Thioredoxin Expression in Primary T-Cell Acute Lymphoblastic Leukemia and Its Therapeutic Implication

Li-en Shao, Mitchell B. Diccianni, Tetsuya Tanaka, Ruby Gribi, Alice L. Yu, Jeanette D. Pullen, Bruce M. Camitta, and John Yu

Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California 92037 [L. S., T. T., R. G., J. Y.]; Department of Pediatrics, University of California at San Diego Medical Center, San Diego, California 92103 [M. B. D., A. L. Y.]; University of Mississippi Medical Center, Jackson, Mississippi 39216 [J. D. P.]; and Midwest Children’s Cancer Center, Milwaukee, Wisconsin 53226 [B. M. C.]

ABSTRACT

Increased expression of intracellular thioredoxin has been implicated in the inhibition of apoptosis and in a decrease in the sensitivity of the malignancies to drug-induced apoptosis. In the present studies, we analyzed expression of thioredoxin in samples from 28 children with T-cell acute lymphoblastic leukemia and analyzed their sensitivity toward inhibition of thioredoxin expression. Thioredoxin was expressed in variable amounts. Higher expression was associated with higher WBC counts. Exogenously added thioredoxin stimulated proliferation of clonogenic cells among the T-cell acute lymphoblastic leukemia samples expressing relatively lower levels of intracellular thioredoxin, whereas there was no effect on the clonogenic cells expressing high levels of thioredoxin. In addition, there was differential sensitivity of the leukemia clonogenic cells toward 1-methylpropyl 2-imidazolyl disulfide, an inhibitor of thioredoxin expression, as compared with normal hematopoietic progenitors. This suggests the possibility of using this approach for treatment. Because overexpression of thioredoxin is associated with resistance to many anticancer drugs, the inhibition of thioredoxin expression may overcome this drug resistance and probably sensitize leukemia cells to other chemotherapeutic agents.

INTRODUCTION

ALL3 is the most common type of cancer in children. The well-known prognostic factors include WBC count, age, gender, and specific cytogenetic changes. The majority of childhood ALL is B-cell lineage. T-ALL comprises approximately 10–15% of all of the cases of ALL. Because T-ALL is a more aggressive disease, patients require more intensive chemotherapy and, should they relapse, their clinical outcome is very dismal. A better understanding of the biology of T-cell leukemia may facilitate the development of a selective therapy that exploits specific biological properties.

Thioredoxin is an approximately M, 12,000 redox protein that plays an important role in cell viability, activation, and proliferation (1). Thioredoxin, known as a dithiol hydrogen donor, is the major reductant for many targets, including ribonucleotide reductase, essential for DNA synthesis (2). It is also required for the interaction between transferrin receptor mRNA and the iron response element-binding protein, serving to promote cellular proliferation (3). Furthermore, thioredoxin regulates the activity of transcription factors including nuclear factor-κB, TFIIIC, BZLF1, and the glucocorticoid receptor (4–7). The binding of Fos/Jun to the AP-1 site is also subject to redox control by redox factor 1, which is reduced by thioredoxin (8, 9). Interestingly, thioredoxin is induced by various stress conditions, such as viral infection (10–12), and is also secreted from the cells. Furthermore, several reports (13, 14) have demonstrated that thioredoxin-transfected cells are more resistant to anticancer drugs than are the control cells. On the other hand, antisense-transfected cells become sensitive again to the same agents.

We investigated the expression of thioredoxin in T-ALL and studied the sensitivity of these samples toward inhibition of thioredoxin expression. The results show that thioredoxin was expressed in variable amounts in T-ALL, with higher expression associated with higher leukemia cell counts. It was also found that the proliferation of T-ALL clonogenic cells from those samples expressing relatively low intracellular thioredoxin was stimulated significantly by the addition of exogenous thioredoxin. In addition, the sensitivity toward an inhibitor of thioredoxin expression varied substantially between the leukemia clonogenic cells and the normal hematopoietic progenitors, suggesting the possibility of using this approach as a potential therapeutic regimen for treatment. Overall, current studies should facilitate a better understanding of the molecular changes in T-ALL and shed light on the potential molecular targets for new treatment and assessment of clinical outcome.

