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TRANSCRIPTIONAL REGULATION OF CYTOKINE EXPRESSION BY DIETHYLDITHIOCARBAMATE IN HUMAN HL-60 PROMYELOCYTIC LEUKEMIA CELLS

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Abstract—Diethyldithiocarbamate (DDTC) is an investigational agent used to ameliorate chemotherapy- or radiotherapy-induced myelosuppression. We studied the effects of DDTC on the regulation of hematopoietic cytokine production in human myeloid cells. The results demonstrated that DDTC decreases proliferation of human HL-60 promyelocytic leukemia cells in a concentration-dependent manner. DDTC treatment also increased interleukin-α (IL-1α), IL-1β, and tumor necrosis factor (TNF) expression in these cells. Similar findings were obtained in normal human peripheral blood monocytes. Peak induction of these cytokines occurred 6–12 hr after exposure to DDTC; levels returned to those in control cells by 24–48 hr in HL-60 cells. This effect was specific for IL-1 and TNF in that there was no detectable increase in IL-3, macrophage colony-stimulating factor or granulocyte/macrophage colony-stimulating factor RNA expression. Transcriptional run-on analysis demonstrated that exposure to DDTC increased the rate of TNF gene transcription in HL-60 cells. These data suggest that the myeloprotective effects of DDTC may be mediated, at least in part, by the induction of TNF, IL-1α, and IL-1β.

Diethyldithiocarbamate (DDTC§) is a small thiolate anion that has been shown to ameliorate myelosuppression caused by several cancer chemotherapeutic drugs without altering their tumoricidal activity [1, 2]. DDTC also induces proliferation of murine hematopoietic stem and granulocyte/macrophage progenitor cells [2], inhibits progression and relieves the constitutional symptoms associated with the acquired immunodeficiency syndrome [3], and functions as an effective radioprotective agent [4]. Although the precise mechanism of action involved in these diverse effects of DDTC is unknown, the ability of this agent to restore the number of hematopoietic cells toward normal levels following a toxic insult [2], the stimulation of DNA synthesis by DDTC in hematopoietic cells and not in tumor cells [1], and the efficacy of very low doses of DDTC [1] have suggested that the mechanism of action of DDTC may involve the induction of certain genes. Indeed, DDTC stimulates the production of interleukin-2 (IL-2) by human peripheral blood lymphocytes [5] and has been shown to enhance production of hematopoietic colony-stimulating activity in the murine long-term bone marrow culture system [6]. Taken together, these findings suggest that the production of certain hematopoietic cytokines may be responsible for the myeloprotective effects of DDTC. This study was undertaken to examine whether the activity of DDTC is mediated via the induction of one or more cytokines, to study the transcription and posttranscriptional regulation of cytokine gene expression by DDTC, and to determine the effects of DDTC on cytokine gene expression in normal, human cells.

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§ Abbreviations: DDTC, diethyldithiocarbamate; IL, interleukin; NSE, α-naphthyl acetate esterase; NBT, nitroblue tetrazolium; M-CSF, macrophage colony-stimulating factor; TNF, tumor necrosis factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; kb, kilobase; NAC, *N*-acetyl-L-cysteine; PDTC, pyrrolidone derivative of dithiocarbamate; and NF-κB, nuclear factor κB.

MATERIALS AND METHODS

Cell culture. Human HL-60 promyelocytic leukemia cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (Sigma Chemical Co., St. Louis, MO). DDTC (Sigma) was dissolved in RPMI 1640 medium and filter-sterilized prior to its addition to the cells. Control cells were treated with sterile RPMI 1640 medium alone. Cytocentrifuge smears of cultured control and drug-treated cells were examined for α-naphthyl acetate esterase (NSE) staining and nitroblue tetrazolium (NBT) reduction [7, 8]. Triplicate cultures were treated and stained, and the percentage of positive cells was determined by counting greater than 100 cells per slide. Viability was assessed by trypan blue exclusion. In certain experiments, cells were treated with cycloheximide (Sigma) or actinomycin-D (Sigma) at a final concentration of 5 μg/mL.

