The EMBO Journal vol.15 no.2 pp.216–225, 1996

Superoxide anion is a natural inhibitor of Fas-mediated cell death

Marie-Véronique Clément and Ivan Stamenkovic

Department of Pathology, Harvard Medical School and Pathology Research, Massachusetts General Hospital, 149 13th Street, Charlestown Navy Yard, Boston, MA 02129, USA

The cell surface receptor Fas is a major trigger of apoptosis. However, expression of the Fas receptor in many tumor cell types does not correlate with sensitivity to Fas-mediated cell death. Because a prooxidant state is a common feature of tumor cells, we examined the role of intracellular reactive oxygen intermediates in the regulation of Fas-mediated cytotoxicity. Our results show that an oxidative stress induced by increasing the intracellular superoxide anion (O_2^-) concentration can abrogate Fas-mediated apoptosis in cells which are constitutively sensitive to Fas. Conversely, an O_2^- concentration decrease is observed to sensitize cells which are naturally resistant to Fas signals. These observations suggest that intracellular O_2^- may play a key role in regulating cell sensitivity to a potentially lethal signal and provide tumor cells with a natural, inducible mechanism of resistance to Fas-mediated apoptosis.

Keywords: apoptosis/Fas/superoxide anion/tumor cells

Introduction

Apoptosis is an essential mechanism for the maintenance of homeostasis in multicellular organisms (Wyllie *et al.*, 1980). Apoptotic cell death can result either from developmentally controlled activation of endogenous execution programs (Ellis *et al.*, 1991) or from transduction of death signals triggered by a wide variety of exogenous stimuli (reviewed in Nagata and Golstein, 1995; Steller, 1995; Thompson, 1995). Potential exogenous triggers of apoptosis range from growth factor withdrawal, to ligand- or antibody-mediated engagement of specific cell surface proteins capable of transducing lethal signals. In most tissues, cell survival relies on the constant supply of survival signals, provided by the extracellular matrix and neighboring cells. Most cells will die if isolated from their tissue of origin unless they are provided with appropriate cytokines (Thompson, 1995). One interpretation of these observations is that most cells will naturally undergo apoptosis unless they receive survival signals, which in the case of isolated cells in culture, can be provided by a variety of growth factors. However, exposure to signals such as those triggered by Fas, which have the potential to induce cell death, does not invariably result in apoptosis, suggesting that cells may possess endogenous mechanisms capable of inhibiting death signals.

The Fas receptor is expressed on the surface of a variety of normal and malignant lymphoid cells as well as non-lymphoid tumors and tumor cell lines (Itoh *et al.*, 1991; Nagata and Golstein, 1995), and its primary function appears to be induction of apoptosis, particularly in the immune system (Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995; Nagata and Golstein, 1995). However, there does not appear to be a strict correlation between Fas expression and susceptibility to Fas-dependent cell death. Notably, engagement of Fas by ligand or antibody in a variety of tumor cell lines and in activated lymphocytes does not induce apoptotic cell death (Owen-Schaub *et al.*, 1994). The mechanisms which determine whether any given cell is sensitive or resistant to Fas are poorly understood. However, elucidation of these mechanisms is required to understand how apoptosis in general, and Fas-mediated apoptosis in particular, is regulated.

One common feature of activated lymphocytes and tumor cells, with respect to quiescent and normal counterparts, is an increase in metabolic rate and reactive oxygen intermediate (ROI) generation. ROIs are produced in all mammalian cells, partly as a result of normal cellular metabolism, and partly due to activation of a variety of ROI-producing enzymes in response to exogenous stimuli. The principal intermediates are superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH·) (Fridovich, 1976, 1978; Cross, 1990; Cross and Jones, 1991). O_2^- is the primary ROI generated by normal cellular metabolism, whereas hydrogen peroxide is a catalytically derived intermediate in the conversion of O_2^- to O_2. Hydroxyl radical is a by-product of both O_2^- and H_2O_2 in iron-dependent radical reactions (Fridovich, 1976, 1978; Halliwell and Gutteridge, 1989). Excessive accumulation of ROIs is toxic (Fridovich, 1978; Behl *et al.*, 1994), and the intracellular level of ROIs is therefore tightly regulated by several small antioxidant molecules which contain sulfhydryl groups and ROI-scavenging enzymes. The principal ROI-scavenging enzymes are superoxide dismutases (SOD), which catalyze dismutation of O_2^- to H_2O_2, and catalase (CAT) and glutathione peroxidase (GPx), which transform H_2O_2 into H_2O and O_2 (Fridovich, 1976, 1978; Hassan, 1988). Antioxidants which remove hydroxyl radical are typically formate, polyunsaturated fatty acids and sugars, such as mannitol (Fridovich, 1976, 1978).

Current opinion holds that ROIs are implicated in apoptosis, and that they may provide effector mechanisms for the final common pathway of programmed cell death (Buttke and Sandstrom, 1994). However, there is evidence that oxidative stress, induced by overproduction or decreased elimination of O_2^-, provides tumor cells with a survival advantage over normal counterparts (Cerutti, 1985). ROIs may therefore have beneficial effects at concentrations which do not overwhelm the endogenous cellular protective mechanisms against ROI-induced damage. In this work, we addressed the possibility that one

of the beneficial effects of ROIs may be induction of resistance to Fas-mediated apoptosis. Our results show that intracellular concentration of O_2^- may be a key regulator of sensitivity to Fas-mediated cell death, and that oxidative stress induced by an increase in O_2^- concentration can block apoptosis triggered by Fas.

Results

Development of a Fas-sensitive human melanoma cell line

We have recently described the development of stable human melanoma cell lines (MC) expressing fusion proteins consisting of the extracellular domain of CD40 or tumor necrosis factor receptor p55 (TNFRp55) and the transmembrane and intracellular domains of Fas (Clément and Stamenkovic, 1994). Stimulation of transfectants expressing CD40–Fas and TNFRp55–Fas fusion proteins with soluble CD40 ligand (CD40L) and recombinant TNFα respectively results in cell death within 6–24 h, mimicking the effect of wild-type Fas triggering (Clément and Stamenkovic, 1994). To demonstrate that TNF–Fas fusion proteins retain their function in other cell lines, we stably expressed TNFRp55–Fas in an unrelated human melanoma, M14, which lacks constitutive expression of Fas and TNFR (data not shown). TNFR–Fas fusion protein provides the advantage of being able to trigger Fas-mediated response by a receptor–ligand interaction using recombinant TNFα as the specific ligand. Several independent clones were obtained which respond to engagement of the fusion protein by ligand by undergoing apoptosis within 6 h (Figure 1). The clone that was found to be the most sensitive, TNF–Fas 4 (henceforth referred to as M14 TF4) was used in subsequent experiments.

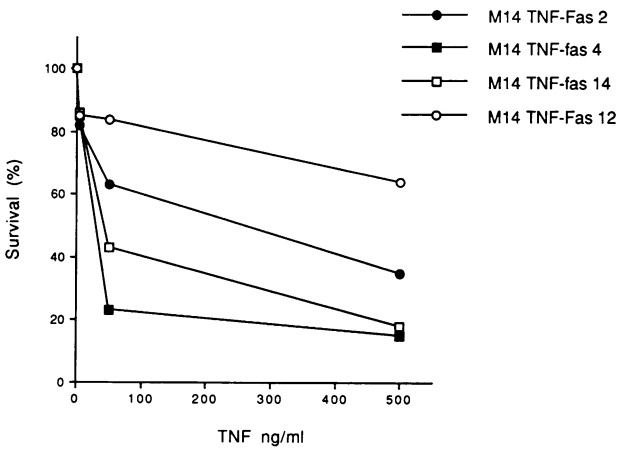

Fig. 1. Sensitivity of M14 TNF-Fas clones to Fas-mediated cell death. M14 TNF–Fas clones were incubated for 6 h at 37°C in DME 5% FBS in the presence of increasing concentrations of recombinant TNFα. Survival was determined by crystal violet staining and is expressed as the percentage of the dye incorporation of cells incubated in medium alone. The parental cell line as well as cells transfected with pSV2neo alone were not affected by a 6 h incubation in the presence of recombinant TNFα.

An increase in intracellular superoxide concentration inhibits Fas-mediated apoptosis

Unlike TNF-induced cell death, where the role of ROIs is well documented (Zimmerman *et al.*, 1989; Schulze-Osthoff *et al.*, 1994), Fas-mediated cytotoxicity does not appear to depend on the generation of ROIs. Neither inhibitors of the mitochondrial respiratory chain nor antioxidants protect cells from Fas-mediated death (Schulze-Osthoff *et al.*, 1994), and killing by Fas is not enhanced by inhibition of CAT or GPx (M.-V.Clément and I.Stamenkovic, unpublished results).

