shock protein; $O_2^{\bullet-}$, superoxide; OD, optical density; ROS, reactive oxygen species; rTNF, recombinant tumor necrosis factor; SOD, superoxide dismutase; TNF, tumor necrosis factor; TNF-R p55, TNF receptor p55.

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