Myeloperoxidase Is a Key Regulator of Oxidative Stress–Mediated Apoptosis in Myeloid Leukemic Cells

Tomonori Nakazato,[1] Morihiko Sagawa,[1] Kenji Yamato,[3] Mingji Xian,[1] Takehiro Yamamoto,[2] Makoto Suematsu,[2] Yasuo Ikeda,[1] and Masahiro Kizaki[1]

Abstract

Purpose: We reported previously that reactive oxygen species (ROS) are key mediators of apoptosis induced by a polyphenol, (-)-epigallocatechin-3-gallate (EGCG), in myeloid leukemic cells. This study aimed to further examine the mechanism of ROS-mediated apoptosis induced by EGCG and its relationship to the heme enzyme myeloperoxidase (MPO).

Experimental Design: We established stably transfected K562 cells expressing wild-type and mutant *MPO*. Then, sensitivity against EGCG and other ROS-inducing agent was examined and further investigated the detailed molecular mechanism of ROS-inducing apoptosis in MPO-positive leukemic cells.

Results: EGCG rapidly induced apoptosis in MPO-positive leukemia cells. Preincubation of myeloid leukemic cells with the MPO-specific inhibitor, 4-aminobenzoic acid hydrazide, and the heme biosynthesis inhibitor, succinylacetone, resulted in inhibition of the intracellular MPO activity, ROS production, and induction of apoptosis following addition of EGCG. Overexpression of MPO sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG. In contrast, an enzymatically inactive MPO mutant–expressing K562 cell could not respond to EGCG, suggesting that MPO is important for determining the sensitivity to EGCG-induced oxidative stress. Hypochlorous acid scavengers and the hydroxyl radical (•OH) scavenger inhibited EGCG-induced apoptosis in myeloid leukemic cells. The fluorescence intensity of both aminophenyl fluorescein–and hydroxyphenyl fluorescein–loaded myeloid leukemic cells significantly increased on stimulation with EGCG, indicating that EGCG generated highly toxic ROS in myeloid leukemic cells.

Conclusions: These results indicated that highly toxic ROS such as •OH generated via the hydrogen peroxide/MPO/halide system induce apoptosis and that ROS may be the direct mediators of EGCG-induced apoptosis in MPO-positive leukemic cells.

Acute myelogenous leukemia (AML) is a heterogeneous group of malignant disorders of hematopoietic progenitor cells by an accumulation of granulocyte and monocyte precursors in the bone marrow and peripheral blood. Despite scientific advances in our understanding of the epidemiologic, genetic, and biological features of AML, the disease remains fatal in a majority of patients and especially in older individuals. The therapeutic approach to AML is usually chemotherapy, but severe side effects and complications, including infection and bleeding induced by the anticancer drugs, are major problems in the clinical setting. Recently, more specifically targeted agents have been developed for the treatment of AML, including anti-CD33 antibodies and immunoconjugate drugs, inhibitors of multidrug resistance proteins, farnesyl transferase inhibitors, tyrosine kinase inhibitors, histone deacetylase, and proteosome inhibitors (1). However, these candidate agents for targeted therapy have yet to be translated into clinical application. In the future, in addition to doing therapeutic trials of these agents, it will be important to identify other highly specific therapeutic agents based on our evolving understanding of the biology of AML.

Myeloperoxidase (MPO), a microbicidal protein in the primary granules of neutrophils, is the hallmark enzyme of the myeloid lineage. Expression of the MPO gene is specific for myeloid precursors and their leukemic counterparts. The percentage of MPO-positive blast cells has been generally considered to be important for the diagnosis of AML. MPO catalyzes the formation of hypochlorous acid (HOCl), a powerful oxidant derived from chloride ions and hydrogen peroxide (H_2O_2). HOCl then may interact with other small molecules including NH_3 to form monochloramines (NH_2Cl) or with other ROS to yield peroxynitrite ($ONOO^-$), hydroxyl radical (•OH), singlet oxygen ($^1O_2$), and ozone (O_3; ref. 2).

Authors' Affiliations: [1]Division of Hematology, Department of Internal Medicine and [2]Department of Biochemistry, Keio University School of Medicine; and [3]Molecular Cellular Oncology and Microbiology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Received 2/26/07; revised 5/21/07; accepted 5/30/07.

Grant support: Ministry of Education, Culture, Sports, Science and Technology of Japan, Takeda Science Foundation, and Keio University Medical Science Fund of Keio University (M. Kizaki).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

Requests for reprints: Masahiro Kizaki, Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan. Phone: 81-3-5363-3785; Fax: 81-3-3353-3515; E-mail: makizaki@sc.itc.keio.ac.jp.

©2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-07-0481

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

There are many species of ROS, but they tend to be considered collectively as "oxidative stress" when their effects in living cells are discussed. However, each species of ROS is likely to have a specific role in living cells. Further, it has been reported that the percentage of MPO-positive blast cells has an effect on the prognostic significance for clinical outcomes of patients with AML (3 – 6). The Japan Adult Leukemia Study Group study showed that patients with >50% MPO-positive blast cells have a significantly better outcome (7). However, the biological significance of the relationship between MPO and prognosis of AML is totally unknown.

We have reported previously that the green tea polyphenol (-)-epigallocatechin-3-gallate (EGCG) rapidly induces apoptosis of AML cells via modulation of reactive oxygen species (ROS) production *in vitro* and *in vivo* (8). In this study, we further investigated the molecular mechanisms of oxidative stress – mediated apoptosis by EGCG and other ROS-producing agents. We found that highly toxic ROS (hROS), such as hydroxyl radical generated via the H_2O_2/MPO/halide system induce apoptosis and that such ROS may directly mediate EGCG-induced apoptosis in myeloid leukemic cells.

Materials and Methods

Cells and cell culture. The human myeloid leukemic cell lines HL-60, U937, Kasumi-1, KG-1, K562, and THP-1 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). The retinoic acid – resistant acute promyelocytic leukemia cell line UF-1 was established in our laboratory from a relapsed patient with acute promyelocytic leukemia who received treatment with all-*trans*-retinoic acid (9). The retinoic acid – sensitive acute promyelocytic leukemia cell line NB4 was a gift of Dr. M. Lanotte (Hôpital St. Louis, Paris, France; ref. 10). Bone marrow or peripheral blood samples from patients with AML were obtained after appropriate Human Protection Committee validation at the Keio University School of Medicine (Tokyo, Japan) and with written informed consent. Mononuclear cells were separated by Lymphoprep (Nycomed Pharma AS). Cells were maintained in RPMI 1640 (Life Technologies) with 10% FCS (Hyclone Laboratories), 100 units/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO_2. The morphology was evaluated by cytospin slide preparations with Giemsa staining and the viability was assessed by trypan blue dye exclusion.

Reagents. Catechin derivative EGCG (Fig. 1A), taurine, methionine, and thiourea were purchased from WAKO Chemical Co. Catalase, manganese superoxide dismutase (MnSOD), 4-aminobenzoic acid hydrazide (ABAH), ebselen, *N*-nitro-L-arginine methyl ester hydrochloride, desferrioxamine, apocynin, succinyl acetone (ScAc), H_2O_2, and arsenic trioxide (As_2O_3) were obtained from Sigma Chemical Co.

Clonogenic assay. Colony growth inhibition of EGCG-treated leukemic cells was analyzed as reported previously (11). Briefly, MPO-sensitive and MPO-resistant leukemic cells were exposed to various concentrations (0-100 μmol/L) of EGCG for 24 h and washed with RPMI 1640. The cells (1,400 per plate) were then plated in triplicated wells in a 12-well plate containing 0.6 mL of Methocult medium (Veritas). The plates were incubated at 37°C with 5% CO_2 for 7 days. Colonies (≧30 cells) were counted with an inverted microscope and the percentage colony growth inhibition compared with the untreated control cells was calculated.

Assays for apoptosis. Apoptosis was determined by morphologic changes as well as by staining with an Annexin V-FITC and propidium iodide double-labeling kit purchased from PharMingen.

Measurement of intracellular generation of ROS. To assess the production of ROS, control and EGCG-treated cells were incubated with 10 μmol/L dichlorodihydrofluorescein diacetate (DCFH-DA; Molecular Probes), which was oxidized to the fluorescent compound, dihydrofluorescein, by cellular ROS. Cells (1×10^5) were stained with 10 μmol/L DCFH-DA for 30 min at 37°C and then washed and resuspended in PBS. The oxidative conversion of DCFH-DA to dihydrofluorescein was measured by flow cytometry (Becton Dickinson).

