Thioredoxin, a Gene Found Overexpressed in Human Cancer, Inhibits Apoptosis in Vitro and in Vivo

Amanda Baker, Claire M. Payne, Margaret M. Briehl, and Garth Powis

Arizona Cancer Center [A. B., G. P.], Department of Microbiology and Immunology and Arizona Research Laboratories [C. M. P.], and Department of Pathology [M. M. B.], University of Arizona, Health Sciences Center, Tucson, Arizona 85724

ABSTRACT

The redox protein thioredoxin plays an important role in controlling cancer cell growth through regulation of DNA synthesis and transcription factor activity. Thioredoxin is overexpressed by a number of human primary cancers and its expression is decreased during dexamethasone-induced apoptosis of mouse WEHI7.2 thymoma cells. We examined the ability of WEHI7.2 cells stably transfected with human thioredoxin cDNA showing increased levels of cytoplasmic thioredoxin to undergo apoptosis in vitro and in vivo. The cells were protected from apoptosis induced by dexamethasone, staurosporine, etoposide, and thapsigargin, but not by N-acetyl-sphingosine. When inoculated into severe combined immunodeficient mice, the trx-transfected cells formed tumors that showed increased growth compared to wild-type, as well as bel-2-transfected, WEHI7.2 cells. The trx- and bel-2-transfected cell tumors both showed less spontaneous apoptosis than tumors formed by the wild-type cells. Unlike tumors formed by the wild-type and bel-2-transfected WEHI7.2 cells, trx-transfected cell tumors did not show growth inhibition upon treatment with dexamethasone. This study suggests that increased thioredoxin expression in human cancers may result in an increased tumor growth through inhibition of spontaneous apoptosis and a decrease in the sensitivity of the tumor to drug-induced apoptosis.

INTRODUCTION

Trx is a low molecular weight redox protein found in both prokaryotic and eukaryotic cells (1). The cysteine residues at the conserved Cys32-Gly-Pro-Cys35-Lys active site of Trx undergo reversible oxidation-reduction catalyzed by the NADPH-dependent selenium-containing flavoprotein Trx reductase (2). Human Trx is a protein of Mr 11,500 with 27% amino acid identity to Escherichia coli but containing three additional Cys residues not found in bacterial Trx that give the human protein unique biological properties (3). Trx was originally studied for its ability to act as a reducing cofactor for ribonucleotide reductase, the first unique step in DNA synthesis (4). More recently, Trx has been shown to exert redox control over a number of transcription factors, including nuclear factor κB (5), transcription factor IIIC (6), BZLF1 (7), and the glucocorticoid receptor (8), and indirectly, through nuclear redox factor Ref-1/HAPE, Trx can regulate AP-1 (Fos/Jun heterodimer; Ref. 9).

Trx is also a growth factor with a unique mechanism of action. Human Trx stimulates the proliferation of both normal fibroblasts and a wide variety of human solid and leukemic cancer cell lines (10, 11). Redox activity is essential for growth stimulation by Trx, and mutant redox-inactive forms of Trx lacking the active site Cys32 and Cys35 residues are devoid of growth stimulating activity (11). Studies with 125I-labeled Trx have revealed no high-affinity binding sites that might suggest receptors for Trx on the surface of cancer cells (12). Trx appears to stimulate cell proliferation by increasing the sensitivity of the cells to growth factors secreted by the cells themselves (12).

We have found that Trx mRNA is elevated compared to paired normal tissue in almost half of the human primary lung and colon tumors we examined (3, 13). Other studies have found increased Trx in human neoplastic cervical squamous epithelium cells and hepatocellular carcinoma (14, 15). We have recently shown that human breast cancer cells transfected with a dominant negative, redox-inactive mutant Trx show reduced anchorage-independent growth in vitro and an almost complete inhibition of tumor formation in vivo (16). Thus, Trx overexpression may be a factor in the growth of some human cancers.

We previously reported that Trx gene expression is decreased during dexamethasone-induced apoptosis of mouse thymoma-derived WEHI7.2 cells (17). To further study the effects of Trx on apoptosis, in this study we stably transfected WEHI7.2 cells with human Trx cDNA and examined the effects on both spontaneous and drug-induced apoptosis in vitro and with the cells growing in scid mice.