To determine whether ROIs may play a role in cell resistance to Fas-mediated death, we sought to manipulate O_2^- concentration in cells which are normally killed by engagement of Fas. Superoxide concentration in mammalian cells is controlled in large part by the activity of the constitutively expressed cytoplasmic Cu/Zn SOD (Fridovich, 1976; Amstad *et al.*, 1991, 1994; Huang *et al.*, 1992; Hiraishi *et al.*, 1994), which rapidly dismutates O_2^- into H_2O_2, helping maintain O_2^- concentration at non-toxic baseline levels (Elroy-Stein *et al.*, 1986). To augment intracellular O_2^- concentration, we used the potent and widely used inhibitor of the Cu/Zn SOD, diethyldithiocarbamate (DDC) (Hiraishi *et al.*, 1992). Using a lucigenin-based chemiluminescence assay, which is specific for O_2^- (Gyllenhammar, 1987; Porter *et al.*, 1992), we assessed the conditions under which DDC induces a significant increase in intracellular O_2^- concentration without affecting cell viability. Incubation of M14 TF4 cells with DDC concentrations up to 1 mM for 1 h resulted in a DDC dose-dependent increase in intracellular O_2^- (Figure 2A). However, a detectable increase in intracellular O_2^- could not be induced by DDC, even at concentrations exceeding 1 mM, in cells which had been exposed to the drug for 18 h (data not shown).

An inverse correlation was found between the intracellular O_2^- concentration and sensitivity of M14 TF4 cells to Fas-mediated death. A 1 h exposure of M14 TF4 cells to increasing concentrations of DDC, before engagement of Fas, was observed to be sufficient to induce a DDC dose-dependent inhibition of Fas-mediated cell death. Moreover, a 2.8-fold increase in intracellular O_2^- concentration, induced by pre-incubation with 1 mM DDC for 1 h, correlated with complete abrogation of M14 TF4 sensitivity to Fas-mediated killing (Figure 2B). The effect was the same whether DDC was maintained in the culture medium throughout the assay or removed from the medium after 1 h of pre-incubation, immediately before engagement of Fas. However, cells which had been exposed to the drug for 18 h remained sensitive to Fas-mediated apoptosis despite pre-incubation with increasing concentrations of fresh DDC (data not shown).

To demonstrate that inhibition of Fas-mediated apoptosis by pre-incubation with DDC can be induced in cells which constitutively express the Fas receptor, and which are sensitive to Fas-mediated cytotoxicity, T cell leukemia Jurkat cells were triggered with anti-Fas mAb following a 1 h exposure to increasing concentrations of DDC. Similar to M14 TF4 cells, 50% of Jurkat cells underwent apoptosis within 5 h of incubation with anti-Fas mAb, while pre-incubation with 1 mM DDC significantly reduced Fas-dependent cell death (Figure 2D). DDC-induced resistance of Jurkat cells to apoptosis was observed to correlate with an increase in intracellular O_2^- concentration (Figure 2C).

One possible explanation of these results is that abroga-

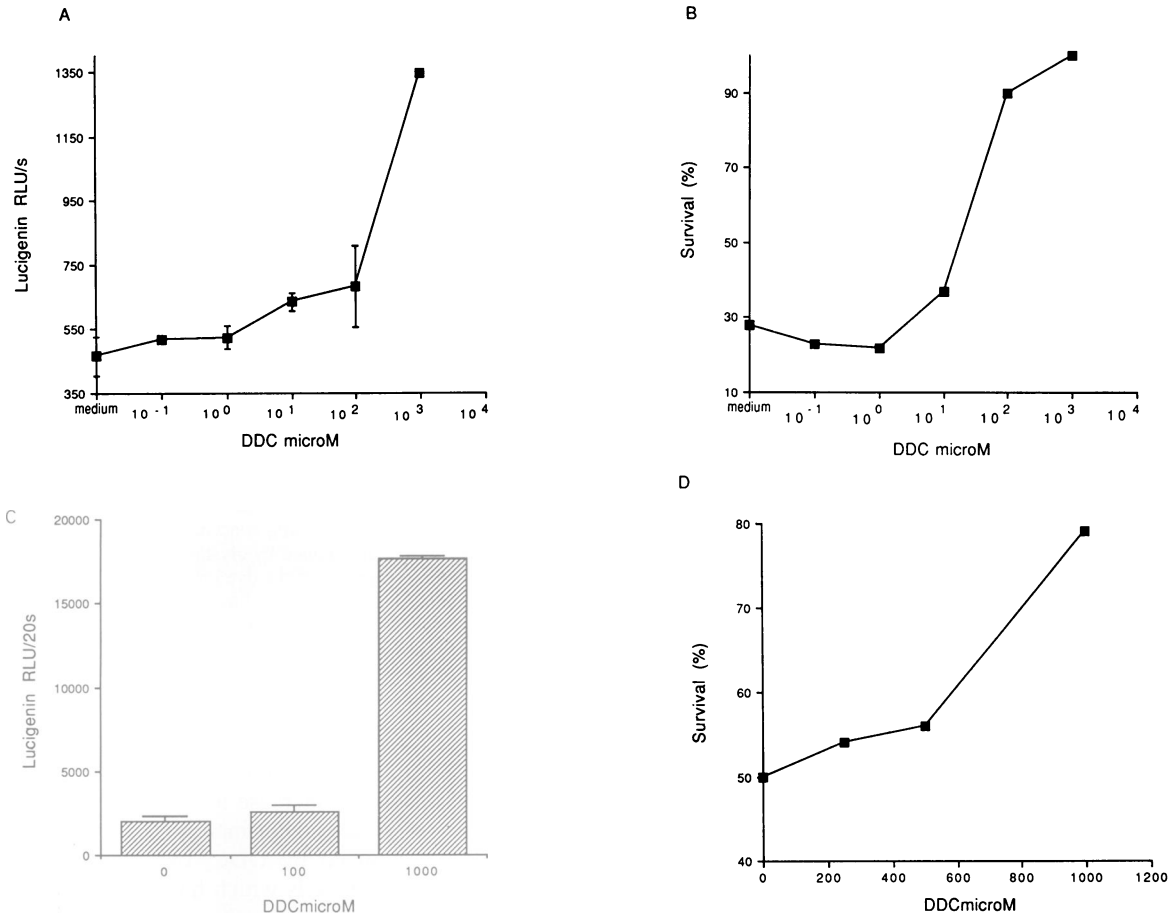

Fig. 2. Effect of DDC on intracellular superoxide accumulation and Fas-mediated cell death. (**A**) 2×10^6 M14 TF4 cells or (**C**) 2×10^6 Jurkat cells were incubated for 1 h in the presence of increasing concentrations of DDC before lucigenin chemiluminescence measurement. Lucigenin chemiluminescence was monitored for 20 s and is expressed as (A) relative light units/s (RLU/s) (± SE) or (C) RLU/20 s (± SE). (**B**) M14 TF4 cells (2×10^4 cells/100 µl) and (**D**) Jurkat cells (5×10^4 cells/100 µl) were seeded onto 96-well plates and incubated with increasing concentrations of DDC for 1 h before triggering of Fas with (B) 30 ng/ml of TNF or (D) 1 µg/ml of anti-Fas mAb. After a 6 h (B) or 5 h (D) incubation time, survival was determined by (B) crystal violet or (D) MTT staining. Survival is expressed as the percentage of dye incorporation by cells exposed to the same DDC concentration but without TNF or anti-Fas mAb.

tion of Fas-mediated cell death following pre-incubation of cells with DDC is due to an O_2^--based oxidative stress resulting from SOD inhibition. To test this possibility, M14 transfectants were triggered with TNF–Fas fusion protein ligand following a 1 h pre-incubation with DDC and either *N*-acetylcysteine (a thiol-containing antioxidant capable of directly inactivating ROIs as well as inducing glutathione production; Meister and Anderson, 1983), or tiron (4,5-dihydro-1,3 benzene disulfonic acid), a more selective scavenger of O_2^- (Pagano *et al.*, 1993; Munzel *et al.*, 1995). Both antioxidants blocked the increase in intracellular O_2^- resulting from Cu/Zn SOD inhibition, and abrogated the corresponding induction of resistance to Fas-mediated apoptosis (Figure 3A–C and data not shown). At the concentrations used to inhibit the oxidative stress induced by SOD inhibition, neither *N*-acetylcysteine nor tiron alone affected cell viability.

To provide further evidence that an increase in intracellular O_2^- is responsible for the inhibition of Fas-mediated cell death, M14 TNF–Fas cells were triggered with TNF in the presence of the herbicide paraquat (1,1′-dimethyl-4,4′-bipyridinum dichloride), a potent generator of intracellular O_2^- (Elroy-Stein *et al.*, 1986; Lee and Hassan, 1986; Krall *et al.*, 1988). Because paraquat is potentially toxic, a serial dilution assay was performed to determine the concentration range within which its presence might be tolerated by cells in culture. Paraquat concentrations of up to 3 mM in cell culture medium were found not to induce cytotoxicity in M14 cells, while markedly increasing intracellular O_2^- (Figure 4A). Consistent with the results obtained by blocking Cu/Zn SOD, addition of paraquat to a maximal concentration of 3 mM, to the cell culture medium, rendered M14 transfectants partially resistant to Fas mediated-cell death (Figure 4B). Similar to the effect of DDC, resistance to Fas was induced whether paraquat was maintained in the culture medium throughout the assay or removed after 1 h of pre-incubation and before engagement of the TNF–Fas fusion protein.

