Regulation of Manganese Superoxide Dismutase and Other Antioxidant Genes in Normal and Leukemic Hematopoietic Cells and Their Relationship to Cytotoxicity by Tumor Necrosis Factor

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Myeloid cells are a major source of superoxide and other oxygen metabolites. As a protective mechanism, cells express antioxidant enzymes including manganese superoxide dismutase (Mn-SOD), copper-zinc SOD (Cu/Zn-SOD), and glutathione peroxidase (GSX-PX). Even though hematopoietic cells are a major source of oxidants, little is known of their expression of antioxidants. We found that seven myeloid leukemic cell lines blocked at different stages of differentiation constitutively expressed Mn-SOD, Cu/Zn-SOD, and GSX-PX RNAs. Level of Mn-SOD activities paralleled levels of Mn-SOD RNA. Terminal differentiation of native HL-60 cells to either granulocytes or macrophages did not alter levels of Mn-SOD RNA but markedly decreased cell division. Myeloid leukemic lines sensitive to cytotoxic effects of tumor necrosis factor (TNF) as well as normal peripheral blood lymphocytes and monocytes, dramatically increased their levels of Mn-SOD RNA in the presence of TNF. In contrast, Cu/Zn-SOD and GSX-PX RNA levels did not increase in these same cells. TNF-resistant leukemic lines had higher constitutive levels of Mn-SOD RNA and activity; and these levels did not change in the presence of TNF. Antisense but not random oligonucleotides to Mn-SOD markedly increased the sensitivity to the inhibitory effects of TNF for both the native HL-60 (TNF-sensitive) and K562 (TNF-resistant) cell lines. Further studies showed that the antisense oligonucleotides entered the cells and resulted in decreased levels of Mn-SOD RNA. The data suggest that Mn-SOD may provide protection against cytotoxicity of TNF in hematopoietic cells.

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GENERATION OF OXYGEN metabolites such as superoxide (O$_2^-$), hydrogen peroxide and hydroxyl radicals are involved in killing microorganisms by leukocytes. However, these same reactive oxygen species may damage cells. To counteract these oxidants, cells have several antioxidant enzymes including superoxide dismutase (SOD; EC 1.15.1.1), glutathione peroxidase (GSX-PX) and catalase. Eukaryotic cells have two forms of SOD: one found in the mitochondrial matrix, the manganese SOD (Mn-SOD), and another found predominantly in the cytosol, the copper-zinc SOD (Cu/Zn-SOD). Prokaryotes have another, iron SOD. These enzymes dismutate superoxide to H$_2$O$_2$, which is then converted to water by either catalase or GSX-PX. Cells of epithelia and mesenchymal origin have an increase in Mn-SOD activity after treatment with tumor necrosis factor (TNF)-α and -β, as well as interleukin-1α (IL-1α) and IL-1β. The GSX-PX uses the reduced glutathione to convert H$_2$O$_2$ to water as well as to convert lipid peroxide to lipid metabolites and eicosanoids. A delicate balance exists between expression of each SOD and GSX-PX to provide cellular resistance to oxidative stress. Surprisingly, even though leukocytes are a major source of superoxide and other by-products of oxygen metabolism, little is known of expression of the enzymatic scavenger mechanisms for these reactive oxygen species in hematopoietic cells. Studies have shown that activation of monocytes increased expression of Mn-SOD and cytoplasmic Cu/Zn-SOD. In addition, the native HL-60 cells decreased their expression of Cu/Zn-SOD as they differentiated to either granulocytes or macrophages.

Cytotoxicity of TNF may be mediated through induction of oxidative radicals such as superoxide as well as H$_2$O$_2$ and •OH. TNF has been shown to be either cytotoxic or cytostatic for a variety of tumor cells. Tumors of epithelial origin as well as fibroblasts usually express very low levels of Mn-SOD RNA, and both TNF and IL-1α induce Mn-SOD RNA. In these epithelial tumor cells, expression of a Mn-SOD cDNA reduced cellular sensitivity to cytotoxicity by TNF. Moreover, antisense Mn-SOD cDNA reduced expression of endogenous Mn-SOD and increased sensitivity of these cells to TNF.

