



Thioredoxin participates in a cell death pathway induced by interferon and retinoid combination

Xinrong Ma^{1,6}, Sreenivasu Karra^{1,6}, Daniel J Lindner^{1,5}, Junbo Hu¹, Sekhar PM Reddy², Adi Kimchi³, Junji Yodoi⁴ and Dhananjaya D Kalvakolanu^{*1}

¹Greenebaum Cancer Center, Department of Microbiology and Immunology, Molecular and Cellular Biology Program, University of Maryland School of Medicine, Baltimore, Maryland, MD 21201 USA; ²Department of Environmental Sciences, The Johns Hopkins University School of Public Health, Baltimore, Maryland, MD, USA; ³Department of Molecular Genetics and Virology, Weizmann Institute of Science, Rehovot, Israel; ⁴Department of Biological Responses, Institute for Virus Research, 53 Kawahara-Cho, Shogin, Japan

Interferons (IFNs) and retinoids are potent tumor growth suppressors. We have shown earlier that the IFN- β and all-trans retinoic acid combination, but not the single agents, induces death in several tumor cell lines. Employing a genetic approach we have recently identified several Genes associated with Retinoid-IFN induced Mortality (GRIM) that mediate the cell death effect of IFN/RA combination. One of the GRIMs, GRIM-12, was identical to human thioredoxin reductase (TR), an enzyme that controls intracellular redox state. To define the participants of TR mediated death pathway we have examined the role of thioredoxin (Trx), its downstream substrate, and its influence on IFN/RA-induced death regulation. Inhibition of the thioredoxin expression by antisense RNA suppressed cell death. Similarly, a mutant Trx1 lacking the critical cysteine residues blocked cell death. In contrast, overexpression of wildtype thioredoxin augmented cell death. This effect of Trx1 was in part due to its ability to augment cell death via caspase-8. The redox inactive Trx1 mutant inhibits the cell death induced by caspase-8 but not caspase-3. These studies identify a novel mechanism of cell death regulation by IFN/RA combination involving redox enzymes. *Oncogene* (2001) 20, 3703–3715.

Keywords: cytokines; vitamin-A; apoptosis; redox control; tumor growth

Introduction

Cytokines protect the host against infectious pathogens and the development and survival of neoplastic cells. The interferon (IFN) family of cytokines stimulates antiviral, antitumor, and immunoregulatory activities. Using the Janus tyrosine kinases (JAK)-Signal Trans-

ducing Activator of Transcription (STAT) pathway (Darnell *et al.*, 1994), IFNs induce the expression of a number of cellular genes, which mediate their diverse actions. A central role for IFNs in neoplastic cell growth suppression was highlighted by observations such as an increased incidence of carcinogen-induced tumors in the IFN- γ receptor^{-/-} and STAT1^{-/-} mice, and a failure to reject the STAT1 null tumors by the immune system (Kaplan *et al.*, 1998). Some IFN-regulated factors such as IRF-1 and ICSBP (IFN-consensus sequence binding protein), act as tumor growth suppressors and mutations in these genes have been implicated in the development of myeloid leukemias (Holtshcke *et al.*, 1996; Willman *et al.*, 1993). Since STAT1 and IRFs are transcription factors, their growth suppressive effects are ultimately dependent on the downstream gene products induced by them. Among these two IFN induced antiviral enzymes, protein kinase R (PKR) and Ribonuclease L (RNaseL) have been implicated in stress-induced and spontaneous apoptosis (Balachandran *et al.*, 1998; Der *et al.*, 1997; Zhou *et al.*, 1997). However, PKR and RNaseL independent growth suppressive actions of IFNs have also been described (Kalvakolanu, 2000; Levy-Strumpf and Kimchi, 1998). IFNs also activate growth suppressive proteins such as pRb (Resnitzky *et al.*, 1992) and down regulate *c-myc*, E2F and cyclin D3 in certain lymphoblastoid tumor cell lines (Raveh *et al.*, 1996; Tiefenbrun *et al.*, 1996). Although IFNs are effective in the therapy of certain leukemias, they are marginally active in the therapy of solid tumors (Gutterman, 1994). To overcome this drawback a number of combination therapies have been developed. Among these, the combination of IFNs with retinoids is highly effective against several tumors (Moore *et al.*, 1994).

Retinoids are vitamin A derivatives, which induce the expression of genes involved in differentiation, growth control and metabolism using specific nuclear receptors (Mangelsdorf and Evans, 1995). In vitamin A deprived animals, high incidence of a number of carcinomas and leukemias (Love and Gudas, 1994) and an abnormal rise in myelopoiesis owing to a loss of spontaneous apoptosis (Kuwata *et al.*, 2000) were

*Correspondence: DV Kalvakolanu

⁵Current address: Taussig Cancer Center, Cleveland Clinic Foundation, Cleveland, Ohio, OH 44195, USA

⁶Contributed equally to this work

Received 22 December 2000; revised 15 March 2001; accepted 19 March 2001

noted. All trans Retinoic Acid (RA), a prototypic retinoid, can either suppress or reverse these effects *in vivo*. RA inhibits the growth of certain neuroblastomas, promyelocytic leukemias, and teratocarcinomas *in vitro* (Love and Gudas, 1994). Two structurally similar but genetically distinct transcription factors, the retinoic acid receptor (RAR) and the retinoid X receptor (RXR) induce the expression of genes involved in growth suppression (Glass *et al.*, 1997). However, the nature of these retinoid-regulated inhibitors of cell growth is unknown. A perplexing number of RAR and RXR isoforms present in mammalian cells (Mangelsdorf and Evans, 1995) seem to mediate this complex process. Based on this, several synthetic and natural retinoids that can differentially activate these receptors have been identified. The growth suppressive effects of synthetic retinoids are mediated by a RAR–RXR dependent and independent manner (Liu *et al.*, 1996; Lotan, 1996). Induction of caspase-dependent cleavage of ubiquitous transcription factor Sp1 and cell death by certain synthetic retinoids occurs independent of transcription (Piedrafito and Pfahl, 1997). Other synthetic retinoids induce cell death by a p53 dependent or caspase dependent manner (Sun *et al.*, 2000). Some retinoids activate translocation of orphan nuclear receptor TR3 from nucleus to the mitochondrion, and the consequent release of cytochrome *c* to induce apoptosis (Li *et al.*, 2000).

Although IFN and RA use different signaling pathways, a cross talk between their growth suppressive pathways exists. PML–RAR α , a mutant chimeric retinoic acid receptor found in certain acute promyelocytic leukemias (Wang *et al.*, 1998) responds to RA. Interestingly, this mutant receptor is induced by IFNs and has been reported to participate in the anticellular action of IFN- α (Chelbi-Alix *et al.*, 1998; Nason-Burchenal *et al.*, 1996). PML forms a nuclear body consisting of several IFN-inducible gene products (Gaboli *et al.*, 1998; Gongora *et al.*, 1997). PML is induced by IFNs and its promoter contains STAT1 binding sites (Pelicano *et al.*, 1997). In certain IFN-resistant cells, RA induces STAT1 levels resulting in an enhancement of IFN-responses (Gianni *et al.*, 1997; Kolla *et al.*, 1997; Matikainen *et al.*, 1997). RA has also been shown to induce some ISGs directly (Pelicano *et al.*, 1997). Indeed, several clinical and experimental studies have shown that the IFN/RA combination is a more potent inhibitor of cell growth than either drug alone (Lindner *et al.*, 1997; Moore *et al.*, 1994).