Finally, a similar inhibitory effect was observed when the TNF–Fas fusion protein in M14 TF4 cells was triggered following a 1 h incubation of the cells in the presence of the xanthine/xanthine oxidase (X/XO) superoxide generating system (Shibanuma *et al.*, 1988) (data not shown).

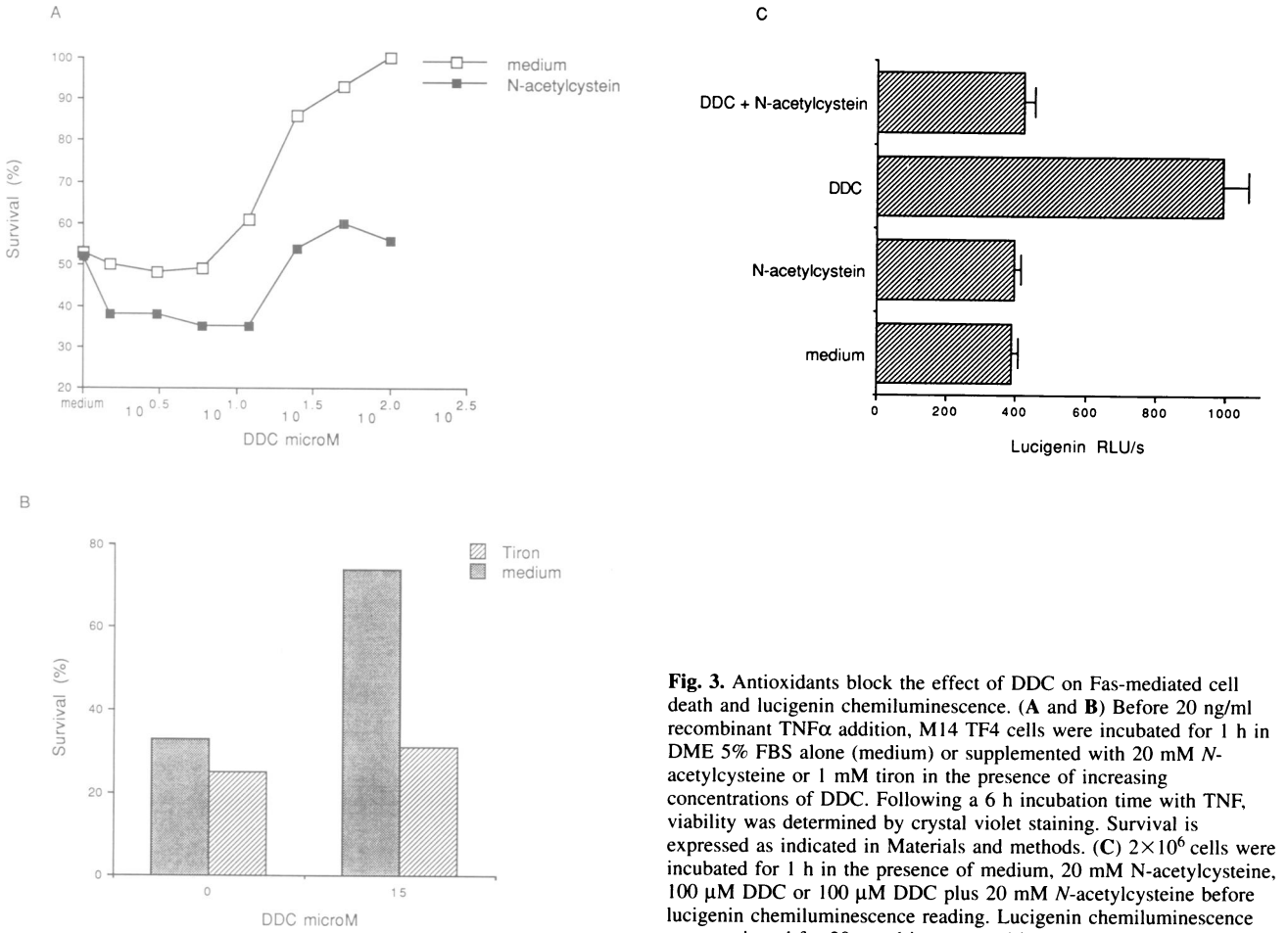

Fig. 3. Antioxidants block the effect of DDC on Fas-mediated cell death and lucigenin chemiluminescence. (**A** and **B**) Before 20 ng/ml recombinant TNFα addition, M14 TF4 cells were incubated for 1 h in DME 5% FBS alone (medium) or supplemented with 20 mM *N*-acetylcysteine or 1 mM tiron in the presence of increasing concentrations of DDC. Following a 6 h incubation time with TNF, viability was determined by crystal violet staining. Survival is expressed as indicated in Materials and methods. (**C**) 2×10^6 cells were incubated for 1 h in the presence of medium, 20 mM N-acetylcysteine, 100 μM DDC or 100 μM DDC plus 20 mM *N*-acetylcysteine before lucigenin chemiluminescence reading. Lucigenin chemiluminescence was monitored for 20 s and is expressed in RLU/s (± SE).

Inhibition of Fas-mediated apoptosis by phorbol esters is linked to enhanced superoxide production

Phorbol esters directly activate protein kinase C (PKC) and promote cell transformation which can be inhibited by antioxidants (reviewed in Cerutti, 1985). In neutrophils, PKC activation has been shown to stimulate NADPH-related oxidases which induce an extra- as well as an intracellular burst of O_2^- (Watson *et al.*, 1991). We therefore addressed the possible role of phorbol ester-induced superoxide-producing enzyme activity in the inhibition of Fas-mediated cell death. Pre-incubation of M14 TF4 cells with phorbol 12-myristate 13-acetate (PMA) and 12,13-phorbol dibutyrate (PDBu) for 30 min resulted in a significant increase in intracellular O_2^- and inhibited Fas-mediated apoptosis (Figure 5A and E; also data not shown). The observed increase in O_2^- concentration and the inhibitory effect on Fas-mediated death were abolished when cells were pretreated with either phorbol ester for 24 h (data not shown), which results in desensitization of PKC (Jin *et al.*, 1992). Similarly, pre-incubation of cells with 10 nM staurosporine or 24 μM bisindolylmaleimide GF109203X (BIM), both of which inhibit PKC (Toullec *et al.*, 1991), abrogated the phorbol ester-induced increase in intracellular O_2^- and inhibition of Fas-mediated apoptosis (Figure 5B, C and E; also data not shown). Although staurosporine inhibits the activity of several kinases (Ruegg and Buirgess, 1989), at nanomolar concentrations used in the present experiments it is suggested preferentially to inhibit PKC. Neither staurosporine nor BIM were observed to be cytotoxic at the above concentrations. Several other kinase inhibitors, including the protein tyrosine kinase inhibitor genistein and the phosphatidyl inositol 3 kinase inhibitor, wortmannin, had no effect on phorbol ester-induced resistance or on unstimulated M14 TF4 sensitivity to Fas (Table I and data not shown).

To address the possibility that phorbol ester-induced augmentation in O_2^- concentration, and the corresponding inhibition of Fas-mediated cell death, might be due to superoxide-producing enzyme activation, M14 TF4 cells were pre-incubated with inhibitors of various classes of O_2^--producing enzymes (Cross and Jones, 1991) before stimulation with phorbol esters, and triggering of Fas (Table I). Inhibitors of xanthine oxidase (allopurinol and oxypurinol), cyclooxygenase (indomethacin) and lipo-oxygenase (nordihydroguaiaretic acid) had no effect on phorbol ester-induced inhibition of Fas-mediated cell death (Table I). In contrast, pre-incubation of M14 transfectants with neopterin and diphenylene iodanium (DPI), two inhibitors of NADPH oxidases (Cross and Jones, 1986; Cross, 1987, 1990; Hancock and Jones, 1987; Kojima *et al.*, 1993), effectively abrogated the phorbol ester-induced increase in O_2^- and the corresponding inhibition of Fas-mediated apoptosis (Table I; Figure 5D and E; also data not shown). Because DPI is known to inhibit nitric oxide synthetase (NOS) in addition to flavoprotein-dependent superoxide-producing enzymes (Stuehr *et al.*, 1991), M14 TF4 cell phorbol ester-induced inhibition of

