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A Redox-Inactive Thioredoxin Reduces Growth and Enhances Apoptosis in WEHI7.2 Cells

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Cancer cell lines transfected with thioredoxin show increased anchorage-independent growth and decreased sensitivity to induction of apoptosis by a number of anticancer drugs. The present studies were undertaken to evaluate further the role of thioredoxin in cell growth and drug-induced apoptosis. A redox-inactive mutant thioredoxin was stably transfected into WEHI7.2 mouse lymphocytic leukemia cells and two clones were examined for growth characteristics and the induction of apoptosis by dexamethasone, etoposide, doxorubicin, and staurosporine. These clones each exhibited a 71% increase in doubling time in solution and a 20 and 75% reduction in colony formation in soft agarose. The transfected cells also showed increased susceptibility to apoptosis induced by dexamethasone, etoposide, doxorubicin, and staurosporine compared with controls. The results of this study suggest that thioredoxin can regulate the growth rate of cells and that thioredoxin is a critical component in the pathway leading to drug-induced apoptosis in WEHI7.2 cells. © 2000 Academic Press

Key Words: **thioredoxin; redox-inactive; apoptosis; dexamethasone; WEHI7.2 cells.**

Human thioredoxin (Trx) is a ubiquitous small (12 kDa) protein that is a substrate for the NADPH-dependent seleno-flavoenzyme thioredoxin reductase (1, 2). Trx is identical to the leukemic cell growth factor, adult T cell leukemia derived factor (ADF) (3). Trx acts intracellularly to provide reducing equivalents to enzymes such as ribonucleotide reductase, the first unique step of DNA synthesis (4), and via thiol-disulfide exchange regulates the activity of some transcription factors including NF-κB (5), the glucocorticoid receptor (6, 7), and, indirectly through Ref-1, AP-1 (8, 9). Trx is also secreted by cells and acts as a growth factor for both leukemic and solid cancer cells (10–13) by increasing the response to endogenously produced growth factors such as interleukin-2 and basic-fibroblast growth factor (11, 14, 15). Trx expression is increased in a number of human primary cancers. Trx mRNA is elevated, sometimes several fold, in almost half of human primary lung and colon tumors examined compared to normal tissue from the same subjects (3, 16). Other studies have found increases levels of Trx protein in human cervical cancer (17), hepatocellular carcinoma (18, 19), squamous cell carcinoma (20), and gastric cancer (21).

Increased expression of Trx augments tumor cell growth. Transfection of mouse NIH 3T3 cells with human Trx increases their growth in culture (22), while mouse WEHI7.2 lymphocytic leukemia cells transfected with human Trx form tumors in immunodefficient mice that grow much more rapidly than tumors formed by wild-type WEHI7.2 cells (23). Increased expression of Trx also inhibits apoptosis. Immunohistochemical studies of human primary gastric carcinomas have shown that increased levels of Trx are negatively correlated with apoptosis as measured by the TUNEL assay (21). Cancer cell lines with increased expression of Trx are resistant to a variety of apoptosis-inducing agents including the anticancer drugs doxorubicin (24), cisplatin, mitomycin C, and etoposide (25, 26). We have previously reported that over expression of Trx in WEHI7.2 cells inhibits the induction of apoptosis by dexamethasone, etoposide, staurosporine, and thapsigargin (23). However, others have reported that although increased Trx expression can, indeed, inhibit cell death in cells treated with superoxide generating agents, Trx does not protect cells from cisplatin (27), vicristine, and colchicine (25). Thus, the role of Trx in the inhibition of apoptosis by these and other agents remains in question. The most rigorous way to evaluate the role of Trx as an anti-apoptotic protein is to separately overexpress and inhibit its function in a cell line and then examine the induction of apoptosis by a variety of agents.

