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ORIGINAL ARTICLE

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Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity

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Abstract *Purpose*: Therapeutic selectivity is one of the most important considerations in cancer chemotherapy. The design of therapeutic strategies to preferentially kill malignant cells while minimizing harmful effects to normal cells depends on our understanding of the biological differences between cancer and normal cells. We have previously demonstrated that certain agents generating reactive oxygen species (ROS) such as 2-methoxyestradiol (2-ME) preferentially kill human leukemia cells without exhibiting significant cytotoxicity in normal lymphocytes. The purpose of the current study was to investigate the biochemical basis for such selective anticancer activity. *Methods*: Flow cytometric analyses were utilized to measure intracellular O_2^- levels and apoptosis. MTT assays were used as indicators of cellular viability. Western blot analysis was used to measure the expression of antioxidant enzymes in cancer and normal cells. *Results*: Malignant cells in general are more active than normal cells in the production of O_2^-, are under intrinsic oxidative stress, and thus are more vulnerable to damage by ROS-generating agents. The intrinsic oxidative stress in cancer cells was associated with the upregulation of SOD and catalase protein expression, likely as a mechanism to tolerate increased ROS stress. The increase in SOD and catalase expression was observed both in primary human leukemia cells and in primary ovarian cancer cells. Both malignant cell types were more sensitive to 2-ME than their normal counterparts, as demonstrated by the significant accumulation of O_2^- and subsequent apoptosis. The administration of ROS scavengers in combination with 2-ME prevented the accumulation of O_2^- and abrogated apoptosis induction. *Conclusions*: O_2^- is an important mediator of 2-ME-induced apoptosis. The increased oxidative stress in cancer cells forces these cells to rely more on antioxidant enzymes such as SOD for O_2^- elimination, thus making the malignant cells more vulnerable to SOD inhibition than normal cells.

Keywords Oxidative stress · Cancer · Therapeutic selectivity · Reactive oxygen species · 2-Methoxyestradiol

Abbreviations *CLL* Chronic lymphocytic leukemia · *2-ME* 2-Methoxyestradiol · *ROS* Reactive oxygen species · *SOD* Superoxide dismutase

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Introduction

Reactive oxygen species (ROS) are chemically reactive molecules, which can be generated endogenously during various cellular metabolic activities. In mammalian cells, mitochondria are the major intracellular source of ROS generation [16, 40]. The overproduction of ROS can result in detrimental cellular damage including lipid peroxidation, DNA adduct formation, protein oxidation and enzyme inactivation, which can all ultimately lead to cell death. Paradoxically, a moderate level of intracellular ROS is thought to be important to maintain

appropriate redox balance and to stimulate cellular proliferation [31, 34, 35, 38]. The continual need for oxygen in numerous biological mechanisms in aerobic cells gives way to the constant metabolic production of ROS. Over the course of evolution, cells have developed a highly regulated antioxidant defense system to maintain appropriate intracellular ROS levels and prevent oxidative damage. This system includes antioxidant enzymes such as superoxide dismutase (SOD), catalase, and various peroxidases that effectively remove these noxious species. SOD is the key enzyme required for the removal of O_2^- by converting it to hydrogen peroxide (H_2O_2), which is further eliminated by catalase and peroxidases [16]. There are two major intracellular forms of the SOD enzyme. CuZnSOD, or SOD1, is primarily found in the cytosol and in the intermembrane space of mitochondria, while MnSOD, or SOD2, is found mainly in the mitochondrial matrix. Both isoforms of the SOD enzyme have been shown to be extremely efficient in scavenging O_2^- and in preventing oxidative damage [16, 32].

Alterations in the cellular ROS status have been shown to play an important role in apoptotic cell death [2, 14, 39]. Supporting this idea are the observations that the reduction of SOD increases cellular sensitivity to ROS-inducing agents including ionizing radiation, doxorubicin, paraquat and hyperthermia [19, 28] and increases cellular injury following focal cerebral ischemia [15, 25, 33]. In addition, the administration of SOD or overexpression of the enzyme has been shown to prevent apoptosis and postischemic injury in a variety of situations [1, 4, 47]. Collectively, these studies indicate that ROS play an important role in apoptosis and that SOD is critical in regulating ROS-mediated cellular damage. These observations also suggest the possibility of damaging cancer cells by increasing free radicals through the inhibition of SOD activity.

We have previously shown that 2-methoxyestradiol (2-ME), an anticancer agent currently in clinical trials, is able to induce a severe accumulation of O_2^- in human leukemia cells by inhibition of SOD [17]. This leads to a preferential killing of the malignant cells. The ability of 2-ME and other steroid derivatives to inhibit SOD as well as the structure-activity relationship has been further characterized by Wood et al. [49]. Interestingly, a recent study has indicated that inhibition of SOD by 2-ME enhances the therapeutic effect of photodynamic therapy [13]. Inhibition of SOD is likely an important mechanism by which 2-ME causes increase of ROS in cancer cells, although caution should be exercised when using various assays for the measurement of SOD activity [20]. Importantly, the same concentrations of 2-ME that effectively kill leukemia cells do not induce significant toxicity in normal lymphocytes [17]. Considering the critical role of SOD in eliminating O_2^-, one would expect that the inhibition of this enzyme would be detrimental to both the normal and cancer cells. Thus, the biochemical basis responsible for this therapeutic selectivity remains unclear at the present time.

The objective of the current study was to investigate the biochemical basis for the selective anticancer activity of 2-ME. We hypothesized that cancer cells, due to their active metabolism associated with the dysregulation of various cellular events, generate high levels of free radicals and thus are under constant oxidative stress. This intrinsic oxidative stress renders the malignant cells more dependent on SOD for the elimination of O_2^-, and thus more sensitive to SOD inhibition by 2-ME. To test this hypothesis, we compared the steady states of cellular O_2^- levels in cancer and normal cells. The protein expression levels of SOD1 and SOD2 in cancer cells were also determined in comparison with those in normal cells. Additionally, the change in cellular O_2^- levels before and after 2-ME incubation was evaluated, and its relationship with apoptosis induction was sought. We demonstrated that primary leukemia cells isolated from patients with chronic lymphocytic leukemia (CLL) possess significantly higher basal levels of intracellular O_2^- and express high levels of SOD1 protein. This intrinsic oxidative state likely renders CLL cells more dependent on SOD for survival and contributes, at least in part, to the increased sensitivity of these cells to 2-ME-induced apoptosis. Similar results were obtained in studies with ovarian cancer cells in comparison with normal ovarian epithelial (NOE) cells.