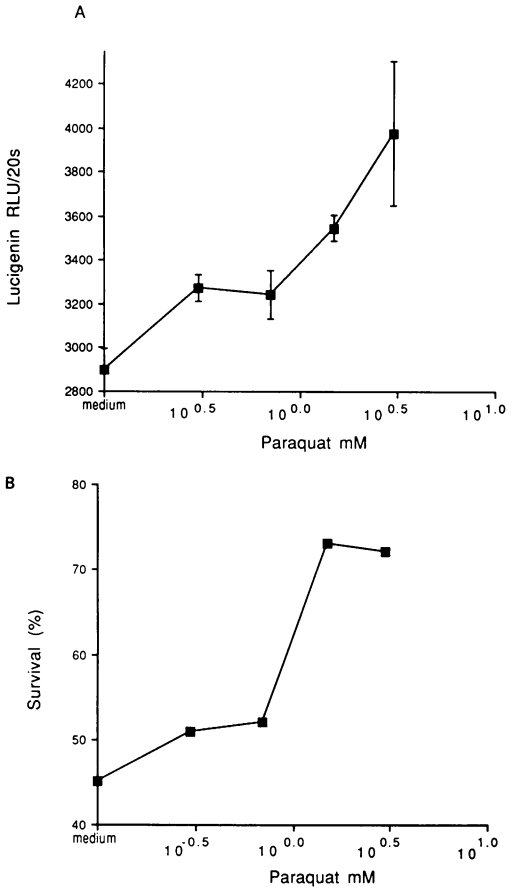

Fig. 4. Effect of paraquat on Fas-mediated cell death and lucigenin chemiluminescence. M14 TF4 cells were incubated with increasing concentrations of paraquat for 1 h before (**A**) measurement of lucigenin chemiluminescence or (**B**) triggering of Fas-mediated cell death. (A) Lucigenin chemiluminescence was monitored for 20 s and is expressed as RLU/20s (± SE). (B) M14 TF4 cell viability was determined by crystal violet staining following a 6 h incubation with 30 ng/ml TNF. Survival is expressed as the percentage of the staining value of cells exposed to the same paraquat concentration in the absence of TNF.

sensitivity to Fas was tested following incubation with *N*-monomethyl-L-arginine and *N*-nitro-L-arginine, two inhibitors of NOS. Inhibition of NOS was not found to alter the PMA-induced inhibition of Fas-mediated death of M14 TF4 cells (Table I). Similarly, NO production induced by sodium nitroprusside was not observed to affect sensitivity to Fas in M14 cells (data not shown), ruling out the possibility that NO plays a role in phorbol ester-induced inhibition of Fas-mediated cell death.

Inhibition of Fas-mediated cell death by intracellular O_2^- is independent of protein synthesis

ROIs have been implicated in transcription factor activation (Shibanuma *et al.*, 1988; Abate *et al.*, 1990; Schreck *et al.*, 1991), raising the possibility that the inhibitory effect of O_2^- on Fas-mediated death may be due to enhanced or *de novo* expression of specific genes. To determine whether O_2^--associated inhibition of Fas-mediated cell death requires protein synthesis, M14 TF4 cells were exposed to increasing concentrations of the SOD inhibitor DDC in the presence or absence of cycloheximide before engagement of the TNFR–Fas fusion protein. No difference in the ability of DDC to inhibit Fas-mediated apoptosis was observed whether cycloheximide was present or not (Figure 6).

Fas-resistant tumor cells can be rendered sensitive by decreasing their intracellular superoxide level

To address the potential role of intracellular superoxide concentration on natural resistance to Fas-mediated cell death, we selected two human cell lines, the bladder carcinoma T24 and the osteosarcoma U20S, both of which constitutively express Fas but are resistant to its potentially lethal signal. Incubation of T24 and U20S cells with anti-Fas mAb induced cell death in only 10–20% of cells at 24 h, compared with 70–80% in Jurkat and M14 TF4 cells. Cell surface expression of Fas in the two cell lines was comparable, and similar to that in Jurkat cells and M14 transfectants (data not shown). Thus, the level of Fas receptor expression alone cannot account for differences in response among the cell lines.

To determine whether the natural resistance of these cells to Fas-mediated cytotoxicity may be due to endogenous O_2^- production by phorbol ester/PKC-inducible flavin oxidases, T24 and U2OS cells were triggered with anti-Fas mAb following a 1 h incubation with either 10 nM staurosporine, 24 μM BIM, 50 μM DPI or 10 μg/ml cycloheximide. Pretreatment with each of the drugs highly sensitized T24 and U2OS cells to Fas-mediated apoptosis, suggesting that PKC activation and O_2^--producing enzymes participate in natural tumor cell resistance to Fas (Figure 7A; also data not shown) and that protein synthesis is required to maintain the resistant state. Sensitization of T24 and U2OS to Fas was observed to be accompanied by a decrease in the level of intracellular O_2^-, the degree of which was augmented the longer the cells were exposed to the drugs (Figure 7B; also data not shown). However, induction of sensitivity to Fas-mediated death in T24 and U2OS cells in the presence of BIM, staurosporine, DPI and cycloheximide was blocked when the intracellular O_2^- concentration was maintained above baseline levels by inhibiting Cu/Zn SOD (Figure 7C). The behavior of T24 and U2OS cells in these experiments was comparable, and results obtained using T24 cells only are shown.

Discussion

In this work we have specifically addressed the role of intracellular O_2^- in the regulation of Fas-mediated cell death. We have used four distinct methods to augment intracellular O_2^- concentration: inhibition of Cu/Zn SOD, paraquat-mediated generation of intracellular O_2^-, X/XO-induced O_2^- production and PKC activation-dependent intracellular O_2^- production. To decrease intracellular O_2^- levels, we have used inhibitors of PKC and of O_2^--producing enzymes. Changes in intracellular O_2^- levels were monitored by the lucigenin-based chemiluminescence assay, shown to be specific for O_2^- (Gyllenhammar, 1987; Porter *et al.*, 1992). Chemiluminescence currently provides the most sensitive means to detect small changes in intracellular ROIs for any given cell type (Porter *et al.*, 1992). Our results show that an increase in intracellular concentration of O_2^- induces resistance to apoptosis triggered by Fas. Conversely, a reduction in O_2^- concentra-

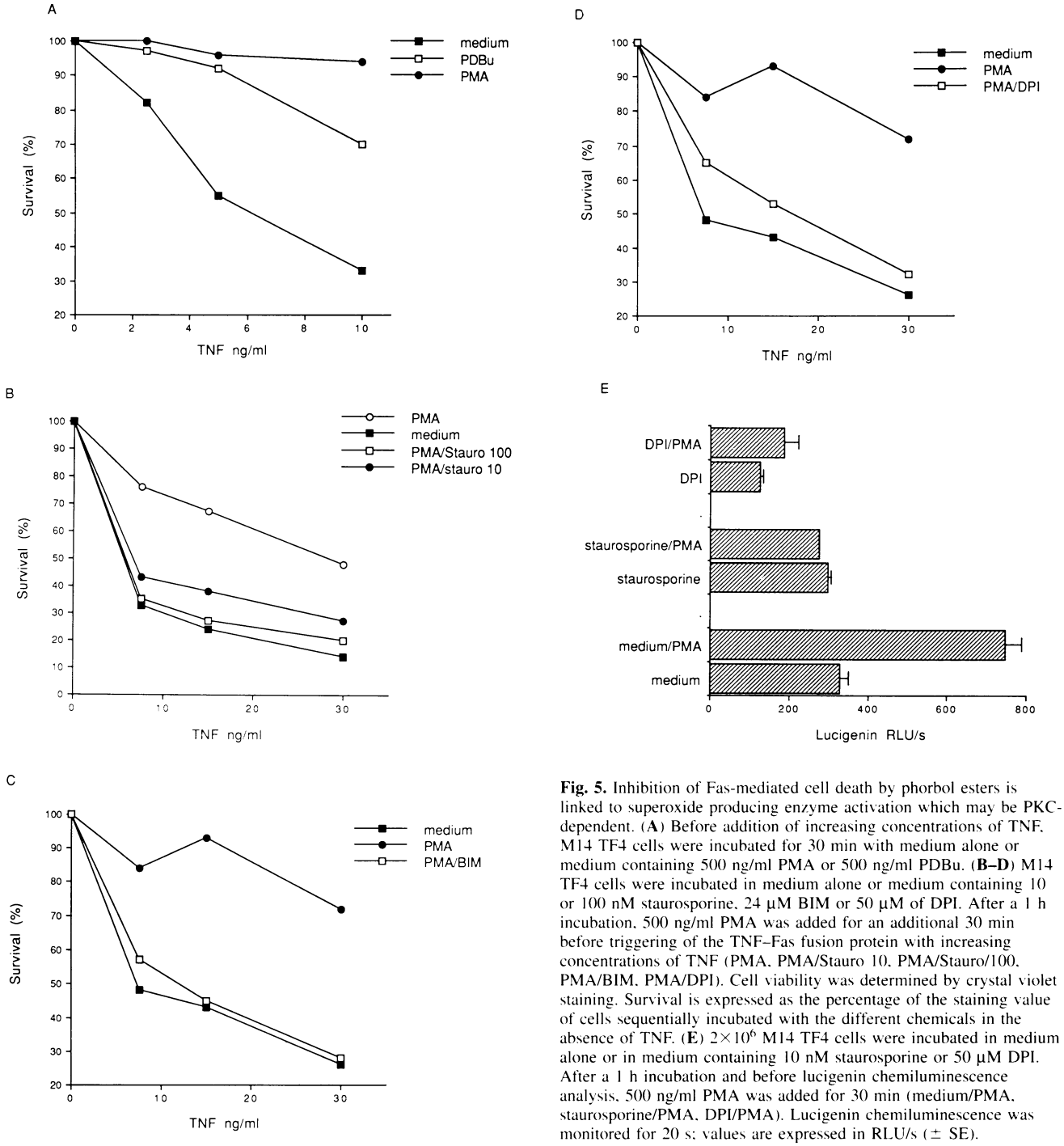

Fig. 5. Inhibition of Fas-mediated cell death by phorbol esters is linked to superoxide producing enzyme activation which may be PKC-dependent. (**A**) Before addition of increasing concentrations of TNF, M14 TF4 cells were incubated for 30 min with medium alone or medium containing 500 ng/ml PMA or 500 ng/ml PDBu. (**B–D**) M14 TF4 cells were incubated in medium alone or medium containing 10 or 100 nM staurosporine, 24 μM BIM or 50 μM of DPI. After a 1 h incubation, 500 ng/ml PMA was added for an additional 30 min before triggering of the TNF–Fas fusion protein with increasing concentrations of TNF (PMA, PMA/Stauro 10, PMA/Stauro/100, PMA/BIM, PMA/DPI). Cell viability was determined by crystal violet staining. Survival is expressed as the percentage of the staining value of cells sequentially incubated with the different chemicals in the absence of TNF. (**E**) 2×10^6 M14 TF4 cells were incubated in medium alone or in medium containing 10 nM staurosporine or 50 μM DPI. After a 1 h incubation and before lucigenin chemiluminescence analysis, 500 ng/ml PMA was added for 30 min (medium/PMA, staurosporine/PMA, DPI/PMA). Lucigenin chemiluminescence was monitored for 20 s; values are expressed in RLU/s (± SE).