In order to evaluate further the role of Trx on cancer cell growth and on anticancer drug-induced apoptosis, we have antagonized the action of native Trx by ex-

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pressing a dominant-negative, redox-inactive mutant human Trx (22) in WEHI7.2 cells. Our results demonstrate that expression of a redox-inactive Trx in WEHI7.2 cells decreases the growth rate in solution and in soft agarose, and renders these cells more sensitive to drug-induced apoptosis.

MATERIALS AND METHODS

Drugs and Reagents

Dexamethasone, staurosporine, etoposide, and doxorubicin were obtained from Sigma Chemical Co. (St. Louis, MO) and stock solutions were prepared in sterile dimethyl sulfoxide (DMSO) or for doxorubicin in sterile water. The stock solutions were stored at −20°C under light-free conditions and diluted to appropriate final concentrations in media just before use. Vehicle controls of water and 0.01% DMSO were included in all experiments and consistently found to be equivalent to drug-free controls in the levels of gene expression, protein expression, and occurrence of apoptosis.

Cell Culture and Drug Exposure

Mouse WEHI7.2 lymphocytic leukemia cells transfected with human Trx have been previously described and the clone selected for this study, Trx5, had a 1.8-fold increase in Trx mRNA (23). Wild-type WEHI7.2 cells were used to establish clonal cell lines expressing a redox-inactive mutant human Trx where the active site Cys-32 and Cys-35 residues have both been replaced by Ser (28), designated Serb (Ser both). The same expression vector (pDC304-neo) was used for the Trx5 and the Serb clones. The Serb cells were established by mixing 10 μg plasmid DNA and 5×10^5 WEHI7.2 cells and electroporating at 4°C, 220 V and 960 μF (Bio-Rad Electroporator, Hercules CA) in 400 μl serum-free Dulbecco's modification of Eagle's medium (DMEM). Cells were selected in 800 U/ml G418 (Life Technologies, Gaithersburg, MD) for 1 week and then plated in 0.3% soft agarose supplemented with 400 U/ml G418. Individual colonies were obtained from the soft agarose following 14 days of growth. All cells were grown in DMEM supplemented with penicillin/streptomycin antibiotic mix (Sigma) and 10% heat-inactivated fetal bovine serum (FBS) (Omega Scientific, Tarzana, CA). Transfected cells were maintained in 400 U/ml G418.

All cultures were maintained under a fully humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cultures were routinely screened for mycoplasma RNA (Gen-Probe, San Diego, CA) and consistently found to be mycoplasma-free. For experimental incubations, cells in log-phase growth were suspended at a density of 1×10^5 cells/ml containing the appropriate concentrations of drug and maintained as described above. Experimental incubations were terminated by pelleting cells at 400*g* for 5 min and aspiration of the media. Cell pellets were used for procedures described below.

Northern Analysis

Total cellular RNA was isolated from 1×10^7 cells using Trizol (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Total RNA (15 μg) was then separated on a 1.2% agarose/formaldehyde gel as previously described (22). The RNA was blotted onto nylon (Schleicher and Schuell, Keene, MD) by upward capillary transfer for 16 h and then cross-linked to the nylon by ultra-violet light. The blot was then hybridized (23) with a thioredoxin cDNA probe. Probes were random primed with [α-^{32}P]dCTP (3000 Ci/mM) (New England Nuclear, Boston, MA) using a kit and protocol from Life Technologies. The blot was then washed extensively in 0.2× SSC/0.1% SDS (1× SSC = 150 mM NaCl, 15 mM sodium citrate) at 65°C and quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA). The blot was subsequently stripped and reprobed with a labeled glyceraldehyde-phosphate dehydrogenase (GAPDH) cDNA utilized as a loading control.

Growth Studies

Suspension culture. Cells were seeded in duplicate in at 0.5×10^5/ml in DMEM supplemented with 10% FBS. Cell number was measured at 24, 48, and 72 h using a hematocytometer and trypan blue exclusion.