Materials and methods

Chemicals

2-ME, phosphate-buffered saline (PBS), Tiron (4,5-dihydroxybenzene disulfonic acid), N-acetylcysteine (NAC), and dimethylsulfoxide (DMSO) were purchased from Sigma. 2-ME was prepared in DMSO as a stock solution of 10 m*M* and kept at −20°C until use. Tiron was prepared fresh in water at a concentration of 10 m*M*. NAC was prepared in PBS at a concentration of 10 m*M* and kept at −20°C until use.

Cell culture

The human leukemia cell line HL-60 was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The ovarian cancer cell lines SKOV3 and OVCAR-3 were obtained from the American Type Culture Collection (ATCC). The SNU251 ovarian cancer cell line was from Dr. Jae-Gahb Park of the Korea cell line bank at the Seoul National University College of Medicine [52]. All ovarian cancer cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS. NOE cells were cultured in a mixture of Medium 199 and MCDB 105 medium (1:1 ratio, both from Sigma), supplemented with 15% FBS, antibiotics (penicillin/streptomycin, Sigma), and 10 ng/ml epidermal growth factor (Sigma). Experiments with primary NOE cells were performed and completed within a 4-week period after the cells were placed in culture from the liquid nitrogen storage. All cells were maintained in an incubator at 37°C with humidified air containing 5% CO_2.

Isolation of leukemia cells and normal lymphocytes

Primary leukemia cells were isolated from peripheral blood samples obtained from patients with CLL after obtaining appropriate informed consent. The procedure for isolation of CLL cells using a

Ficoll density gradient method has been described previously [18]. The isolated leukemia cells were washed twice with PBS, and then resuspended in RPMI 1640 medium containing 10% FBS and antibiotics (penicillin/streptomycin, Sigma). Normal lymphocytes were isolated from blood samples from healthy donors using the same isolation procedures as for the CLL samples. All samples were cultured in an incubator at 37°C in humidified air containing 5% CO_2.

Morphological analyses

To examine changes in cell morphology, cytospins were prepared from the HL-60 control cells and 2-ME-treated cells by centrifuging at 550 rpm for 5 min onto glass slides. The slides were fixed with 100% methanol for 45 min, air-dried, and then stained with Wright's Giemsa stain solution (Biochemical Sciences, Swedesboro, N.J.). Cells were then examined for morphological changes and microscopic photographs were taken using a ×20 objective lens (Nikon, Japan).

Measurement of cellular superoxide

Superoxide was measured using hydroethidine (HEt, Molecular Probes). HEt emits a light-blue fluorescence, and upon interaction with O_2^-, is converted to ethidium (Et), which intercalates into the DNA and emits a red fluorescence. This fluorescent dye is commonly used for detection of O_2^- [12]. Intracellular O_2^- was quantitated by flow cytometry analysis of HEt-stained cells as described previously [17]. All flow data were analyzed using the Becton Dickinson CellQuest Pro software package.

MTT assays

NOE and SKOV3 cells were plated at a density of 2000 cells per well in a 96-well flat bottomed plate and allowed to attach to the culture surface overnight. The following day, the medium was aspirated off and 200 µl of drug-containing medium was added. After 72 h, 50 µl of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT reagent, 3 mg/ml) was added to each well and incubated for 3 h. At the end of the incubation, the MTT-containing medium was aspirated off and 200 µl DMSO was added to each well. After 10 min, optical density was determined using a MultiSkan plate reader (LabSystems) at a wavelength of 570 nm.

Measurement of apoptosis

Terminal deoxynucleotide transferase dUTP nick end labeling (TUNEL) and annexin V-FITC assays were used as indicators of apoptosis. An ApoDirect kit was used for the TUNEL assay, following the procedures recommended by the manufacturer (Phoenix Flow Systems). Briefly, cells were fixed with 1% paraformaldehyde for 30 min on ice and washed with PBS. The cells were then suspended in 70% ethanol and stored at −20°C for at least 18 h. The cells were then washed twice in PBS and incubated in a solution containing TdT enzyme and FITC-dUTP for 1 h at 37°C. After washing, the samples were analyzed by a flow cytometer (FACSCalibur, Becton Dickinson). For Annexin V-FITC labeling, cells were harvested and washed once with PBS. The cell pellets were resuspended in binding buffer containing 10 m*M* Hepes, pH 7.4, 140 m*M* NaCl, and 2.5 m*M* $CaCl_2$. FITC-conjugated annexin-V (BD-Pharmingen) was then added to the cell suspension and the cells were gently vortexed, incubated in the dark for 15 min at room temperature, and then immediately analyzed using a FACSCalibur flow cytometer (Becton Dickinson). The data were analyzed using the Becton Dickinson Cell Quest Pro software.

Protein expression

The expression of SOD1, SOD2, and catalase was determined using a Western blot analysis. CLL cells were harvested and lysed using a freshly prepared buffer containing 1% Triton X (Bio-Rad), 300 m*M* NaCl, 0.5% deoxycholate, 25 m*M* Hepes, pH 7.5, 20 m*M* glycerol PO_4, 0.1% sodium dodecyl sulfate (SDS), 0.5 m*M* DTT, 1.5 m*M* $MgCl_2$, 0.2 m*M* EDTA, and a protease inhibitor cocktail. Primary ovarian tumor samples were homogenized and lysates prepared using the above buffer. Ovarian cancer cell lines and NOE cells were harvested and lysed using a freshly prepared buffer containing 50 m*M* Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue, and 2.5% β-mercaptoethanol. Cell lysates were then heated at 95°C for at least 10 min and 20 µg protein was loaded directly onto the gel. All samples were separated using 12% SDS-PAGE and transferred to a nitrocellulose membrane. Polyclonal sheep anti-MnSOD (Biodesign), polyclonal sheep anti-CuZnSOD (Calbiochem), polyclonal rabbit anti-catalase (Calbiochem), and monoclonal mouse anti-β-actin (Sigma) antibodies were used for protein detection by Western blotting.

