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(−)-Epigallocatechin-3-*O*-gallate Induces Nonapoptotic Cell Death in Leukemia Cells Independent of the 67 kDa Laminin Receptor

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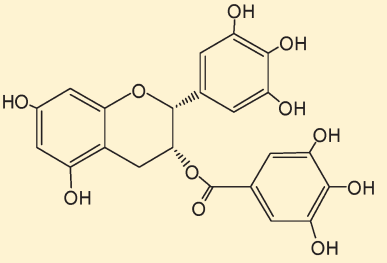
ABSTRACT: The 67 kDa laminin receptor (67 LR) mediates (−)-epigallocatechin-3-*O*-gallate (**1**; EGCG)−67 LR direct action only at physiological concentrations. The relevancy of biological effects of **1** at physiological concentrations to 67 LR was investigated in myeloid and lymphoid leukemia cells using flow cytometric analysis. It was shown that physiological concentrations of **1** suppressed the cell growth of HL60 myeloid leukemia cells and Raji lymphoid leukemic cells independent of 67 LR expression. Moreover, there was no discernible change in the levels of intracellular reactive oxygen species, characteristics of apoptosis such as phosphatidylserine translocation and activated caspase-3. The activity of **1** at physiological concentrations does not depend on direct 67 LR-mediated actions, and this compound induces necrosis-like death of promyelocytic leukemia and non-Hodgkin's lymphoma cells.



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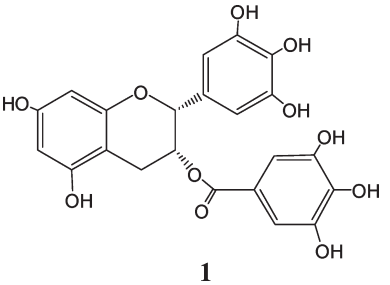
(−)-Epigallocatechin-3-*O*-gallate (**1**; EGCG), the major catechin present in green tea [(*Camellia sinensis* (L.) O. Kuntze (Theaceae)], has attracted much attention due to its potential. Epidemiological studies have indicated that the consumption of green tea may lead to decreased risk of carcinogenesis.[1] As **1** is a biologically active polyphenol presenting antioxidant activities and cytotoxic properties, the chemopreventive and chemotherapeutic effects of **1** in various malignancies have been reported.[2−5]

The cytotoxic effects of **1** include inhibition of angiogenesis, suppression of cell proliferation, apoptosis induction through the modulation of reactive oxygen species (ROS), induction of actin rearrangement, and the blocking of epidermal growth factor receptor (EGFR) autophosphorylation.[3−6] Since the pharmacological behavior of **1** depends on its concentration, with those removed from physiological concentrations (approximately up to 10 μM) the direct action of **1** is not reflected.[7] The 67 kDa laminin receptor (67 LR) has been identified as a direct surface receptor for **1** because it is a potent mediator in the direct action of this catechin.[8−10] The 67 LR is a non-integrin laminin receptor highly associated with lipid rafts.[10] The expression levels of 67 LR strongly correlate with the invasion and metastasis of carcinoma (colon, breast, stomach, liver, lung, and ovary).[11] However, little information is available on the expression of 67 LR in human leukemia, except the association of 67 LR to the monocytic differentiation in leukemia.[11,12] Concerning **1**−67 LR-mediated action, distinct cytotoxic properties of EGCG at physiological concentrations have been revealed.[9,10] Tachibana et al. suggested that treatment with high concentrations of **1** induces ROS generation rather than most **1**−67 LR activities.[8] In contrast, studying the mechanism of the antiproliferative action of **1** in myeloid leukemia cells has revealed that ROS are key mediators of the apoptosis, and they are correlated with the heme enzyme myeloperoxidase (MPO).[13] There have been no reports regarding the growth inhibitory effects and molecular mechanisms of **1** on leukemia cells studied from the point of **1**−67 LR-mediated action, whereas 67 LR has been identified as a direct surface receptor for EGCG. In addition, although the biological properties of **1** depend significantly on cell type and its concentration, as mentioned in earlier reports, careful experimental examination is still needed. The autoxidation of **1** occurs rapidly without antioxidant reagents because the stability of EGCG has not been ascertained under cell culture conditions and its half-life is relatively short.[6,14]

In the present study, the cytotoxic properties of **1** were examined at physiological concentrations in the HL60, K562, and UT-7 myeloid leukemia cell lines as well as Raji lymphoid leukemia cells. The relevancy of 67 LR was investigated to the biological effects of **1** at physiological concentrations in leukemia cells.