Cell lysate preparation and Western blotting. Cells were collected by centrifugation at 700 × *g* for 10 min and then the pellets were resuspended in lysis buffer [1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride, 40 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl] at 4°C for 15 min. Protein concentrations were determined using a protein assay DC system (Bio-Rad). Cell lysates (15 μg protein per lane) were fractionated in 12.5% SDS-polyacrylamide gels before transfer to the membranes (Immobilon-P membranes, Millipore) using a standard protocol. Antibody binding was detected by using an enhanced chemiluminescence kit for Western blotting detection with hyper-ECL film (Amersham). Blots were stained with Coomassie brilliant blue to confirm that there were equal amounts of protein extract on each lane. The following antibodies were used in this study: anti-MPO (Upstate Biotechnology) and anti-β-actin (Santa Cruz Biotechnology).

MPO staining and measurement of MPO activity. A 3,3′-diaminobenzidine staining kit (Muto Chemical Co.) was used for cytochemical staining of MPO according to the manufacturer's instructions. Briefly, the peroxidase reaction was developed with 0.05% 3,3′-diaminobenzidine in 50 mmol/L Tris-HCl (pH 7.6) with 0.03% H_2O_2 for 1 to 2.5 min. The sections were counterstained with hematoxylin, dehydrated, and mounted. Cytospin slides were prepared and stained with Giemsa. Oxidized 3,3′-diaminobenzidine (a brown, highly insoluble indamine polymer) is visible under light microscopy. Cells were harvested by centrifugation, washed with PBS, and resuspended in

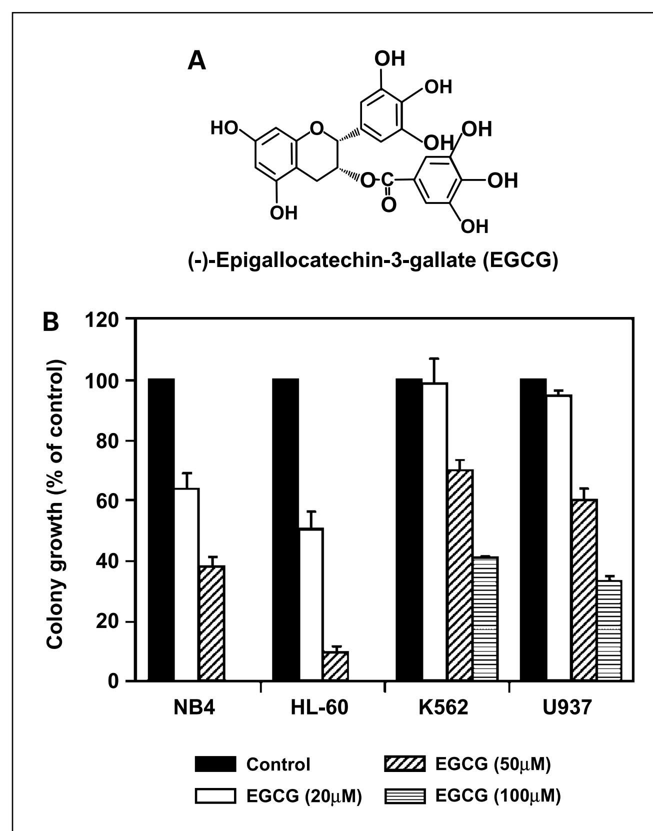

Fig. 1. *A,* structures of EGCG. *B,* EGCG inhibited the proliferation of myeloid leukemic cells. Various myeloid leukemic cells (NB4, HL-60, U937, and K562) were treated with EGCG for various concentrations (0-100 μmol/L) for 24 h. Clonogenic assay was done as described in Materials and Methods. Columns, mean of three different experiments; bars, SD (within 5% of the mean).

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

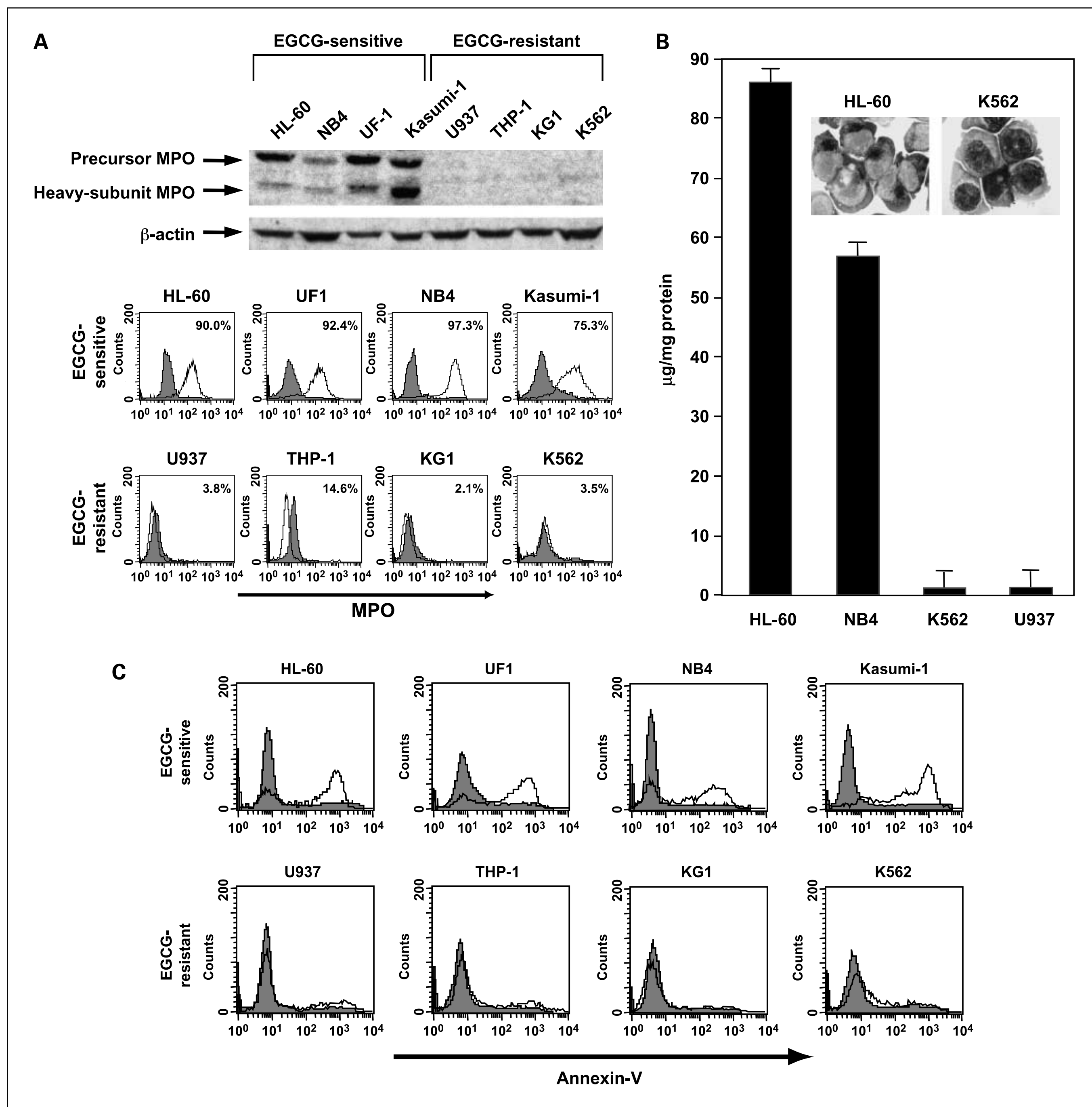

Fig. 2. Expression of MPO protein in various myeloid leukemic cells. *A,* MPO protein expression was measured using Western blotting. Rabbit polyclonal anti-MPO antibody detected precursor MPO at 85 to 90 kDa (immature MPO) and glycosylated heavy subunit at ~59 kDa (mature MPO) in EGCG-sensitive cell lines (*top*). Detection of intracellular MPO protein by fluorescence-activated cell sorting. For fixation and permeabilization of the cells, an Intraprep cell permeabilization kit was used. Intracellular MPO protein was detected by using FITC-conjugated anti-MPO antibody. Percentage of MPO-positive cells was expressed in the top right corner (*bottom*). *B,* MPO activity of various myeloid leukemic cells. MPO activity of the cells was measured by using a MPO ELISA kit according to the manufacturer's instructions. MPO activity was detected by cytochemical staining in HL-60 and K562 cells. Cytospin slides were prepared and cytoplasmic MPO was stained by using a 3,3′-diaminobenzidine staining kit. Then, each slide was stained with Giemsa. Original magnification, ×1,000 (*inset*). *C,* detection of apoptotic cells by Annexin V staining. Cells were cultured with 50 μmol/L EGCG for 24 h, stained with Annexin V-FITC, and analyzed by flow cytometry. Three independent experiments were done and all gave similar results.