Peripheral blood monocytes were isolated from by-products of normal donor platelet pheresis

by Ficoll–Hypaque gradient sedimentation and adherence to plastic culture flasks for 30 min at 37° in the presence of 10% fetal bovine serum. The nonadherent cells were removed by vigorous washes with phosphate-buffered saline (PBS) and the remaining adherent cells were incubated for 48 hr in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Northern blot analysis. Total cellular RNA was isolated using the guanidine isothiocyanate/cesium chloride method [9]. RNA (15 or 20 μg) was separated in a 1% agarose/formaldehyde gel, transferred to nitrocellulose paper (Schleicher & Schuell, Keene, NH), and hybridized to the following ^{32}P-labeled DNA probes: (1) the 0.66-kilobase (kb) HindIII/HincII IL-1α human cDNA fragment isolated from the huIL-1αH2H3 plasmid ([10]; courtesy of Immunex Research and Development Corp., Seattle, WA); (2) the 0.57-kb SstI/PvuII IL-1β human cDNA fragment isolated from the huIL-1β:pGEM1 plasmid ([10]; courtesy of Immunex); (3) the 1.0-kb BamHI human IL-3 cDNA fragment purified from the phSRA22-1 plasmid ([11]; American Type Culture Collection, Rockville, MD); (4) the 0.57-kb AccI/EcoRI human macrophage colony-stimulating factor (M-CSF) cDNA fragment isolated from the pc-CSF-12 plasmid [12]; (5) the 1.9-kb BamHI/PstI human cDNA fragment of tumor necrosis factor (TNF) isolated from the pE4 plasmid [13]; (6) the 0.8-kb XhoI human granulocyte/macrophage colony-stimulating factor (GM-CSF) cDNA fragment isolated from the p91023 plasmid ([14]; courtesy of Genetics Institute, Cambridge, MA); and (7) the 2.0-kb PstI β-actin cDNA purified from the pA1 plasmid [15].

Run-on transcriptional analyses. HL-60 cells (10^8 cells per treatment) were treated with 200 μM DDTC for varying times, the nuclei were isolated by gentle lysis in 0.5% NP-40 buffer, and newly elongated RNA was radiolabeled with [α-^{32}P]UTP as described [16, 17]. The RNA was purified by Sephadex G-50 column separation and hybridized to plasmid DNAs containing various cloned inserts after digestion with restriction endonucleases as follows: (1) the 2.0-kb PstI fragment of the chicken β-actin pA1 plasmid (positive control, Ref. 15); (2) the 1.1-kb BamHI insert of the human β-globin gene (negative control, Ref. 18); (3) the 1.9-kb BamHI/PstI insert of the human TNF cDNA [13]; (4) the 0.66-kb HindIII/HincII fragment of the human IL-1α cDNA [10]; and (5) the 0.57-kb SstI/PvuII fragment of the human IL-1β cDNA [10]. The digested DNA was run in a 1% agarose gel, transferred to nitrocellulose filters and hybridized with ^{32}P-labeled RNA as previously described [16, 17]. Autoradiographic bands were scanned using an LKB UltroScan XL laser densitometer and analyzed using the LKB GelScan XL software package. The intensity of hybridization was normalized against β-actin expression.

Statistical analysis. Statistical analysis of the data was performed using a one-tailed Student's *t*-test. The statistics were determined using the mean ± SD of the three replicate experiments. $P < 0.05$ was used as the level of significance.

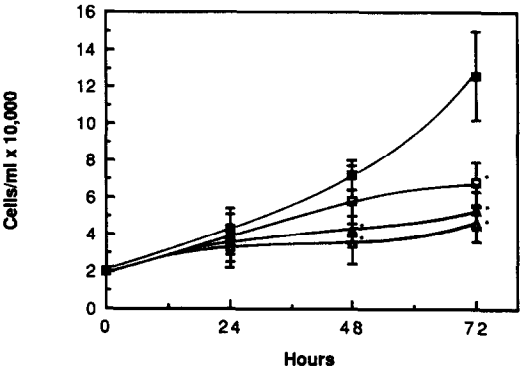

Fig. 1. Effects of DDTC on HL-60 cell proliferation. HL-60 cells (1×10^5 cells/mL) were grown in culture and treated with vehicle for control cells (■), or with 50 μM (□), 200 μM (▲), or 1000 μM (△) DDTC. At the specified times, an aliquot of cells was removed to determine the number of viable cells. The means ± SD are shown for three replicate experiments. Key: (*) significantly different from control, $P < 0.05$.

RESULTS AND DISCUSSION

The effects of DDTC were first studied in HL-60 promyelocytic leukemia cells. DDTC inhibited the growth of HL-60 cells in a concentration- and time-dependent manner (Fig. 1). Exposure to 50 μM DDTC was associated with a significant decrease in cellular proliferation at 72 hr with a cellular density that was 55% of that observed in vehicle-treated control cell cultures. At the higher concentrations of 200 and 1000 μM DDTC, cellular proliferation declined more rapidly reaching densities at 48 hr of 59 and 49%, respectively, that in control cultures. This inhibition of proliferation was associated with no detectable induction of HL-60 cellular differentiation as determined by NSE and NBT staining or cellular adherence (data not shown).