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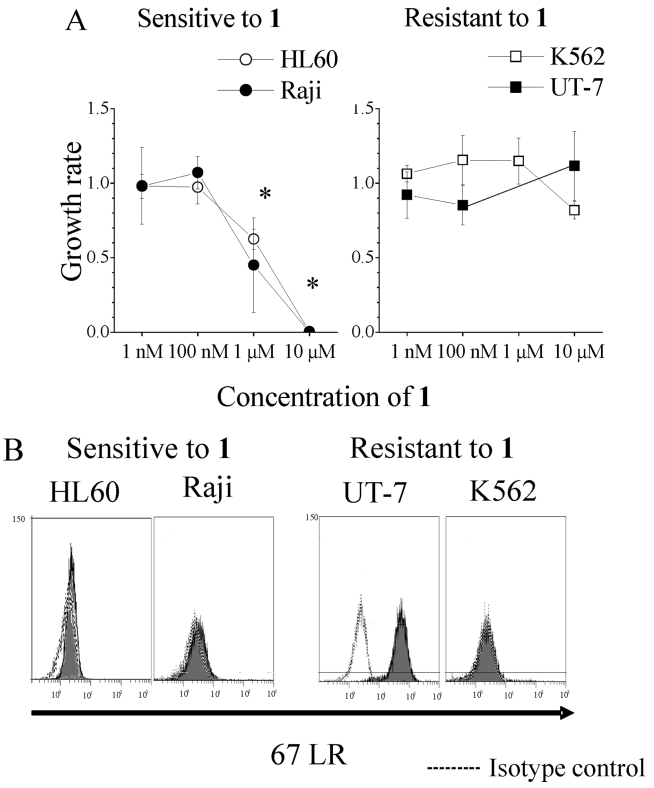

Figure 1. Sensitivity to EGCG (**1**) and expression of 67 LR in leukemia cells. (A) Cells were cultured in various concentrations of **1** for 7 days; subsequently, cells in each condition were harvested and diluted in trypan blue. Cell growth was estimated according to the Experimental Section. The growth rate was determined relative to viable cells (viable cells exposed to **1**/viable nontreated cells). (B) Exponentially growing cells were used to measure 67 LR expression on the cell surface by flow cytometry. Representative cytograms are shown. (*$p < 0.05$ by Mann–Whitney's *U* test).

RESULTS AND DISCUSSION

The 67 LR Expression Level Is Irrelevant to Sensitivity toward EGCG (1)-Induced Growth Inhibition. In order to investigate the sensitivity to **1**, cells were treated with various concentrations (1 nM–10 μM) of this compound (Figure 1A). Although the growth rates of UT-7 and K562 cells were not significantly inhibited by **1** even at 10 μM, a dose-dependent growth inhibition was observed in HL60 and Raji cells. To substantiate that the growth-suppressive effects of **1** require 67 LR on the cell surface, the expression levels of 67 LR were examined. However, there are few 67 LRs in HL60 and K562 cells, in accordance with an earlier report,[15] and no relationships between the sensitivity to **1** and the expression levels of 67 LR were demonstrated (Figure 1B).