Results

Previous studies had demonstrated that 2-ME preferentially kills leukemia cells by causing an accumulation of O_2^- in malignant cells through its inhibition of SOD [17]. In the current investigation, human leukemia HL-60 cells treated with 2-ME showed a typical apoptotic morphology 24 h after drug incubation (Fig. 1A, B). A two-parameter flow cytometry analysis was then used to further evaluate the relationship between intracellular O_2^- accumulation and apoptotic events during 2-ME incubation in vitro. Cells were treated with 2 µ*M* of 2-ME for various times and then double-stained with hydroethidine and annexin V-FITC for O_2^- (red fluorescence) and apoptosis (green fluorescence), respectively. As shown in Fig. 1C, D, a substantial amount of O_2^- accumulation was observed 6 h after the addition of 2-ME to the HL-60 cells, as evidenced by a shift of the cell population from the lower-left quadrant upward along the *y*-axis (superoxide fluorescent intensity on a log scale). Quantitative analysis revealed that the intracellular O_2^- increased from 12 to 20 arbitrary units (mean fluorescent intensity, Fig. 1F), which represented a 70% increase in cellular O_2^- content. Kolmogorov-Smirnov analysis revealed that this increase was statistically significant ($D = 0.67$). However, no detectable drug-induced apoptosis was seen at this time (6 h), as evidenced by the lack of change in annexin-V-FITC labeling (Fig. 1D, *x*-axis). In the continuous presence of 2-ME, O_2^- levels remained elevated up to 24 h, whereas a significant number of annexin V-positive cells did not appear until 16 h (Fig. 1G), and cell death was further increased at 24 h (Fig. 1E). The temporal relationship between O_2^- build-up and cell death during 2-ME incubation clearly indicates that O_2^- accumulation occurred well before the onset of apoptosis (Fig. 1G). This is consistent with our previous observation that 2-ME induced apoptosis through a free-radical-mediated mechanism.

To investigate the biochemical basis for the selective killing of leukemia cells by 2-ME, the steady state of cellular O_2^- levels were first examined in primary

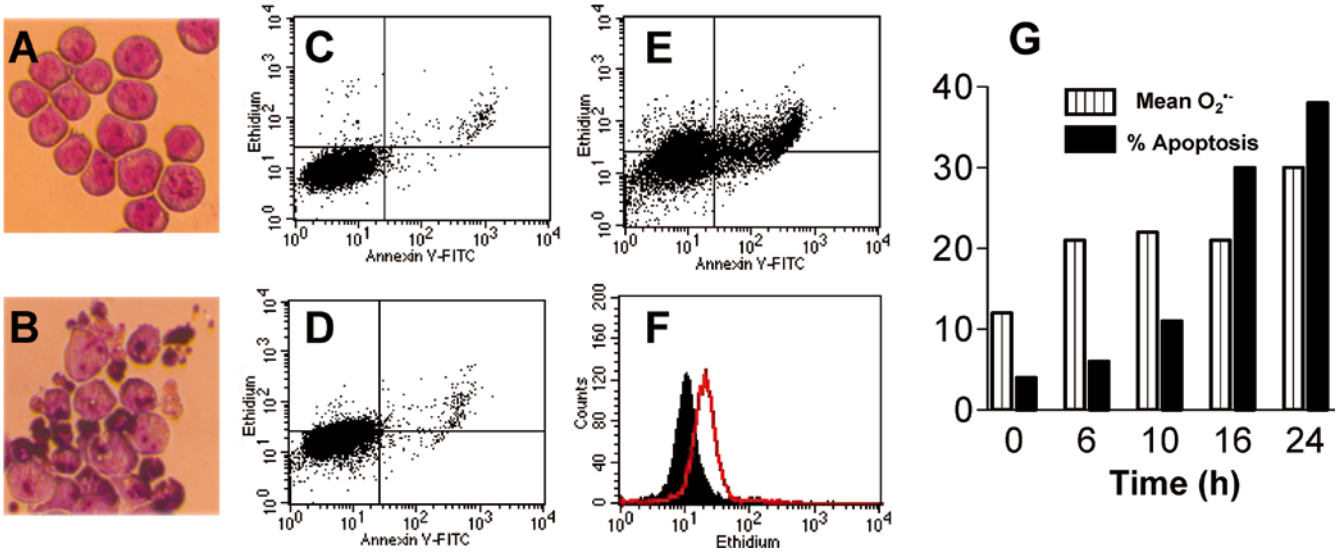
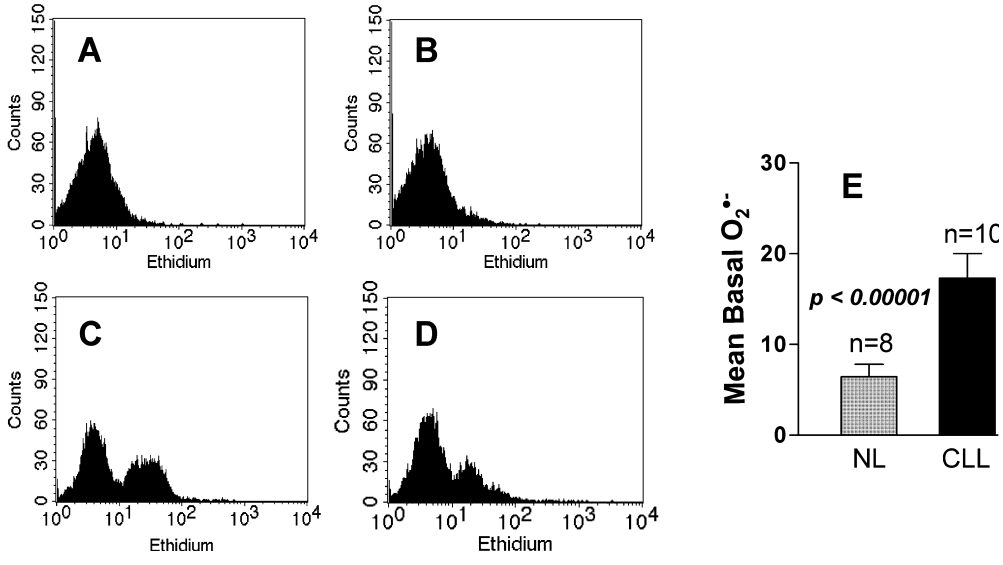


Fig. 1A–G Induction of O_2^- accumulation and apoptosis by 2-ME in HL-60 cells. HL-60 leukemia cells were treated without (**A**) or with 2-ME for 24 h (**B**), stained with Wright's Giemsa stain solution, and photographed. **C–G** Temporal relationship between cellular O_2^- accumulation and apoptosis in HL-60 cells incubated with 2 μ*M* 2-ME measured by double-staining with hydroethidine and annexin-V-FITC conjugate: **C** control; **D** 6 h; **E** 24 h; **F** histogram overlay for O_2^- accumulation in the control cells (*shaded histogram*) and the 2-ME-treated cells (6 h, *unshaded histogram*) from the same experiment; **G** accumulation of O_2^- occurred before cells undergoing apoptosis (*hatched bars* cellular O_2^- content in arbitrary units, *solid bars* percent of cells undergoing apoptosis)

leukemia cells isolated from patients with CLL in comparison with that of peripheral normal lymphocytes from healthy donors. Figure 2 shows the flow cytometric analysis of O_2^- in two representative normal lymphocyte samples (Fig. 2A, B) and two CLL patient samples (Fig. 2C, D). The mean basal O_2^- levels appeared to be much higher in CLL cells than in normal lymphocytes. Figure 2E summarizes the mean and standard deviation of the basal O_2^- levels determined in ten different CLL patient samples and normal lymphocytes from eight healthy donors. The mean basal O_2^- levels in CLL cells were significantly higher than in normal lymphocytes ($P < 0.00001$).