We have shown earlier that the IFN/RA combination, but not the single agents, causes cell death *in vitro* and suppresses tumor growth *in vivo* (Lindner *et al.*, 1997). Furthermore, no detectable activation of IFN-stimulated PKR or RNaseL and no change in p53 levels, and pRb phosphorylation status occurred under these conditions (Hofmann *et al.*, 1998). These data suggested the existence of novel IFN/RA-regulated mechanisms of cell death. To identify the genes responsible for cell death and define their mechanism of action, we employed an antisense technical knock-out approach (Deiss and Kimchi, 1991). In this

approach specific death-associated genes are identified by their ability to confer a growth advantage to cells in the presence of death inducers, when expressed in an antisense orientation. This resulted in the identification of several Genes associated with Retinoid-IFN induced Mortality (GRIM) (Hofmann *et al.*, 1998). One of the GRIMs, GRIM-12, was identical to human thioredoxin reductase (TR), an intracellular redox regulatory enzyme (Hofmann *et al.*, 1998). In this study, we show that thioredoxin 1 (Trx1), the downstream substrate of TR, mediates some of the death functions. The activation of certain members of the caspase family is regulated by IFN/RA combination through thioredoxin. We show that the wildtype, but not redox inactive Trx1 mutant promotes cell death by stimulating caspase-8 activation.

Results

Role of Trx in the IFN/RA induced cell death

Our previous studies have shown that thioredoxin reductase is critical for cell death to occur in human breast carcinoma cells, following IFN- β /RA treatment. Since mammalian TR can reduce a variety of substrates (Arner and Holmgren, 2000), we have examined the role of its well-known substrate, Trx1, in cell death regulation. Although a recent member of this family, Trx2, has been described (Spyrou *et al.*, 1997) this study will primarily focus on the well-characterized substrate Trx1. Therefore, MCF-7 cells were transfected with mammalian episomal vector pTKO1 or the same vector carrying the human Trx1 gene in an antisense orientation. Following this cells were selected with Hygromycin B in the presence or absence of human IFN- β (500 U/ml) and RA for 3 weeks. Cells transfected with pTKO1 vector perished following IFN/RA treatment as evidence by a lack of colony formation (Figure 1a). In contrast, a significant number of cell colonies survived in the pTKO1-Trx transfected plates under similar conditions. The lack of colonies in pTKO1 transfected plates was not due to toxic effect of the transfection protocol, because a number of colonies have survived in the presence of Hygromycin alone. Interestingly, a fraction of pTKO1-Trx transfected cells survived the toxic effect of IFN- β /RA. Thus, antisense Trx offers a partial protection against cell death.

The cell protective function of antisense Trx was also confirmed in two independent assays. These are: (1) a colorimetric growth assay that quantifies cell number and (2) annexin-V binding assay, which quantifies apoptotic cells. Equal numbers of cells were plated and exposed to IFN/RA combination for 7 days. Cell growth was compared to the untreated controls for each cell line. IFN/RA treatment of pTKO1 transfected cells caused cell death as measured in the colorimetric assay. Under the same conditions, cells expressing the antisense Trx1 were resistant to cell death (Figure 1b). Similarly, significantly higher

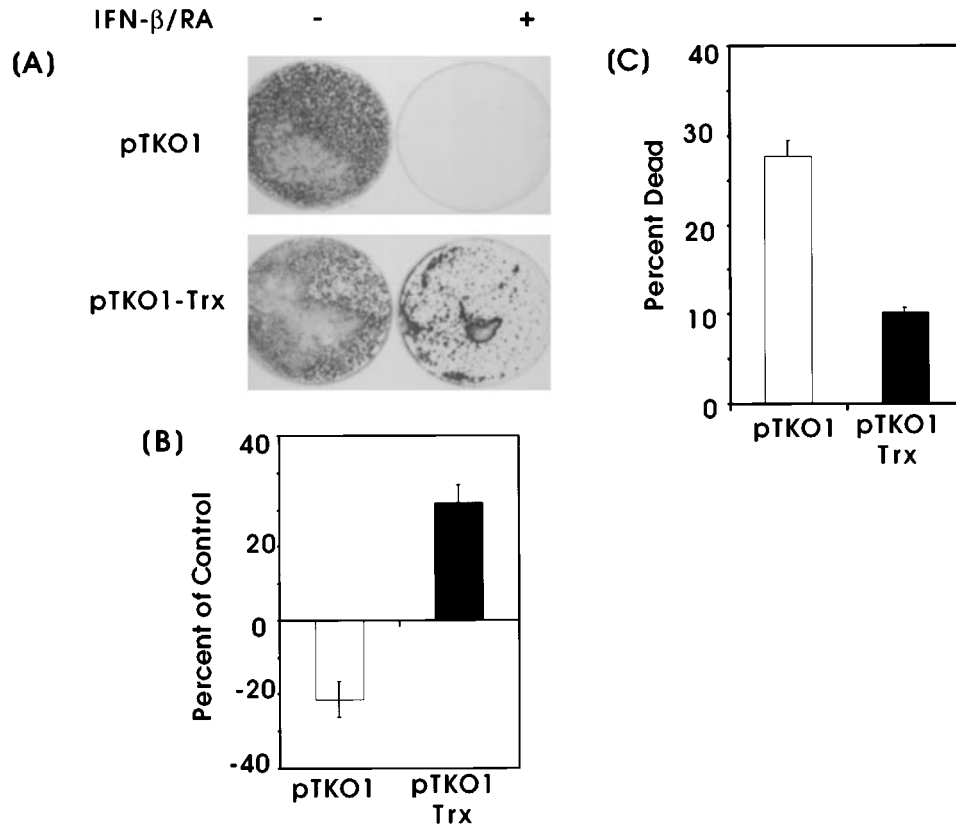


Figure 1 Antisense Trx1 confers resistance against IFN/RA induced cell death. (a) MCF-7 cells were transfected with episomal vector pTKO1 or the same vector carrying the Trx1 cDNA in an antisense orientation were selected for 2 weeks with Hygromycin B (100 $\mu\text{g/ml}$), in the absence (-) or presence (+) of human IFN- β (500 U/ml) and RA (1 μM). The plates were then fixed, stained with Sulforhodamine B prior to photography. In (b) the same cells were used in a colorimetric growth assay (see Materials and methods) for quantifying the differences in death sensitivities of the cell lines expressing vector and antisense Trx1. Cells were treated with IFN and RA as described above for 5 days. Absorbance values for 0 and 100% growth in this experiment are 0.162 and 0.602, respectively. Each data point represents mean \pm s.e. of 12 replicates. (c) Cells treated with IFN/RA were stained with annexin-V as described under Materials and methods. The percentage of annexin-V positive cells was quantified using FACS analysis. Each bar represents mean \pm s.e. of triplicates

annexin-V positive cells were observed in pTKO1-transfected cultures than in the antisense Trx transfected cells (Figure 1c). These data suggest that Trx1 plays a role in IFN/RA induced cell death.

Inhibition of cell death was due to down regulation of Trx levels

We next determined whether the survival was due to the expression and the consequent downregulation of cellular Trx levels. Total RNA was prepared from IFN/RA treated MCF-7 cells transfected with pTKO1 and antisense-Trx1 and analysed by Northern blotting. These blots were probed with ^{32}P -labeled human Trx1 cDNA. As expected a mRNA corresponding to the endogenous Trx1 (E) was detected in both cell types (Figure 2a). However, an additional band corresponding to the antisense Trx mRNA was found only pTKO1-Trx transfected cells. This mRNA moves to a higher position on the gel (indicated by a filled triangle) than the endogenous mRNA due to the fact that vector derived

untranslated sequence is added to the 3' of the antisense gene.