The effects of DDTC on HL-60 cell growth were associated with induction of cytokine expression. Levels of IL-1α and IL-1β mRNA were undetectable in vehicle-treated HL-60 cells (Fig. 2A). However, 6 hr following the administration of 200 μM DDTC, IL-1α transcripts were increased and reached maximal levels at 12 hr after drug treatment. The induction of IL-1β mRNA showed a similar response. The level of IL-1β transcripts was increased 6 hr following DDTC treatment and remained elevated up to 24 hr after drug administration (Fig. 2A). The effects of DDTC on IL-1α and β expression were transient and the levels of these transcripts decreased to nearly that in control cells by 48 hr. A broad range of DDTC concentrations effectively increased IL-1α and β mRNA levels (Fig. 2B). For example, treatment of HL-60 cells with 50 μM DDTC was associated with induction of these transcripts while maximal responses were observed at 500 μM.

Although IL-3 is known to stimulate the proliferation of hematopoietic stem and granulocyte/macrophage progenitor cells [19], DDTC treatment was not associated with increased IL-3 mRNA levels (data not shown). However, TNF and IL-1 share

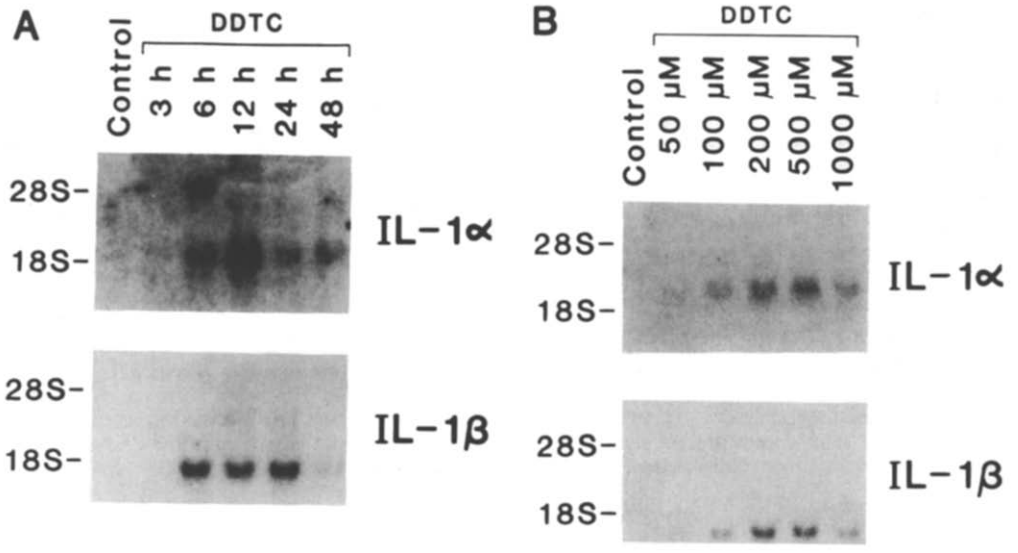

Fig. 2. Effects of DDTC on IL-1α and IL-1β mRNA levels in HL-60 cells. (A) Northern blot analysis was performed on mRNA from HL-60 cells treated with 200 μM DDTC for the indicated times. Control cells were treated with drug vehicle alone. Total cellular RNA (20 μg/lane) was hybridized to a ^{32}P-labeled IL-1α or IL-1β human cDNA probe. (B) Similar analysis of mRNA from HL-60 cells treated with the indicated concentration of DDTC (or vehicle for control cells) for 6 hr.