MATERIALS AND METHODS

Cells. Human wild-type Trx cDNA was prepared as described previously, cloned into the NotI site of the pDC304neo mammalian transfection vector (16) and transfected by electroporation into mouse WEHI7.2 thymoma-derived cells (18). Transfected cells were maintained at culture densities up to 10⁶ cells/ml in DMEM containing 10% fetal bovine serum supplemented with 800 µg/ml G418 sulfate, and clones were isolated in soft agarose and then maintained in 200 µg/ml G418 sulfate. All studies were conducted on clonal lines between passages 3 and 20. Stably transfected bel-2 WEHI7.2 cells (W.Hb12 cells) were obtained from Dr. Roger Miesfeld (University of Arizona, Tucson, AZ; Ref. 19). Drugs were added at a culture density of 1 × 10⁵ to 2 × 10⁵ cells/ml. Stock solutions (10 mM) of dexamethasone were prepared in ethanol, whereas staurosporine, etoposide, thapsigargin, and N-acetyl-sphingosine were prepared in DMSO. Further dilutions were made using culture medium.

mRNA Expression. Northern blot hybridization analysis was performed as described previously using a full-length [α-32P]dCTP-labeled human Trx cDNA probe (3). Blots were quantified using a Molecular Dynamics PhosphorImager.

Glucocorticoid Receptors. The level of functional glucocorticoid receptors was assessed using a transient cotransfection of cells with a glucocorticoid response element/CAT reporter plasmid (pmnCAT; Ref. 20) and β-galactosidase. After a 22-h recovery period, the cells were treated with 1 µM dexamethasone, and CAT protein was measured after an additional 24 h using a CAT ELISA (Boehringer Mannheim, Indianapolis, IN). An aliquot of the transfected cells was stained for β-galactosidase activity and CAT activity normalized for transfection efficiency.

Apoptosis. Apoptosis was measured by an ELISA for histone-associated DNA fragments (21), by morphology and by flow cytometry (22). The criteria used for the morphological identification of apoptotic cells included condensation and margination of the chromatin with the formation of crescents, cell shrinkage, increased staining, nuclear fragmentation, cytoplasmic vacuolization, and apoptotic body formation. Cells were incubated with 20 µg/ml 7-amino actinomycin D for 30 min at 4°C before being analyzed by flow cytometry.

Immunofluorescence Staining. Cells were centrifuged onto 0.17-mm thick quartz coverslips, air dried for 10 min, fixed with 4% methanol-free formaldehyde for 20 min at room temperature, washed for 15 min in PBS, pH 7.2. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 2/7/97; accepted 9/17/97.
thioredoxin prevents apoptosis

Thioredoxin (Trx) is a small, ubiquitously expressed protein that plays a crucial role in cell survival by preventing apoptosis. In this study, the expression of Trx was increased in mammalian cells, and the effects of this increase on cell survival were investigated.

### RESULTS

WEHI7.2 cells were stably transfected with human Trx cDNA in the pDC304neo mammalian transfection vector. We examined multiple clones and found the maximal increase in Trx mRNA compared to endogenous levels of mouse Trx mRNA, was 1.8-fold for clones Trx5 and Trx6 (Fig. 1A). As determined by immunofluorescent staining and confocal microscopy, the trx-transfected cells showed increased levels of Trx (Fig. 1A). The relative fluorescence intensity of wild-type WEHI7.2 cells (±SE; n = 2) was 1.00 ± 0.05; of TrxS, 1.87 ± 0.11 (P < 0.001 compared to wild type); and of Trx6, 2.15 ±0.14 (P < 0.001 compared to wild type). Trx-like fluorescent staining was observed in the nucleus as well as the cytoplasm of the cells (Fig. 1C). In the wild-type cells, 60.1 ± 5.1% of the fluorescent staining was in the nucleus, in the Trx5 cells it was 59.8 ± 2.5%, and in the Trx6 cells it was 36.1 ± 1.8%.