MATERIALS AND METHODS

Primary Leukemic Cells. Peripheral blood or bone marrow samples were obtained from 28 newly diagnosed patients with childhood T-ALL who enrolled in Pediatric Oncology Group protocol #9400. Informed consent was obtained from the patients and/or their parents. The content of lymphoblasts was generally >95%, as determined by Wright stain. The MNC fraction was isolated from these leukemia samples by Ficoll-Paque density gradient centrifugation (Pharmacia, Piscataway, NJ). In some cases, MNCs of T-ALL samples were cryopreserved and stored in liquid nitrogen before use in the studies. Viability on thawing was generally greater than 80%, as determined by trypan blue dye exclusion.

Western Blot Analysis. The MNC fraction of leukemia samples was lysed for 1 h in lysis solution of 1% NP40, 1 mM EDTA, and 150 mM NaCl in 50 mM Tris buffer (pH 8.0) containing 20 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 5 μg/ml antipratin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 0.5 μg/ml NαP-Tosyl-L-lysine chloromethyl ketone (Sigma Chemical Co., St. Louis, MO). After centrifugation for 5 min at 4°C and 12,000 X g, the supernatant was harvested and stored at −80°C until further analysis. About 10–20 μg of each protein extract was analyzed by electrophoresis on a 15% polyacrylamide/SDS gel and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). After blocking in Blotto solution (i.e., 5% nonfat milk, 0.2% Tween 20, 0.05% NP40, 150 mM NaCl, and 10 mM Tris-HCl, pH 8.0) for 1 h at room temperature, the membranes were incubated overnight at 4°C with 1:2000 dilution of rabbit antiserum directed against B-cell leukemia virus type-I.
Leukemia Colony Assay. Approximately $5 \times 10^5$ primary leukemia cells were cultured in 35-mm Petri dishes at $37^\circ C$, 5% CO$_2$ in Iscove’s modified Dulbecco’s medium (ICN Biomedicals, Aurora, OH) supplemented with 1.0% methylcellulose, 0.1 $\mu$g/ml thymidine, 15% horse serum, 15% FCS, 1% BSA, 10 ng/ml PMA, and 100 units/ml recombinant human IL-2 (Immunex, Seattle, WA; also see Ref. 15). Two dishes were set up for each individual data point/experiment. T-ALL colonies with 50 or more cells were enumerated after incubation of the dishes for 10–14 days at $37^\circ C$. To examine the effect of recombinant human thioredoxin (American Diagnostica, Greenwich, CT), PMA was omitted in the cultures, and 0.5 $\mu$g/ml of recombinant thioredoxin was added. On the other hand, for the inhibitor studies, a series of increasing concentrations of the inhibitor compound IV-2 from 0.01 to 30 $\mu$g/ml was added in regular leukemia colony cultures, and the concentration of IV-2 resulting in 50% inhibition of colony formation in the cultures (IC$_{50}$) was calculated.

Clonogenic Assay for Normal Hematopoietic Progenitor Cells. Cultures of hematopoietic progenitor cells were performed as described in previous publications (16). Briefly, 2 $\times 10^5$ MNCs from normal bone marrow donors were plated in Petri dishes at $37^\circ C$ in the presence of increasing concentrations of compound IV-2. At day 14, BFU-E was identified as a large aggregate of more than 64 hemoglobinized cells or as clusters of three or more subcolonies consisting of eight or more hemoglobinized cells/subcolony (16). The CFU-GM was enumerated as a group of more than 50 granulocytic/monocytic translucent cells (16).

Statistical Analysis of Data. Comparisons among groups of data were made with Student’s t test or nonparametric ANOVA (17). Correlation analysis was performed by linear regression and Pearson correlation with GraphPad InStat software (GraphPad Software Inc., San Diego, CA). A P of less than 0.05% was considered statistically significant.

RESULTS

Increased Thioredoxin Expression in Primary Leukemia from Patients with T-ALL. The levels of thioredoxin protein in leukemic samples were measured in the Western blots in a semiquantitative manner. Using different amounts of recombinant thioredoxin, the sensitivity of this semiquantitative assay was in the range of 0.5 to 15 pmol (see Ref. 18).