To determine whether antisense mRNA expression caused a depletion of Trx protein, Western blotting was performed using human Trx1 specific antibodies. Equal amounts of cell extracts from cells described above were used in this experiment. As shown in Figure 2b, although the Trx protein was detected in both cases, its levels were significantly suppressed (7–8-fold) in the pTKO1-Trx cells compared to the vector transfected cells. These blots were also probed with a monoclonal antibody against actin protein to ensure the presence of comparable amounts of protein in both the lanes.

Trx is involved in the IFN/RA induced death pathway

To further demonstrate the role for Trx in IFN/RA induced cell death MCF-7 cells were stably transfected with the expression vectors carrying wildtype or mutant Trx. The mutant bore serine residues in the place of critical cysteines at positions 32 and 35. Two

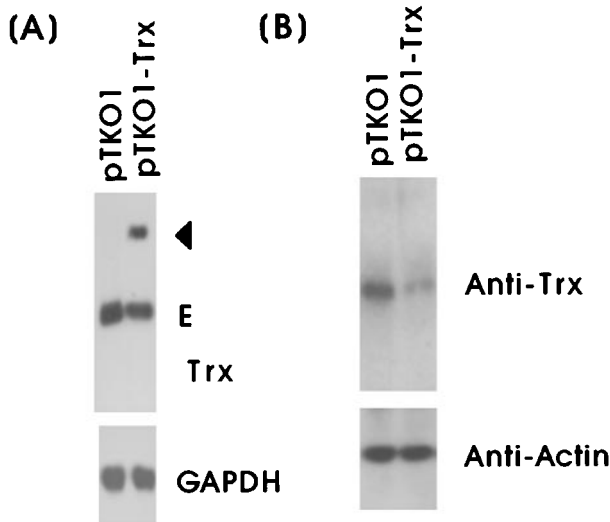


Figure 2 Cells were treated as described in Figure 1a and the expression of Trx1 RNA and protein was analysed. (a) Expression of antisense Trx1 mRNA. Comparable amount of total RNA (35 μ g) from each cell line was Northern blotted and probed with a 32 P-labeled human Trx1 cDNA. The endogenous (sense) and antisense Trx1 mRNAs were indicated by the letter E and a filled triangle, respectively. The same blot reprobed with GAPDH cDNA was shown in the lower portion of panel (a). (b) Expression antisense Trx1 mRNA depresses Trx1 protein levels. Total protein extracts (50 μ g) from the indicated cell lines were Western blotted using Trx1 specific antibodies

sets of plates were set up in each case. In one case, the number of G418 resistant colonies formed (Figure 3a) after transfection was determined by staining the transfected plates with Sulforhodamine B and in the other, the resultant colonies were pooled and used in the experiments. Transfection of expression vector alone in to MCF-7 cells resulted in the formation of \sim 150 G418-resistant colonies (bar 1). A significantly reduced number of colonies (\sim 33% fewer) formed upon transfection of cells with wildtype Trx1 (bar 2). In contrast, plates transfected with the mutant yielded a higher number of colonies (35% more) than in the control (bar 3 in Figure 3a). These data suggest that overexpression of wildtype Trx is cytotoxic while the mutant is protective. Cells expressing wildtype Trx1, survived after selection with G418, grew slower than those expressing either the empty vector or mutant Trx (Figure 3b). These data indicate that expression of moderate levels of Trx1 impedes cell growth.

We next examined whether these differential effects on cell growth were due to the expression of wildtype and mutant Trx1. Total RNA was extracted from cell lines stably transfected with Trx1 or its mutant and a comparable amount of RNA was employed in Northern blot analysis. These blots were probed with human Trx1 probe. Cells transfected with the mutant Trx had a 12-fold high level of Trx mRNA compared to the vector transfected ones (Figure 3c, compare bars 1 and 2). In contrast a sixfold more Trx mRNA was found in the wildtype Trx transfected cells than those with the vector alone. Similarly, we also examined the expres-

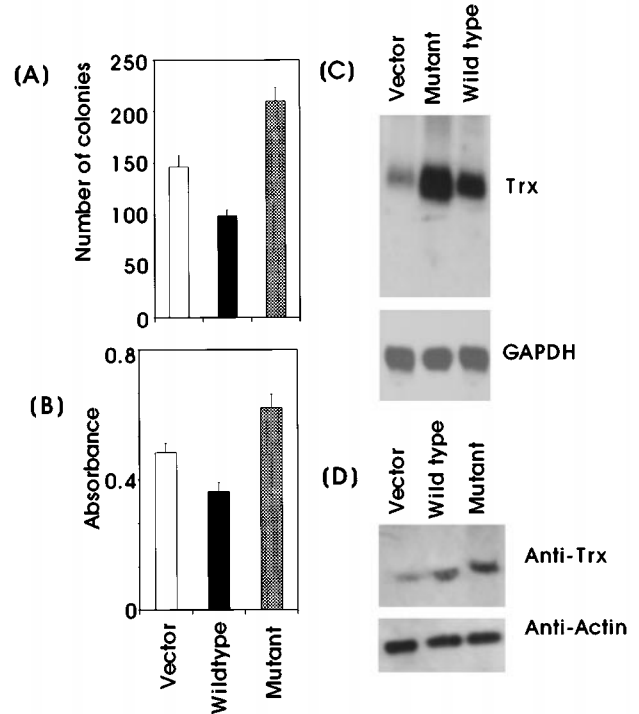


Figure 3 Death promoting effect of Trx1. (a) Overexpression of Trx1 results in a loss of cell viability. MCF-7 cells were transfected with equimolar amounts of pDSR α mammalian expression vector bearing Trx1 or its mutant. Along with these plasmids pSV2-Neo vector (0.5 μ g) was cotransfected for selecting the transfectants. After 3 weeks of selection with G418 (1 mg/ml), the surviving colonies were counted. Each bar represents mean \pm s.e. of triplicates. (b) Trx1 expressing cells grow slower than the vector transfected ones. Equal number of cells (2000/well), stably expressing various Trx1 or the mutant, were plated and cell growth was monitored after 5 days using the sulforhodamine B binding assay as in the Materials and methods. Each bar represents mean \pm s.e. of six replicates. (c) Expression of transfected Trx1 gene in MCF-7 cells. Total RNA (35 μ g) from the indicated cell lines was Northern blotted and probed with the indicated probes (on the left). (d) The expression of Trx1 protein in transfectants. Total protein (50 μ g) was Western blotted and the blots were probed with antibodies against human Trx1. Lower portion of this blot shows the same blot probed with antibodies against actin

sion of Trx protein by Western blotting. The wildtype and mutant Trx expressing cells had three- and sevenfold more Trx1 protein, respectively (Figure 3d). The lower level of wildtype Trx protein in transfected cells suggests that a higher level of this protein is cytotoxic. This suggestion is also consistent with the observation that mutant Trx1 is expressed at a higher level than the wildtype Trx1. These cells grew better than those overexpressing wildtype Trx1 gene (Figure 3a,b).

Overexpression of Trx promotes cell death

Based on the above results we determined whether cells expressing wildtype Trx1 were more sensitive to the death-inductive effects of IFN/RA combination than those expressing the vector or mutant Trx1. In the first

set, an equal number of cells expressing the wildtype or the mutant Trx1 were exposed to the IFN- β /RA combination for 2 weeks. The cell colonies were visualized after fixation and staining with Sulforhodamine B (Figure 4a). As expected wildtype Trx expressing cells were killed by the IFN/RA. Conversely, the cells carrying mutant Trx were resistant to cell death. However, prolonged treatment (4 weeks) or higher concentration of IFN/RA (2000 U/ml and 1 μ M) caused cell death in mutant Trx1 expressing cells (data not shown). These observations suggest that Trx1 is one of the players in this death pathway and the IFN/RA combination can induce cell death in a Trx1-independent manner, depending on the strength of the death signal.