As shown in Fig. 2, primary CLL cells contained two subpopulations with different O_2^- levels, as indicated by the two distinct peaks in the histogram (Fig. 2C, D). We speculated that two possibilities might be responsible for this bimodal distribution: (1) the isolated CLL cells were contaminated with other cell types that have a distinct O_2^- content, or (2) the CLL cells actually consist of two subpopulations of leukemia cells with different cellular O_2^- content. To distinguish these two possibilities, we

Fig. 2A–E Comparison of O_2^- levels in primary leukemia cells and normal lymphocytes. Basal intracellular O_2^- levels were determined by flow cytometry using hydroethidine staining. Two representative normal lymphocyte samples (**A, B**) and two representative CLL leukemia samples (**C, D**) are shown. **E** O_2^- content (means + SD) of a total of ten CLL samples from different patients and eight normal lymphocyte samples



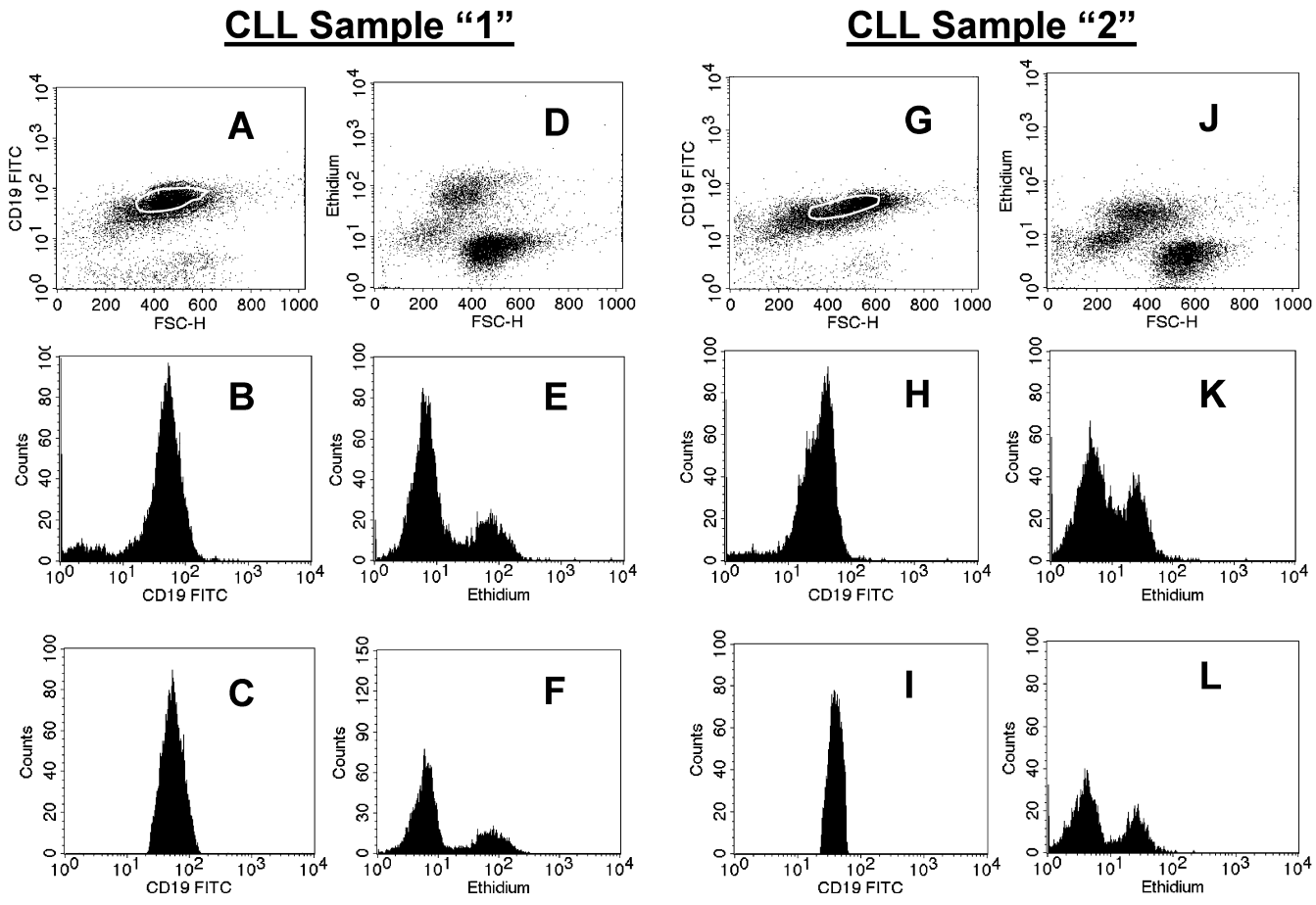


Fig. 3A–L Heterogeneous cellular O_2^- content in primary CLL cells. CLL cells from two different patients were double-stained in vitro for a B-cell marker (using anti-CD19-FITC) and for cellular O_2^- (using hydroethidine). **A, G** CD19 dot plots; **B, H** corresponding ungated histograms. **D, J** HEt dot plots; **E, K** corresponding ungated histograms for cellular O_2^-. Cells were gated for a population uniform in CD19 expression (**A, G** *white circles*), and the resulting CD19 histograms (**C, I**) and cellular O_2^- histograms (**F, L**) for the gated, homogeneous CD19 population are shown