Soft agarose. Cells were seeded at 1000 cells/ml (0.5 ml/well) in triplicate in 0.3% agarose in DMEM supplemented with 10% FBS and incubated for 7 days. Subsequently, 500 μl of a 1 mg/ml solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride hydrate (Aldrich, Milwaukee, WI) was added to each well and incubated overnight. Colonies greater than 60 μm in diameter were counted using Stratagene's EagleEye image analyzer and software (Stratagene, La Jolla, CA).

Viability. Cells were seeded in duplicate at 1×10^5/ml in DMEM supplemented with 10% FBS. Total viable cell number was determined every 8 h for 72 h using a hematocytometer and trypan blue exclusion.

Apoptosis Quantitation

The annexin V apoptosis kit and protocol from Boehringer Mannheim (Boehringer Mannheim, Indianapolis, IN) was utilized with minor modifications. Briefly, cell pellets containing 1×10^6 cells were washed with PBS and resuspended in 20 μl diluted FITC-annexin V/propidium iodide stain. Cells were incubated at 22°C for 15 min and then diluted with 500 μl buffer supplied with the kit. Ten thousand cells were analysed by flow cytometry for each condition.

Cell Morphology

Following drug treatment, cytocentrifuge slides were prepared containing 8×10^4 cells/slide. Slides were stained with 20% Wright-Giemsa and apoptosis was scored by counting the percentage of apoptotic cells in 3 random fields.

RESULTS

Establishment of Redox-Inactive Trx (Serb) Transfected Cell Lines

Wild-type (wt) WEHI7.2 cells were transfected with the pDC304neo vector containing a cDNA encoding a human redox-inactive Trx. Five clones, referred to as Serb1 through 5, were examined for expression of Trx at the mRNA level and compared to the empty vector control (Neo3) and wt WEHI7.2 cells (Fig. 1). Subsequent experiments examining growth and apoptosis utilized the Serb1 and Serb5 cell lines. When normalized to a GAPDH control, the message level of redox-inactive Trx was 11.2- and 9.2-fold above the message level of native Trx (Serb1 and Serb5, respectively). Interestingly, expression of the redox-inactive Trx increased the expression of native Trx mRNA. Compared to both the wt and Neo3 WEHI7.2 cells, native Trx mRNA expression was increased 1.6- and 2-fold in the Serb1 and Serb5 cell lines.

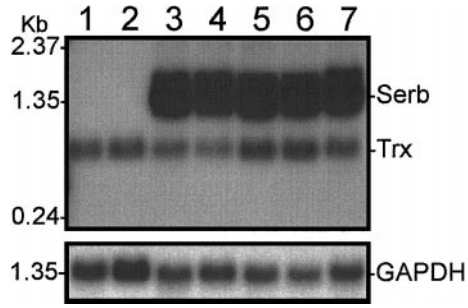

FIG. 1. Northern blot of WEHI7.2 cells probed with a Trx and GAPDH cDNA. WEHI7.2 cells were transfected with the empty vector or the redox-inactive Trx cDNA and outgrowths from individual colonies were examined for Trx and GAPDH expression. Lane 1, wild-type parental cells; lane 2, empty vector cells (Neo3); lanes 3 through 7, redox-inactive Trx cells lines (Serb1 to 5). Native Trx is the lower band, while the dominant-negative Trx is the upper band. Message size is indicated on the left side of the blot.

Growth Studies

The doubling time of the wt, Neo3, and Trx overexpressing Trx5 cells in suspension culture were 14 h for all 3 cell lines. In contrast, the Serb1 and Serb5 cells had doubling times of 24 h (Fig. 2A). Each cell line was also seeded in soft agarose and scored for colony formation after 7 days. This assay is thought to more closely model tumor formation *in vivo* (22). After 7 days, the Trx5 cells formed larger and more numerous colonies, and the Serb1 and Serb5 cells formed fewer and smaller colonies than the wt and Neo3 cells (Fig. 2B). Because the doubling time of the Serb cell lines was greater than that of the Neo3 or Trx5 cells, the Serb1 and Serb5 colonies were again counted after 12 days. Even with the added time, the colonies formed by the Serb cells were fewer and smaller in size that the control colonies at 7 days (not shown).