tion in cells which are naturally resistant to Fas induces sensitivity to Fas-mediated cell death. Thus, contrary to the toxic effect of ROIs at concentrations which overwhelm cellular antioxidant capacity, an O_2^--based oxidative stress can render cells resistant to a major trigger of apoptosis.

Inhibition of Fas-mediated apoptosis by an artificially induced increase in intracellular O_2^-

Inhibition of O_2^- dismutation was achieved using DDC, which is currently the best-characterized inhibitor of Cu/Zn-SOD, both *in vitro* and *in vivo* (Ogino *et al.*, 1990; Iqbal and Whitney, 1991; Hiraishi *et al.*, 1992; Oyama *et al.*, 1994; Rothstein *et al.*, 1994). Pre-incubation of M14 transfectants and Jurkat cells with 1 mM DDC for 1 h was observed to augment intracellular O_2^- and to strongly inhibit Fas-mediated death. Presence of DDC was not required at the time of TNF–Fas fusion protein or Fas receptor engagement. In contrast, when M14 transfectants were exposed to DDC for 18 h and then incubated with fresh DDC 1 h before engagement of Fas, no increase in intracellular O_2^- or in resistance to Fas was induced. These findings are consistent with the possibility that during a prolonged exposure to a SOD inhibitor, cells adapt to the oxidative stress by adjusting the abundance and activity of antioxidants which help re-establish baseline intracellular O_2^- levels. Because Cu/Zn SOD is already

Table I. Effect of reactive oxygen intermediate producing enzyme and kinase inhibitors on PMA-induced inhibition of Fas-mediated death

Reagents	Enzyme inhibited	Range of concentrations used	Inhibition of PKC-induced resistance to Fas-mediated cell death
Allopurinol	Xanthine oxidase	25 μM to 2.5 mM	–
Oxypurinol	Xanthine oxidase	5 μM to 500 μM	–
Indomethacin	Cyclooxygenase	50 μM to 5 mM	–
Nordihydroguaiaretic acid	Lipooxygenase	0.5 μM to 50 μM	–
Neopterin	NADPH oxidase	2 μM to 2 mM	+
DPI	NADPH oxidase and flavoprotein using enzymes	0.50 μM to 50 μM	++
N-nitro-L-arginine	NO synthetase	5 μM to 500 μM	–
N-monomethyl-L-arginine	NO synthetase	5 μM to 500 μM	–
Genistein	protein tyrosine kinase	6 μM to 200 μM	–
Wortmannin	phosphatidyl inositol 3 kinase	500 nM	–

M14 TF4 transfectants were incubated for 1 h in the presence of either allopurinol, oxypurinol, indomethacin, nordihydroguaiaretic acid, neopterin, DPI, *N*-nitro-L-arginine, *N*-monomethyl-L-arginine, genistein, wortmannin before adding 500 ng/ml PMA. After an additional 30 min incubation in the presence of PMA, 20 ng/ml of recombinant TNFα were added, and following a 6 h incubation cell death was assessed by crystal violet staining. –: cell survival after incubation with the reagent followed by PMA treatment was equal to survival after PMA treatment alone. +: cell survival after incubation with the reagent followed by PMA treatment was 40–50% of the survival after PMA treatment alone. ++: cell survival after incubation with the reagent followed by PMA treatment was equal to survival obtained without PMA treatment.

inhibited by prolonged exposure to DDC, addition of fresh DDC would not be expected to alter intracellular O_2^- levels or any biological process dependent on O_2^-. These observations suggest that Fas-mediated cell death is inhibited by the O_2^--based oxidative stress induced by SOD inhibition and not by a reactive antioxidant state in response to the oxidative stress or by an unrelated property of DDC itself. This notion is further supported by the observations that resistance to Fas-mediated death induced by DDC is abolished by *N*-acetylcysteine and the O_2^- scavenger, tiron.

The second approach used to augment intracellular concentration of O_2^- consisted of exposing M14 TF4 cells to paraquat, which generates intracellular O_2^-. Paraquat forms a stable, yet oxygen-sensitive paraquat radical (PQ^+), which reacts very rapidly with oxygen to generate O_2^- (Krall *et al.*, 1988). In M14 TF4 cells, a 3 mM concentration of paraquat in cell culture medium was observed to augment intracellular O_2^- without inducing cytotoxicity. Similar to the effect of inhibiting SOD, Fas-mediated apoptosis was dramatically reduced following pre-incubation of cells with paraquat, before engaging the TNF–Fas fusion protein. Exposure of cells to the superoxide-generating system composed of X/XO, had an effect comparable with that of paraquat. Taken together, these observations provide a strong argument that induction of an O_2^--based oxidative stress before engagement of Fas can promote cellular resistance to Fas-mediated death.

Intracellular sources of superoxide

Superoxide is produced at at least three distinct sites within the cell, the mitochondria, cytosol and plasma membrane. Plasma membrane-bound flavin oxidases, including NADPH oxidase (Nauseef *et al.*, 1991; Fialkow *et al.*, 1993; Chanock *et al.*, 1994; Massey, 1994) constitute an important, inducible source of intracellular O_2^-. Although the NADPH oxidase complex is associated primarily with professional phagocytes, where it induces the oxidative burst, related superoxide-producing oxidases are reported to be present in other cell types (Cross and Jones, 1991), and their activity is typically augmented in

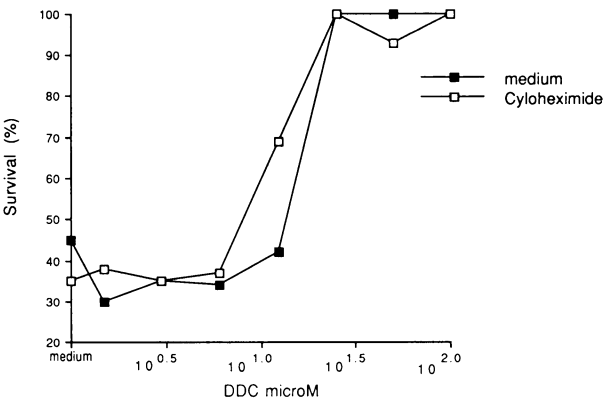

Fig. 6. The effect of cycloheximide on inhibition of Fas-mediated cell death by DDC. M14 TF4 cells were incubated for 1 h with increasing concentrations of DDC in medium alone or in medium containing 10 μg/ml cycloheximide, before addition of 30 ng/ml TNF. After a 6 h incubation, cell viability was determined by crystal violet staining. Survival is expressed as percentage of the staining value obtained in presence or absence of cycloheximide but without TNF.

metabolically active cells (Meier *et al.*, 1989; Cross and Jones, 1991; Watson *et al.*, 1991). That flavin oxidases may provide one source of O_2^- capable of regulating Fas-mediated cytotoxicity is supported by the observation that an inhibitor of flavin oxidases, such as DPI, sensitizes cells which are naturally resistant to Fas or in which resistance to Fas is induced by phorbol esters. Because flavin oxidase activity is stimulated by PKC (Watson *et al.*, 1991), it is conceivable that flavin oxidase activation provides one mechanism for the observed PMA/PKC-induced inhibition of Fas-mediated apoptosis. The precise involvement of PKC in regulating O_2^--dependent resistance to apoptosis, however, will require further elucidation. It is noteworthy that DPI-induced sensitivity to Fas in cells which are naturally resistant can be abrogated by DDC-mediated inhibition of Cu/Zn-SOD. This observation indicates that in the presence of inhibitors of SOD, sources of O_2^- other than flavin