50 μL PBS containing Triton X-100 (0.2%) and phenylmethylsulfonyl fluoride (1 mmol/L). Protein concentrations were determined using a Bio-Rad protein assay. MPO activity was measured by using a MPO ELISA kit (Calbiochem) according to the manufacturer's instructions.

Measurement of MPO expression by flow cytometry. Control and EGCG-treated cells were prepared for flow cytometry. Cytoplasmic staining was done to detect the expression of MPO. For fixation and permeabilization of the cells, an Intraprep cell permeabilization kit

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

(Immunotech) was used. Cells (1×10^6) were incubated with FITC-conjugated anti-MPO antibody (Beckman Coulter) for 30 min at 4°C, and isotype-matched mouse IgG served as a control. Flow cytometric analysis was done by using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson).

Transfection of K562 cells with normal and mutant MPO cDNAs. Plasmids with normal and mutated cDNA for *MPO* were transfected into log-phase K562 cells by Amaxa Nucleofector apparatus (Amaxa) according to the manufacturer's procedure. Cells were washed, resuspended at 1×10^6 in 100 μL Cell Line Nucleofector V buffer (Amaxa) with 20 μg pREP10 plasmid only as a control (K562/Cont) and containing normal or mutated cDNA, and then electroporated. After electroporation, cells were immediately cultured with complete medium in 12-well plates at 37°C. By site-directed mutagenesis, we introduced changes affecting His-502 (CAC) to Ala (GCC) in pro-MPO (K562/H502A). His-502 in pro-MPO is located at calcium-binding domain of the enzyme, and it has been reported that this site is important for the enzymatic activity (12). Stable transfectants (K562/Cont, K562/MPO, or K562/H502A) were selected using 0.7 mg/mL neomycin for 48 h after transfection and stable lines expressing the desired protein for up to 6 weeks were obtained 72 h after selection.

Detection of hROS. To selectively detect the hROS, such as •OH and $ONOO^-$, cells were loaded with aminophenyl fluorescein (APF) or hydroxyphenyl fluorescein (HPF; 10 μmol/L) by incubation for 30 min at room temperature according to the previously published method (13). APF and HPF themselves are not highly fluorescent, but when reacted with hROS, APF and HPF exhibit strong dose-dependent fluorescence. Furthermore, using these probes together, hypochlorite (OCl^-) can be selectively detected from •OH and $ONOO^-$. HPF/APF can be used to differentiate hROS from H_2O_2, nitric oxide (NO), and

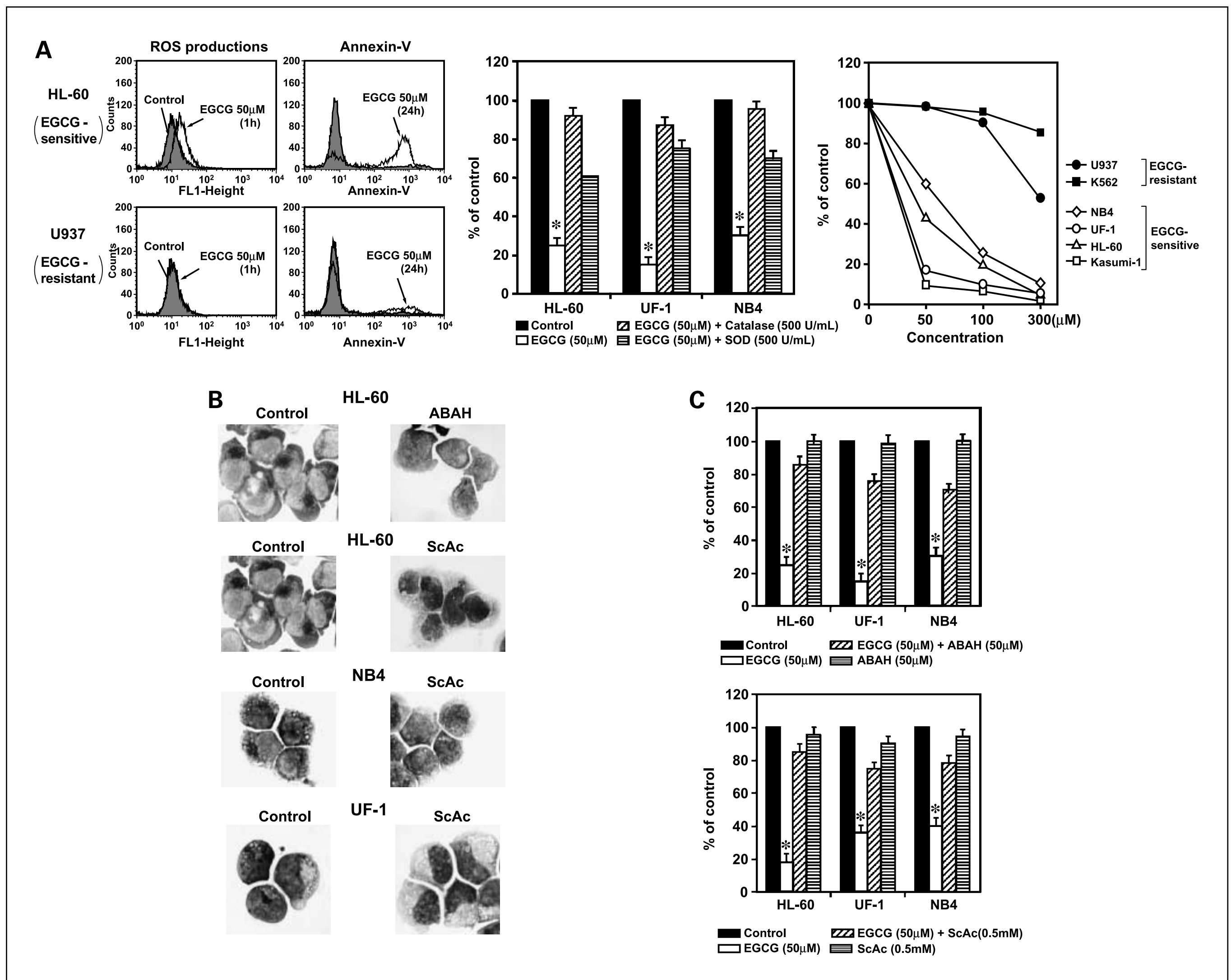

Fig. 3. EGCG induced apoptosis via the production of ROS in myeloid leukemic cells. *A, left,* detection of apoptotic cells by Annexin V staining. Cells were cultured with 50 μmol/L EGCG for 24 h, stained with Annexin V-FITC, and analyzed by flow cytometry. Three independent experiments were done and all gave similar results. To determine the intracellular concentration of ROS, leukemic cells were cultured with DCFH-DA and the fluorescence was measured by flow cytometry. Leukemic cells were treated for 1 h with 50 μmol/L EGCG. *Middle,* the antioxidants, catalase and MnSOD, blocked EGCG-induced cell growth in MPO-positive leukemic cells. Cells were treated with 50 μmol/L EGCG alone or together with 500 units/mL catalase or 500 units/mL MnSOD for 24 h. Cell viability was measured by trypan blue dye exclusion. Columns, mean of at least three different experiments; bars, SD. *, $P < 0.01$. *Right,* direct effect of H_2O_2 on the cellular growth of leukemic cells. Cells were treated with H_2O_2 (0-300 μmol/L) for 24 h. Cell viability was assessed by trypan blue dye exclusion test. Points, mean of three different experiments; bars, SD (within 5% of the mean). *B,* MPO activity was detected by cytochemical staining. Pretreatment of HL-60 cells with 100 μmol/L ABAH and 0.5 mmol/L ScAc for 24 and 3 h, respectively, significantly inhibited MPO enzymatical activity. *C,* ABAH blocked EGCG-induced cell growth in MPO-positive leukemic cells. Cells were treated with 50 μmol/L EGCG alone or together with 100 μmol/L ABAH for 24 h. Cell viability was measured by trypan blue dye exclusion. Columns, mean of at least three different experiments; bars, SD. *, $P < 0.01$ (*top*).