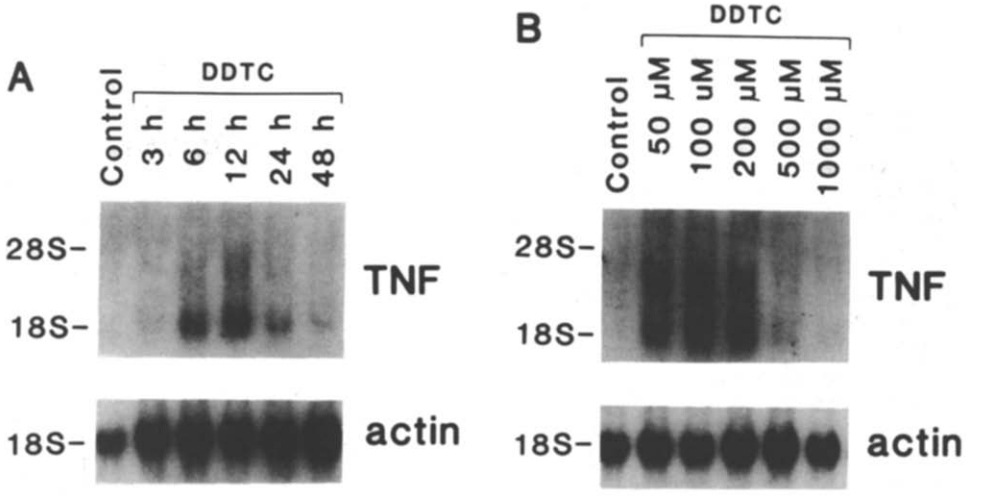

Fig. 3. Effects of DDTC on TNF mRNA levels in HL-60 cells. (A) Northern blot analysis of RNA levels from HL-60 cells treated with 200 μM DDTC for the specified times. Control cells were treated with drug vehicle alone. Total cellular RNA (20 μg/lane) was hybridized to ^{32}P-labeled TNF or β-actin cDNA probes. (B) Similar analysis was performed on RNA from HL-60 cells treated with various concentrations of DDTC (or vehicle for control cells) for 6 hr.

many similar biologic activities and evidence suggests that TNF stimulates IL-1 production [20]. Conversely, TNF production is induced by IL-1 [21]. We therefore studied the effects of DDTC on TNF expression. As shown in Fig. 3A, treatment of HL-60 cells with DDTC resulted in an increase in TNF mRNA levels. The kinetics of TNF mRNA were similar to that seen with IL-1β. Treatment of HL-60 cells with 200 μM DDTC for 3 hr increased TNF transcripts, and maximal levels were detectable 12 hr after drug treatment. Although longer exposures were associated with down-regulation, TNF mRNA levels remained slightly elevated at 48 hr compared to that in control cells. Increased TNF mRNA levels were observed at DDTC concentrations of 50–500 μM (Fig. 3B).

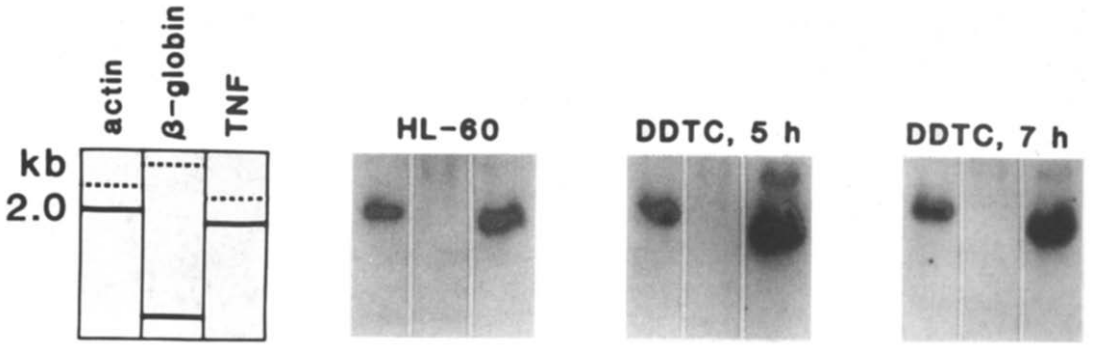

Fig. 4. Effects of DDTC on the transcriptional rate of TNF gene expression. HL-60 cells were treated with 200 μM DDTC for the indicated times. Nuclei were isolated, and ^{32}P-labeled RNA was prepared and hybridized to plasmid DNAs containing 2 μg of β-actin, β-globin and TNF cDNA. In the schematic diagram, the solid lines represent the relative positions of the cDNA inserts, and the dashed lines indicate the relative position of the plasmid vectors.

DDTC had no detectable effect on GM-CSF or macrophage colony-stimulating factor (M-CSF) mRNA levels (data not shown). These findings indicated that the effects of DDTC are selective for IL-1 and TNF. Furthermore, the inhibition of cellular proliferation (Fig. 1), and thus generalized transcription, and the absence of a time- or concentration-dependent change in actin mRNA transcripts (Fig. 3, A and B) support the specificity of this cellular response to DDTC.