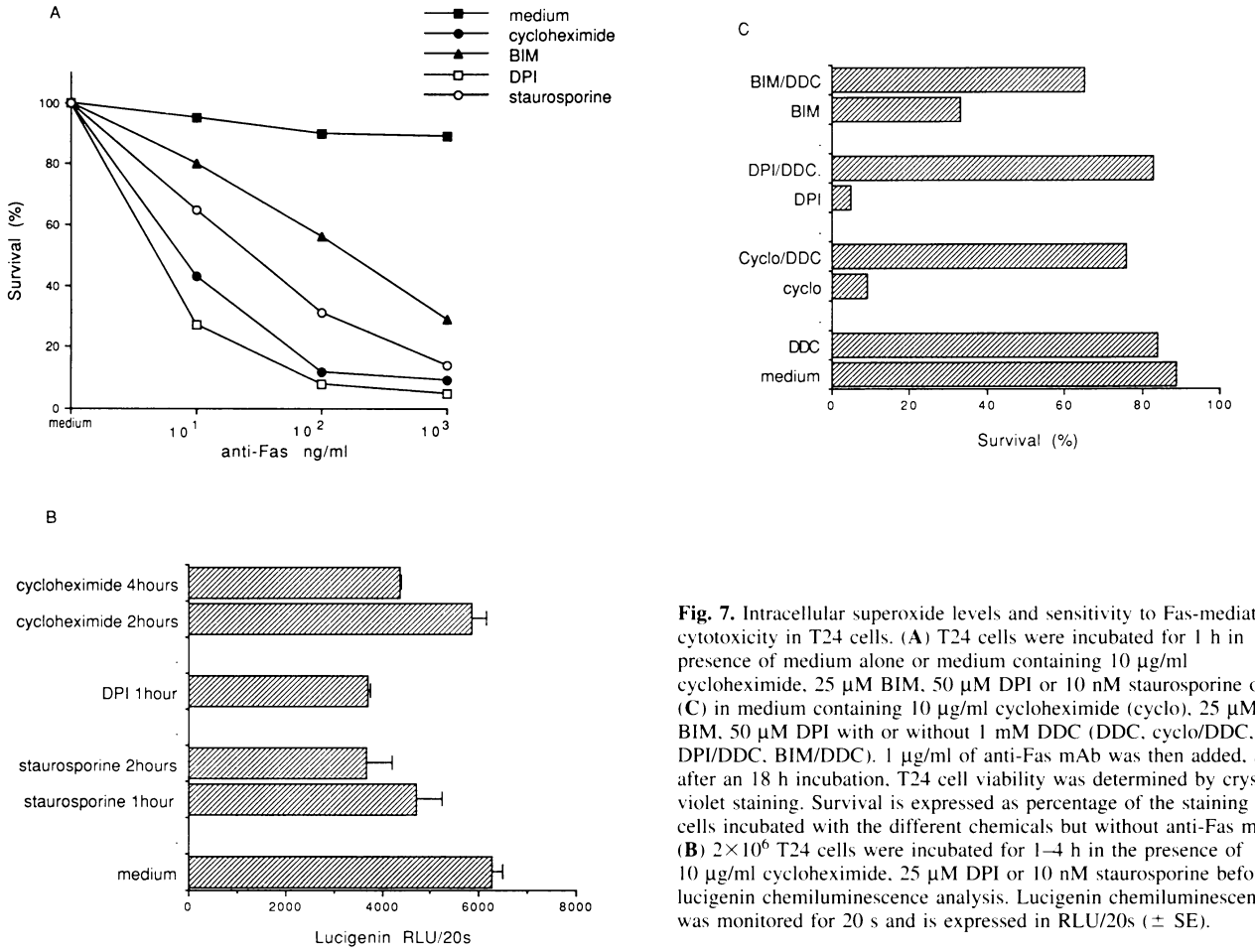

Fig. 7. Intracellular superoxide levels and sensitivity to Fas-mediated cytotoxicity in T24 cells. (**A**) T24 cells were incubated for 1 h in presence of medium alone or medium containing 10 μg/ml cycloheximide, 25 μM BIM, 50 μM DPI or 10 nM staurosporine or (**C**) in medium containing 10 μg/ml cycloheximide (cyclo), 25 μM BIM, 50 μM DPI with or without 1 mM DDC (DDC, cyclo/DDC, DPI/DDC, BIM/DDC). 1 μg/ml of anti-Fas mAb was then added, and after an 18 h incubation, T24 cell viability was determined by crystal violet staining. Survival is expressed as percentage of the staining of cells incubated with the different chemicals but without anti-Fas mAb. (**B**) 2×10^6 T24 cells were incubated for 1–4 h in the presence of 10 μg/ml cycloheximide, 25 μM DPI or 10 nM staurosporine before lucigenin chemiluminescence analysis. Lucigenin chemiluminescence was monitored for 20 s and is expressed in RLU/20s (± SE).

oxidases are sufficient to attain intracellular concentrations which confer resistance to Fas-mediated death.

A role for O_2^- in natural resistance to Fas-mediated apoptosis

Functionally active Fas is expressed on a wide variety of normal and malignant cells, many of which are resistant to its cytotoxic signals. Tumor cells typically exhibit a prooxidant state, characterized by elevated intracellular ROIs, and more specifically, O_2^- concentration (Cerutti, 1985). The prooxidant state is thought to be implicated in DNA damage and mutagenesis which are associated with malignancies and underlie many of the properties of tumor cells, including their invasive and metastatic proclivity. It is also well established that in most cases, the prooxidant state confers a growth advantage to tumor cells, which is inhibited by antioxidants (Cerutti, 1985). The growth advantage with which tumor cells are endowed is often more closely related to an intrinsic resistance to apoptosis than to an increased proliferative capacity. Our results are consistent with the possibility that a prooxidant state may provide one natural mechanism for tumor cell resistance to apoptosis mediated by Fas.

How might O_2^- induce resistance to Fas signals? O_2^-, similar to other ROIs, is a small, ubiquitous molecule, thereby bearing some of the characteristics of second messengers and there is good evidence that ROIs can promote transcription factor activity and regulate expression of a variety of genes (Shibanuma *et al.*, 1988; Abate *et al.*, 1990). However, in view of the fact that O_2^- blocks Fas-mediated apoptosis in the presence of inhibitors of protein synthesis and protein kinase activity, it seems improbable that its effect relies on specific gene activation or on protein phosphorylation. More likely possibilities are that O_2^- may regulate interaction between Fas and intracellular Fas-binding proteins (Chinnaiyan *et al.*, 1995; Stanger *et al.*, 1995), which may be required in Fas-mediated death, or, alternatively, inhibit the activity of ICE-type proteases (Thornberry *et al.*, 1992) which are proposed to provide the final death signal common to most triggers of apoptosis (Miura *et al.*, 1993; Yuan *et al.*, 1993) and which have been directly implicated in Fas signaling (Enari *et al.*, 1995; Tewari *et al.*, 1995). Elucidation of the mechanism by which O_2^- inhibits Fas-mediated cell death will be important in resolving the molecular function of Fas and may have broad implications in understanding the regulation of apoptosis in general.

Materials and methods

Cell lines

M14 TNF–Fas transfectants were developed in the human melanomas M14 (A.Bartolazzi and I.Stamenkovic, unpublished results). The human bladder carcinoma T24 and Jurkat cell line were purchased from ATCC (Rockville, MD). The human osteosarcoma U20S was donated by Dan Haber of the Cancer Center at Massachusetts General Hospital. M14 TNF–Fas clones were cultured in DME/5% fetal bovine serum (FBS) (Irvine Scientific, CA) supplemented with 1 mg/ml G418 (Gibco-BRL, Gaithersburg, MD). T24 and U2OS were maintained in culture in

DME/5% FBS and Jurkat cells in RPMI/10% FBS. Peripheral blood lymphocytes were obtained from healthy donors and separated on Ficoll–Hypaque using standard procedures. Activation of T cells was performed by adding 1 μg/ml phytohemagglutinin A (PHA) (Sigma Chemical Co., St Louis, MO) to the culture medium (RPMI, 5% FBS) for 4–9 days.

Development of stable transfectants

M14 melanoma cells were stably transfected with the TNF–Fas expression plasmid described previously (Clément and Stamenkovic, 1994). The TNF–Fas expression plasmid was added to 0.5 ml of a 10^7 cells/ml suspension along with the pSV2Neo selection vector at a ratio 15:1 μg. Transfection was performed by electroporation, using a Gene Pulser (Bio-Rad Laboratories, Richmond, CA), at 625 V/cm, 960 μF. Cells were seeded onto culture plates in DME/20% FBS (Irvine Scientific), and 48 h later, the medium was replaced by fresh DME/5% FBS supplemented with 1 mg/ml G418 (Gibco-BRL). Resistant clones were tested for TNFRp55 expression by indirect immunofluorescence.

Immunofluorescence

M14 TNF–Fas transfectants were incubated with 1 μg of the anti TNFRp55 antibody htr9 (Clément and Stamenkovic, 1994) in 100 μl of PBS/5% FBS for 1 h at 4°C, washed, and incubated with fluorescein-conjugated affinity-purified goat anti-mouse antibody (Cappel, Malvern, PA) for 30 min at 4°C. Cells were washed, resuspended in PBS and analyzed by flow cytometry on a FACS (Becton Dickinson, Mountain view, CA). Clones showing a 3-fold increase in mean fluorescence intensity with respect to the parental cell line were considered positive.