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

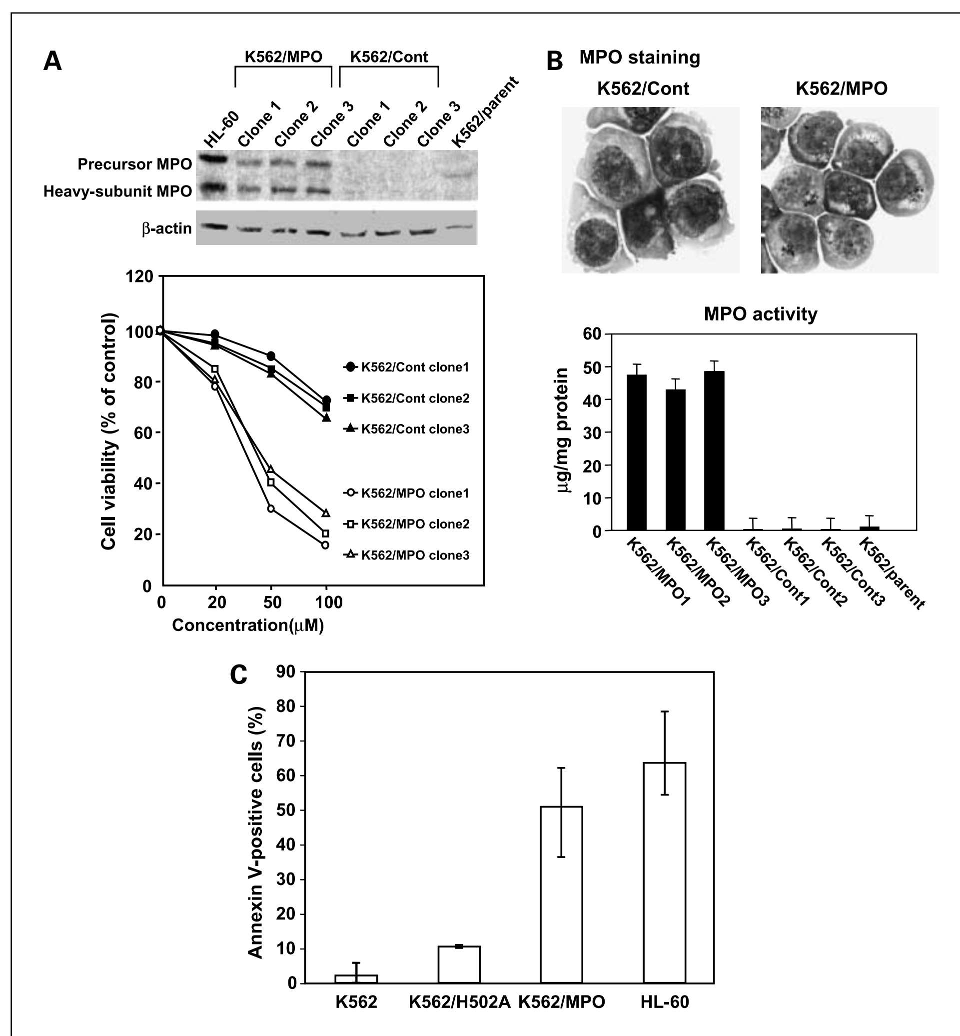

Fig. 4. Overexpression of MPO in K562 cells enhanced MPO activity and ROS production and sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG. *A, top,* MPO protein expression in MPO-transfected K562 cells (K562/MPO cells). Western blotting and fluorescence-activated cell sorting analysis showed MPO protein expression in K562/MPO cells, but MPO protein was not detected in K562/Cont cells or parent K562 cells. *Bottom,* overexpression of MPO in K562 cells sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG. Cells were treated with 50 μmol/L EGCG for 24 h. Cell viability was measured by trypan blue dye exclusion. Points, mean of at least three different experiments; bars, SD. *B,* measurement of MPO activity by cytochemical staining (*top*) and ELISA (*bottom*). K562/MPO cells synthesized enzymatically active MPO protein. In contrast, K562/Cont cells expressed only a low level of MPO activity, which was similar to the level in parent K562 cells. Enzymatically active MPO protein in K562/MPO cells was detected by cytochemical staining. *C,* EGCG-induced apoptosis in K562/H502A cells. The indicated cells were cultured with EGCG (50 μmol/L) for 24 h and then stained with Annexin V-FITC and analyzed by flow cytometry.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

O_2^-. Dye-loaded cells were treated with EGCG, and fluorescence images were acquired twice in each experiment (before and 30 min after the treatment with EGCG) using an LSM510 confocal laser scanning unit. The excitation wavelength was 488 nm, and the emission was filtered using a 505 to 550 nm barrier filter.

Statistical analysis. Results are expressed as mean ± SD. The Student's *t* test was used to compare quantitative data population with normal distribution and equal variance. $P < 0.05$ was considered statistically significant otherwise specified.

Results

EGCG significantly inhibited cellular proliferation of MPO-positive myeloid leukemic cells. We first investigated the effects of EGCG on the cellular proliferation of various leukemic cell lines (HL-60, NB4, UF-1, Kasumi-1, K562, and U937) by a clonogenic assay. EGCG inhibited the cellular proliferation of all leukemic cells in a dose-dependent manner (Fig. 1B; data not shown). Interestingly, EGCG was particularly sensitive to NB4 and HL-60 leukemic cells (Fig. 1B), all of which expressed MPO protein (Fig. 2A). These MPO-positive cell lines also showed strong MPO activity as detected by MPO ELISA assay and cytochemical staining (Fig. 2B, *inset*). Annexinn V–positive apoptotic fractions were detected 24 h after exposure to EGCG in all MPO-positive leukemic cell lines (Fig. 2C). These results showed that EGCG-induced growth inhibition of MPO-positive leukemic cells was mediated by apoptosis. In contrast, EGCG failed to induce apoptosis in MPO-negative leukemic cell lines (Fig. 2C).

Effects of EGCG on primary cells from patients with various types of AML. Among the myeloid leukemic cell lines, MPO-positive leukemic cells were more sensitive to EGCG compared with MPO-negative leukemic cells. We therefore analyzed the association between the sensitivity to EGCG and the expression of MPO in freshly isolated cells from patients with AML and found that the expression of active MPO also contributed to the sensitivity to EGCG-induced apoptosis in the primary myeloid leukemic cells (Supplementary Fig. S1).

ROS production triggers EGCG-induced apoptosis in MPO-positive leukemic cells. We have reported previously that EGCG induces apoptosis of myeloid leukemic cells via modulation of ROS production *in vitro* and *in vivo* (8). To investigate the role of ROS in EGCG-induced apoptosis in myeloid leukemic cells, we analyzed the production of intracellular ROS in both MPO-positive and MPO-negative cells. Treatment with EGCG caused a significant ROS production in the MPO-positive HL-60 cell line. In contrast, intracellular ROS production was less modulated by EGCG in MPO-negative U937 cells (Fig. 3A, *left*). Enhancement of cellular antioxidant status by preincubation

of MPO-positive leukemic cells with a superoxide (O_2^-) and a H_2O_2 scavenger, catalase and MnSOD, respectively, largely suppressed EGCG-induced ROS production and cell growth (Fig. 3A, *middle*). In addition, H_2O_2 itself induced growth inhibition of MPO-positive leukemic cells (Fig. 3A, *right*). These results indicate that EGCG-induced apoptosis is mediated by the production of H_2O_2 and O_2^-.

To investigate the role of MPO in ROS-mediated apoptosis induced by EGCG, we further investigated the effects of the MPO-specific inhibitor ABAH on proliferation of various leukemic cells. ABAH represents a highly selective, mechanism-based inhibitor for MPO, which is first oxidized by the enzyme and then causes its irreversible inactivation (14). Preincubation of MPO-positive myeloid leukemic cells (HL-60, NB4, and UF-1) with ABAH resulted in significant inhibition of intracellular MPO activity, ROS production, and apoptosis following addition of EGCG (Fig. 3B; Fig. 3C, *top*).