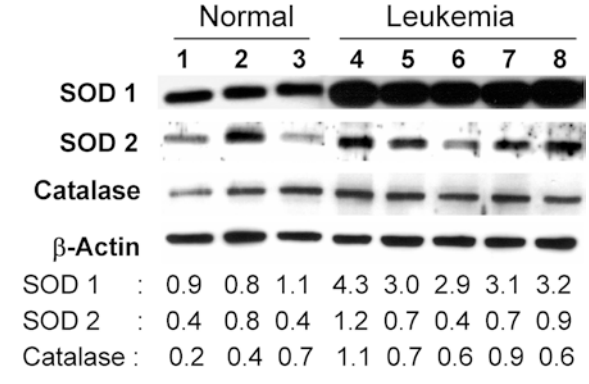


Fig. 4 Increases in SOD1, SOD2, and catalase expression in primary CLL cells. Protein lysates of CLL cells and normal lymphocytes were analyzed for expression of SOD1, SOD2, and catalase proteins by Western blotting, using the respective antibodies. β-Actin was also blotted as a protein loading control: *lanes 1–3* normal lymphocytes from three different healthy donors, *lanes 4–8* CLL cells isolated from five different patients. Values below each lane are the ratio of each protein divided by the β-actin band intensity

double-stained the CLL samples with hydroethidine for O_2^- (red fluorescence) and with a FITC-labeled antibody specific for CD19 (a surface marker for B lymphocytes) for a gating analysis. Figure 3 shows the results of such analysis in two representative CLL samples. The ungated CLL cell population again showed a bimodal distribution of cellular O_2^- content (Fig. 3D, E, J, K). Although gating of CD19 resulted in a relatively homogeneous $CD19^+$ subpopulation (Fig. 3C, I), this gated subpopulation still contained two distinct O_2^- peaks (Fig. 3F, L) similar to those observed in the ungated cell population. Thus, it appears that there is heterogeneity in O_2^- content within the $CD19^+$ B-CLL cells. This is in contrast with primary normal lymphocytes, which appeared as a single peak in their cellular O_2^- measurement.

Because SOD is the key enzyme involved in the metabolic elimination of O_2^-, Western blot analyses were performed to compare the SOD expression levels in CLL cells versus normal lymphocytes. As illustrated in Fig. 4, primary leukemia cells isolated from five CLL patients uniformly showed a significantly higher SOD1 expression than normal lymphocytes isolated from three healthy donors. β-Actin was used as a loading control and confirmed that a similar quantity of protein was loaded in each lane. The numbers shown below each

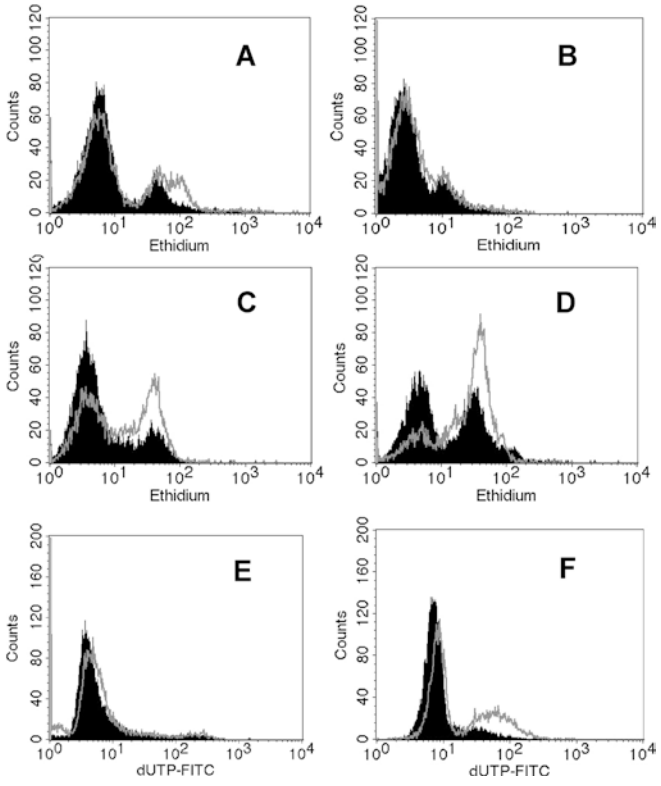


Fig. 5A–F Selective induction of O_2^- accumulation and cell death by 2-ME in CLL cells. CLL cells and normal lymphocytes were incubated for 24 h with 30 μ*M* 2-ME, and O_2^- levels were measured by flow cytometry analysis using hydroethidine. Data for two normal lymphocyte samples (**A, B**) and two CLL samples (**C, D**) are presented (*shaded histograms* control cells, *unshaded histograms* cells treated with 2-ME). **E, F** TUNEL staining is also shown for one normal lymphocyte sample (**E**) and one CLL sample (**F**) at 24 h (*shaded histograms* control cells, *unshaded histograms* cells treated with 2-ME)

sample indicate the ratio of the protein band intensity to the *β*-actin band intensity. Interestingly, the expression of SOD2 was heterogeneous among CLL patients and normal individuals, although the CLL cells seemed to generally possess higher levels of SOD2 protein than normal lymphocytes. The increase of SOD in CLL cells seems to reflect the intrinsic oxidative stress in these cells. This is consistent with the results shown in Fig. 2, and suggests that CLL cells may heavily rely on SOD to eliminate the high levels of toxic O_2^-. The expression of another antioxidant enzyme, catalase, in CLL cells and normal lymphocytes was also investigated by Western blot analysis. As shown in Fig. 4, the catalase expression was generally higher in CLL cells compared with normal lymphocytes, although there appeared to be some heterogeneous expression among different individuals. The increase in SOD and catalase expression in CLL cells reflects the cellular response to intrinsic oxidative stress in the malignant cells.

When CLL cells were incubated with 2-ME, a significant accumulation of O_2^- was detected in the leukemia cells (Fig. 5C, D). This was in contrast to the normal lymphocytes, which showed only slight increases of O_2^- in response to the same concentrations of 2-ME (Fig. 5A, B). Thus, it appears that the active generation of O_2^- in CLL cells renders them much more vulnerable to SOD inhibition by 2-ME. The increase in intracellular O_2^- in CLL cells subsequently caused apoptosis, as evidenced by DNA strand breaks revealed by the TUNEL assay (Fig. 5F). Conversely, there was little evidence of cell death in normal lymphocytes treated with the same concentration of 2-ME (Fig. 5E).