Although the above data indicated the sensitivity and resistance of the cells expressing wildtype and mutant Trx, respectively, they did not reveal the relative differences between the death sensitivities of wildtype and vector expressing cells. Therefore, MCF-7 cells stably transfected with the vector, wildtype, and mutant, were exposed to IFN/RA combination and cell growth was monitored in short term assays. Cell growth was measured on two different time points after IFN/RA treatment and the data were expressed as a per cent of the untreated control. Although both the vector and wildtype Trx1 expressing cells were inhibited by IFN/RA the latter were threefold more sensitive than the former in a 4-day growth assay

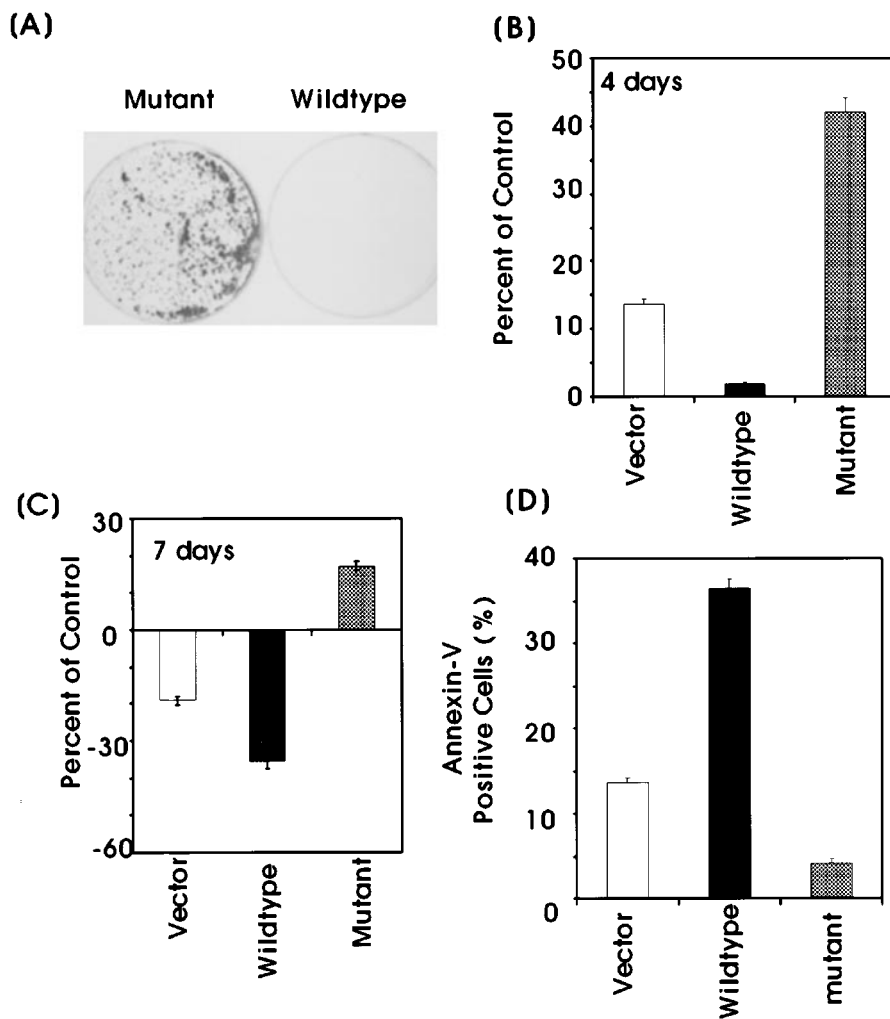


Figure 4 MCF-7 cells transfected with mutant Trx1 become resistant to IFN/RA induced death (a). MCF-7 cells (10^5) expressing various plasmids were selected with G418 (1 mg/ml), IFN- β (500 U/ml) and RA (1 μ M) for 2 weeks and then with G418 alone for 1 week. Colonies surviving on the plates were fixed and stained with sulforhodamine B as described above. (b), (c) Cell death induction by the IFN- β /RA combination. Cell growth was measured using sulforhodamine B as described in Materials and methods (Skehan *et al.*, 1990). Each data point represents mean \pm s.e. of six replicates. The absorbance value for 0% growth is 0.168, 0.154, and 0.166 for the vector, wildtype Trx1 and mutant expressing cells respectively. The 100% growth values for days 4 and 7 days are 0.574, 0.401, 0.768, and 0.835, 0.712, 0.953 and, respectively. Values on negative scale indicate death of initially plated cells. Each data point is mean \pm s.e. of six replicates. (d) shows the percentage of cell death as measured by annexin-V staining. MCF-7 cells were exposed to various treatments for 5 days and then stained with propidium iodide and FITC-labeled annexin-V. Annexin positive cells were scored by FACS analysis and expressed as a per cent of total number of cells. Each bar represents mean \pm s.e. of triplicates

(Figure 4b), as measured by the amount of growth occurred. Importantly, the cells transfected with wild-type Trx1 were twofold more sensitive to IFN/RA induced death (negative values on the y-axis) compared to the vector transfected ones as revealed in a 7-day assay. In contrast, cells transfected with the mutant did not die after IFN/RA treatment (Figure 4c). These observations were also confirmed by annexin-V staining (Figure 4d). In contrast to the mutant transfected cells, a robust death activation occurred in the vector and wildtype Trx1 transfected cells with IFN/RA treatment. The wildtype Trx1 expressing cultures bound 2–3-fold more annexin-V than the vector expressing cells. These data indicate that the mutant Trx1 interferes with IFN/RA-induced apoptosis.

Higher TR activity in the Trx1 transfected cells

We next examined whether the hypersensitivity of cells to IFN/RA induced death activation was due to an enhancement of TR activity. An equal quantity of protein from cells expressing the vector, wildtype, and mutant Trx1 was used for determining the TR activity (Figure 5a). The wildtype Trx1 expressing cells had twofold more TR activity than the vector expressing ones. In contrast, the mutant expressing cells had a significantly lower enzymatic activity. In this experiment no exogenous Trx was added to the lysates. Any activity seen is due to endogenous Trx. Thus, an increase in enzymatic activity corresponds to cellular hypersensitivity to IFN/RA induced death.

It can be argued that the increase and decrease in the TR activities of the wildtype and mutant Trx1 expressing cells, respectively, is due to differential

catalytic activities of TR in these cells. Therefore, we have determined the relative activities using exogenous Trx as substrate. In this experiment the basal absorbance values observed in the absence of exogenous Trx was subtracted from the experimental values. As shown in Figure 5b, no differences in the catalytic activity of TR from the vector, wildtype and mutant Trx1 transfected cells were noted.

Trx1 activates cell death

Since the above experiments indicated that overexpression of Trx1 causes reduction in the number of G418 resistant colonies formed (Figure 3) and indirectly indicated a loss of cell viability, the effect of Trx1 overexpression on cell survival was determined using a transient assay. MCF-7 cells were transfected with CMV- β -galactosidase and the expression vectors for wildtype Trx1 or the mutant. Three days after transfection, cells were fixed and stained with X-gal to determine the percentage of dead cells. Blue colored cells with a normal epithelial morphology were considered live and those with a rounded appearance were considered dead. The percentage of cell death was determined from the ratio of dead blue cells to the total blue cells. As shown in Figure 6, wildtype but not mutant Trx1 caused a dose dependent cell death. Vector alone did not cause significant cell death. Although the percentage of cell death was smaller it was distinctly different from the controls. At 2 μ g, wildtype Trx1 caused a sevenfold increase in cell death compared to the vector transfected cells (bar 1), but not with the mutant. Similar data was obtained in T47D breast carcinoma cells (data not presented). Thus, Trx1, when overexpressed, activates cell death in different tumor cell lines.