Chemicals

Staurosporine, genistein and recombinant TNFα were purchased from Upstate Biotechnology Inc. (Lake Placid, NY), BIM, PMA and PDBu from Calbiochem-Novabiochem Corporation (San Diego, CA) and DPI from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). All other compounds used in this study, including DDC, methyl viologen (paraquat), *N*-acetylcysteine, tiron, allopurinol, oxypurinol, indomethacin, nordihydroguaiaretic acid, neopterin, *N*-nitro-L-arginine, *N*-monomethyl-L-arginine, wortmannin and lucigenin were purchased from Sigma Chemical Co. (St Louis, MO). Staurosporine, BIM, DPI, tiron, PMA, PDBu, genistein were diluted in dimethylsulfoxide (DMSO). Stock solutions were at least 100×. A 1/100 dilution of DMSO neither altered cell viability nor inhibited the Fas-mediated signal. Lucigenin stock solution was diluted in distilled water. All the other chemicals were directly diluted in the culture medium.

Cytotoxicity assays

To trigger Fas-mediated cytotoxicity, M14 TNF-Fas clones and Jurkat cell line were respectively incubated with 5–500 ng/ml of recombinant TNFα or with 1 μg/ml anti-Fas mAb (Upstate Biotechnology Inc.) for 5–6 h. T24 and U2OS were incubated with 0.1–1 μg/ml anti-Fas mAb for 18 h. Pre-incubation with all the chemicals except PMA and PDBu was always performed for 1 h before triggering of Fas signals.

Crystal violet staining was used to assess M14 TNF-Fas clone, T24 and U2OS cell viability following Fas triggering. Crystal violet assays were performed in 96-well microtiter plates. 2×10^4 cells/well were plated in DME/5% FBS, 24 h before the test was performed. Medium was then aspirated and replaced with fresh DME 5% FBS alone or containing different drugs before adding recombinant TNFα or anti-Fas antibody. After various incubation times, medium was aspirated and replaced for 10 min by 50 μl of 0.75% crystal violet in a 50% ethanol, 0.25% NaCl and 1.75% formaldehyde solution. Cells were then washed with water, air-dried, and the dye eluted with PBS/1% SDS. Cell viability was assessed by dye absorbance determined by optical density (OD) measurement at 595 nm on an automated ELISA reader. Survival was calculated as the mean of triplicate OD values of cells incubated with the specific ligand (TNFα or anti-Fas antibody) divided by the mean of triplicate OD values of cells incubated in control medium, and expressed as a percentage. In each case, the control medium included exactly the same ingredients as the test medium, with the exception of TNFα and anti-Fas mAb. The standard error (SE) of the mean of triplicate OD values never exceeded 5%. The data presented show one representative experiment from a minimum of three.

Jurkat cell viability was assessed using the MTT assay. MTT assays were done in 96-well microtiter plates. 5×10^4 cells/well of Jurkat cells or 4- to 9-day PHA-activated lymphocytes were plated in the presence of RPMI/5% FBS alone or containing various concentrations of staurosporine, DPI, PMA or DDC. Following a 1 h incubation, 1 μg/ml anti-Fas antibody was added. Following respective incubation time, 10 μl of a 5 mg/ml MTT (Sigma) stock solution in PBS was added per well and the incubation continued for 2 h at 37°C. Finally, elution of the precipitate was performed using 100 μl of 15% SDS and 0.02 M HCl. Following a 5 h incubation at room temperature, absorption values at 595 nm were determined in an automated ELISA reader. Survival was calculated as mean of triplicate OD determinations of cells incubated in presence of anti-Fas antibody divided by the mean of triplicate OD determinations of cells incubated in control medium and is expressed as a percentage. The SE of the mean of triplicate OD values never exceeded 5%.

Chemiluminescence assay

Lucigenin chemiluminescence assays were performed as described by Porter *et al.* (1992). 2×10^6 cells were seeded onto 100 mm tissue culture dishes 24 h before the experiment was performed. Incubation in the presence of the different chemicals before the lucigenin chemiluminescence reading was performed at 37°C in the culture dishes. Cells were then washed once with PBS, trypsinized (trypsin, Irvine Scientific, CA), transferred to a sample cuvette and centrifuged at 2000 r.p.m. at room temperature for 1 min. Pellets were resuspended and cells permeabilized in 400 μl of 1× releasing agent (Sigma). 100 μl of 850 μM lucigenin stock solution, was injected automatically before the reading. Chemiluminescence was monitored for 20 s in a Lumat LB 9501 luminometer (Wallac Inc., Gaithersburg, MD). Data are shown in relative light units per second (RLU/s) or per 20 s (RLU/20s) ± SE from two independent measurements.

Acknowledgements

We thank Dan Haber and Shyamala Maheshawan for the generous gift of U20S cells and Robert McCluskey and Pierre Vassalli for valuable discussions and comments. This work was supported by USPH grant CA55735. I.S. is a Scholar of the Leukemia Society of America.

References

Abate,C., Patel,L., Rauscher,R.J.III and Curran,T. (1990) Redox regulation of Fos and Jun DNA-binding activity *in vitro*. *Science*, **249**, 1157–1161.

Amstad,P., Alexander,P., Shah,G., Mirault,M.-E., Moret,R., Zbinden,I. and Cerutti,P. (1991) The balance between Cu,Zn-superoxide dismutase and catalase affects the sensitivity of mouse epidermal cells to oxidative stress. *Biochem. J.*, **30**, 9305–9313.

Amstad,P., Moret,R. and Cerutti,P. (1994) Glutathione peroxidase compensates for the hypersensitivity of Cu,Zn-superoxide dismutase overproducers to oxidant stress. *J. Biol. Chem.*, **269**, 1606–1609.

Behl,C., Davis,J.B., Lesley,R. and Schubert,D. (1994) Hydrogen peroxide mediates amyloid β protein toxicity. *Cell*, **77**, 817–827.

Brunner,T. *et al.* (1995) Cell-autonomous Fas (CD95)/Fas-ligand interaction mediated activation-induced apoptosis in T cell hybridomas. *Nature*, **373**, 441–444.

Buttke,T.M. and Sandstrom,P.A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today*, **15**, 7–10.

Cerutti,P.A. (1985) Prooxidant states and tumor promotion. *Science*, **227**, 375–381.

Chanock,S.J., Benna,J.E., Smith,R.M. and Babior,B.M. (1994) The respiratory burst oxidase. *J. Biol. Chem.*, **269**, 24519–24522.

Chinnaiyan,A.M., O'Rourke,K., Tewari,M. and Dixit,V.M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. *Cell*, **81**, 505–512.

Clément,M.-V. and Stamenkovic,I. (1994) Fas and TNF receptor-mediated cell death: similarities and distinctions. *J. Exp. Med.*, **180**, 557–567.

Cross,A.R. (1987) The inhibitory effects of some iodonium compounds on the superoxide generating system of neutrophils and their failure to inhibit diaphorase activity. *Biochem. Pharmacol.*, **36**, 489–493.

Cross,A.R. (1990) Inhibitors of the leukocyte superoxide generating oxidase: mechanisms of action and methods for their elucidation. *Free Radical Biol. Med.*, **8**, 71–93.

Cross,A.R. and Jones,O.T.G. (1986) The effect of the inhibitor diphenylene iodonium on the superoxide generating system of neutrophils. Specific labelling of a component polypeptide of the oxidase. *Biochem. J.*, **237**, 111–116.

Cross,A.R. and Jones,O.T.G. (1991) Enzymic mechanisms of superoxide production. *Biochim. Biophys. Acta*, **1057**, 281–298.

Dhein,J., Walczak,H., Baumler,C., Debatin,K.-M. and Krammer,P.H.

(1995) Autocrine T-cell suicide mediated by APO-1/(Fas/CD95). *Nature*, **373**, 438–441.

Ellis,R.E., Yuan,J. and Horvitz,H.R. (1991) Mechanisms and functions of cell death. *Annu. Rev. Cell Biol.*, **7**, 663–698.

Elroy-Stein,O., Bernstein,Y. and Groner,Y. (1986) Overproduction of human Cu/Zn-superoxide dismutase in transfected cells: extenuation of paraquat-mediated cytotoxicity and enhancement of lipid peroxidation. *EMBO J.*, **5**, 615–622.

Enari,M., Hug,H. and Nagata,S. (1995) Involvement of an ICE-like protease in Fas-mediated apoptosis. *Nature*, **375**, 78–81.

Fialkow,L., Chan,S.K., Grinstein,S. and Downey,G.P. (1993) Regulation of tyrosine phosphorylation in neutrophils by the NADPH oxidase: role of reactive oxygen intermediates. *J. Biol. Chem.*, **268**, 17131–17137.

Fridovich,I. (1976) Oxygen radical, hydrogen peroxide, and oxygen toxicity. In Pryor,W.A. (ed.), *Free Radicals in Biology*. Academic Press, London, pp. 239–277.

Fridovich,I. (1978) The biology of oxygen radicals. *Science*, **201**, 875–880.

Gyllenhammar,H. (1987) Lucigenin chemiluminescence in the assessment of neutrophil superoxide production. *J. Immunol. Methods*, **97**, 209–220.