To further investigate the role of O_2^- in 2-ME-induced apoptosis in CLL cells, we used free radical scavengers in combination with 2-ME and evaluated the effect of these antioxidants on cell sensitivity to 2-ME. As shown in Fig. 6, when CLL cells were treated with 2-ME alone, there was a substantial accumulation of intracellular O_2^- as revealed by a shift of the ethidium fluorescent intensity to the right (red histogram). Coadministration of the free radical scavengers tiron [27] or NAC with 2-ME effectively diminished this free radical accumulation and shifted the fluorescence back to almost the control level (green and blue histogram,

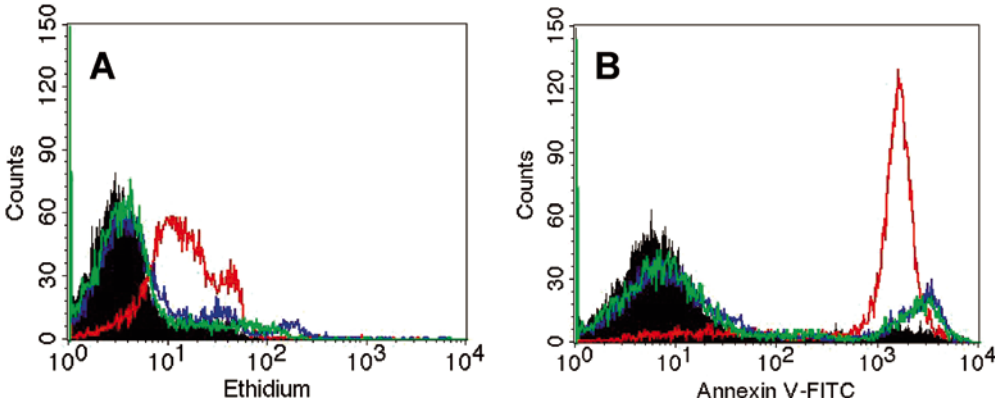


Fig. 6A, B Abrogation of 2-ME-induced O_2^- accumulation and apoptosis by ROS scavengers. CLL cells were treated with 30 μ*M* 2-ME in the presence and absence of Tiron (5 m*M*) or NAC (5 m*M*). A portion of the cells was analyzed for O_2^- levels using hydroethidine staining and flow cytometry analysis at 24 h (**A**). The second portion of cells was analyzed for apoptosis using annexin-V-FITC labeling (**B**). *Black histograms* control, *red curve* 2-ME alone, *green curve* 2-ME + Tiron, *blue curve* 2-ME + NAC

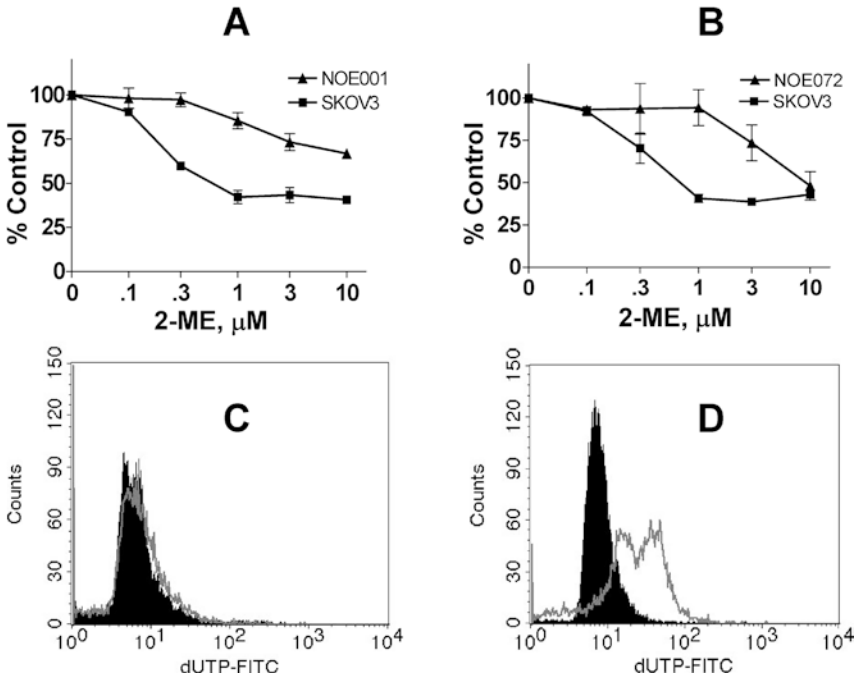


Fig. 7A–D Effect of 2-ME on cell survival in human ovarian cancer cells (SKOV3) and NOE cells. The sensitivity of SKOV3 cells to various concentrations of 2-ME was compared with that of NOE (NOE001 and NOE072 denote cultured NOE cells from two different individuals) by the MTT assay (**A, B**) as described in Materials and methods. The TUNEL assay was also used to compare the apoptotic response to 2-ME (1 μ*M*, 24 h) in NOE cells (**C**) and SKOV3 cells (**D**). *Shaded histograms* control, *unshaded histograms* drug-treated samples

respectively). CLL cells treated with 2-ME alone underwent massive apoptosis at 48 h, as indicated by the appearance of the annexin-V-FITC positive peak (Fig. 6B, red histogram). The presence of either tiron or NAC significantly suppressed 2-ME-induced cell death, as evidenced by a drastic decrease in annexin-V-positive labeling (Fig. 6B, green and red peaks, respectively). Taken together, these results indicate that O_2^- accumulation is a necessary and critical event in 2-ME-induced apoptosis in CLL cells.

An important issue in this study was to determine if the selective anticancer activity of 2-ME is specific for leukemia cells or if this selectivity also holds true in other types of cancer. We therefore tested the effect of 2-ME on ovarian cancer cells (SKOV3) in comparison with two batches of NOE cells (NOE001 and NOE072). Figure 7A, B illustrates the differential effect of 2-ME on SKOV3 cells versus NOE cells. MTT assays revealed that the ovarian cancer cells were sensitive to growth inhibition by 2-ME, with an estimated IC_{50} value of approximately 0.7 μ*M* (72-h incubation), whereas the NOE cells were relatively resistant to 2-ME, with an estimated IC_{50} value of greater than 10 μ*M*. The preferential cytotoxic effect of 2-ME in the malignant cells was further confirmed using the TUNEL assay as an indicator of apoptosis. SKOV3 cells treated with 2-ME for 24 h exhibited a positive TUNEL signal in a substantial portion of the cell population (Fig. 7D). In contrast, the same drug incubation did not induce a significant increase of TUNEL signal in the NOE cells (Fig. 7C), further confirming the selective anticancer activity of 2-ME in this solid tumor model.