Intracellular MPO and Oxidative Stress Are Unrelated to the Effects of EGCG (1) at Pharmacological Concentrations. Since the high sensitivity of HL60 cells toward **1** is attributed to MPO-mediated oxidative stress and these ROS accumulate at 1–3 h treatment of **1**,[3,13] the MPO expression levels were confirmed in HL60, Raji, and K562 cells (Figure 2A). In the case of HL60 and K562 cells, the observations made were in accordance with an earlier report that demonstrated a correlation between the sensitivity to **1** and MPO expression levels.[13] In contrast, no discernible differences were observed among the levels of intracellular ROS in cells treated with 10 μM **1** for 1 and 3 h (Figure 2B and data not shown). However, pretreatment with 10 mM NAC neutralized the suppressive effects of **1** against HL60 cells (Figure 2C). In contrast to myeloid leukemia cells, few MPO proteins were observed in Raji cells and 10 mM NAC could not attenuate the suppressive effects of **1** against these cells (Figure 2C).

Antioxidant Agents Neutralized Growth-Suppressive Effects of EGCG (1). Since the autoxidation of **1** occurs rapidly under cell culture conditions without antioxidant reagents, treatment of **1** was conducted in the presence of SOD (5 U·mL^{-1}), catalase (30 U·mL^{-1}), and mixtures of these agents (Mix), according to earlier reports.[6,14] As shown in Figure 3A, the growth suppression of Raji and HL60 cells treated with 10 μM **1** was not neutralized by SOD or catalase alone after 48 h incubation. However, when treated with the culture medium along with the mix, not only viability but also the sub-G1 phase of the cell cycle were neutralized at 48 h (45.9% vs 2.6% in HL60 cells) (Figure 3B). The neutralizing effects of a combination of SOD and catalase on the growth inhibition by **1** revealed that this property depends strongly on the molecular state of **1** itself rather than on the intracellular ROS. Culture conditions with antioxidant agents were adopted in some earlier studies since **1** itself is unstable in the culture medium as autoxidation occurs.[6,14] For example, SOD (5 U·mL^{-1}) is proposed to prevent the production of EGCG dimer from O^-,[6] and the combination of SOD (5 U·mL^{-1}) and catalase (30 U·mL^{-1}) stabilized **1** in culture.[12] When EGCG was stabilized by SOD, the inhibitory effect on cell growth was enhanced significantly in KYSE 150 esophageal carcinoma cells.[6] However, antioxidant agents protected the EGCG-sensitive HL60 and Raji leukemia cells (Figure 3). These observations suggest that a few ROSs, namely, O^- and H_2O_2, react with **1**, and the subsequent products seem to be key mediators for the observed cytotoxic effects against HL60 and Raji cells. Therefore, **1** itself is not thought to be a modulator of cytotoxic activity in the present study. These conflicting pharmacological effects of **1** may depend not only on the state of the molecules of **1** but also on the cell type. Yamamoto et al. demonstrated that ROS production by high concentrations of EGCG (50–200 μM) was pronounced only in OSC-2 and OSC-4 oral carcinoma cells not in primary epidermal keratinocytes.[16] Moreover, our previous study has shown that low concentrations of **1** (0.01–10 μM) have no toxicity, and 10–100 nM levels of EGCG induced the enhancement of colony formation and radio-protective effects against human megakaryocytic progenitor cells.[17] It is necessary to limit its potential for cumulative myelosuppression in therapeutic strategies,[18,19] because hematopoietic toxicity is often a dose-limiting factor in the administration of chemotherapeutic agents in cancer therapy, especially in leukemia patients. As shown in earlier reports, the action of [^{3}H]-**1** localized in the cytosol as well as the nuclei was observed in PC-9 lung cancer cells,[20] and its direct binding to DNA and RNA was confirmed.[21] However, these interferences with the basic machinery of DNA synthesis and cell division do not explain the lack of toxicity against human primary cells or the differential impact among cancer cells and normal cells.[16,17]

EGCG (1)-Induced Cell Death Lacks Phosphatidylserine Translocation and Active Caspase-3. To verify **1**-induced apoptosis in cells sensitive to this catechin, translocation of phosphatidylserine (PS) and active caspase-3 was analyzed because their activation are key signals in the apoptotic pathway.[22–24] As shown in Figure 4A, the passage of PS translocation was not