Previous studies have reported the induction of TNF and IL-1 gene transcription by multiple agents [22–25]. Run-on transcriptional assays in isolated nuclei from HL-60 cells treated with DDTC were performed to determine the mechanisms that regulate IL-1 and TNF gene expression. The actin gene was actively transcribed and the β-globin gene was transcriptionally inactive in both untreated and DDTC-treated HL-60 cells (Fig. 4). A low level of TNF gene transcription was detectable in untreated HL-60 cells, while exposure to DDTC for 5 and 7 hr increased TNF gene transcription 2.9- and 1.6-fold, respectively (Fig. 4). In contrast, detectable levels of IL-1α and IL-1β gene transcription were not obtained in untreated or DDTC-treated HL-60 cells using this assay, and we were unable to determine relative transcriptional rates of these genes (data not shown).

The effects of protein synthesis inhibition of IL-1 and TNF gene expression were also studied in HL-60 cells exposed to DDTC. Cycloheximide alone was associated with increased expression of IL-1α, IL-1β and TNF mRNA (Fig. 5). Treatment of cells with DDTC and cycloheximide was associated with similar increases in these transcripts as compared to treatment with cycloheximide alone (Fig. 5). These findings suggest that induction of cytokine mRNA by DDTC is independent of new protein synthesis.

To study the posttranscriptional regulation of IL-1 and TNF gene expression, cells were treated with DDTC for 12 hr to induce gene expression and then exposed to actinomycin-D for various times to inhibit further transcription. The half-life of TNF mRNA as determined by densitometric scanning was 24 min

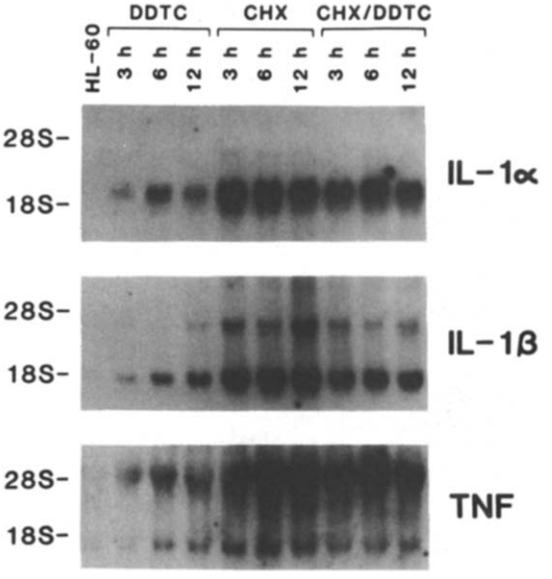

Fig. 5. Effect of cycloheximide on IL-1 and TNF mRNA levels in HL-60 cells. Cells were treated with 200 μM DDTC in the absence or presence of 5 μg/mL cycloheximide (CHX). Total cellular RNA (20 μg/lane) was isolated after 3, 6, and 12 hr and analyzed by hybridization to ^{32}P-labeled IL-1α, IL-1β or TNF cDNA probes.

(Fig. 6). Inhibition of both protein synthesis and transcription was not associated with an increase in the half-life of TNF mRNA ($T_{1/2}$ = 25 min, Fig. 6). Similar results were demonstrated for IL-1α and IL-1β (Fig. 6). Taken together, these findings suggest that posttranscriptional mechanisms involving stability of mRNA have little, if any, role in the regulation of IL-1 and TNF by DDTC and support the findings that IL-1 and TNF gene expression is transcriptionally regulated by DDTC (*vide supra*).

Further studies were performed to determine whether DDTC also stimulates cytokine production