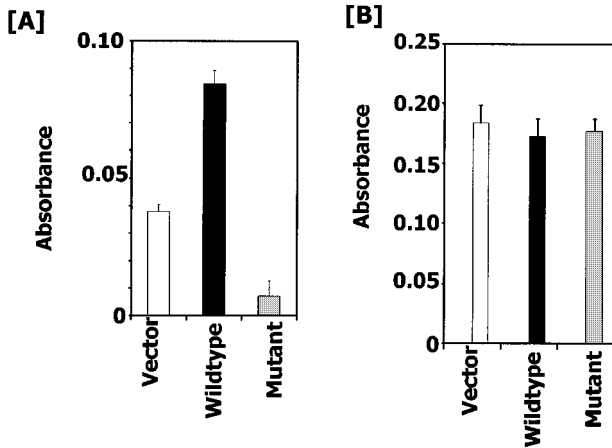


Figure 5 Thioredoxin reductase activity in cells expressing various Trx1 mutants. Data indicates mean \pm s.e. of triplicates. In (a), the basal TR activity using endogenous Trx was measured using 50 μ g of total cell extract. In (b), the catalytic activity of TR in the extracts of various cell lines was measured using exogenous Trx as substrate. In each case background enzymatic activity (in the absence of exogenous Trx) was measured and subtracted from the experimental values. In this experiment 20 μ g of cell extract was used. Each bar represents mean \pm s.e. of triplicates. The absorbance was in the linear range compared to control enzyme, TR

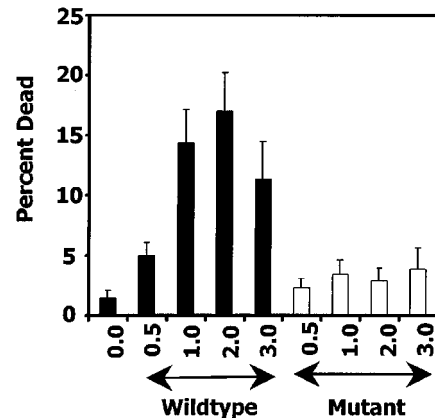


Figure 6 Induction of cell death by Trx1 in MCF-7 cells as measured by a transfection assay. (a) Cells were transfected with a β -galactosidase reporter and expression vectors for Trx1 or the mutant. Seventy-two hours later, cells were stained with x-gal and the number of dead cells was determined. Numbers on the X-axis indicate the micrograms of expression plasmid. Total amount of DNA transfected into cells was kept constant (4 μ g) by adding pcDNA3.1 vector, where required

IFN/RA induced cell death is dependent on caspase activation

Since most cell death signals culminate in caspase activation and our recent studies indicated that caspases are activated during IFN/RA regulated cell death, we next examined whether the Trx1 induced cell death was dependent on caspases. For this purpose, a chemical caspase inhibitor z-VAD-fmk and a biological caspase inhibitor C-FLIP were employed in the subsequent experiments (Figure 7). In the first experiment MCF-7 expressing wildtype Trx1 were treated with IFN/RA in the presence and absence of z-VAD-fmk. While cell death was clearly noted in the absence of the inhibitor, it was strongly suppressed in its presence (Figure 7a). Similar results

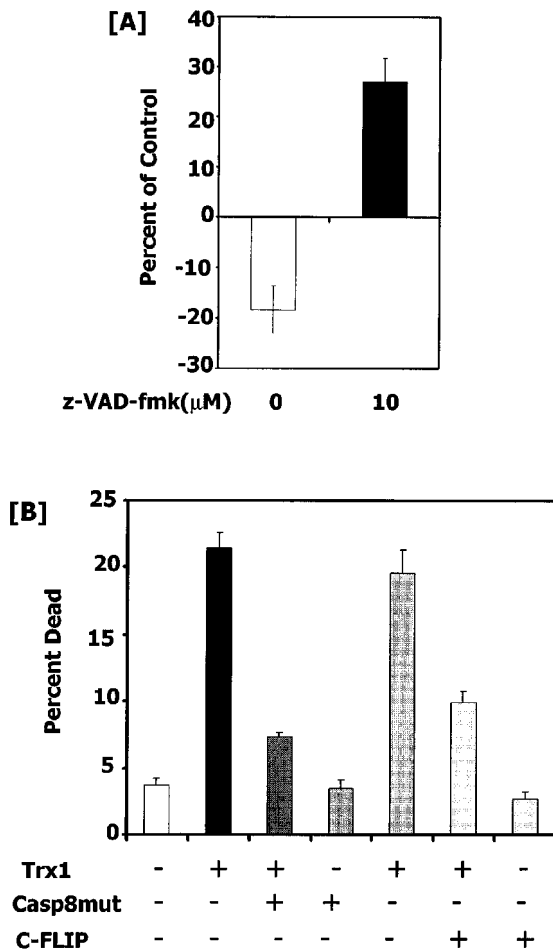


Figure 7 Effect of caspase inhibitors on IFN/RA induced death. SRB binding assay was performed with cells exposed to IFN- β (500 U/ml), RA (1 μM) or their combination for 7 days. MCF-7 cells expressing wildtype Trx1 were pre-treated with z-VAD-fmk (10 μM) and then with IFN/RA combination. The 0 and 100% growth values in this experiment are: 0.157 and 0.913, respectively. Panel B Inhibition of Trx1 induced cell death by a mutant Caspase-8 or C-FLIP. MCF-7 cells were transfected with a β -galactosidase reporter (1 μg) of Trx1 (2 μg). Along with these 1 μg of either a mutant caspase-8 or C-FLIP was co-transfected. Percentage of death was calculated as described above. pcDNA 3.1 was used to normalize the total amount of DNA transfected

were obtained with annexin-V binding assays (data not shown).

Since z-VAD-fmk is a general caspase inhibitor, we have examined the influence of C-FLIP (Thome *et al.*, 1997) and a catalytically inactive caspase-8 mutant on Trx1 induced cell death. C-FLIP suppresses death receptor-induced apoptosis by interfering with caspase-8 functions. It is a structural homolog of caspase-8 but lacks the critical cysteine at the active site. MCF-7 cells were transiently transfected with a CMV- β -galactosidase expression vector and wildtype Trx1. Along with these plasmids the expression vector carrying a catalytically inactive caspase-8 mutant (C \rightarrow A) or C-FLIP was also transfected. The percentage of death was determined following staining with X-gal (Figure 7b). Transfection of Trx1 caused 22% death. However, co-expression of the caspase-8 mutant significantly suppressed such cell death. Expression of caspase-8 mutant alone did not cause any significant increases in death compared to the vector transfected control. In a similar manner, C-FLIP also inhibited Trx1 activated cell death. These data suggest that Trx1 induced cell death is dependent on caspases, in particular caspase-8.