Sensitivity of leukemic cells to TNF might be influenced by their expression of antioxidant enzymes such as Mn-SOD. To date, few studies have investigated the expression of antioxidants in hematopoietic cells and modulation of these antioxidants by TNF. In this study we analyzed the expression of RNA coding for Mn-SOD and several other antioxidant enzymes in various normal and leukemic hematopoietic cells and the expression of Mn-SOD activity in these same cells. The effect that TNF has on expression of Mn-SOD and several other antioxidant genes was also examined in these cells. Finally, we examined the effect of Mn-SOD antisense oligonucleotides on the sensitivity of clonal hematopoietic cells to TNF.

MATERIALS AND METHODS

Cells. Leukemic cell lines used in this study were as follows: KG-1 (myeloblasts), native HL-60 (promyelocytes), ML-3 (early...
myelomonoblasts), THP-1 and U937 (monoblasts), and K562 and HEL (early myeloid/erythroid blast cells). WI38 (normal human embryonic lung fibroblasts) and Lu-CSF-1 cells (human adenocarcinoma of lung) (obtained from American Type Tissue Culture Collection [ATCC], Bethesda, MD) were also used. The TNF-resistant native HL-60 cells were developed in our laboratory. Briefly, native HL-60 cells were plated at 1 x 10^5 cells per 100-mm dish in alpha medium with 10% fetal bovine serum (FBS), and TNF was added to the cultures at increasing concentrations from 20, 50, 100, 300, to 1,000 U/mL each for 3-week intervals. During culture with TNF, medium containing freshly prepared TNF was added every 3 days. After exposure to 1,000 U/mL TNF, the TNF-resistant native HL-60 cells were isolated by growing the cells in 1,000 U/mL TNF in soft gel culture, and an individual clone (HL-60R) was expanded in liquid culture. These cells expressed normal levels of TNF receptors (data not shown).16

Mononuclear blood cells were isolated by Ficoll-Hypaque (Pharmacia, Piscataway, NJ) density separation from heparinized venous blood obtained from healthy donors after receiving informed consent. These cells were washed several times with phosphate-buffered saline (PBS). Monocytes were removed by their ability to adhere by culturing cells on plastic dishes for 3 hours. The nonadherent, mononuclear fraction contained greater than 90% lymphocytes, as determined by light microscopy of Wright-Giemsa-stained slide preparation. The adherent cells were greater than 95% monocytes as determined by morphologic examination of cytoplasmic granules.

Chemicals. Recombinant human TNFα (5.6 x 10^6 U/mg protein) was supplied by Genentech (San Francisco, CA). Specific activity was assayed by examining cytolysis on actinomycin D-treated L929 fibroblasts.17 All trans-retinoic acid (RA), dimethyl sulfoxide (DMSO), and 12-O-tetradecanoylphorbol 13 acetate (TPA) were purchased from Sigma Chemical Co, St Louis, MO, dissolved in either ethanol or acetone and stored at -20°C. The 1,25-dihydroxyvitamin D₃ ([1,25(OH)₂D₃]), provided by Dr M. Usokovic (Hoffmann-LaRoche, Nutley, NJ) was dissolved in ethanol and stored at -20°C.

Northern blot analysis. Total cellular RNA was extracted from hematopoietic cell lines by hot phenol extraction.18 Twenty micrograms of RNA per sample was electrophoresed on formaldehydeagarose gels (Bethesda Research Laboratories, Gaithersberg, MD); and transferred to nylon membrane (ICN Biomedicals Inc, Irvine, CA). Hybridization with the labeled probes was for 16 to 24 hours at 42°C in 50% formamide, 2X SSC (1X SSC = 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0, 5X Denhardts, 0.1% sodium dodecyl sulfate (SDS); 10% dextran sulfate (Sigma Co)). Filters were washed to a stringency of 0.1X SSC at 65°C and exposed to Kodak XAR film (Eastman Kodak, Rochester, NY). Autoradiograms were exposed for 1 to 7 days. Modulation in levels of SOD RNA was quantified by initial standardization to the amount of β-actin-specific transcripts. The relative density of β-actin–specific and SOD-specific transcripts in the different lanes was first determined by laser densitometry using multiple exposure of blots; and the ratio of SOD/β-actin in the control lane was assigned to be the basal level. The fold-stimulation in the experimental lanes was calculated by multiplying the ratio of density of transcripts (SOD/β-actin) by the reciprocal of the ratio of the base level.