Compared to both wild-type or vector-alone-transfected cells, the trx-transfected WEHI7.2 cells were resistant to apoptosis induced by 1 μM dexamethasone as measured by histone-associated DNA fragmentation (Fig. 2A) or by flow cytometry (Fig. 2B). Histological examination of the WEHI7.2 cells revealed a classic apoptotic morphology in response to dexamethasone. However, only a small fraction of the cells undergo apoptosis at any one time, and they rapidly progress to fragmented cells. For this reason, results are expressed as relative apoptosis rather than percentage of apoptotic cells. Glucocorticoid receptor activity measured using a glucocorticoid receptor/CAT reporter plasmid was not decreased in the trx-transfected cells (results of three studies not shown). We also studied the effect of trx transfection on other agents known to induce apoptosis (Table 1). Compared to vector-alone-transfected cells, trx-transfected cells were resistant to apoptosis induced by staurosporine, a general kinase inhibitor (23); by a cell-permeant sphingosine analogue, N-acetyl sphingosine (24); by thapsigargin, which blocks the uptake of intracellular Ca²⁺ resulting in an increase in intracellular free Ca²⁺ concentration (19); and by etoposide, a topoisoasemerase II inhibitor.

---

**Fig. 1. A. Northern blot hybridization analysis of total RNA extracted from: wild-type mouse WEHI7.2 cells; from pDC304neo vector-alone transfected WEHI7.2 cells (Neo); and from the trx-transfected WEHI7.2 clones Trx5 and Trx6. A full-length 32P-labeled trx cDNA probe was used for hybridization. Top band, transfected human Trx mRNA; bottom band, mouse Trx mRNA. The values on the right show the position of molecular weight markers (kb). B. Fluorescence immunohistochemical staining of Trx in cells using immunofluorescence-purified rabbit antihuman Trx polyclonal antibody, biotinylated goat anti-rabbit IgG fluorescent streptavidin fluorochrome, and laser scanning confocal microscopy. 1, wild-type WEHI7.2 cells; 2, pDC304neo vector-alone transfected WEHI7.2 cells; 3, Trx5 trx-transfected cells; 4, Trx6 trx-transfected cells. C. Fluorescence immunohistochemical staining of Trx using Cy5-streptavidin fluorochrome and YOYO-1 to counterstain nuclear DNA showing that Trx is present in the cytoplasm and the nucleus of wild-type WEHI7.2 cells (5) and Trx6 trx-transfected cells (6).**
Fig. 2. Effects of trx and bcl-2 transfection in WEHI7.2 cells on dexamethasone-induced apoptosis. A, apoptosis measured by an ELISA for histone-associated DNA fragments, expressed as relative nucleosomal enrichment. Wild, wild-type WEHI7.2 cells; Neo, pDC304neo vector-alone-transfected WEHI7.2, W.Hb12, bcl-2-transfected WEHI7.2, Trx5 and Trx6, trx-transfected WEHI7.2 cells. The cells were treated with 0.01% ethanol vehicle (■) or 1 μM dexamethasone (▲), and apoptosis was measured 24 h later. Columns, mean of four determinations; bars, SE. * P < 0.05 compared to Neo control. B, apoptosis measured by flow cytometry showing typical results. Regions R1, R2, and R3 of the scattergrams are live nonapoptotic, early apoptotic, and late apoptotic cells, respectively. 1, pDC304neo vector-alone-transfected control cells; 2, pDC304neo vector-alone-transfected cells treated for 48 h with 1 μM dexamethasone; 3, Trx6 trx-transfected WEHI7.2 cells; 4, Trx6 cells treated for 48 h with 1 μM dexamethasone.

(25). WEHI7.2 cells transfected with the bcl-2 antiapoptotic proto-oncogene (W.HB12 cells) showed a similar pattern of protection against apoptosis induced by the various agents as did the trx-transfected cells (Table 1).