A heme biosynthesis inhibitor significantly inhibited MPO activity and blocked EGCG-induced apoptosis. ScAc, a potent inhibitor of 5-aminolevulinic acid dehydratase, has been reported to be an inhibitor of heme biosynthesis in MPO processing (15, 16). It has also been reported that treatment of HL-60 cells with ScAc resulted in a loss of MPO enzymatic activity and disruption of the posttranslational processing of the enzyme (17). Untreated myeloid leukemic cells expressed both precursor (85-90 kDa) and heavy subunit (mature) MPO (60 kDa). In contrast, ScAc-treated cells expressed large amounts of precursor MPO but only very small amounts of mature MPO (Supplementary Fig. S2, *top*). Furthermore, ScAc significantly inhibited intracellular MPO activity, ROS production, and induction of apoptosis following addition of EGCG to MPO-positive myeloid leukemic cells (Fig. 3C, *bottom*; Supplementary Fig. S2, *bottom*). However, both ABAH and ScAc did not affect cellular growth of EGCG-treated MPO-negative parent K562 cells (data not shown). These results strongly suggest that MPO plays a central role in EGCG-induced apoptosis.

Overexpression of normal MPO in K562 cells enhanced MPO activity and ROS production and sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG. To investigate the role of MPO in EGCG-induced apoptosis, we transfected the full-length *MPO* cDNA and empty vector into MPO-negative K562 cells and designated these cells as K562/MPO and K562/Cont, respectively. MPO protein was expressed in the

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

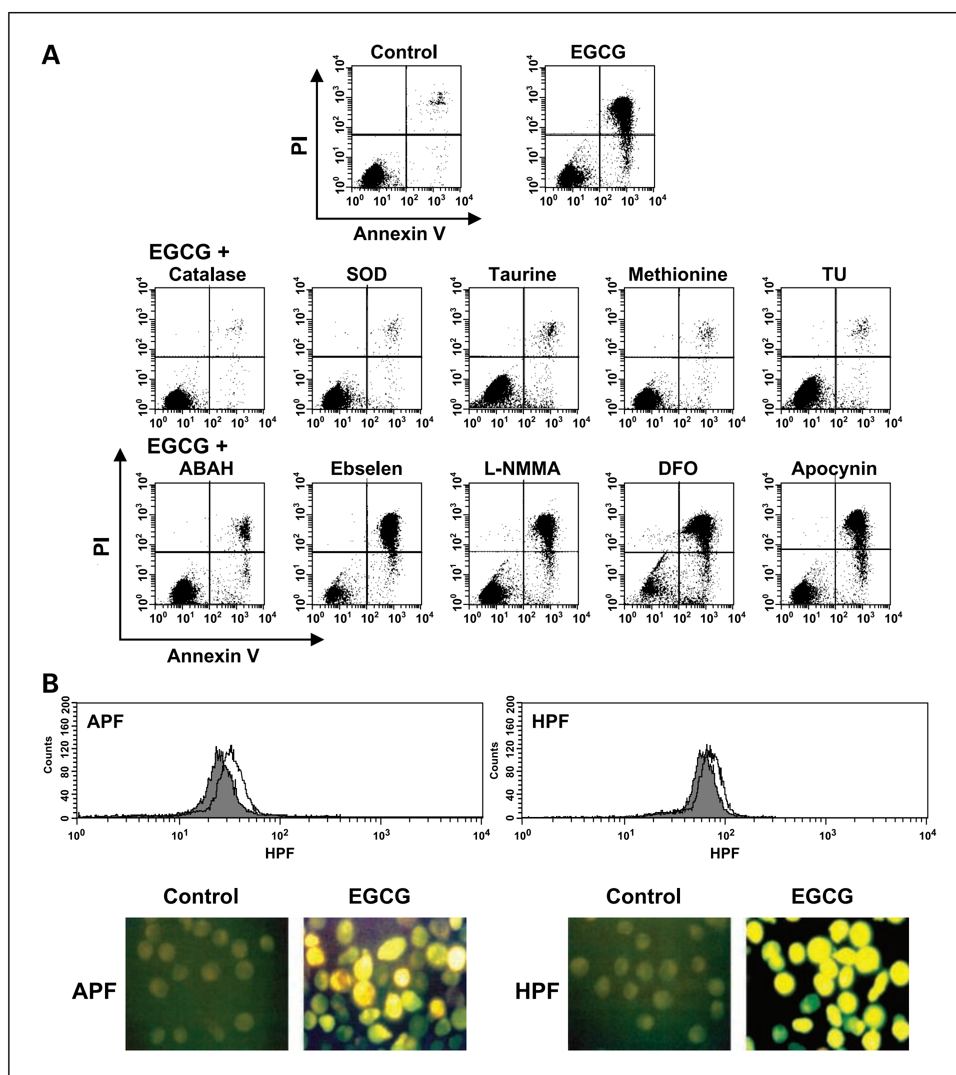

Fig. 5. Various antioxidants blocked EGCG-induced apoptosis. *A,* the addition of HOCl scavengers (methionine and taurine) inhibited EGCG-induced apoptosis in MPO-positive (EGCG sensitive) myeloid leukemic cells. HL-60 cells were treated with 50 μmol/L EGCG alone or together with various antioxidants for 24 h. The antioxidants were as follows: 500 units/mL catalase (H_2O_2 scavenger), 500 units/mL MnSOD (O_2^- scavenger), 25 mmol/L taurine (HOCl scavenger), 25 mmol/L methionine (HOCl scavenger), 25 mmol/L thiourea (*TU*; •OH scavenger), 100 μmol/L ABAH (MPO inhibitor), 4 μmol/L ebselen ($ONOO^-$ scavenger), 1 mmol/L N^G-monomethyl-L-arginine (*L-NMMA*; inducible nitric oxide synthase inhibitor), 100 μmol/L desferrioxamine (*DFO*; iron chelator), and 200 μmol/L apocynin (NADPH inhibitor). Apoptotic cells were detected by double staining with Annexin V and propidium iodide (*PI*). Three independent experiments were done and all gave similar results. *B,* hROS were selectively detected by staining with the novel fluorescence probes APF and HPF. Cells were seeded onto a glass-bottomed dish and then loaded with APF or HPF (10 μmol/L) by incubation for 30 min at room temperature. Dye-loaded cells were treated with EGCG. The fluorescence intensity images were acquired twice in each experiment (before and 30 min after the treatment with EGCG) using an LSM510 confocal laser scanning unit and flow cytometry.