In Fig. 1A, typical results are presented according to the levels of thioredoxin expressed in these cells (Lanes 2–6). Expression of thioredoxin protein ranged from less than 1.5-fold to more than 9-fold greater than the expression in normal MNCs (Fig. 1A). The fold increase of thioredoxin expression was calculated individually relative to normal MNC samples from healthy donors ($n = 5$) and normalized against the amount of $\beta$-actin in the same analysis.

A Kruskal-Wallis test of all of the data in Fig. 1B indicated that the variations of thioredoxin expression in these samples and the normal values in healthy donors are extremely significant ($P = 0.001$). The T-ALL samples could be separated into two major subtypes: group A with relatively low expressions of intracellular thioredoxin (≤3.0-fold increase) and group B with relatively high expressions of intracellular thioredoxin (>3-fold increase; Fig. 1B). The nonparametric ANOVA did not reveal significantly different levels of thioredoxin repression in group A as compared with that in the controls ($P < 0.05$). On the other hand, the expression of thioredoxin in group B is significantly higher than that in the controls and those in group A (both $P < 0.001$; Fig. 1B).

The expression of thioredoxin in T-ALL cells was also analyzed and compared with the level of thioredoxin expression in thymocytes from five nonleukemic patients. The conclusion about the enhanced expression of thioredoxin among leukemia cells relative to its expression in thymocytes was similar to those observed in Fig. 1B using MNCs (data not shown).

Effect of Exogenous Thioredoxin on Leukemia Colony Formation. Thioredoxin could be released from cells and function as an extracellular growth or autocrine factor for normal and tumor cells (10, 19, 20). Thus, it would be of interest to examine whether exogenously added thioredoxin stimulates proliferation of T-ALL cells. In early experiments using $[^{3}H]$thymidine incorporation assay, no difference in cell proliferation in 3-day cultures was observed between samples of T-ALL leukemia cells with and without the addition of exogenous thioredoxin, suggesting a lack of effect on the bulk of leukemia cells by thioredoxin (data not shown). Next, we considered that an in vitro leukemia colony-forming assay, where
clonogenic leukemia cells can be analyzed, may offer a more appropriate system to evaluate the influence of thioredoxin on cell growth. The clonogenic cells of leukemia samples form colonies in response to growth factors such as IL-2 and PMA, which induces expression of IL-2 receptors. Because thioredoxin is known to increase the expression of IL-2 receptor α chain, we hypothesized that exogenous thioredoxin may be able to stimulate leukemia colony formation in the absence of PMA added to the cultures. As shown in Fig. 2, there was a striking difference in the in vitro leukemia colony-forming assay according to the state of expression of intracellular thioredoxin in the cells. Notably, we found that an enhancing effect of thioredoxin in these cultures for leukemia colony formation (244.2 ± 7.4%) was observed in the leukemia samples that had very low levels of intracellular thioredoxin expression (mean fold increase 1.8 ± 0.4 as compared with control). In contrast, no enhancement in leukemia colony formation (104.1 ± 3.3%) was observed in samples with higher thioredoxin expression (e.g., 7.3 ± 1.3-fold increase).

Effect of Inhibitor for Thioredoxin Expression on ALL Colony Formation. Because thioredoxin is a critical regulator for the growth of some human cancers, Obiang et al. and Powis et al. examined the biological activity of asymmetrical disulfides in an attempt to determine their potential for redox regulation. A series of alkyl 2-imidazolyl disulfides inhibited thioredoxin-dependent cell proliferation (23). It was also shown that incubation of thioredoxin with these disulfides results in an irreversible inhibition of the thioredoxin as a substrate for reduction by thioredoxin reductase (24).