Trx1 modulates caspase-8 induced cell death

Because Trx1-activated cell death was suppressed in the presence of caspase-8 mutant or C-FLIP, it was of interest to examine whether Trx1 targets specific caspases during induction of cell death. We have chosen two specific caspases, caspase-8, and caspase-3 for this study. A number of studies have been shown that these enzymes participate in the initial and terminal phases of cell death, respectively, in response to extracellular death activators. Cells were transfected with RSV- β -galactosidase and caspase-8 expression vectors. Along with these either wildtype Trx1 or the mutant expression vector was cotransfected. Thirty-six hours later cells were stained with X-gal and the percent of cell death was determined as described in the Methods section. As shown in Figure 8a, transfection of wildtype Trx1 caused a significant cell death, as determined by the number of dead cells expressing β -galactosidase. In this experiment, we employed a suboptimal concentration of wildtype Trx1 to distinguish its stimulatory effect on caspase-8 induced death. Caspase-8 overexpression itself caused cell death. Cotransfection of expression vector for wildtype Trx1 augmented cell death significantly higher than either plasmid alone, indicating a synergistic effect. In contrast, the mutant Trx1 strongly interfered with the death inductive effect of caspase-8.

The IFN/RA inducible cell death did not involve internucleosomal DNA fragmentation in MCF-7 cells (Hofmann *et al.*, 1998). MCF-7 cells do not express endogenous caspase-3 protein due to a deletion of 47-nucleotides in the first exon of caspase-3 gene (Janicke *et al.*, 1998). Although, IFN/RA combination does not

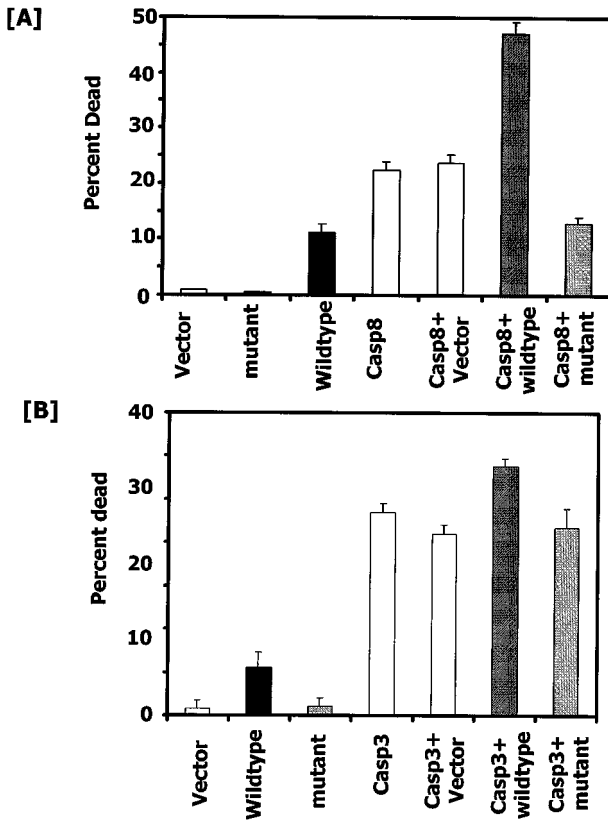


Figure 8 Effect of Trx1 on caspase induced cell death. Expression vector for caspase-8 (a) or caspase-3 (b) was co-transfected with the β -galactosidase reporter gene into MCF-7 cells. One microgram each of caspase and β -galactosidase reporters were used in this experiment. Specific effector plasmids, indicated on the X-axis, were co-expressed to determine their effects on caspase induced cell death. The amount (1 μ g) of wildtype or the mutant Trx1 DNA transfected was kept at a suboptimal level to distinguish the differences between various controls. Total amount of DNA transfected into cells was kept constant (3 μ g) by adding pcDNA3.1 vector, where required

require caspase-3 for inducing cell death in MCF-7 cells, it may be required in other cell types for death to occur in response to this combination. Since MCF-7 cells lack the endogenous caspase-3, they offer a good model to test the effect of Trx1 on the co-transfected caspase-3. Therefore, MCF-7 cells were transfected with a caspase-3 expression vector along with the RSV- β -galactosidase reporter plasmid. In addition to these plasmids either wildtype Trx1 or the mutant was co-expressed to examine their influence on caspase-3 induced cell death (Figure 8b). As anticipated caspase-3 expression caused an increase in cell death and expression vector alone had no significant effect on it. Although wildtype Trx1 marginally increased caspase-3-dependent cell death, the mutant did not inhibit the caspase-3 induced death. The higher amount of caspase-3 induced death in this case may be due to the fact that caspase-3 is a terminal enzyme in the death pathway and it can potentially activate mid-level caspases, such as caspase-9 through an amplification of

caspase cascades (Green and Reed, 1998; Gross *et al.*, 1999).

Activation of caspase-8 during IFN/RA induced cell death

To directly test whether caspases-8 was activated during IFN/RA induced cell death, Western blot analyses were performed using specific antibodies. MCF-7 cells expressing various Trx1 mutants were treated with the IFN/RA combination for 5 days. Cell lysates were prepared and used in Western blot analyses. As shown in Figure 9, Caspase-8 was activated in vector-transfected MCF-7 cells upon IFN/RA treatment. In the cells expressing wildtype Trx1, a higher magnitude of caspase-8 activation occurred as evidenced by the conversion of procaspase-8 into active form. In contrast, no activation of caspase-8 occurred in cells expressing the mutant Trx1. We also determined the cleavage of poly ADP ribose polymerase (PARP), a marker of caspase-mediated apoptosis in these cells. IFN/RA combination induced the cleavage of PARP in the vector-transfected cells. A similar but enhanced cleavage of PARP was noted in cells expressing wildtype Trx1. PARP cleavage was not detected in cells expressing mutant Trx1 (Figure 9, bottom panel).

Discussion

Several anti-oxidant mechanisms govern the survival of mammalian cells. These include superoxide dismutase, catalase, glutathione, thioredoxin, selenoproteins and the thiol-specific antioxidant (Berlett and Stadtman, 1997; Nakamura *et al.*, 1997). Some of these protect the cells, while the others suppress abnormal cell growth. Thioredoxin (Trx) participates in a wide-array of activities including cell growth, transcriptional and immune functions (Arner and Holmgren, 2000). Thioredoxins are ubiquitous proteins that mediate not only redox but also non-redox functions (Arner and Holmgren, 2000). The conserved cysteine moieties of

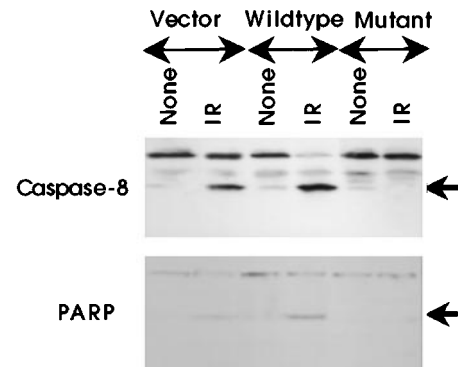


Figure 9 MCF-7 cells stably transfected with various Trx1 expression vectors were treated with IFN/RA (IR) combination for 5 days. IFN- β :500 U/ml and RA: 1 μ M. Cell lysates were prepared and Western blot analyses, with the indicated antibodies, were performed using (100 μ g) of protein from each sample. Cleavage products were indicated with arrows