The flow cytometric analysis for cellular O_2^- described above was used to examine the effect of 2-ME on O_2^- accumulation in SKOV3 and NOE cells. As shown in Fig. 8, SKOV3 ovarian cancer cells displayed a significant accumulation in O_2^- following incubation with 2-ME (Fig. 8B, log scale), whereas only a slight but detectable increase in O_2^- was observed in the NOE cells (Fig. 8A). This pattern of selective increase in cellular O_2^- in the ovarian cancer cells was similar to that observed in the experiments with CLL cells and normal lymphocytes. Western blot analysis showed that SKOV3 cells contained much higher levels of SOD1 and SOD2 (Fig. 8C, lane 6) than did NOE cells (lanes 1 and 2). We also determined SOD1 and SOD2 levels in several other human ovarian cancer lines (lanes 3–5). This revealed that the malignant ovarian cells generally expressed higher levels of SOD1. As in leukemia cells, the levels of SOD2 expression varied among samples, with a trend of higher expression in the cancer cells.

To directly compare the expression of SOD and catalase in primary ovarian tumor samples and NOE cells, protein extracts from primary human ovarian cancer tissue and primary NOE cells were analyzed side by side on a Western blot. As shown in Fig. 9, there was a clear increase in the levels of both SOD1 and SOD2 protein expression in the primary tumor samples compared with the NOE cells. This observation further confirms the findings in ovarian cancer cell lines shown in Fig. 8. Additionally, the primary tumor samples also exhibit a higher expression level of catalase. Taken together, these findings further support the conclusions that ovarian cancer cells are under increased oxidative stress, rely upon the high expression of SOD and other antioxidant enzymes for survival, and thus are susceptible to SOD inhibition by 2-ME.

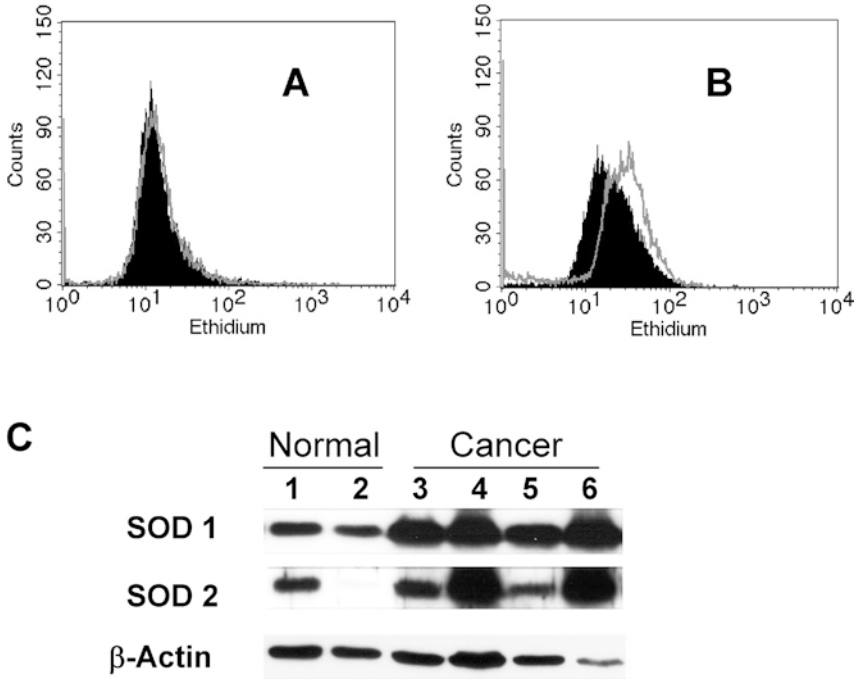


Fig. 8A–C Effect of 2-ME on O_2^- accumulation in NOE cells (**A**) and SKOV3 cells (**B**). Cells were incubated with 1 μ*M* 2-ME and O_2^- levels were measured at 16 h as described in Materials and Methods (*shaded histograms* control, *unshaded histograms* 2-ME-treated samples). **C** The cellular SOD protein levels for NOE cells, SKOV3 cells, and several other ovarian cancer lines (OVCAR-3, DOV3, and SNU251) were also measured by Western blot analysis: *lanes 1 and 2* two different batches of NOE cells, *lane 3* OVCAR-3 cells, *lane 4* DOV3 cells, *lane 5* SNU251 cells, *lane 6* SKOV3 cells

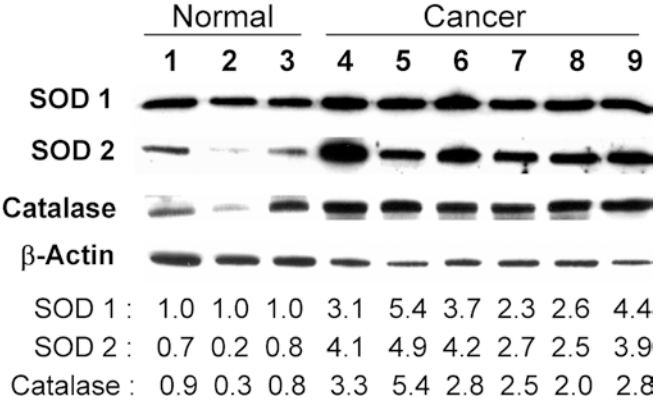


Fig. 9 Increases in expression of SOD1, SOD2, and catalase in primary ovarian cancer tissues. Protein lysates of primary ovarian cancer tissues and primary NOE cells were analyzed for expression of SOD1, SOD2, and catalase proteins by Western blotting using the respective antibodies. β-Actin was used as a protein loading control: *lanes 1–3* three different batches of NOE cells, *lanes 4–9* primary ovarian tumor samples from six different patients. Values below each lane are the ratio of each protein band intensity divided by the β-actin band intensity as determined by densitometry

Discussion

O_2^- is the major free radical species produced during normal aerobic metabolism and serves as a precursor for the formation of other ROS [16]. Mild increases in O_2^- levels have been shown to play an important role in mediating cellular proliferation [31, 34, 35, 38]. However, an excessive accumulation of cellular O_2^- is cytotoxic and has been well-characterized as an inducer of apoptosis in a variety of cell types [3, 11, 39, 41]. These observations suggest an apparent dual role for ROS in affecting cell growth and cell death. Recently, it has been suggested that increases in ROS following antineoplastic therapies induce the expression of antioxidants such as SOD [26]. The overproduction of ROS in turn exhausts the capacity of SOD and other adaptive antioxidant defenses. A "threshold concept" for cancer therapy has been proposed to explain the dual effects of oxygen radicals [26]. In cancer cells, if ROS levels reach the "threshold level" that overwhelms the antioxidant capacity, irreversible damage occurs and apoptosis is initiated. The present study demonstrates that inhibition of SOD by 2-ME provides an effective pharmacological measure to induce severe oxidative damage to malignant cells.