A human Mn-SOD cDNA probe (EcoRI-EcoRI; 1.0 kb) was provided by Dr K. Madsushi (National Cancer Institute, Bethesda, MD) and human GSX-PX cDNA probe (Pst I-EcoRI; 0.6 kb) was provided by Dr M. Mirault (Quebec, Canada). A human Cu/Zn-SOD cDNA probe (BanHI-BanHI; 0.8 kb) was obtained from ATCC; and β-actin probe was EcoRI-BamHI (0.7 kb) fragment from plasmid pZIP A-3ut.19 These probes were 32P-labeled by random priming.

Cellular incorporation of radioactive thymidine. The native HL-60 cells and peripheral blood lymphocytes were incubated with 1 μCi [3H]thymidine for 4 hours at 37°C; cells were washed twice in PBS, precipitated in 5% trichloroacetic acid (TCA) (30 mmol/L Na₂HPO₄) at 4°C for 1 hour, filtered onto glass microfilter membrane (Whatman, Hillsboro, OR), washed in 3% TCA (30 mmol/L Na₂HPO₄) and heated at 80°C for 1 hour. Samples were assayed by liquid scintillation counter.

Mn-SOD activity. The Mn-SOD activity was assayed according to the method reported by Oberley and Spitz.21 The ability of xanthine-xanthine oxidase generated O₂ to reduce nitroblue tetrazolium (NBT) at a constant rate was used as a competitive Mn-SOD inhibition assay. The rate of NBT reduction was monitored spectrophotometrically at 560 nm. One unit of SOD was defined as the number of cells (per 10⁶ cells) required to inhibit by 50%, the maximal NBT reduction. To inhibit the Cu/Zn-SOD activity, we used 5 mmol/L sodium cyanide (Sigma Co) and determined the activity of Mn-SOD.

Agar cloning assay using antisense oligonucleotides to Mn-SOD. Antisense and random oligonucleotides (oligos) to the human Mn-SOD gene were purchased from Genosys Biotechnologies, Inc (Woodlands, TX); antisense oligonucleotides (which included the transcriptional start site) were 5'–TGCGCGGCTCAA-CATGTGCCT-3'; the random oligonucleotides (control) were: 5’–GGCGAGTGTTCGCGAAACA-3’ The K562 and native HL-60 cells (1 x 10⁶/mL) were incubated with either antisense or random oligonucleotides (30 μmol/L) in Iscove’s modified Dulbecco’s medium (Sigma Chemical Co) without serum at 37°C for 36 hours. After the incubation, additional oligonucleotides (30 μmol/L), alpha medium and FBS (1% vol/vol for K562 cells, 3% vol/vol for native HL-60 cells) were added to cells (1 x 10⁶/mL) and grown in 0.3% agar (Difco Laboratories, Inc, Detroit, MI) either with or without TNF. Cultures were incubated with 5% CO₂ at 37°C and colonies were counted after 7 days. The data were compared by Student’s t-test.

Cellular uptake and stability of nucleotides. To examine oligonucleotide uptake and stability by native HL-60 cells, 5’–end-labeled antisense oligonucleotides to Mn-SOD gene were incubated for 36 hours at 37°C in PBS alone and in the presence of native HL-60 cells in alpha medium with 3% heat-inactivated FBS. The oligonucleotides were end-labeled with T4 polynucleotide kinase (Bethesda Research Laboratories, Bethesda, MD) and α-[32P]adenosine triphosphate (ATP) to a specific activity of 1 x 10⁶ cpm/μg RNA. Native HL-60 cells (5 x 10⁴) in 100 μL alpha medium with heat-inactivated FBS were exposed to 10 μmol/L of 5’–end-labeled oligonucleotides for 36 hours at 37°C. The cells were washed twice and supernatant saved (extracellular fraction). Nonidet-P40 (NP-40) lysis buffer (10 mmol/L Tris.HCl, pH 8.0, 140 mmol/L NaCl, 15 mmol/L MgCl₂, 0.5% NP-40 [Sigma]) was then added, and nuclei were separated by centrifugation. This phase represented the cytoplasmic fraction and it was extracted twice with phenol/chloroform, and precipitated overnight in 0.3 mol/L Na acetate (pH 5.2) and 2 vol absolute ethanol. The precipitate was then electrophoresed in 7 mol/L urea/20% polyacrylamide gel and autoradiographed.