The effects of IV-2 on colony formation of primary T-ALL cells were determined using the in vitro leukemia colony assay as described (15). Increasing concentrations of compound IV-2 from 0.01 to 30 μM were added to the 35-mm Petri dishes containing methylcellulose and other additives for leukemia colony-forming cultures. After 14 days, colonies were counted, and the IC50 was determined. On the basis of a study of 19 samples from patients, it was found that the leukemia colony formation of primary T-ALL samples is sensitive to inhibition by compound IV-2, with an average IC50 of 3.2 ± 1.3 μM (Fig. 3). The average IC50 of T-ALL clonogenic cells toward compound IV-2 were very significantly different from the IC50 of normal hematopoietic progenitor cells (e.g., CFU-GM and BFU-E) from healthy donors (9.4 ± 0.5 and 9.6 ± 0.5 μM, respectively, in Fig. 3; both P < 0.0001 in unpaired t test). Such differential sensitivity between leukemia cells and normal hematopoietic progenitors suggests the possibility of using disulfide compounds for the treatment of human T-cell leukemia.

Relationship between Increase of Thioredoxin Expression and White Cell Count. Recent studies (25) to develop uniform risk criteria for children with ALL resulted in a consensus based mainly on age of patients and WBC count at diagnosis. As shown in Fig. 4A, the fold increase of thioredoxin expression is statistically correlated with WBC at diagnosis among these 28 patients. The correlation coefficient, r, in Pearson correlation analysis is 0.46 with 95% confidence interval shown in the figure; and the two-tailed P is 0.01, which is considered to be significantly correlated. When grouped as presented in Fig. 1B, these data also showed the trend of association of higher thioredoxin expression with increasing number of white cells (Fig. 4B). An unpaired t test indicated that variations in white blood counts among these groups are significant (P = 0.03).

DISCUSSION

Our studies have shown that thioredoxin expression is increased in leukemic cells from most children with newly diagnosed T-ALL. Human thioredoxin was cloned as an adult T-cell leukemia-derived factor, produced by HTLV-I (26–28). This factor was also shown to induce the expression of high-affinity IL-2 receptor α chain and Tac antigen (22). Unlike adult T-cell leukemia, which is caused by HTLV-I, childhood T-ALL has no known viral etiology. However, it is interesting to observe that childhood T-ALL cells expressed high levels of thioredoxin. Levels of thioredoxin protein or mRNA were also elevated in primary tumors or cell lines from patients with breast, cervical squamous cell carcinoma, hepatoma, lung, and colon tumors (11, 12, 18, 29).

Thioredoxin, as part of a general protein disulfide-reducing system, plays an important role in maintaining the redox environment in the cell (30, 31). It is induced by oxidative stresses, serving as an endogenous antioxidant and providing cytoprotective activity. Thioredoxin is a multifunctional protein. It serves as a dithiol hydrogen donor for many target proteins, including ribonucleotide reductase, which is important for cell proliferation. Thioredoxin increases DNA-binding of redox-sensitive transcription factors such as nuclear factor-κB and thereby regulates gene transcription (4–7). In addition, thioredoxin stimulates the redox factor 1 activity (8, 9), which acts as a transcription activator for p53 (32). Moreover, cotransfection with thioredoxin and p53 enhances induction of p21 protein (33). It is thus far, however, unclear whether endogenous thioredoxin indeed mediates these cell-cycle regulatory events in the cells. It was speculated that the increased expression of thioredoxin in tumor cells might reflect a compensatory mechanism for p53 dysfunction, as is common in most tumors (33). However, we have shown previously (34) that p53 mutation is rare in T-ALL at diagnosis.

On the other hand, many studies (35) have reported that overexpression of thioredoxin in cells suppresses apoptosis. Gallegos et al. (36) have shown that breast cancer cells stably transfected with
thioredoxin show increased anchorage-independent growth, whereas a redox-inactive mutant of thioredoxin acts in a dominant-negative manner to inhibit proliferation and xenograft tumor formation in mice. It was also found that thioredoxin expression is decreased during dexamethasone-induced apoptosis of thymoma cells (35). Furthermore, the increased levels of thioredoxin were shown to be associated with resistance to cisplatin, mitomycin C, doxorubicin, and etoposide (13, 14). Together with the findings of high levels of thioredoxin expression in tumors, these studies suggest that increased thioredoxin expression may lead to an increased tumor growth through inhibition of spontaneous apoptosis and a decrease in the sensitivity of cancer cells to drug-induced apoptosis. Intriguingly, a recent study (37) showed that caspase-3 activity in cell lysate was suppressed by a thiol-oxidant, diamide, but restored by thio-reducing agents including thioredoxin. This observation seems to be at odds with the fact that thioredoxin protects cells against apoptosis and the increase in caspase activity is responsible for the execution of apoptosis. To solve this dilemma, Baker et al. (38) suggested that thioredoxin might reduce thiol groups on other proteins in the cells that negatively regulate...
caspase activity indirectly. They showed that the \textit{in vitro} activation of caspase-3 is a general feature of many reduced proteins and not a specific action of thioredoxin (38).