Halliwell,B. and Gutteridge,J.M.C. (1989) *Free Radicals in Biology and Medicine*. Second Edition. Clarendon Press, Oxford.

Hancock,J.T. and Jones,O.T.G. (1987) The inhibition by diphenylene iodonium and its analogues of superoxide generation by macrophages. *Biochem. J.*, **242**, 103–107.

Hassan,H.M. (1988) Biosynthesis and regulation of superoxide dismutases. *Free Radical Biol. Med.*, **5**, 377–385.

Hiraishi,H., Terano,A., Razandi,M., Sugimoto,T., Harada,T. and Ivey,K.J. (1992) Role of cellular superoxide dismutase against reactive oxygen metabolite injury in cultured bovine aortic endothelial cells. *J. Biol. Chem.*, **267**, 14812–14817.

Hiraishi,H., Terano,A., Sugimoto,T., Harada,T., Razandi,M. and Ivey,K.J. (1994) Protective role of intracellular superoxide dismutase against extracellular oxidants in cultured rat gastric cells. *J. Clin. Invest.*, **93**, 331–338.

Huang,T.-T., Carlson,E.J., Leadon,S.A. and Epstein,C.J. (1992) Relationship of resistance to oxygen free radicals to CuZn-superoxide dismutase activity in transgenic, transfected, and trisomic cells. *FASEB J.*, **6**, 903–910.

Iqbal,J. and Whitney,P. (1991) Use of cyanide and diethyldithiocarbamate in the assay of superoxide dismutases. *Free Radical Biol. Med.*, **10**, 69–77.

Itoh,N., Yonehara,S., Ishii,A., Yonehara,M., Mizushima,S.I., Sameshima,M., Hase,A., Seto,Y. and Nagata,S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. *Cell*, **66**, 233.

Jin,L.-W., Inaba,K. and Saitoh,T. (1992) The involvement of protein kinase C in activation-induced cell death in T-cell hybridoma. *Cell. Immunol.*, **144**, 217–227.

Ju,S.-T., Panka,D.J., Cui,H., Ettinger,R., El-Khatib,M., Sherr,D.H., Stanger,B.Z. and Marshak-Rothstein,A. (1995) Fas(CD95)/FasL interactions required for programmed cell death after T-cell activation. *Nature*, **373**, 444–448.

Kojima,S., Nomura,T., Icho,T., Kajiwara,Y., Kitabatake,K. and Kubota,K. (1993) Inhibitory effect of neopterin on NADPH-dependent superoxide-generating oxidase of rat peritoneal macrophages. *FEBS Lett.*, **329**, 125–128.

Krall,J., Bagley,A.C., Mullenbach,G.T., Hallewell,R.A. and Lynch,R.E. (1988) Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *J. Biol. Chem.*, **263**, 1910–1914.

Lee,F.J. and Hassan,H.M. (1986) Biosynthesis of superoxide dismutase in *Saccharomyces cerevisiae*: effects of paraquat and copper. *J. Free Radical Biol. Med.*, **1**, 319–325.

Massey,V. (1994) Activation of molecular oxygen by flavins and flavoproteins. *J. Biol. Chem.*, **269**, 22459–22462.

Meier,B., Radeke,H.H., Selle,S., Younes,M., Sies,H., Resch,K. and Habermehl,G.G. (1989) Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumor necrosis factor α. *Biochem. J.*, **263**, 539–545.

Meister,A. and Anderson,M.E. (1983) Glutathione. *Annu. Rev. Biochem.*, **52**, 711–760.

Miura,M., Zhu,H., Rotello,R., Hartwieg,E.A. and Yuan,J. (1993) Induction of apoptosis in fibroblasts by IL-1b-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell*, **75**, 653–550.

Munzel,T., Sayegh,H., Freeman,B.A., Tarpey,M.M. and Harrison,D.G. (1995) Evidence for enhanced vascular superoxide anion production in nitrate tolerance. A novel mechanism underlying tolerance and cross-tolerance. *J. Clin. Invest.*, **95**, 187–194.

Nagata,S. and Golstein,P. (1995) The Fas death factor. *Science*, **267**, 1449–1456.

Nauseef,W.M., Volpp,B.D., McCormick,S., Leidal,K.G. and Clark,R.A. (1991) Assembly of the neutrophil respiratory burst oxidase: protein kinase C promotes cytoskeletal and membrane association of cytosolic oxidase components. *J. Biol. Chem.*, **266**, 5911–5917.

Ogino,K., Hobara,T., Kawamoto,T., Kobayashi,H., Iwamoto,S., Oka,S. and Okazaki,Y. (1990) Mechanism of diethyldithiocarbamate-induced gastric ulcer formation in the rat. *Pharmacol. Toxicol.*, **66**, 133–137.

Owen-Schaub,L.B., Radinsky,R., Kruzel,E., Berry,K. and Yonehara,S. (1994) Anti-Fas on nonhematopoietic tumors: levels of Fas/APO-1 and bcl-2 are not predictive of biological responsiveness. *Cancer Res.*, **54**, 1580–1586.

Oyama,Y., Furukawa,K., Chikahisa,L. and Hatakeyama,Y. (1994) Effect of *N,N*-diethyldithiocarbamate on ionomycin-induced increase in oxidation of cellular 2',7'-dichlorofluorescin in dissociated cerebellar neurons. *Brain Res.*, **660**, 158–161.

Pagano,P.J., Tornheim,K. and Cohen,R.A. (1993) Superoxide anion production by rabbit thoracic aorta: effect of endothelium-derived nitric oxide. *Am. J. Physiol.*, **265**, H707–712.

Porter,C.D., Parka,M.H., Collins,M.K.L., Levinsky,R.J. and Kinnon,C. (1992) Superoxide production by normal and chronic granulomatous disease (CGD) patient-derived EBV-transformed B cell lines measured by chemiluminescence-based assays. *J. Immunol. Methods*, **155**, 151–157.

Rothstein,J.D., Bristol,L.A., Hosler,B., Brown,R.H.,Jr and Kuncl,R.W. (1994) Chronic inhibition of superoxide dismutase produces apoptotic death of spinal neurons. *Proc. Natl Acad. Sci. USA*, **91**, 4155–4159.

Ruegg,U.T. and Buirgess,G.M. (1989) Staurosporine, K252 and UCN-01: potent but non-specific inhibitors of protein kinases. *Trends Pharmacol. Sci.*, **10**, 218–220.

Schreck,R., Rieber,P. and Baeuerle,P.A. (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *EMBO J.*, **10**, 2247–2258.

Schulze-Osthoff,K., Krammer,P. and Droge,W. (1994) Divergent signalling via APO-1/Fas and the TNF receptor, two homologous molecules involved in physiological cell death. *EMBO J.*, **13**, 4587–4596.

Shibanuma,M., Kuroki,T. and Nose,K. (1988) Induction of DNA replication and expression of proto-oncogene c-myc and c-fos in quiescent Balb/3T3 cells by xanthine/xanthine oxidase. *Oncogene*, **3**, 17–21.

Stanger,B.Z., Leder,P., Lee,T.-H., Kim,E. and Seed,B. (1995) RIP: a novel protein containing a death domain that interacts with Fas/APO-1 (CD95) in yeast and causes cell death. *Cell*, **81**, 513–523.

Steller,H. (1995) Mechanisms and genes of cellular suicide. *Science*, **267**, 1445–1449.

Stuehr,D.J., Fasehun,O.A., Kwon,N.S., Gross,S.S., Gonzalez,J.A., Levi,R. and Nathan,C.F. (1991) Inhibition of macrophage and endothelial cell nitric oxide synthetase by diphenylieneiodonium and its analogs. *FASEB J.*, **5**, 98–103.

Tewari,M., Quan,L.T., O'Rourke,K., Desnoyers,S., Zeng,Z., Beidler,D.R., Poirier,G.G., Salvesen,G.S. and Dixit,V.M. (1995) Yama/CPP32β, a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell*, **81**, 801–809.

Thompson,C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462.

Thornberry,N.A. *et al.* (1992) A novel heterodimeric cysteine protease is required for interleukin-1b processing in monocytes. *Nature*, **356**, 768–774.

Toullec,D. *et al.* (1991) The bisindolemaleimide GF109203X is a potent and selective inhibitor of protein kinase C. *J. Biol. Chem.*, **266**, 15771–15781.

Watson,F., Robinson,J. and Edwards,S.W. (1991) Protein kinase C-dependent and -independent activation of the NADPH oxidase of human neutrophils. *J. Biol. Chem.*, **266**, 7432–7439.

Wyllie,A.H., Kerr,J.F.R. and Currie,A.R. (1980) Cell death: the significance of apoptosis. *Int. Rev. Cytol.*, **68**, 251–306.

Yuan,J., Shaham,S., Ledoux,S., Ellis,H.M. and Horvitz,H.R. (1993) The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1-β-converting enzyme. *Cell*, **75**, 641–652.

Zimmerman,R.J., Chan,A. and Leadon,S.A. (1989) Oxidative damage in murine tumor cells treated *in vitro* by recombinant human tumor necrosis factor. *Cancer Res.*, **49**, 1644–1648.

Received on September 10, 1995; revised on September 20, 1995