Semiquantitative analysis of Mn-SOD RNA by reverse transcriptase polymerase chain reaction (RT-PCR). Native HL-60 cells were incubated either with or without antisense oligonucleotides
RESULTS

Expression of RNA for Mn-SOD and other antioxidant enzymes in human myeloid leukemic cell lines. We examined Mn-SOD, Cu/Zn-SOD and GSX-PX RNA accumulation in cells from human myeloid leukemia cell lines blocked at different stages of differentiation (early and late myeloblasts [KG-1, native HL-60]; monoblasts [U937, THP-1]; erythroblasts [K562, HEL]) (Fig 1). Constitutive expression of Mn-SOD RNA was detected in each myeloid cell line. Transcripts were 4.0 and 1.0 kb, and no variant sizes were observed in any cell line. Hybridization of the same blot with a 32P-labeled β-actin DNA probe showed that comparable amounts of RNA were present in each lane (Fig 1). The THP-1, K562, and HEL cells had the greatest accumulation of Mn-SOD RNA. Expression of Cu/Zn-SOD and GSX-PX RNA was also detectable in each myeloid line. Four lines (KG-1, ML-3, THP-1, and HEL) expressed the greatest levels of Cu/Zn-SOD RNA. The ML-3 and THP-1 lines expressed slightly less GSX-PX RNA than the other five lines.

Expression of Mn-SOD activity in human leukemic cell lines. The Mn-SOD activity of cells each of the myeloid lines in general paralleled their Mn-SOD RNA levels (Table 1). The three lines (THP-1, K562, and HEL) with the highest level of expression of Mn-SOD RNA had the highest content of Mn-SOD (22-26 U/10^6 cells). No clear correlation between cell type and quantity of Mn-SOD was noted, except that two of three of the high-expressor lines were erythroid in phenotype. As summarized in Table 1 from prior studies by us, the three lines (THP-1, K562, and HEL) with the highest levels of SOD, were resistant to the cytotoxic/cytostatic effects of TNF. Also, all the cell lines expressed a comparable number of high-affinity TNF receptors.

Expression of Mn-SOD and Cu/Zn-SOD RNAs and 3H-thymidine incorporation during differentiation of native HL-60 cells. The native HL-60 cells can terminally differentiate along the granulocytic pathway2; after 4 days exposure to either DMSO (1.25%) or RA (10^{-8} mol/L), approximately 70% to 80% of the native HL-60 cells matured towards granulocytic cells, as shown by morphology and ability to reduce NBT (data not shown). Also, native HL-60 cells can differentiate along the macrophage pathway22, exposure to either 1,25(OH)2D3 (10^{-7} mol/L) or TPA (10^{-8} mol/L) for 4 days induced approximately 85% to 95% of native HL-60 cells to differentiate toward morphologic and functional macrophage like cells as measured by positive staining with α-naphthyl butyrate esterase (data not shown). Native HL-60 cells and those differentiated toward either granulocytes or macrophages had comparable levels of Mn-SOD RNA, (Fig 2A). The granulocytic and macrophage-like native HL-60 cells had 80% to 95% lower levels of 3H-thymidine incorporation as compared with the wild-type
Table 1. Mn-SOD Content of Various Myeloid Lines