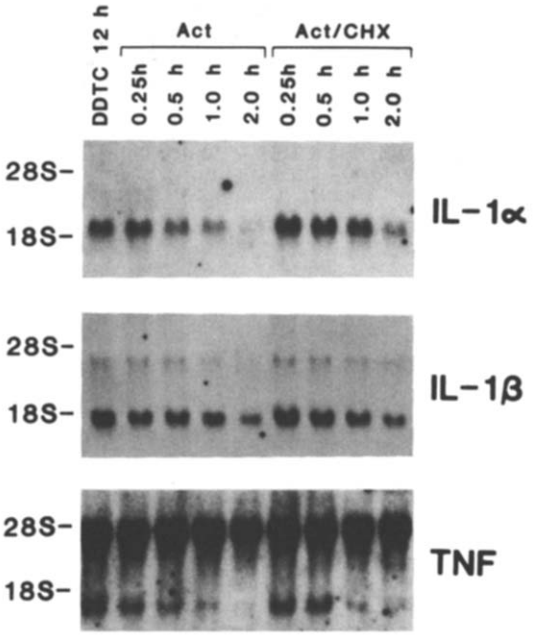

Fig. 6. Effect of cycloheximide on mRNA stability in DDTC-treated HL-60 cells. HL-60 cells were treated with 200 μM DDTC for 12 hr followed by 5 μg/mL actinomycin-D (Act) or actinomycin-D and cycloheximide (CHX). Cells were harvested at the indicated times (after the addition of actinomycin-D) and analyzed for IL-1α, IL-1β and TNF transcripts as described.

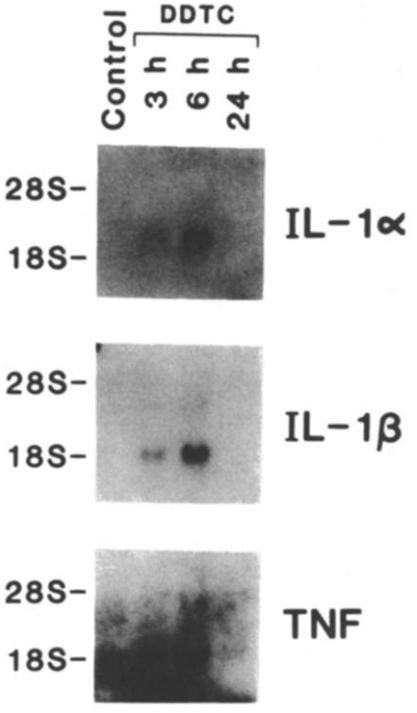

Fig. 7. Effects of DDTC on IL-1α, IL-1β, and TNF in normal human peripheral blood monocytes. A Northern blot analysis of RNA levels was performed in normal, human monocytes treated with 200 μM DDTC for 3, 6 or 24 hr. Control cells were treated with drug vehicle alone. Total cellular RNA (15 μg/lane) was hybridized to ^{32}P-labeled human IL-1α, IL-1β, or TNF cDNA probes.

in normal myeloid cells. Treatment of human peripheral blood monocytes with 200 μM DDTC for 3 hr was associated with an increase in IL-1α, IL-1β, and TNF mRNA levels (Fig. 7). Peak levels of these transcripts were detectable 6 hr after drug administration. As in HL-60 cells, these effects of DDTC were transient and expression declined to control levels at 24 hr (Fig. 7). Taken together, these findings indicated that DDTC induces IL-1 and TNF gene expression in both transformed as well as in normal myeloid cells.

We have demonstrated that DDTC regulates expression of IL-1 and TNF in human myeloid cells. In this regard, IL-1 is known to initiate the release of factors stimulating the proliferation of pleuripotent stem and granulocyte/macrophage progenitor cells [26], two populations of cells whose survival is increased when DDTC is administered after a myelosuppressive cancer chemotherapeutic drug [2]. Both IL-1 and TNF are also effective radioprotectors of bone marrow progenitor cells [27–31], suggesting that the myeloprotective effects of DDTC may be due to the expression of these cytokines. Furthermore, the observation that TNF stimulates production of GM-CSF by certain cell types [32, 33] suggests that GM-CSF may be indirectly involved in the hematologic response to DDTC. Moreover, recent studies have demonstrated autoregulatory mechanisms involved in the induction of both TNF and IL-1 [34–36]. Thus, the wide range of activity associated with DDTC may be due, at least in part, to the stimulation of IL-1 and TNF which have both direct effects and can, in turn, induce the production of other hematopoietic cytokines.

Recent studies have demonstrated that *N*-acetyl-L-cysteine (NAC), a well-characterized antioxidant, and other thiol compounds including the pyrrolidone derivative of dithiocarbamate (PDTC) block the activation of nuclear factor-κB (NF-κB) by hydrogen peroxide in Jurkat T cells and mouse fibroblasts [37, 38]. In additional studies, NAC blocked the DNA binding of NF-κB and activation of the human innumodeficiency virus long terminal repeat after stimulation with TNF and phorbol 12-myristate 13-acetate [39]. In the present study, stimulation with DDTC was associated with increases in TNF and IL-1 transcripts in human HL-60 myeloid cells and peripheral blood monocytes. Taken together, these findings suggest that regulatory mechanisms of gene expression involving thiol compounds may be unique for different genes and/or for different cell types.

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