the sequence, Cys-Gly-Pro-Cys, are necessary for the redox function of thioredoxin (Arner and Holmgren, 2000). Several thioredoxins or thioredoxin-like proteins exist in mammalian cells (Kurooka *et al.*, 1997; Lee *et al.*, 1998b; Spyrou *et al.*, 1997), whose individual physiologic roles have not been clearly understood. The most-well characterized member of these thioredoxins, Trx1, is critical for mammalian development because a homozygous deletion of this gene causes embryonic lethality in mice (Matsui *et al.*, 1996). Trx also plays a regulatory role in yeast, *Drosophila* and *Xenopus* cell division. The Trx homolog of *Drosophila*, *deadhead*, is essential for female meiosis and early embryonic development (Salz *et al.*, 1994), but not for DNA synthesis *in vivo* (Pellicena-Palle *et al.*, 1997). Thioredoxin inhibits DNA synthesis in the fertilized *Xenopus* eggs (Hartman *et al.*, 1993). In yeast lacking the Trx gene an increase in the frequency of mitotic cell cycle occurs (Muller, 1991). Loss of TR gene relieves p53-dependent growth suppression in yeast (Casso and Beach, 1996; Pearson and Merrill, 1998). While these studies highlighted the essential role of thioredoxin system in cell growth suppression, the exact targets TR:Trx affects have not been defined clearly. Since these enzymes modify their substrates in an extremely transient manner and no stable prosthetic groups are involved in this process (Arner and Holmgren, 2000), it has been difficult to define their targets. Therefore, application of genetic methods will be useful in identifying the downstream effectors.

The redox status of Trx is modified by an upstream regulatory enzyme thioredoxin reductase (TR). This gene is now known as *TR1* and is expressed ubiquitously in the mammalian tissues (Arner and Holmgren, 2000). Recently, two other TR homologs *TR2* and *TR3* have been identified using database searches or by biochemical purification (Lee *et al.*, 1999; Sun *et al.*, 1999). The latter two are expressed in a tissue and organelle specific manner. The specific roles of these new members of TR family enzymes are unclear at this stage. Mammalian TR has broader substrate specificity, unlike its prokaryotic homologs (Arner and Holmgren, 2000). For example, it can reduce unrelated compounds such as selenite, alloxan, 5,5'-dithiobis(2-nitorbenzoic acid), in addition to Trx. It is likely that each TR exhibits a narrow-substrate specificity and acts in a localized manner.

Using the antisense knockout approach we have shown earlier that *TR1* is critical for the cell death controlled by IFN/RA and IFN/Tamoxifen combination (Hofmann *et al.*, 1998; Lindner *et al.*, 2000). In this approach the death associated genes are identified based on functional inactivation (Deiss and Kimchi, 1991; Hofmann *et al.*, 1998). Over expression of *TR1* led to a significant decrease in the viable cell colony formation. Although some cells expressing moderate levels of *TR1* were viable, they grew slower than the vector-transfected cells and were hypersensitive to death induction by IFN/RA (Hofmann *et al.*, 1998). That *TR1* exerts growth suppressive functions *via* apoptosis in the presence of exogenous ligands is also

consistent with other studies which showed that Trx1, when inactivated in a similar manner, suppresses IFN- γ induced cell death (Deiss and Kimchi, 1991), and certain tumor cells that hyper produce Trx grow poorly (Rubartelli *et al.*, 1995). Although overexpression of Trx1 in clonal isolates of certain transformed cells promotes growth, the majority of the cells over-expressing Trx1 did not survive (Gallegos *et al.*, 1996). Lastly, RA induces the transcription of the Trx1 gene and enforced expression of Trx1 causes cell death in lung epithelial cells (SPM Reddy and R Wu, unpublished data). Collectively these data indicate that TR1:Trx1 can regulate cell death in mammalian cells. In contrast, some studies have shown that certain HTLV-transformed lymphocytes secrete Trx in high amounts and grow rapidly indicating a cytokine-like function for this protein (Tagaya *et al.*, 1989). Other studies have shown that Trx inhibits the apoptotic activity of ASK1/MKCK5 (Saitoh *et al.*, 1998). In this case Trx appears to be functioning as a chaperone that controls the apoptotic activity (Liu *et al.*, 2000), by preventing the oxidation of ASK1 and the consequent loss of its activity.

In this study we have shown that antisense expression and a redox-inactive Trx1 mutant clearly interfered with cell death. Conversely, over expression of wildtype Trx1 promoted cell death. These results are in contrast to other studies, which reported a growth-promoting role for Trx1. These contrasting observations raise the question: how can a growth-promoting factor regulate diverse effects? The reasons for these differences could be manifold: (1), unlike the previous studies which employed supra-physiologically over-expressed wildtype Trx1, we show that down regulation of physiological Trx1 levels promotes cell death; (2), there was no selection pressure on the cells in the previous studies; (3), the growth promoting effect of Trx1 was cell type dependent. For example, while secreted Trx was growth promoting in HTLV-transformed cells (Tagaya *et al.*, 1989), it was inhibitory for hepatocellular carcinomas (Rubartelli *et al.*, 1995). In a similar manner EGF (Cao *et al.*, 2000; Chin *et al.*, 1997), oncoprotein *c-myc* (Evan *et al.*, 1992; Hermeking and Eick, 1994; Wagner *et al.*, 1994) and transcription factor E2F (Field *et al.*, 1996; Pierce *et al.*, 1999), which promote cell growth, can also induce apoptosis depending on the physiologic status of the cell. Indeed, E2F has been shown to act as a transcriptional inhibitor in the presence of RA (Lee *et al.*, 1998a). Additionally, p202, an IFN-induced protein suppresses the DNA binding and transcriptional activity of E2F by a direct interaction (Choubey *et al.*, 1996). Thus, Trx can participate in both growth stimulatory and suppressive functions, depending on the physiologic context and influence of extracellular agents.

Cell death in multicellular organisms is controlled by a 'central machinery' consisting of caspases and BCL proteins (Green and Reed, 1998; Gross *et al.*, 1999). It is clear that a number of disparate signals feed into this central regulatory unit during death activation. This

suggests the existence of a signal specific dedicated switch(es) that turns on this machinery. We show here that one such switch, Trx1, targets caspase-8 during cell death. Evidence presented in this study suggests this property of Trx1: (1) transient transfection of wildtype Trx1 but not redox-inactive mutant can promote cell death (Figure 6); (2) catalytically inactive caspase-8 or its inhibitor, C-FLIP block Trx1 induced cell death (Figure 7); (3) coexpression of wildtype Trx1 with caspase-8 augments cell death and the mutant blocks it (Figure 8); (4) IFN/RA induced caspase activation and cell death was blocked upon overexpression of mutant Trx1 (Figure 9).

The post-translational modifications of death machinery constituents by pro- and anti-death signals in part could account for its regulation. For example, phosphorylation of caspase-9 inhibits its biological activity (Cardone *et al.*, 1998). Phosphorylation of BAD, a member of BCL family, by Akt and Protein kinase A inhibits its death promoting activity (Datta *et al.*, 1997; Harada *et al.*, 1999; Kennedy *et al.*, 1999). Recently, N-myristoylation of proapoptotic protein BID has been shown to permit its docking to the mitochondrial membranes and cell death (Zha *et al.*, 2000). Little is known about other potential post-translational mechanisms of caspase regulation. One post-translational mechanism that can modulate caspase activity is their catalytic site. The cysteine residue of the active site is critical for these enzymes to execute cells in response to exogenous death signals (Green and Reed, 1998; Gross *et al.*, 1999). Indeed, a cysteine \rightarrow alanine (C \rightarrow A) mutant of caspase-8 blocked cell death (Figure 7). Data obtained with a limited number of caspases used in this study show that caspase-8, but not caspase-3, is selectively activated by Trx1 (Figures 8). Although, a recent study has shown that caspase-3 activity can be restored upon incubation with Trx1 (Ueda *et al.*, 1998), a mutant Trx1 was unable to inhibit caspase-3 function in our study. Interestingly, a mutant Trx1 lacking the cysteine residues was unable to inhibit caspase-3. These data suggest that substrates other than Trx1 maintain the redox state caspase-3 *in vivo*. The differential effect of Trx1 on caspases may also be due to regulation of caspase activities by its modulator TR in a localized manner. Consistent with our studies evidence for redox regulation of caspase functions has been recently reported. For example, nitric oxide-mediated oxidation and S-nitrosylation of active site cysteine have been implicated in inhibition of caspase-3 activity (Lipton, 1999; Mohr *et al.*, 1997). Depletion of cellular glutathione levels enhances the apoptotic effect of TNF- α in certain hepatoma cells (Pierce *et al.*, 2000). Treatment of cells with reducing agents augmented Fas-induced cell death and enhanced caspase-3 activity (Sen *et al.*, 1999). Furthermore, a number of redox enzymes have been identified as potent mediators of p53-induced death (Polyak *et al.*, 1997). These data highlight the importance of redox control in cell death. More importantly, Trx1 now provides a link between death machinery and IFNs.