Dexamethasone-Induced Apoptosis

Dexamethasone-induced apoptosis was assessed at 48 h by labeling cells with fluorescein-conjugated annexin V followed by flow cytometry (Fig. 3). Trx5 cells were significantly less sensitive to dexamethasone-induced apoptosis compared to the wt and empty vector cells, confirming previous reports (23). Serb5 cells were significantly more sensitive to dexamethasone-induced apoptosis at all concentrations except the highest (**$P < 0.001$). Histological examination of cytospin slides prepared from each cell line at each concentration confirmed the results of the flow cytometry (not shown). Serb5 cells at 50, 100, and 500 nM dexamethasone had undergone extensive apoptosis (>99%), while the wt and Neo3 cells were also severely depleted in number at 100 and 500 nM. In contrast, Trx5 cells were numerous at all concentrations of dexamethasone.

The time course of cell death induced by 20 nM dexamethasone over a 72-h period was examined. Beginning with 1×10^6 cells, the total number of viable cells was determined every 8 h by counting cells on a hemacytometer and trypan blue exclusion (Fig. 4). The wt, Neo3, and Trx5 cells grow well, but slower than untreated cells, and the total cell number peaked at 24 h. The Serb4 cell number peaked at 18 h with marked apoptosis at later time points. Interestingly, the cell number of both the wt and Neo3 cells declined rapidly after 24 h, but the Trx5 cell number did not decline from 24 to 72 h of treatment. With increasing time, the Trx5 cells became more crowded and cell debris accumulated indicating that the growth and death rates were approximately balanced.

Doxorubicin-, Etoposide-, and Staurosporine-Induced Apoptosis

Wild-type, Neo3, and Serb5 cells were also treated with 5 nM doxorubicin, 250 nM etoposide, and 20 nM staurosporine for 24 h and apoptosis measured by annexin V staining and flow cytometry (Fig. 5). Compared

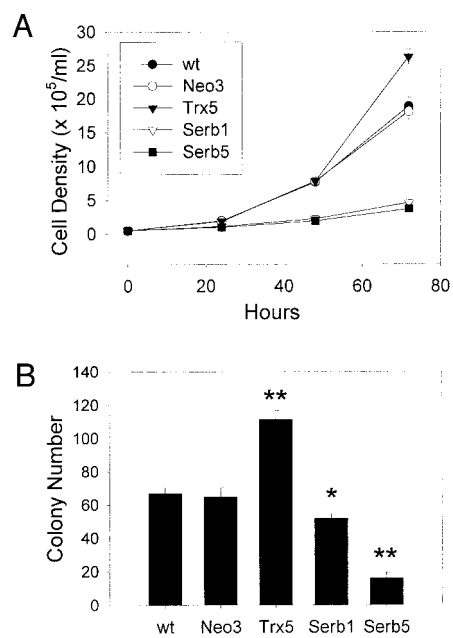

FIG. 2. Growth rates of wild-type WEHI7.2 cells, cells containing the empty vector (Neo3), overexpressing Trx (Trx5), or expressing the redox-inactive Trx (Serb1 and Serb5) in suspension culture (A) and soft agarose (B). (A) Cells were plated at an initial density of 0.5×10^5 cell/ml and were counted after 24, 48, and 72 h. Time points represent the mean of triplicate platings ± standard deviation (SD). (B) Cells were plated in 0.3% agarose at 1000 cells/ml (0.5 ml/well in a 24-well plate) and colonies greater than 60 μm were counted after 7 days. Trx5 cells formed significantly more colonies than the wt and empty vector cells (**$P < 0.001$), while the Serb1 and Serb5 cells formed significantly fewer and smaller colonies compared to control cells (*$P < 0.01$ and **$P < 0.001$, respectively). Data represent the means of quadruplicate platings ± SD. Two additional experiments yielded similar results.