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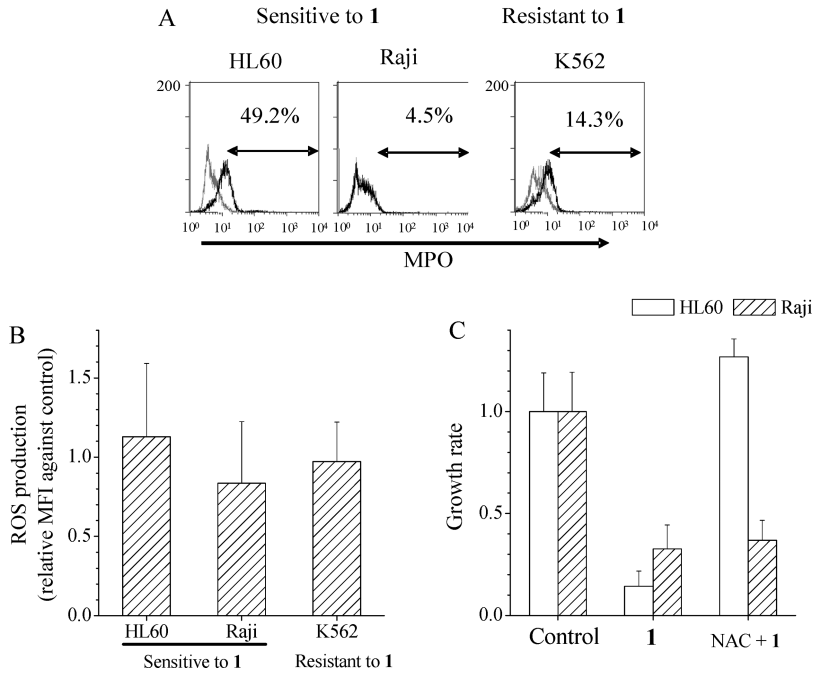

Figure 2. Intracellular MPO expression and ROS production induced by EGCG (**1**) treatment. (A) Exponentially growing cells were used to measure intracellular MPO expression by flow cytometry. Representative cytograms are shown. (B) ROS production was monitored by flow cytometry. Cells were treated with 10 μM **1** for 3 h; then intracellular ROS was probed using H_2DCFDA. Values are expressed as means ± SD of more than three separate experiments. (C) Cells treated with NAC were preincubated with 10 mM NAC for 30 min at 37 °C. Subsequently, cells were treated with 10 μM EGCG (**1**) for 48 h; then cell growth was estimated according to the Experimental Section. Growth rate was determined relative to viable cells (viable cells exposed to **1**/viable nontreated cells). Values are means ± SD of more than three separate experiments in triplicate wells.