<table>
<thead>
<tr>
<th>Myeloid Cell Lines</th>
<th>Mn-SOD Activity (U/10^6 cells)</th>
<th>Mn-SOD mRNA*</th>
<th>TNF Receptors</th>
<th>Clonal Inhibition of Cells by TNF (ED_{50}, pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KG-1 (myeloblast)</td>
<td>11.5 ± 0.2</td>
<td>+</td>
<td>676</td>
<td>31</td>
</tr>
<tr>
<td>HL-60 (late myeloblast)</td>
<td>10.3 ± 0.5</td>
<td>+</td>
<td>776</td>
<td>42</td>
</tr>
<tr>
<td>ML-3 (myelomonoblast)</td>
<td>ND</td>
<td>+</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>U937 (monoblast)</td>
<td>10.2 ± 0.8</td>
<td>++</td>
<td>550</td>
<td>22</td>
</tr>
<tr>
<td>THP-1 (monoblast)</td>
<td>21.9 ± 0.4</td>
<td>+++</td>
<td>1,870</td>
<td>94</td>
</tr>
<tr>
<td>K562 (early myeloid/erythroid blast)</td>
<td>21.1 ± 0.5</td>
<td>+++</td>
<td>537</td>
<td>28</td>
</tr>
<tr>
<td>HEL (early myeloid/erythroid blast)</td>
<td>25.9 ± 1.1</td>
<td>+++</td>
<td>517</td>
<td>37</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.

* Approximate levels of Mn-SOD mRNA from Fig 1.
† Concentration that gave half-maximal binding of TNF-α. Results previously reported.
‡ TNF concentration required to inhibit growth of half the colonies. Results previously reported.

native HL-60 cells (Fig 2B). Time-course studies in native HL-60 cells showed that Cu/Zn-SOD RNA started to decrease 6 hours after addition of TPA (data not shown). These studies showed that either slowly or nondividing, myeloid cells that had undergone differentiation, had little change in their levels of Mn-SOD RNA.

Effect of TNF on RNA levels of Mn-SOD and other antioxidant enzymes in various hematopoietic cells. We evaluated the effect of TNF on a variety of normal and leukemic hematopoietic cells as well as human embryonic lung fibroblasts (Figs 3A through D and 4A and B). Human embryonic lung fibroblasts (Fig 3A), peripheral blood lymphocytes (PBL; Fig 3C) and monocytes (Fig 3D) expressed a low level of Mn-SOD RNA, whereas these cells exposed to TNF had a 5.3-, 5.3-, and 2.7-fold induction of Mn-SOD RNA, respectively. TNF induced a 3.8-fold increased level of Mn-SOD RNA in U937 (TNF-sensitive, Fig 3B). The HL-60R and K562 (both resistant to cytotoxic effects of TNF) constitutively expressed Mn-SOD RNA; and TNF did not increase levels of these transcripts (Fig 3A and B). In contrast, expression of GSX-PX transcripts in response to TNF decreased in PBL and were unchanged in U937 and K562 cells (Fig 3B and C).

Both dose- and time-response studies were performed to

Fig 2. (A) Mn-SOD and Cu/Zn-SOD RNA levels in native HL-60 during their differentiation to either granulocytelike or macrophagelike cells. Native HL-60 cells were exposed for 4 days to either 1.25% DMSO, 10^{-8} mol/L RA (both produce granulocytic differentiation) or to either 10^{-7} mol/L 1,25(OH)_{2}D_{3} or 10^{-8} mol/L TPA (both induce macrophagelike differentiation). RNA analysis was performed as described in Materials and Methods. Blot was sequentially hybridized with ^{32}P-labeled Mn-SOD, Cu/Zn-SOD, and β-actin cDNAs. (B) DNA synthesis was measured by ^{3}H-thymidine incorporation (per 10^6 cells) in wild-type, granulocytelike or macrophagelike native HL-60 cells. The inducers are indicated on the abscissa. Results represent the mean ± SE of triplicate cultures. Same cell cultures were used for the analysis of both RNA and ^{3}H-thymidine incorporation.
analyze in greater detail the effects of TNF on the RNA and protein expression of antioxidant enzymes, using TNF-sensitive native (HL-60) and TNF-resistant (K562) cell lines. In native HL-60 cells, TNF induced both Mn-SOD RNA and Mn-SOD activity in a dose-dependent fashion (Figs 4A and 5). In these same cells, levels of Cu/Zn-SOD RNA slightly decreased and those of GSX-PX RNA also slightly decreased at 10^3 and 10^4 U/mL of TNF (Fig 4A). Time-dependent analysis showed that native HL-60 cells expressed greater than twofold higher levels of both Mn-SOD RNA and Mn-SOD activity within 30 minutes of exposure to TNF (10^3 U/mL) compared with control native HL-60 cells, and levels remained stable for at least 8 hours (Figs 4B and 5). The levels of both Mn-SOD RNA and Mn-SOD activity were initially high in TNF-resistant K562 cells, and did not change with exposure to TNF in dose- (Figs 4D and 5) and time-response (Figs 4D and 5) studies.