When inoculated into scid mice, the trx-transfected WEHI7.2 cells formed tumors that grew more rapidly than tumors formed by either wild-type or bcl-2-transfected WEHI7.2 cells (Fig. 3A). Upon histological examination, tumors formed by the wild-type cells showed fields of apoptotic cells adjacent to fields of viable cells, as well as apoptotic cells admixed with viable-appearing cells (Fig. 3B). The cells undergoing apoptosis exhibited the classic appearance of condensed and margined chromatin, some in the form of crescents, and a dense cytoplasm accompanied by vacuolization. The trx-transfected WEHI7.2 cell tumors showed minimal numbers of cells undergoing
Table 1 Effect of trx and bcl-2 transfection on apoptosis induced by different agents

<table>
<thead>
<tr>
<th></th>
<th>Neo</th>
<th>Trx5α</th>
<th>Trx6α</th>
<th>W.Hb12α</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dexamethasone</td>
<td>19.2 ± 0.6</td>
<td>4.4 ± 0.7</td>
<td>7.7 ± 1.2</td>
<td>0.7 ± 0.0</td>
</tr>
<tr>
<td>Stauroporine</td>
<td>58.3 ± 9.8</td>
<td>5.3 ± 0.0</td>
<td>9.9 ± 1.1</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td>N-Acetyl-sphingosine</td>
<td>50.8 ± 7.1</td>
<td>11.1 ± 9.8</td>
<td>22.6 ± 4.5</td>
<td>5.5 ± 1.3</td>
</tr>
<tr>
<td>Etoposide</td>
<td>162.5 ± 12.1</td>
<td>6.0 ± 0.6</td>
<td>20.9 ± 4.1</td>
<td>4.5 ± 0.7</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>4.3 ± 0.9</td>
<td>2.3 ± 1.8</td>
<td>1.8 ± 1.2</td>
<td>0.9 ± 0.6</td>
</tr>
</tbody>
</table>

* p < 0.05 compared to vector-alone transfected control cells.

apoptosis scattered throughout the tumor mass. Tumors formed by bcl-2-transfected WEHI7.2 cells also showed very few cells undergoing apoptosis (not shown). Areas of necrosis were seen in wild-type, trx-transfected, and bcl-2-transfected cell tumors, usually adjacent to fields of viable-appearing tumor cells or, in the case of the wild-type cells, adjacent to areas that show extensive apoptosis or next to viable-appearing cells. Treatment of the mice with dexamethasone starting at day 9 had no effect on the growth of the trx-transfected cell tumors but markedly inhibited the growth of the wild-type tumors and the bcl-2-transfected cell tumors (Fig. 3A). Histological examination revealed no evidence of increased apoptosis caused by dexamethasone treatment of wild-type, trx-transfected, or bcl-2-transfected cell tumors.

DISCUSSION

WEHI7.2 cells stably transfected with human trx showed a maximal increase of 1.8-fold in Trx mRNA compared to endogenous levels of mouse Trx mRNA. This relatively low level of overexpression is similar to our experience with trx transfection of mouse NIH 3T3 cells and human MCF-7 breast cancer cells (16), suggesting that higher levels of unregulated trx expression may be toxic to cells. As deter-
The finding that Trx is present in the cytoplasm and the nucleus of cells confirms an earlier immunohistochemical study using conventional light microscopy of cervical tumor cells that reported cytoplasmic, nuclear, or cytoplasmic and nuclear localization of Trx (14). This is an important observation because Trx may be able to directly reduce redox-regulated nuclear transcription factors, such as AP-1 (Fos/Jun heterodimer; Ref. 9). If Trx can enter the nucleus, it may not need other nuclear redox factors, such as Ref-1/HAP1, as has been suggested (9).

The trx-transfected cells were resistant to apoptosis induced by dexamethasone. Trx has been reported to be necessary for assembly of the glucocorticoid receptor (8). However, glucocorticoid receptor activity was not decreased in the transfected cells, suggesting that the effects of Trx on apoptosis appear to lie downstream of the glucocorticoid receptor. The trx-transfected cells also showed resistance to apoptosis induced by staurosporine, etoposide, N-acetyl-sphinogosine, and thapsigargin. Exogenously added human Trx has been reported to inhibit apoptosis induced by tumor necrosis factor α in U937 human lymphoma cells (26). However, we found that exogenously added human Trx did not protect WEHI7.2 cells against apoptosis induced by dexamethasone (27). Tumor necrosis factor α and dexamethasone are thought to trigger apoptosis by different signaling pathways. It may also be that exogenous Trx is not taken up by WEHI7.2 cells. We have found that other tumor cells take up Trx poorly, if at all (12). Clearly, an increase in intracellular Trx achieved by transfection of trx in the present study is associated with resistance of the WEHI7.2 cells to apoptosis induced by dexamethasone and other agents.