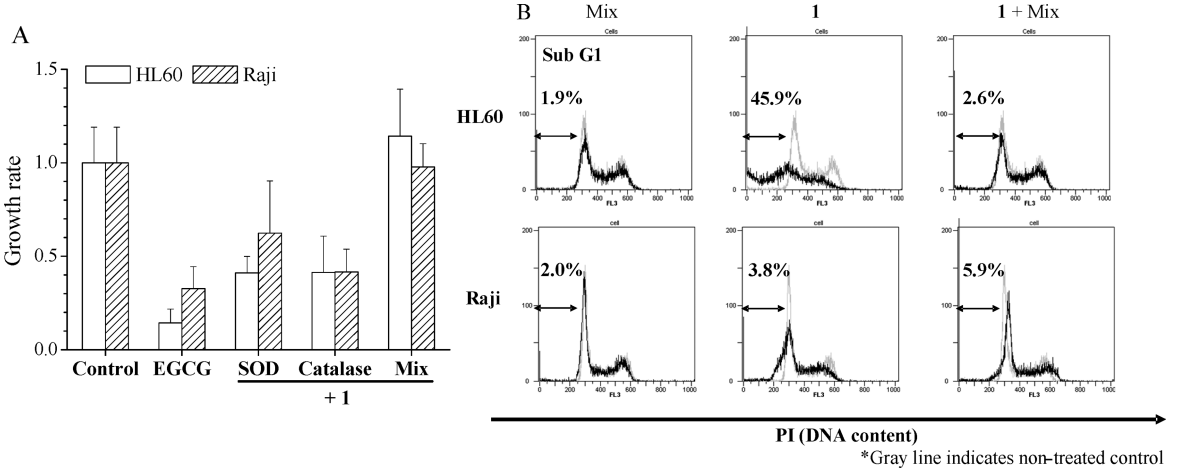

Figure 3. Growth-suppressive effects of EGCG (**1**) stabilized by antioxidant agents. (A) Cells were treated with 10 μM **1** in medium containing SOD (5 U·mL^{-1}) or catalase (30 U·mL^{-1}), or a mixture of SOD and catalase (Mix) for 48 h. Subsequently, cells in each condition were harvested, and cell growth was estimated according to the Experimental Section. Growth rate was determined relative to viable cells (viable cells exposed to **1**/viable nontreated cells). Values are means ± SD of more than three separate experiments in triplicate wells. (B) Cells in each condition were subjected to cell cycle analysis using PI staining according to the procedure described in the Experimental Section. Representative cytograms are shown.

observed in **1**-treated Raji cells, although rituximab, a monoclonal antibody to the CD20 antigen that has demonstrated cytotoxic effects in non-Hodgkin's lymphoma,[23] induced remarkable PS translocation in Raji cells. In contrast, time-dependent increase of necrotic population was observed in **1**-treated Raji cells, and the cytotoxic effects of **1** preferably depend on necrotic fractions [annexin V (−)/PI (+)], especially at 24 h treatments (6.1% by rituximab vs 14.9% by **1**). In addition, PS translocation was not observed in HL60 cells treated with 10 μM **1** (data not shown), whereas there were similar growth-suppressive effects among each treatment at 24 h (Figure 4B). Furthermore, there was no remarkable expression of active caspase-3 in each cell line treated with **1** at the indicated time course. However, 4 Gy irradiation, which induces DNA damage and apoptosis, triggered caspase-3-dependent apoptosis (Figure 4C). Thus, there were no biological characteristics of apoptosis in EGCG-induced cell death.

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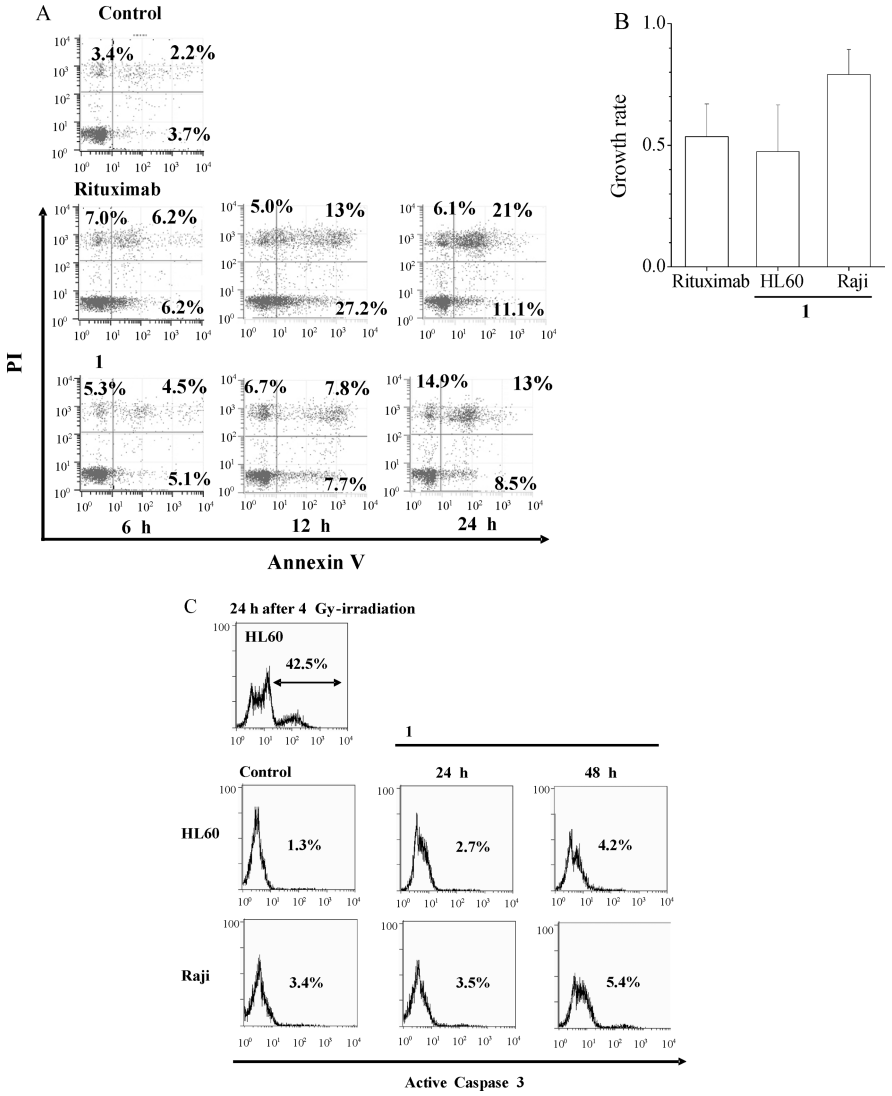