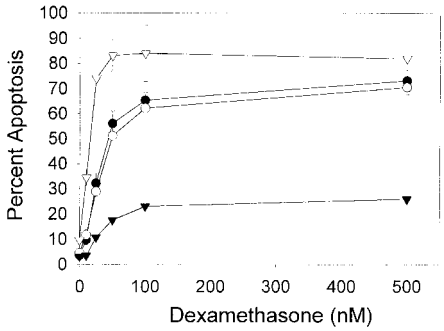

FIG. 3. Quantitation of dexamethasone-induced apoptosis. Wild-type (●), Neo3 (○), Trx5 (▼), and Serb5 (▽) WEHI7.2 cells were treated with dexamethasone (10–500 nM) for 48 h. Apoptosis was assessed by fluorescein-labeled annexin V binding to phosphatidylserine on the outer surface of the cell membrane and followed by flow cytometry. Data represent the means of duplicate determinations from 3 separate experiments ± SD.

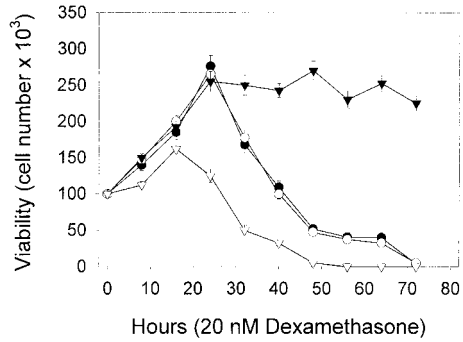

FIG. 4. Viability time course. Wild-type (●), Neo3 (○), Trx5 (▼), and Serb5 (▽) WEHI7.2 cells were treated with 20 nM dexamethasone for 72 h. Cells were plated at 1×10^5 cell/ml. Cell number was determined every 8 h by hemacytometer and viability was assessed by trypan blue exclusion. Viability of wild-type cells was equivalent the empty vector control cells.

to the wt and empty vector control cells, the Serb5 cells were significantly more sensitive to drug-induced apoptosis by doxorubicin, etoposide, and staurosporine ($^*P < 0.005$). Cytospin preparations of each cell line were stained and the percentage of apoptotic cells confirmed by visual examination (not shown).

DISCUSSION

We have previously reported that the overexpression of Trx, by as little as 2-fold, in WEHI7.2 leukemic cells protects these cells from cell death induced by a variety of cytotoxic agents (23). In order to investigate further the role of Trx in protecting leukemic cells from drug-induced apoptosis, we inhibited the function of native Trx by expressing a redox-inactive Trx in WEHI7.2 cells. Unlike native Trx, the mutant Trx is not a substrate for reduction by thioredoxin reductase (28). We observed several biological consequences of expressing a redox-inactive Trx. Firstly, native Trx message was upregulated when normalized to GAPDH. Secondly, the growth rate of cells expressing the redox-inactive Trx was dramatically slower in suspension culture and in soft agarose. Lastly, cells expressing the redox-inactive Trx were more susceptible to drug-induced apoptosis.

The observation that expression of the redox-inactive Trx increased the doubling time and reduced colony formation suggest that native Trx plays an important role in the proliferation of WEHI7.2 cells. Indeed, Trx was first identified as an extracellular growth factor for leukemic cells (10–13). Export of the redox-inactive Trx might result in reduced growth rates and inhibition of colony and tumor formation. Interestingly, the majority of the overexpressed Trx in MCF-7 cells appears to be secreted into the media (22). However, when recombinant human redox-inactive Trx was added to Swiss 3T3 fibroblasts *in vitro*, no effects on cellular proliferation were observed (28). Thus, exactly how expression of a redox-inactive Trx inhibits the growth of WEHI7.2 cells remains unclear.