The pattern of resistance to drug-induced apoptosis caused by trx transfection is similar to that produced by transfection with the human proto-oncogene bel-2. Bcl-2 is believed to exert its inhibitory effects upstream of the activation of the cytosine aspartate proteases cascade (caspase) responsible for the final stages of apoptosis (28). The protective effects of Bcl-2 against apoptosis have been suggested to involve an antioxidant mechanism (29), although this is disputed based on the ability of Bcl-2 to block apoptosis caused by agents that are thought not to act by an oxidant mechanism (30) or caused by hypoxia (31). The antioxidants N-acetyl-cysteine, pyrrolidine dithiocarbamate, Trolox (a water-soluble vitamin E analogue), and butylated hydroxytoluene protect rat thymocytes against drug-induced apoptosis (32, 33). We have previously reported that Trolox, catalase, and superoxide dismutase protect murine WEHI7.2 cells against dexamethasone-induced apoptosis (27). It is intriguing, therefore, that trx, a gene that codes for a known redox-active protein, also inhibits apoptosis. The mechanism by which Trx inhibits apoptosis remains to be established, but its pattern of antiapoptotic activity similar to Bcl-2 suggests that it also may act upstream of the cytosine proteases.

WEHI7.2 cells transfected with trx formed tumors in scid mice that grew considerably faster than tumors formed by the wild-type parental cells or by bel-2-transfected cells. This may be due, in part, to a decreased rate of spontaneous apoptosis that occurred in the trx-transfected cell tumors. High levels of Bcl-2 have been found in a wide variety of human cancers (34). Although transfection with bel-2 is known to confer resistance to apoptosis induced by anticancer drugs and radiation, the effects of bel-2 on tumor growth are less clear. Transfection with bel-2 gives a survival advantage to cells in culture (35). Transgenic mice overexpressing Bcl-2 under transcriptional regulation of the immunoglobulin heavy chain enhancer develop benign lymphoma that eventually progresses to high-grade malignant disease (36). This suggests that bel-2 also provides a survival advantage to cells in vivo but that an additional change, most frequently rearrangement of mvc (36), is necessary for tumor growth. Our studies using WEHI7.2 thymoma cells show that bel-2-transfected cells formed tumors that grew faster than tumors formed by wild-type WEHI7.2 cells. This may be due to a reduction in the rate of spontaneous apoptosis observed in the bel-2-transfected cell tumors compared to the wild-type tumors. It was not possible to distinguish a difference in the rates of spontaneous apoptosis between the trx and bel-2-transfected cell tumors. Paradoxically, the bel-2-transfected cell tumors still showed growth inhibition by high-dose dexamethasone treatment, as did wild-type cell tumors. There was no evidence for increased apoptosis caused by dexamethasone treatment of wild-type, trx-transfected, or bel-2-transfected cell tumors, so the possibility remains that in vivo dexamethasone does not inhibit tumor growth in vivo by a mechanism that involves increasing the rate of apoptosis.

In summary, we have shown that transfection with trx, a gene found to be overexpressed in a number of human cancers, can inhibit apoptosis of cancer cells in culture induced by a variety of agents. In animals, the trx-transfected cancer cells show an increased growth, decreased spontaneous apoptosis, and decreased sensitivity to apoptosis induced by dexamethasone. If similar effects occur in patient tumors, then trx could be a new human proto-oncogene.

ACKNOWLEDGMENTS

We thank Dr. Mikel Aickin for statistical analysis.

REFERENCES

14. Fujii, S., Nambu, Y., Nonogaki, H., Konishi, I., Mori, T., Masutani, H., and Yodoi, J. Coexpression of adult T-cell leukemia-derived factor, a human thioredoxin homo-
Thioredoxin Prevents Apoptosis


Thioredoxin, a Gene Found Overexpressed in Human Cancer, Inhibits Apoptosis in Vitro and in Vivo

Amanda Baker, Claire M. Payne, Margaret M. Briehl, et al.


Updated version Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/22/5162

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.