Figure 4. Determination of EGCG (**1**)-induced cell death. (A) Raji cells treated with 10 $\mu g \cdot mL^{-1}$ rituximab or 10 μM **1** for the indicated periods were costained with PI and annexin V-FITC, which specifically detected PS translocation, as described in the Experimental Section. Subsequently, cells were then examined by flow cytometry. Annexin (−)/PI (+), upper left, shows necrosis; annexin (+)/PI (−), lower right, shows early apoptotic feature; and annexin (+)/PI (+), upper right, shows late apoptosis or secondary necrosis. (B) Cells were treated with 10 μg/mL rituximab or 10 μM **1** for 48 h. Subsequently cells in each condition were harvested, and cell growth was estimated according to the Experimental Section. Growth rate was determined relative to viable cells (viable cells exposed to **1**/viable nontreated cells). Values are means ± SD of more than three separate experiments. (C) Cells treated with 4 Gy irradiation or 10 μM **1** for indicated periods were analyzed for intracellular active caspase-3 expression as described in the Experimental Section. Representative cytograms are shown.

Collectively, it has been demonstrated for the first time that **1** induces nonapoptotic cell death in leukemic cells at physiological concentrations independent of 67 LR. This study is also the first report of the cytotoxic effects of **1** against Raji cells. Although there are numerous reports studying the cytotoxic effects of **1** against several malignant cells, their experimental designs have been divided mainly into two groups: (a) low concentrations of **1** (1−10 μM) with 67 LR and (b) high concentrations of **1** (more than 20 μM) generating ROS and interfering in various signal-related molecular systems.[3−7,13,22] Furthermore, a common final phase of apoptosis has been attributed to the cytotoxic effects of **1**. Recently, Iwasaki et al. suggested that high concentration of **1** (greater than 50 μM) caused caspase-independent necrosis-like cell death in K562 chronic myelogenous leukemia cells.[25] In accordance with this report, the deletion of apoptotic characteristics such as PS translocation and activated caspase-3 support necrosis-like death induction by low concentrations of **1** (Figure 4). Unfortunately, there are no reports investigating the low dose **1**−67 LR mechanism in the presence of antioxidant agents. The present results raise the notion that the stability of **1** is not usually determined under the experimental conditions used. Therefore, to elucidate the cytotoxic mechanism of **1**, it is important to clarify which molecular form (radical, dimer, unmodified, or other) is responsible for the **1**-induced biological effects.

In conclusion, although further studies are required to clarify the key mediator for the pharmacological properties of **1** in EGCG-sensitive HL60 leukemia and Raji lymphoma cells, the present data suggest that this compound clearly shows cytotoxic effects at physiological concentrations (1−10 μM) without 67 LR and induces caspase-independent nonapoptotic cell death.