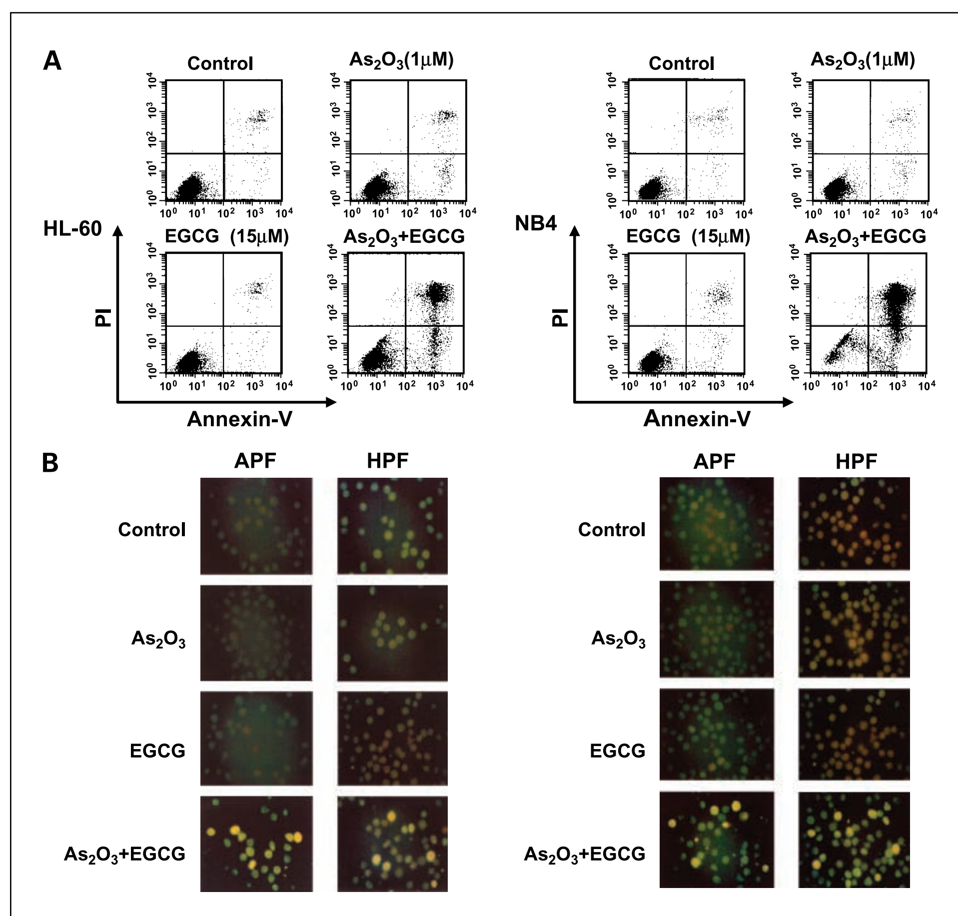

Fig. 6. *A,* EGCG potentiated As_2O_3-mediated apoptosis in myeloid leukemic cells. Cells were cultured in the absence or the presence of As_2O_3 (1 μmol/L), EGCG (15 μmol/L), or (As_2O_3 + EGCG) for 48 h. Apoptotic cells were detected by Annexin V and propidium iodide double staining and analyzed by fluorescence-activated cell sorting. Three independent experiments were done and all gave similar results. *B,* the combination of As_2O_3 and EGCG resulted in a higher level of hROS production than did either As_2O_3 or EGCG alone. To detect hROS, the novel fluorescence probes APF and HPF were used.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

three independent K562/MPO clones but not in the K562/Cont clones or parent K562 cells based on Western blotting and flow cytometry analysis (Fig. 4A, *top*). In addition, K562/MPO cells exhibited significantly more MPO activity, but K562/Cont and parent K562 cells showed very little MPO activity (Fig. 4B). K562/MPO cells were positive for cytochemical staining (Fig. 4B, *top*), suggesting that K562 cells transfected with normal MPO cDNA synthesized the enzymatically active MPO protein. In contrast to K562/Cont cells, K562/MPO cells enhanced MPO activity and ROS production and sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG (Fig. 4A, *bottom*; Supplementary Fig. S3). In addition, catalase, SOD, and MPO inhibitor ABAH inhibited the EGCG-induced suppression of cell growth in K562/MPO cells (Supplementary Fig. S3).

To confirm the role of MPO on EGCG-induced apoptosis in K562 cells, we made stably transfected K562 cells expressing mutant MPO (K562/H502A). It has been reported that no MPO activity was detected in the cells infected with the virus containing MPO/H502A cDNA (12). EGCG induced apoptosis of K562/MPO and MPO-positive HL-60 cells; however, EGCG did not induce apoptosis of MPO-negative parent K562 cells as well as K562/H502A cells expressing enzymatically defective MPO activity (Fig. 4C). These results suggested that MPO expression and its activity determined the sensitivity to EGCG in myeloid leukemic cells.

hROS are key regulators of EGCG-induced apoptosis. MPO catalyzes the formation of HOCl, a powerful oxidant formed from chloride ions and H_2O_2. We next examined the relationship between EGCG-induced apoptosis and the H_2O_2/MPO/halide system in MPO-positive myeloid leukemic cells. H_2O_2 scavenger (catalase), O_2^- scavenger (MnSOD), HOCl scavenger (taurine and methionine), and ABAH significantly blocked EGCG-induced apoptosis in HL-60 cells (Fig. 5A), indicating that H_2O_2, O_2^-, HOCl, and MPO all participate in EGCG-induced apoptosis. Interestingly, the •OH scavengers, thiourea and dimethyl thiourea, also inhibited EGCG-induced apoptosis in HL-60 cells. Thiourea and its derivative, dimethyl thiourea, have been widely introduced as a specific scavenger of •OH (18). These results suggest that hydroxyl radical also may play a key role in EGCG-induced apoptosis. Therefore, we further used the novel fluorescence probes APF and HPF, which can selectively detect hROS, to determine the more detailed mechanism of MPO-mediated apoptosis in myeloid leukemic cells (13). It is noteworthy that the fluorescence intensity of both APF- and HPF-loaded HL-60 cells greatly increased on stimulation with EGCG (Fig. 5B). Our results suggested that EGCG generated hROS (•OH and $ONOO^-$) intracellularly in MPO-positive HL-60 cells. Both NO synthesis inhibitor (N^G-monomethyl-L-arginine) and peroxynitrite scavenger (ebselen) failed to block this apoptosis (Fig. 5A), suggesting that $ONOO^-$ is not a mediator of MPO-mediated apoptosis. Taken together, these observations indicated that hROS such as the hydroxyl radical generated via the H_2O_2/MPO/halide system induce apoptosis and that such ROS may be the direct mediators of EGCG-induced apoptosis in MPO-positive myeloid leukemic cells.

EGCG markedly enhances As_2O_3-mediated apoptosis in myeloid leukemic cells. As_2O_3 has been reported to inhibit the proliferation of human acute promyelocytic leukemia cells by inducing apoptosis via intracellular ROS production (19 – 21). We thus tested the possibility of using a ROS-generating agent, EGCG, to enhance the activity of As_2O_3. The combination of low-dose As_2O_3 (1 μmol/L) and EGCG (15 μmol/L) resulted in a significant increase in apoptosis compared with low-dose As_2O_3 or EGCG treatment alone in HL-60 cells (Fig. 6A). We also found that the combination of low-dose As_2O_3 and EGCG resulted in a higher level of hROS production than did either As_2O_3 or EGCG alone (Fig. 6B). Treatment of HL-60 cells with antioxidants blocked the combination of low-dose As_2O_3 and EGCG-induced hROS production and apoptosis (Fig. 6B). These results suggested that EGCG increased the production of hROS and enhanced As_2O_3-induced cytotoxicity.

Discussion

Redox reactions regulate a broad array of signal transduction pathways. Various ROS, including O_2^-, H_2O_2, •OH, and $ONOO^-$, are now thought of as signaling molecules that are mobilized in response to various stimuli (22 – 24). ROS modulate Ca^{2+} signaling and protein phosphorylation events and thereby function as regulators for various biological processes, such as gene expression, cell growth, differentiation, and chemotaxis (25). ROS are known to mediate apoptosis in many different cell types. Indeed, most anticancer drugs kill their target cells at least in part through the generation of elevated amounts of intracellular ROS (26 – 28). ROS can stimulate proapoptotic signal molecules, such as apoptosis signal regulating kinase 1, c-Jun NH_2-terminal kinase, and p38 mitogen-activated protein kinase, activate the p53 protein pathway, or initiate the mitochondrial apoptotic cascade (29, 30). The various ROS can exert different effects according to their nature and to their intracellular levels, which are determined by both their production rate and the activity of antioxidant enzymes.

We have reported previously that green tea polyphenol, EGCG, rapidly induces apoptosis of AML cells via modulation of ROS production *in vitro* and *in vivo* (8). EGCG has been shown to inhibit cellular proliferation and induce apoptosis of various cancer cells (31 – 35). Although EGCG is generally well known as an antioxidant, it can also behave as a pro-oxidant under certain conditions (36). Recently, it has also been reported that EGCG may induce the production of H_2O_2 in the culture medium (37). In this study, we found that EGCG rapidly induced apoptosis of MPO-positive myeloid leukemic cell lines (HL-60, UF-1, NB4, and Kasumi-1), whereas EGCG failed to induce apoptosis of MPO-negative leukemia cells (U937, THP-1, KG-1, and K562). The MPO-specific inhibitor ABAH and the heme biosynthesis inhibitor ScAc resulted in significant inhibition of intracellular MPO activity and ROS production and apoptosis following addition of EGCG. In addition, overexpression of normal MPO enhanced MPO activity and ROS production and sensitized EGCG-resistant K562 cells to apoptosis induced by EGCG. These results suggest that MPO plays a key role in the oxidative stress – mediated apoptosis induced by EGCG.

MPO is a heme protein that is abundant in the granules of hematopoietic cells, including neutrophils, neutrophil precursors, and macrophages, where its activity in the presence of H_2O_2 is important in the killing of ingested organisms (2). It is contained in the neutrophil primary azurophilic granules, which appear at the promyelocyte stage of myeloid maturation, and this fact explains its abundance in the HL-60, NB4, and UF-1 cell lines. It was recently reported that a drug-induced cytotoxicity was correlated with the amount of MPO in HL-60 cells (38, 39). However, the role of MPO in oxidative stress – induced apoptosis of myeloid leukemic cells is still unclear.