Materials and methods

Reagents

Restriction and DNA modifying enzymes (NE Biolabs); G418 Sulfate, IPTG and Lipofectamine plus (Life Technologies); nitrocellulose membranes, ECL reagents and horseradish peroxidase coupled to anti-rabbit or anti mouse antibodies (Amersham Pharmacia Inc); human IFN- β_{ser} (Berlex Inc.), mouse monoclonal antibodies against actin (Sigma Inc.) and thioredoxin (Serotec Inc.) and rabbit polyclonal antibodies against caspase-8; polyADP ribose polymerase (Santa Cruz Biotech Inc.) were employed in these studies. Fresh stocks of all-trans retinoic acid (Sigma) were prepared in ethanol and added to cultures under subdued light.

Cell culture

MCF-7 cells were cultured in phenol red free EMEM supplemented with 5% charcoal stripped fetal bovine serum (CSFBS) and 10^{-11} M estradiol during treatment with IFN- β and all trans retinoic acid (RA). Cells were grown in phenol red free media 24 h before treatments were initiated.

Plasmids

Episomal vector pTKO1 contains the human thioredoxin cDNA in antisense orientation, under the control of an IFN-stimulated promoter (Deiss and Kimchi, 1991). Mammalian expression vectors carrying caspase-8 and caspase-3 and the corresponding mutants were provided by Emad Alnemri, Thomas Jefferson University, Philadelphia, USA (Alnemri *et al.*, 1995). The wildtype and redox inactive mutant of Trx1 cloned in the mammalian expression vector pDSR α were described elsewhere (Hirota *et al.*, 1997). In the mutant Trx1 the cysteine residues at positions 32 and 35 were substituted with serines.

Cell growth assay

Cells (2000/well) were seeded into 96-well plates. Various inhibitory agents were added and growth was monitored using a colorimetric assay, which quantifies cell numbers (Skehan *et al.*, 1990). Each group of treatments had eight replicates. Cells were fixed with trichloroacetic acid (final concentration 10%) at 4°C for 1 h at the end of the experiment and stained with 0.4% Sulforhodamine B (Sigma). The bound dye was eluted with 100 μ l of Tris-HCl (pH 10.5) and the absorbance was monitored at 570 nm. One plate was fixed with TCA, 10 h after plating the cells. Absorbance obtained with this plate was considered as 0% growth. Absorbance obtained with untreated cells was considered as 100% growth. An increase and decrease of A₅₇₀ values in the experimental wells relative to the 0% value indicates cell growth and death, respectively. When plotted as a percentage of untreated control growth and death values appear on the positive and negative scales of the Y-axis, respectively.

Death assays

Cell death was also determined using an alternate method, the Annexin-V binding assays. Following treatment with the indicated agents, cells were stained using a commercially available kit (Trevigen Inc) per manufacturer's recommendation. Cells were incubated with FITC tagged Annexin-V and

propidium iodide. FITC-stained cells were considered as apoptotic and were quantified using Becton-Dickinson fluorescence activated cell sorter. Annexin-V positive cells (dead) were scored and expressed as a percentage of total number of cells.

Transient transfection assays

Cell death was also monitored in a transient transfection assay. Since the transfection efficiency is only around 20%, it is important to score for the transfected cells. Therefore, a β -galactosidase reporter gene driven by the enhancers of either Rous Sarcoma Viral (RSV) long terminal repeat or the Cytomegalovirus (CMV) was co-transfected along with the effector plasmids. This plasmid permits the detection of transfected cells after staining with X-gal. Briefly, cells were transfected with the indicated plasmids ($\sim 1 \mu\text{g}$) using the Lipofectamine plus reagent. Wherever multiple plasmids were transfected, total amount of DNA transfected was kept to a maximum of 2–4 μg , depending on the experiment. At the end of the experiment they were stained with a commercially available *in situ* β -galactosidase detection kit containing X-gal (Stratagene Inc) and the number of cells stained blue was determined microscopically. Cells with flat attached epithelial morphology were considered alive and those with a rounded and detached appearance were considered dead. Multiple fields were scanned and a total of 300–400 cells were counted to obtain statistically significant numbers.

Enzymatic assay

Thioredoxin reductase activity was determined as described (Holmgren and Bjornstedt, 1995). Cell extracts were prepared after IFN/RA treatment by freeze-thaw lysis. Twenty micrograms of extract was incubated with insulin, NADPH and thioredoxin in 0.2 M HEPES, pH 7.6 for 20 min at 37°C. Reactions were terminated after the addition of 6 M guanidinium hydrochloride/0.4 mg/ml dithiobis(2-nitrobenzoic acid) prepared in 0.2 M Tris, pH 8.0. Absorbance at 412 nm was measured. In each case a corresponding control without Trx was used to determine the basal level of TR activity (due to endogenous Trx and NADPH). Where indicated absorbance values obtained from these controls

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were subtracted from those obtained with the reactions that contained Trx and NADPH. A negative control reaction without cell extracts but with all the reaction components was also employed. Triplicate samples were measured for enzymatic activity. Pure TR was used as a positive control.

Northern and Western blot analyses

Total RNA was extracted and comparable amounts of RNA from each cell line/treatment were separated on a 1% formaldehyde-agarose gel. These blots were probed with ^{32}P -labeled human Trx1 and glyceraldehyde phosphate 3-dehydrogenase (GAPDH) cDNAs. These blots were exposed to X-ray films to detect the expression. Gene expression was quantified using a Molecular Dynamics phosphorimager.

Equal quantities of cell extracts were separated on 12% SDS-PAGE and Western blotted onto nylon membranes. Specific first antibodies were incubated with the blots after blocking as described in our previous publications (Hofmann et al., 1998). These blots were washed and incubated with an appropriate second antibody tagged with horseradish peroxidase. Protein bands were visualized using a commercially available enhanced chemiluminescence (ECL) kit (Amersham Inc).

Abbreviations

FLIP, FLICE-inhibitory protein; GRIM, Genes associated with Retinoid-IFN induced mortality; IFN, Interferon; JAK, Janus tyrosine kinase; RA, all trans retinoic acid; SRB, Sulforhodamine B; STAT, Signal transducing activator of transcription; TR, thioredoxin reductase; Trx, thioredoxin

Acknowledgments

These studies are supported by the National Cancer Institute grants CA 78282 and CA 71401 to DV Kalvakolanu and National Institutes of Health grant HL-58122 to SPM Reddy. We thank E Alnemri, and J Tschopp, for providing plasmids used in this study.

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