Antisense oligonucleotides to Mn-SOD enhanced TNF-mediated clonal inhibition of both K562 and native HL-60 cells. Incubation with antisense oligonucleotides to Mn-SOD significantly enhanced the clonal inhibition mediated by TNF for both the TNF-resistant (K562) and TNF-sensitive (native HL-60) cell lines as compared with clonal growth of these cells cultured with random oligonucleotides and TNF (P < .005) (Fig 6A and B). For example, K562 formed about 450 colonies. TNF alone (5,000 U/mL) or TNF plus random oligonucleotides had no effect on colony formation. However, TNF (5,000 U/mL) plus Mn-SOD antisense oligonucleotides decreased K562 colony formation about 67-90%. Also, TNF (25 U/mL) plus Mn-SOD antisense oligonucleotides decreased HL-60 colony formation by about 50% compared with plates having TNF plus random oligonucleotides, and about 60% compared with plates with TNF alone.

Effects of antisense oligonucleotides to Mn-SOD on the expression of Mn-SOD and β-actin RNA. To verify that
Fig 4. Northern analysis of RNA from native HL-60 (TNF-sensitive) and K562 (TNF-resistant) cells exposed in a dose- and time-response fashion to TNF. (A) Native HL-60 and (C) K562: Cells were exposed to increasing concentrations of TNF (0-10^4 U/mL) for 8 hours and evaluated by Northern analysis using ^32P-labeled Mn-SOD, Cu/Zn-SOD, GSX-PX, and ^32P-actin cDNAs. (B) Native HL-60 and (D) K562: Cells were cultured with TNF (10^3 U/mL) for 0 to 8 hours. Total RNA was isolated and Northern analysis was performed as described in Materials and Methods.

The Mn-SOD antisense oligonucleotides entered the cells, the Mn-SOD oligonucleotides were radiolabeled and incubated with native HL-60 cells (Fig 7A). Denaturing gel electrophoresis of the labeled oligonucleotides from extracellular medium and in the cytoplasm of the cultured cells demonstrated that the oligonucleotides were stable in the culture medium, and entered and were stable for ≥36 hours in the cells.

We used the technique of RT-PCR analysis to demonstrate the effects of antisense oligonucleotides on the expression of Mn-SOD RNA in the cells. As shown in Fig 7B, Mn-SOD antisense oligonucleotides substantially reduced Mn-SOD RNA levels (lane 2). ^32P-actin was amplified simultaneously and showed comparable amounts of RNA were present in cells not containing (lane 1) and containing (lane 2) the Mn-SOD antisense oligonucleotides.

DISCUSSION

In this study we investigated both expression of Mn-SOD as well as Cu/Zn-SOD and GSX-PX in normal and leukemic cells, and the relationship between Mn-SOD expression and TNF cytotoxicity in myeloid leukemic cells. We found by Northern blot analysis that a wide variety of human hematopoietic cell lines blocked at different stages of differentiation (myeloblasts, promyelocytes, myelomonocytes, monoblasts, and erythroblasts) as well as normal human
lymphocytes and macrophages expressed the 1.0 kb and the alternatively spliced 4.0 kb Mn-SOD RNA. Also, Cu/Zn-SOD and GSX-PX RNAs were expressed in these cells. Levels of Mn-SOD activity correlated with levels of expression of Mn-SOD RNA. Slowly or nondividing, terminally differentiated HL-60 granulocytes and macrophages expressed RNA coding for Mn-SOD at a level nearly equivalent to native HL-60 cells.