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Since **1** has presented no toxicity against human hematopoietic stem/progenitor cells,[17] this compound could be an effective agent against promyelocytic leukemia and non-Hodgkin's lymphoma.

EXPERIMENTAL SECTION

Reagents. (−)-Epigallocatechin-3-*O*-gallate (**1**; EGCG) (≥95% pure) was purchased from Sigma (St. Louis, MO) and was diluted in PBS to a concentration of 10 mM. Superoxide dismutase (SOD) was purchased from Nacalai Tesque (Kyoto, Japan) and diluted in PBS to 5000 U·mL^{-1}. Catalase was purchased from Wako (Osaka, Japan) and diluted in PBS to 3000 U·mL^{-1}. Each reagent was distilled with a 0.2 μm disposable syringe filter (Iwaki, Chiba, Japan), then subdivided, sealed, and stored at −20 °C in the dark. *N*-Acetyl-L-cysteine (NAC) was obtained from Sigma and diluted in PBS just before use with a 0.2 μm disposable syringe filter (Iwaki). Recombinant human thrombopoietin (TPO) was obtained from Biosource (Invitrogen, Carlsbad, CA).

Cell Lines. Three human tumor cell lines, Raji (Burkitt EBV-infected non-Hodgkin's lymphoma cells), HL60 (promyelocytic leukemia cells), and K562 (chronic myeloid leukemia cells), were purchased from RIKEN Bio-Resource Center (Tsukuba, Japan). UT-7 (histiocytic lymphoma) cells were kindly provided by Dr. N. Komastu (Juntendo University, Tokyo, Japan). Raji cells and HL60 cells were maintained in a RPMI 1640 continuous culture (Gibco Invitrogen), K562 cells were maintained in F-12 (Gibco Invitrogen), and UT-7 cells were maintained in IMDM (Gibco Invitrogen). These media were supplemented with 10% heat-inactivated fetal bovine serum (Bio Serum, Hiroshima, Japan) and penicillin/streptomycin (Gibco Invitrogen), and later the cells were incubated in a humidified atmosphere at 37 °C with 5% CO_2. In the case of UT-7, TPO (10 ng·mL^{-1}) was added to support viability. All of cell lines used in the present study exhibited approximately 90% viability, and the doubling times were 20−26 h.

Cell Growth Inhibition Assay to Determine Inhibition by EGCG (1). Cells were seeded in a 35 mm culture dish (Iwaki) with 2 mL of culture medium at 2×10^3 cells·mL^{-1}. After 7 days of culture, cells were harvested and diluted in trypan blue (Nacalai Tesque, Kyoto, Japan), which distinguishes viable cells from damaged or dead ones. Cell growth was investigated by microscopic observations, and counting of viable cells was conducted by means of a Burker-Turk hemacytometer (SLGC, Saitama, Japan).

Cell Growth Inhibition Assay to Determine Inhibition by EGCG (1) with Antioxidant Agents (SOD, Catalase, NAC). SOD (5 U·mL^{-1}), catalase (30 U·mL^{-1}), or a combination of these antioxidant agents was added to the culture medium. Cells were preincubated for 30 min at 37 °C with 10 mM NAC. Subsequently, these cells were seeded in a 24-well tissue culture plate (Falcon, Franklin Lakes, NJ) with 500 μL of the culture medium at 1×10^5 cells·mL^{-1} (Raji cells) or 5×10^4 cells·mL^{-1} (HL60). After determined periods, cells in each condition were harvested and diluted in trypan blue. Cell growth was investigated by microscopic observations, and counting of viable cells was conducted

Determination of 67 LR Expression by Flow Cytometry. Cells were seeded in a 35 mm culture dish (Iwaki) with 2 mL of culture medium at 1×10^5 cells·mL^{-1} and incubated for 24 h. The harvested cells were treated with 0.5% BSA/PBS containing mouse anti-67 LR IgM antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h on ice and were then washed and resuspended in 0.5% BSA/PBS containing anti-mouse IgM-FITC antibody (Santa Cruz Biotechnology) for 30 min at 4 °C in the dark. The expression levels of 67 LR were determined using a fluorescence cell analyzer (EPICS XL, Beckman-Coulter, Fullerton, CA).