We also found that WEHI7.2 cells expressing the redox-inactive Trx were significantly more susceptible to drug-induced apoptosis than either empty vector or wt cells. This observation complements previous experiments showing that even a slight overexpression of Trx imparts a significant protection from drug-induced apoptosis by a variety of agents (23).

The mechanisms of action by which dexamethasone, doxorubicin, etoposide, and staurosporine induce cell death include generation of reactive oxygen species (ROS), DNA damage, inhibition of topoisomerase, and a general inhibitor of kinases. However, it is noteworthy that all 4 agents examined have been reported to generate ROS, even though this may not be their main

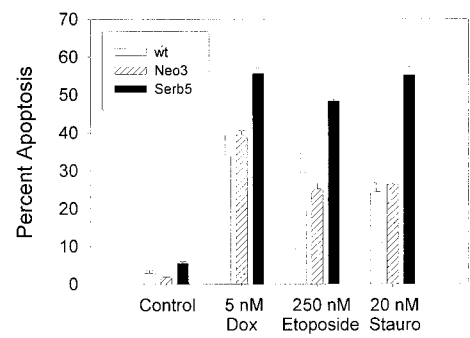

FIG. 5. Induction of apoptosis by doxorubicin, etoposide, and staurosporine. Wild-type, Neo3, and Serb5 WEHI7.2 cells were treated with 5 nM doxorubicin (Dox), 250 nM etoposide, and 20 nM staurosporine (Stauro) for 24 h. Apoptosis was assessed by fluorescein-labeled annexin V binding to phosphatidylserine on the outer surface of the cell membrane and flow cytometry. Data represent the means of duplicate determinations from 3 separate experiments ± SD. Wild-type cells exhibited rates of cell death equivalent to the empty vector control cells.

mechanism of cell killing (29–32). Indeed, dexamethasone treatment of WEHI7.2 cells has been shown to down regulate anti-oxidant proteins such as Trx, catalase, diaphorase, and manganese superoxide dismutase (33), but the exact mechanism whereby it induces cell death is unknown. It is possible that the generation of ROS contributes to the cytotoxicity of many different agents. Trx can scavenge peroxides via Trx peroxidase and help repair oxidative damage to cellular proteins (34, 35) and cells transfected with Trx peroxidase have been reported to show decreased sensitivity to H_2O_2-induced apoptosis (36). Thus, the redox-inactive mutant Trx might inhibit the Trx peroxidase pathway leading to increased apoptosis.

Another mechanism to explain the protective effect of Trx against apoptosis is activation of the transcription factors, nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1), which inhibit drug-induced apoptosis (37) and stimulate proliferation (38), respectively. We have reported that MCF-7 cells that overexpress Trx exhibit a several-fold increase in basal NF-κB and AP-1 activity (8). Trx also binds to and inactivates apoptosis signal-regulating kinase-1 (ASK-1), a pro-apoptotic protein (39). Upon oxidation of Trx, ASK-1 is released, facilitating cell death. The redox-inactive mutant Trx would not inhibit ASK1, and this may also play a role in enhancing drug-induced apoptosis.

In conclusion, we have found that mouse WEHI7.2 lymphocytic leukemia cells expressing a redox-inactive human Trx are significantly more susceptible to apoptosis induced by dexamethasone, doxorubicin, etoposide and staurosporine than either vector alone vector or wild-type cells. It has previously been reported that increased thioredoxin expression decreases the sensitivity of WEHI7.2 cells to apoptosis induced by a variety of chemical agents. Taken together, these observations strongly suggest that Trx is a critical component of cellular defense to drug-induced apoptosis. Trx levels are increased in a number of human cancers and, thus, Trx makes an attractive target for drugs to inhibit cancer cell growth and to sensitize cancer cells to drug-induced apoptosis.

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