Growing evidence suggests that cancer cells produce high levels of ROS and are constantly under oxidative stress [24, 44, 45, 46]. For instance, elevated rates of H_2O_2 generation have been detected in seven human cancer cell lines (up to 0.5 nmol/10^4 cells per hour) [44]. In separate studies, quantitation of O_2^- in primary blood samples from 30 patients with different types of leukemia revealed that the generation of O_2^- was significantly increased compared to normal controls [9]. The increase in O_2^- generation appears to be a general phenomenon associated with various leukemias, since there was no significant difference among different types of leukemia [9]. Furthermore, elevated oxidative modifications in DNA, proteins, and lipids have been detected in various primary cancer tissues including renal cell carcinoma, mammary invasive ductal carcinoma, and colorectal adenocarcinomas [5, 26, 42, 45, 46, 48], further suggesting that cancer cells are inherently under oxidative stress.

The mechanism responsible for increased ROS production in cancer cells is not clear at the present time.

Active metabolism and the dysregulation of cell growth likely contribute to the overproduction of ROS in malignant cells. Another possibility is an alteration in the mitochondrial electron transport chain in cancer cells. It is known that cancer cells have increased glycolytic activity (Warburg effect), and this possibly reflects a less-efficient production of ATP in mitochondria (respiration injury), thus forcing the cancer cells to increase glycolysis to meet the ATP need [42, 48]. A lower mitochondrial respiratory efficiency may result in more free radical production due to a "leakage" of electrons from the respiratory chain. When an electron is captured by molecular oxygen, the superoxide radical is formed. Thus, the inherent oxidative stress in cancer cells is likely due to increased metabolism and a possible dysfunction of the mitochondria. The metabolic differences between cancer and normal cells provide a biochemical basis for therapeutic strategies that exploit these characteristics by preferentially increasing ROS in cancer cells to a toxic level.

It is of interest to note that when normal lymphocytes or NOE cells were incubated with 2-ME, only a slight increase of cellular O_2^- was observed. Based on the mechanism of action of 2-ME, one would expect an increase in free radical accumulation in the normal cells as well with a prolonged inhibition of SOD by 2-ME. However, this was not observed in normal lymphocytes or NOE cells. The most likely explanation is that when normal cells are under oxidative stress, they are able to downregulate their mitochondrial respiratory activity so that fewer O_2^- radicals are generated in the cells. In fact, in a separate set of preliminary experiments, we have observed a decrease in oxygen consumption (about 30%), an indication of mitochondrial respiratory activity, in normal lymphocytes several hours after incubation with 2-ME, with no apoptosis detected up to 48 h. This downregulation of mitochondrial respiration was absent in leukemia cells, since the malignant cells continued to consume the high level of oxygen when they were incubated with 2-ME under the same conditions. Thus, the ability of normal cells to downregulate their oxidative activity when exposed to ROS stress may be a mechanism to protect the cells from further production of ROS and thus oxidative damage. The molecular basis for the downregulation of mitochondrial respiration in response to oxidative stress in normal cells is not clear at the present time, and is an important subject for future investigation.

The bimodal distribution of cellular O_2^- in primary CLL cells is an interesting observation. The double peaks seen in the CLL samples (Figs. 2 and 3) suggest that in the CLL cell population there appear to be two distinct subpopulations that generate different levels of superoxide. Gating by a CD19 marker indicated that both subpopulations were within the B cell lineage (Fig. 3). We consistently observed this phenomenon in more than 50% of the CLL samples tested ($n = 46$). The reason for this phenomenon is unclear. It seems to be unique to CLL cells, since this was not seen in primary lymphocytes, cultured leukemia cells (HL-60, ML-1), or ovarian cancer cells.

Importantly, we consistently detected a significant increase in SOD protein expression in cancer cells compared to their normal counterparts. This increase is likely a consequence of a cellular adaptation to intrinsic oxidative stress. The upregulation of SOD as a mechanism to counteract oxidative stress has been observed in several experimental systems [5, 8, 51]. The increase in SOD has been observed in many different types of cancer cells including squamous cell cancers of the larynx and oral cavity, gastric cancer, central nervous system cancers, and lung cancer [6, 7, 29, 37, 50]. Increased SOD has also been detected in mesothelioma cells using various techniques including Northern blot, Western blot, and enzyme activity assays [21, 22]. In addition, Kokoglu et al. have shown that in leukemia, SOD levels are significantly higher, using the epinephrine method for measuring SOD activity [23]. Devi et al. have also found that CuZnSOD activity is generally increased in several types of human leukemia cells [9]. However, SOD expression may vary among different cancer cells/tissues, and in some cases may even decrease in certain cancer cells [10, 30, 36, 43].

It should be noted that relatively high levels of intracellular free radicals were seen in the cancer cells even though high SOD expression levels were present in these cells. Without this increase in SOD expression, the cellular O_2^- in the cancer cells would theoretically have been much higher than the steady-state levels detected. Thus, the high level of intrinsic oxidative stress in cancer cells suggests that the malignant cells may heavily depend on SOD for the elimination of O_2^- to keep the ROS levels within a tolerable range. Because of the increased basal level of O_2^- and the increased SOD expression, it would be expected that the production of intracellular H_2O_2 would likewise increase based on the role of SOD in converting O_2^- to H_2O_2. This would force the cell to upregulate the expression of H_2O_2-eliminating enzymes such as catalase and glutathione peroxidase. Indeed, we observed a significant increase in catalase expression as well as SOD1 and SOD2 in primary CLL cells and primary ovarian cancer tissues from patients. Thus, it is likely that the oxidative stress in cancer cells occurs in vivo.

Our study demonstrated that it is possible to take advantage of the biological differences between cancer cells and normal cells to selectively kill the malignant cells. The disparities in ROS generation and metabolism in cancer cells versus normal cells provides a biochemical basis to develop new therapeutic strategies to preferentially increase ROS to a toxic level in cancer cells. Therapeutic manipulation of ROS levels may involve inhibition of ROS metabolic enzymes such as SOD, or using agents that directly or indirectly increase ROS production. Certain anticancer agents including doxorubicin, bleomycin, and arsenic trioxide kill cancer cells via mechanisms involving ROS generation. These agents

are currently used in the clinical treatment of cancer, and their combinations with 2-ME, a new anticancer agent currently in clinical trials, may be a logical strategy to enhance therapeutic activity. Further evaluation of such a biochemical based therapeutic strategy in preclinical and clinical settings is warranted.

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