Some cell types are sensitive and others are resistant to the cytotoxic/cytostatic effects of TNF; how the antiprolif-
Fig 7. Effects of Mn-SOD antisense oligonucleotides on the expression of Mn-SOD RNA. (A) Cellular uptake and stability of end-labeled antisense Mn-SOD oligonucleotides. The 21 base nucleotide was incubated in PBS (lane 1), in culture medium with heat-inactivated serum (lane 2), and in the presence of native HL-60 cells for 36 hours at 37°C. The extracellular medium was removed and the cell lysed. The cell lysates (cytoplasmic fraction) and extracellular fractions were ethanol precipitated, and the oligonucleotides loaded onto lanes 3 and 4, respectively. (B) Specific reduction of Mn-SOD RNA in Mn-SOD antisense oligonucleotides-treated native HL-60 cells. Native HL-60 cells were incubated either with or without Mn-SOD antisense oligonucleotides and then total RNA was extracted. One-microgram of RNA was reverse transcribed to cDNA and amplified by PCR with primer pairs specific for Mn-SOD and β-actin as an internal control; the amplification products were subjected to PAGE and stained with ethidium bromide, which resulted in 350 bp and 853 bp (Mn-SOD) and β-actin) bands. Lane 1: untreated native HL-60 cells. Lane 2: Mn-SOD antisense oligonucleotides cultured native HL-60 cells. Bottom lanes show the results of Southern blot hybridization of the PCR products, with probes specific for Mn-SOD (upper bands) and β-actin genes (lower bands).

The ferative effects of TNF are mediated remains unclear. Initial, ground-breaking experiments correlated levels of Mn-SOD RNA with the ability of TNF to inhibit growth of epithelial tumor cell lines. Those cells with high levels of Mn-SOD were resistant to the antiproliferative effects of TNF, and those with low levels were sensitive to TNF. The findings of the studies suggested to the investigators that some of the activity of TNF was mediated by production of toxic oxygen products. We previously identified several myeloid leukemic lines that were sensitive and several that were resistant to the growth inhibitory effects of TNF (Table 1). In addition, we previously showed that all of these hematopoietic lines expressed high affinity TNF receptors (Table 1). Four of the leukemic cell lines (HEL, K562, THP-1, and HL-60R) were resistant to TNF and expressed about twofold higher levels of Mn-SOD activity as compared with the TNF-sensitive leukemic cells. Furthermore, the Mn-SOD activity rose in native HL-60 cells (TNF-sensitive) to the same level as found in K562 cells (TNF-resistant) after exposure for 0.5 hours to TNF. Nevertheless, these results are consonant with several previous studies, which showed that a 1.0-hour pulse-exposure of native HL-60 cells to a low concentration of TNF inhibited the clonal growth of about 90% of these cells. Those cells that survived the exposure to TNF seemed to become resistant to TNF. Other experiments showed that fibroblasts that were adapted to be resistant to oxidative stresses, and only increased their Mn-SOD and Cu/Zn-SOD activities about twofold as compared with sensitive fibroblasts. To pursue the relationship between Mn-SOD and cytotoxic/cytostatic effects of TNF, we performed antisense oligonucleotide experiments. Antisense oligonucleotides to Mn-SOD but not random oligonucleotides, significantly increased the sensitivity of both the native HL-60 and the TNF-resistant K562 clonogenic cells to the growth-inhibitory effects of TNF (Figs 6A and B). These antisense oligonucleotides were taken up by the cells and resulted in decreased levels of Mn-SOD RNA (Figs 7A and B). These studies suggest that Mn-SOD provides protection of leukemic cells against the cytotoxic/cytostatic effects of TNF. However, the susceptibility of cells to effects of TNF
probably can be explained only by intracellular levels of Mn-SOD. Several studies have shown the importance of GSX-PX7 and intracellular levels of glutathione27 in determining resistance to superoxide and/or TNF. Our laboratory found that clonal hematopoietic progenitor cells of transgenic mice having increased expression of Cu/Zn-SOD were resistant to TNF.28 Recently, we also showed that myeloid colony-forming cells that were incapable of making superoxide were resistant to TNF.29 TNF probably increases levels of superoxide in hematopoietic cells, which can rapidly injure these cells unless they are protected by one of several antioxidant enzyme pathways.

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Regulation of manganese superoxide dismutase and other antioxidant genes in normal and leukemic hematopoietic cells and their relationship to cytotoxicity by tumor necrosis factor

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