Cell Cycle Analysis. Cells were treated with 10 μM **1** (with or without antioxidant agents) for the periods indicated. The harvested cells were treated with PBS containing 0.1% Triton X-100 (Wako) for 5 min on ice and stained with propidium iodide (PI; 50 μg·mL^{-1}, Sigma). Analysis of the cell cycle distribution was performed using a Cell Lab Quanta Sc MPL (Beckman-Coulter, CA).

Measurement of Myeloperoxidase Expression by Flow Cytometry. To detect basal intracellular MPO, cells were seeded in a 35 mm culture dish (Iwaki) with 2 mL of culture medium at 1×10^5 cells·mL^{-1} and incubated for 24 h. The harvested cells were treated with the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA), according to the manufacturer's instructions. In brief, cells were washed twice with ice-cold PBS and suspended in Cytofix/Cytoperm solution. After 20 min incubation on ice, cells were pelleted and aspirated. Subsequently, cells were washed twice with Perm/Wash buffer (wash-buffer) at room temperature. Cells were suspended in wash-buffer containing 1% (v/v) mouse anti-MPO mAb (Santa Cruz Biotechnology) and incubated for 1 h at room temperature. After the primary antibody reaction, cells were washed twice with wash-buffer, suspended in wash-buffer containing 1% (v/v) goat anti-mouse IgG-FITC antibody (Santa Cruz Biotechnology), and incubated for 1 h at room temperature in the dark. After the secondary antibody reaction, cells were washed twice with wash-buffer, suspended in PBS, and then detected using Cell Lab Quanta Sc MPL (Beckman-Coulter).

Detection of Intracellular ROS. To assess the generation of reactive oxygen species, control and cells treated with **1** were incubated with 5 μM H_2-DCFDA (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. In brief, cells (1×10^5) were stained with H_2-DCFDA for 25 min at 37 °C, then washed and resuspended in PBS. The fluorescence adduct yielded due to oxidation inside the cells was measured using a Cell Lab Quanta SC MPL apparatus (Beckman-Coulter).

Detection of Apoptosis. The extent of apoptosis was determined by annexin V-FITC and PI staining, according to the manufacturer's instructions. In brief, cells were suspended with 100 μL of binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and stained with annexin V-FITC for 10 min at room temperature in the dark. After washing with binding buffer, cells were resuspended in binding buffer containing PI (20 μg·mL^{-1}). Apoptotic cells were determined using the Cell Lab Quanta SC MPL apparatus. In the annexin V/PI quadrant gating, annexin V (−)/PI (−), annexin V (−)/PI (+), annexin V (+)/PI (−), and annexin V (+)/PI (+) represented the fraction of viable cells, necrotic cells, early apoptotic cells, and late apoptotic/secondary necrotic cells, respectively.

Detection of Active Caspase-3. Active caspase-3 was detected using the FITC-conjugated monoclonal active caspase-3 antibody apoptosis kit I (BD Bioscience), according to the manufacturer's instructions. In brief, cells were washed twice with ice-cold PBS and suspended in Cytofix/Cytoperm solution. After 20 min incubation on ice, cells were pelleted and aspirated, then rinsed with wash-buffer at room temperature. Cells were suspended in wash-buffer containing 5% (v/v) FITC-conjugated antiactive caspase-3 antibody for 40 min at room temperature in the dark. Cells were rinsed with wash-buffer and analyzed using the Cell Lab Quanta SC MPL apparatus.

Statistical Analysis. The significance of differences between the control and experimental groups was determined using Mann−Whitney's *U* test depending on the data distribution. Statistical analysis was performed using the Excel 2003 software program (Microsoft) with the add-in software Statcel 2.

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