Age and WBC are important prognostic factors in children with ALL (25). Recent studies (39), however, indicate that these prognostic factors are significantly less important in T-ALL than in B-precursor ALL. Knowledge of the molecular abnormalities in leukemic cells may provide better keys to understanding the treatment successes and failures in childhood leukemia. Because increased expression of thioredoxin in transfected cells is related to resistance to chemotherapy, it is possible that overexpression of thioredoxin may be associated with poor clinical outcome. However, whether thioredoxin is an independent prognostic factor awaits further study in a larger group of T-ALL patients for a clinical correlative analysis.

Thioredoxin is secreted by cells through a unique “leaderless” pathway at a high rate (40, 41). Exogenously added thioredoxin was also reported to stimulate cell proliferation by enhancing the sensitivity of the cells to cytokines (42). Thioredoxin induces expression of high affinity IL-2 receptor (22) and has comitogenicity with IL-1, IL-2, and IL-4 (19, 27, 43). In the present study, we found that the addition of exogenous thioredoxin stimulates the proliferation of ALL clonogenic cells in the absence of PMA, which has generally been considered a required component of the culture media, possibly because of its ability to induce the expression of IL-2 receptor in the leukemia cells.

The enhancing effect of exogenous thioredoxin was observed in the group of T-ALL samples that had a relatively low level of thioredoxin expression but not in the cells expressing high levels of intracellular thioredoxin. Conceivably, thioredoxin is secreted from high-expressing cells in the stimulated clonogenic progenitors. This agrees with the observation that thioredoxin stimulates cell proliferation of lymphocytes (including HTLV-1 and EBV-transformed cells) and other nonlymphoid cells (11, 19, 41). On the other hand, both normal liver cells and the hepatoma cell line, HepG2, synthesize thioredoxin; however, only the former secretes abundant thioredoxin extracellularly and recombinant thioredoxin was reported to inhibit the proliferation of HepG2 cells (41). These results indicate that different cell types respond differently to variations in the intracellular redox potential.

We have also shown that except for clonogenic cells, the bulk of leukemia blasts are not activated by thioredoxin to undergo cellular proliferation and DNA synthesis, as measured by a thymidine incorporation assay. It is believed that these colony-forming cells from ALL, though comprising only 0.05 to 1.5% of the bulk blood blasts, represent the \textit{in vitro} counterparts of the \textit{in vivo} ALL blast progenitors (44). High numbers of residual clonogenic ALL blasts in remission bone marrow of T-ALL patients usually reflects poor prognosis (45). Therefore, the observed leukemia-enhancing activity by thioredoxin for these clonogenic leukemia cells may have clinical relevance.

Leukemia colony formations of primary T-ALL are sensitive to inhibition by compound IV-2, with an IC$_{50}$ of 3.2 μM, substantially lower than the IC$_{50}$s for normal hematopoietic progenitors such as CPU-GM and BFU-E. Such differential sensitivity suggests the possibility of using disulfide compounds for treatment of human leukemia. Because overexpression of thioredoxin has been found to be correlated with resistance to many anticancer drugs, the inhibition of thioredoxin expression may overcome this drug resistance and may further sensitize leukemia cells to chemotherapeutic agents synergistically.

Characterization of the molecular changes in T-ALL will facilitate the development of a selective therapy that exploits specific biological properties and thereby improves the outlook for this disease. Our findings that thioredoxin was expressed in variable amounts in T-ALL with higher expression associated with higher WBC counts and that there was differential sensitivity toward the disulfide compound IV-2 between primary T-ALL and normal hematopoietic progenitors should facilitate a better understanding of the clinical significance of thioredoxin in this childhood malignancy and shed light on the potential targets for new treatment and assessment of clinical outcome.

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