To determine the species responsible for the MPO-mediated apoptosis induced by EGCG, we examined the detailed effects of various antioxidants on the apoptosis in this investigation. As judged from results indicating radical scavengers, O_2^- (MnSOD), H_2O_2 (catalase), HOCl or monochloramines (methionine and taurine), and •OH scavengers (thiourea and dimethyl thiourea) are involved in the mechanisms for EGCG-induced apoptosis. Considering that O_2^- or H_2O_2 are primary species of ROS generated through oxidase and oxygenase systems, the MPO-derived chlorinated oxidants and •OH resultantly generated from them seemed to account for ROS responsible for triggering the apoptosis. Among •OH-generating systems in biological systems, the reaction of NO with O_2^- is unlikely to be involved in the mechanisms because neither an inhibitor of NO synthesis, N^G-monomethyl-L-arginine, nor a peroxynitrite scavenger, ebselen, blocked the apoptosis. Furthermore, Fenton reaction involving free ion is also unlikely to be involved in the mechanisms, thus far as judged from the absence of the effects of deferoxamine, an iron chelator (data not shown). Excluding these two possibilities of •OH-generating systems, we hypothesized that EGCG induces the production of H_2O_2, and then H_2O_2 is converted to HOCl by MPO ($H_2O_2 + Cl^- + H^+ \rightarrow H_2O + HOCl$). HOCl per se is unlikely to account for the ultimate species responsible for apoptosis inducer because MPO-induced apoptosis was blocked by an O_2^- scavenger (SOD) and an •OH scavenger (thiourea) in MPO-positive leukemic cells. If HOCl acts directly to induce the apoptosis of leukemic cells, SOD should have an accelerating rather than an inhibitory effect due to the induction of H_2O_2 by the treatment with SOD and the •OH scavenger. Subsequent interaction of HOCl with O_2^- causes generation of hydroxyl radical ($HOCl + O_2^- \rightarrow \cdot OH + Cl^- + O_2$) that may directly induce apoptosis of MPO-positive leukemic cells. It has reported that •OH participates in various biological processes (40). •OH can damage DNA bases and mediates redox alteration of cell membrane Ca^{2+} channels, resulting in the induction of apoptosis in HL-60 cells (41). However, because of the lack of an effective method for directly detecting •OH, its participation in these events has been established only indirectly by several scavengers. Several fluorescence probes to detect ROS, such as DCFH and dihydrorhodamine 123, have been developed, but both can react with oxidizing species (O_2^-, H_2O_2, NO, ferrous ion, and others). In addition, DCFH is easily auto-oxidized, resulting in a spontaneous increase in fluorescence on exposure to light (42). Therefore, it is not to be appropriate probes for detecting a specific oxidizing species in cells, such as H_2O_2 or NO, but rather they should be considered as detecting a broad range of oxidizing reactions that may be increased during intracellular oxidative stress. To investigate the molecular mechanisms of oxidative stress – mediated apoptosis by EGCG and its relationship to MPO, we used novel fluorescence probes (AFP and HPF) in this study. The

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

fluorescence intensity of both APF- and HPF-loaded HL-60 cells greatly increased on stimulation with EGCG. These results suggested that EGCG generated hROS in MPO-positive HL-60 cells. Taken together, these observations indicated that hROS such as hydroxyl radical generated via the H_2O_2/MPO/halide system induce apoptosis and that such ROS may be direct mediators of EGCG-induced apoptosis in MPO-positive myeloid leukemic cells.

Oxidative damage has been suggested to be a key mechanism by which As_2O_3 causes cell death (19 – 21). As_2O_3-induced apoptosis is associated with the generation of ROS in several experimental models. Antioxidants and free radical scavengers are able to inhibit apoptosis induced by As_2O_3. These observations suggest the possibility of developing new therapeutic strategies using the free radical-mediated mechanism of As_2O_3 to selectively kill cancer cells. Based on the ability of both EGCG and As_2O_3 to cause free radical generation in cells, we hypothesized that the combination of low-dose As_2O_3 (1 μmol/L) and EGCG (15 μmol/L) should enhance the cytotoxic activity. The combination of low-dose As_2O_3 and EGCG resulted in a significant increase in apoptosis compared with As_2O_3 or EGCG treatment alone in MPO-positive myeloid leukemic cell lines. We also found that the combination of As_2O_3 and EGCG resulted in higher levels of hROS than did As_2O_3 or EGCG alone. Furthermore, treatment of myeloid leukemic cells with various antioxidants completely blocked the combination of As_2O_3- and EGCG-induced apoptosis. These findings suggest that low-dose As_2O_3-EGCG combination treatment enhances apoptosis through an increase in the production of hROS in myeloid leukemic cells. In the clinical setting, low-dose EGCG will enhance positive responses and will limit the toxicity of As_2O_3.

Many cytotoxic drugs function selectively to kill cancer cells by the abrogation of proliferative signals, leading to cell death, and numerous reports have shown that ROS are generated following treatment with these drugs (43, 44). For example, anticancer drugs such as the anthracyclines daunorubicin or doxorubicin have been shown to induce apoptosis in various tumor cells via ROS generation (45). It is indeed possible to combine EGCG with ROS-generating agents, such as As_2O_3, anthracycline, bortezomib, or 2-methoxyestradiol (known as a SOD inhibitor; ref. 46) to enhance therapeutic activity and overcome drug resistance.

Because MPO is a major component of myeloid precursor cells, the enzymatic activity of MPO is used to classify acute leukemias (47). Several studies have shown that the percentage of MPO-positive blast cells is a strong independent factor in AML (3 – 6). The Japan Adult Leukemia Study Group reported that overall survival and disease-free survival were significantly better in a high-MPO group than a low-MPO group (7). Our experimental results strongly support the clinical significance of MPO. MPO may be important to determine the sensitivity to oxidative stress – mediated apoptosis of myeloid leukemic cells by several anticancer drugs. According to our findings, it is expected that ROS-generating agents should be selected to overcome drug resistance in high-MPO AML cells. Furthermore, differentiation-inducing agents such as all-*trans*-retinoic acid or several growth factors that trigger the induction of MPO may enhance the production of hROS and the cytotoxicity of ROS-generating agents in low-MPO or even in MPO-negative AML cells.

We have herein proposed a novel strategy by which EGCG and other ROS-generating agents may serve as an enhancer of chemotherapy via MPO-mediated production of hROS in myeloid leukemic cells. Furthermore, the combination of differentiation-inducing agents and ROS-generating agents may enhance the MPO-mediated production of hROS and their cytotoxicity. ''MPO-targeted therapy'' will lead to new insights that should help in overcoming drug resistance in refractory AML.

Acknowledgments

We thank Chika Nakabayashi for her helpful technical assistance.

References

1. Tallman MS, Gilliland DG, Rowe JM. Drug therapy for acute myeloid leukemia. Blood 2005;106: 1154 – 63.
2. Hampton MB, Kettle AJ, Winterbourn CC. Inside the neutrophil phagosome: oxidants, myeloperoxidase, and bacterial killing. Blood 1998;92:3007 – 17.
3. Takubo T, Kubota Y, Oguma S, et al. Classification of acute non-lymphocytic leukemia according to the distribution picture of peroxidase activity and cell size: correlation between the classification and therapeutic response. Blood Cells 1983;9:501 – 14.
4. Matsuo T, Cox C, Bennett JM. Prognostic significance of myeloperoxidase positivity of blast cells in acute myeloblastic leukemia without maturation (FAB:M1): an ECOG study. Hematol Pathol 1989;3: 153 – 8.
5. Suic M, Boban D, Markovic-Glamocak M, Petrovecki M, Marusic M, Labar B. Prognostic significance of cytochemical analysis of leukemic M2 blasts. Med Oncol Tumor Pharmacother 1992;9:41 – 5.
6. Kuriyama K, Tomonaga M, Kobayashi T, et al. Morphological diagnosis of the Japan adult leukemia study group acute myeloid leukemia protocols: central review. Int J Hematol 2001;73:93 – 9.
7. Matsuo T, Kuriyama K, Miyazaki Y, et al. The percentage of myeloperoxidase-positive blast cells is a strong independent prognostic factor in acute myeloid leukemia, even in the patients with normal karyotype. Leukemia 2003;17:1538 – 43.
8. Nakazato T, Ito K, Miyakawa Y, et al. Catechin, a green tea component, rapidly induces apoptosis of myeloid leukemic cells via modulation of reactive oxygen species production *in vitro* and inhibits tumor growth *in vivo*. Haematologica 2005;90:317 – 25.
9. Kizaki M, Matsushita H, Takayama N, et al. Establishment and characterization of a novel acute promyelocytic leukemia cell line (UF-1) with retinoic acid-resistant features. Blood 1996;88:1824 – 33.
10. Lanotte M, Martin-Thouvenin V, Najman S, Balerini P, Valensi F, Berger R. NB4, a maturation inducible cell line with t(15;17) marker isolated from a human acute promyelocytic leukemia (M3). Blood 1991;77: 1080 – 6.
11. Fiskus W, Pranpat M, Balasis M, et al. Cotreatment with vorinostat (suberoylanilide hydroxamic acid) enhances activity of dasatinib (BMS-354825) against imatinib mesylate-sensitive or imatinib mesylate-resistant chronic myelogenous leukemic cells. Clin Cancer Res 2006;12:5869 – 77.
12. Shin K, Hasegawa H, Lonnerdel B. Mutations affecting the calcium-binding site of myeloperoxidase and lactoperoxidase. Biochem Biophys Res Commun 2001;281:1024 – 9.
13. Setsukinai K, Urano Y, Kakinuma K, Majima HJ, Nagano T. Development of novel fluorescence probes that can reliably detect reactive oxygen species and distinguish specific species. J Biol Chem 2002;278: 3170 – 5.
14. Kettle AJ, Gedye CA, Winterbourn CC. Mechanism of inactivation of myeloperoxidase by 4-aminobenzoic acid hydrazide. Biochem J 1997;321:503 – 8.
15. Castaneda VL, Parmley RT, Pinnix IB, Raju SG, Guzman GS, Kinkade JM, Jr. Ultrastructural, immunochemical, and cytochemical study of myeloperoxidase in myeloid leukemia HL-60 cells following treatment with succinylacetone, an inhibitor of heme biosynthesis. Exp Hematol 1992;20:916 – 24.
16. Nauseef WM, McCormick S, Yi H. Roles of heme insertion and the mannose-6-phosphate receptor in processing of the human myeloid lysosomal enzyme, myeloperoxidase. Blood 1992;80:2622 – 33.
17. PinniX IB, Guzman GS, Bonkovsky HL, Zaki SR, Kinkade JM, Jr. The post-translational processing of myeloperoxidase is regulated by the availability of heme. Arch Biochem Biophys 1994;312:447 – 58.
18. Zhu B-Z, Antholine WA, Frei B. Thiourea protects against copper-induced oxidative damage by formation of a redox-inactive thiourea-copper complex. Free Radic Biol Med 2002;32:1333 – 8.
19. Jing Y, Dai J, Chalmens-Redman RME, Tatton WG, Waxman S. Arsenic trioxide selectively induces acute

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024

promyelocytic leukemia cell apoptosis via a hydrogen peroxide-dependent pathway. Blood 1999;94:2102–11.

20. Dai J, Weinberg RS, Waxman S, Jing Y. Malignant cells can be sensitized to undergo growth inhibition and apoptosis by arsenic trioxide through modulation of the glutathione redox system. Blood 1999;93:268–77.

21. Carmosino I, Latagliata R, Avvisati G, et al. Arsenic trioxide in the treatment of advanced acute promyelocytic leukemia. Haematologica 2004;89:615–7.

22. Sauer H, Wartenberg M, Hescheler J. Reactive oxygen species as intracellular messengers during cell growth and differentiation. Cell Physiol Biochem 2001;11:173–86.

23. Finkel T. Oxidant signals and oxidative stress. Curr Opin Cell Biol 2003;15:247–54.

24. Herr I, Debatin KM. Cellular stress response and apoptosis in cancer therapy. Blood 2001;98:2603–14.

25. Bigelow DC, Squier TC. Redox modulation of cellular signaling and metabolism through reversible oxidation of methionine sensors in calcium regulatory proteins. Biochem Biophys Acta 2005;1703:121–34.

26. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. Immunology Today 1994;15:7–10.

27. Jacobson MD. Reactive oxygen species and programmed cell death. Trends Biochem Sci 1996;21:83–6.

28. Clutton S. The importance of oxidative stress in apoptosis. Br Med Bull 1997;53:662–8.

29. Ueda S, Masutani H, Nakamura H, Tanaka T, Ueno M, Yodoi J. Redox control of cell death. Antioxid Redox Signal 2002;4:405–14.

30. Tobiume K, Matsuzawa A, Takahashi T, et al. ASK1 is required for sustained activations of JNK/p38 MAP kinases and apoptosis. EMBO Rep 2001;2:222–8.

31. Lea MA, Xiao Q, Sadhukhan AK, Cottle S, Wang ZY, Yang CS. Inhibitory effects of tea extracts and (-)-epigallocatechin gallate on DNA synthesis and proliferation of hepatoma and erythroleukemia cells. Cancer Lett 1996;68:231–6.

32. Islam S, Islam N, Kermode T, et al. Involvement of caspase-3 in epigallocatechin-3-gallate-mediated apoptosis of human chondrosarcoma cells. Biochem Biophys Res Commun 2000;270:793–7.

33. Ahmad N, Feyes DK, Nieminen AL, Agarwal R, Mukhtar H. Green tea constituent epigallocatechin-3-gallate and induction of apoptosis and cell cycle arrest in human carcinoma cells. J Natl Cancer Inst 1997;89:1881–6.

34. Bertolini F, Fusetti L, Cinieri S, Martinelli G, Pruneri G. Inhibition of angiogenesis and induction of endothelial and tumor cell apoptosis by green tea in animal models of human high-grade non-Hodgkin's lymphoma. Leukemia 2000;14:1477–82.

35. Pisters AMW, Newman RA, Coldman B, et al. Phase I trial of oral green tea extract in adult patients with solid tumors. J Clin Oncol 2001;19:1830–8.

36. Sergediene E, Jönsson K, Szymusiak H, Tyrakowska B, Rietjens IMCM, Cenas N. Prooxidant toxicity of polyphenolic antioxidants to HL-60 cells: description of quantitative structure-activity relationship. FEBS Lett 1999;462:392–6.

37. Nakagawa H, Hasumi K, Woo JT, Nagai K, Wachi M. Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (-)-epigallocatechin gallate. Carcinogenesis 2004;25:1567–74.

38. Bruno JG, Herman TS, Cano VL, Stribling L, Kiel JL. Selective cytotoxicity of 3-amino-L-tyrosine correlates with peroxidase activity. In Vito Cell Biol Anim 1999;35:376–82.

39. Wagner BA, Buettner GR, Oberley LW, Darby CJ, Burns CP. Myeloperoxidase is involved in H_2O_2-induced apoptosis of HL60 human leukemia cells. J Biol Chem 2000;275:22461–9.

40. Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med 1999;26:463–71.

41. Ren JG, Xia HL, Just T, Dai YR. Hydroxyl radical-induced apoptosis in human tumor cells is associated with telomere shortening but not telomerase inhibition and caspase activation. FEBS Lett 2001;19:123–32.

42. Bilski P, Belanger AG, Chignell CF. Photosensitized oxidation of 2′,7′-dichlorofluorescin: singlet oxygen does not contribute to the formation of fluorescent oxidation product 2′,7′-dichlorofluorescein. Free Radic Biol Med 2002;33:938–46.

43. Hileman EO, Liu J, Albitar M, Keating MJ, Huang P. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. Cancer Chemother Pharmacol 2004;53:209–19.

44. Mansat-de Mas V, Bezombes C, Quillet-Mary A, et al. Implication of radical oxygen species in ceramide generation, c-Jun N-terminal kinase activation and apoptosis induced by daunorubicin. Mol Pharmacol 1999;56:867–74.

45. Panaretakis T, Pokrovskaja K, Shoshan MC, Grander D. Activation of Bak, Bax, and BH3-only proteins in the apoptotic response to doxorubicin. J Biol Chem 2002;277:44317–26.

46. Huang P, Feng L, Oldham EA, Keating MJ, Plunkett W. Superoxide dismutase as a target for the selective killing of cancer cells. Nature 2000;407:390–5.

47. Bennett JM, Catovsky D, Daniel MT, et al. Proposals for the classification of acute leukemias. French-American-British (FAB) co-operative group. Br J Haematol 1976;33:451–8.

Downloaded from http://aacrjournals.org/clincancerres/article-pdf/13/18/5436/1972521/5436.pdf by guest on 04 December 2024