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

Background and Objectives. The aim of this study was to investigate the possibility of green tea polyphenol, (-)-epigallocatechin-3-gallate (EGCG) as a novel therapeutic agent for patients with myeloid leukemia.

Design and Methods. We investigated the effects of EGCG on the induction of apoptosis in leukemic cells in vitro and in vivo. We further examined the molecular mechanisms of EGCG-induced apoptosis in myeloid leukemic cells.

Results. EGCG rapidly induced apoptotic cell death in retinoic acid (RA)-resistant acute promyelocytic leukemia (APL), UF-1 cells within 3 h. EGCG-induced apoptosis in UF-1 cells was associated with the loss of mitochondrial transmembrane potentials (ΔΨm) and activation of caspase-3 and -9. Elevation of intracellular reactive oxygen species (ROS) production was also demonstrated during EGCG-induced apoptosis of UF-1 as well as fresh myeloid leukemic cells. In NOD/SCID mice transplanted with UF-1 cells, EGCG effectively inhibited tumor growth in vivo, and the number of mitoses among the cells significantly decreased in comparison to the number in control mice cells.

Interpretation and Conclusions. In summary, EGCG has potential as a novel therapeutic agent for myeloid leukemia via induction of apoptosis mediated by modification of the redox system.

Key words: green tea, catechin, apoptosis, leukemic cells, reactive oxygen species.

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Design and Methods

Cells and cell culture
The RA-resistant APL cell line UF-1 was established in our laboratory from a patient with relapsed APL who had received ATRA, and the RA-sensitive NB4 promyelocytic leukemia cell line was a gift from Dr. M. Lanotte (Hôpital St Louis, Paris, France). The human myeloid leukemia cell lines HL-60, U937, K562, and KUB12 were obtained from the Japan Cancer Research Resources Bank (Tokyo, Japan). Bone marrow or peripheral blood samples from 6 newly diagnosed patients with acute myelogenous leukemia (AML) were obtained according to appropriate Human Protection Committee validation and with informed consent. Mononuclear cells were separated by Lymphoprep (Nycomed Pharma As, Oslo, Norway). The percentage of leukemic blast cells was more than 80% of the mononuclear cells. Cells were maintained in RPMI 1640 medium (GIBCO-BRL, Gaitherburg, MD, USA) with 15% fetal calf serum (Hyclone Laboratories, Logan, MT, USA), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere with 5% CO2.

Reagents
Various catechin derivatives, including EC, ECG, EGC, and EGCG, were purchased from WAKO Chemical Co. (Tokyo, Japan). Catechin derivatives were dissolved in DMSO and none of the cultures contained more than 0.1% DMSO. Controls were run using 0.1% DMSO and this concentration of diluent had no effect. N-acetyl-L-cysteine (NAC), rotenone, and myxothiazol were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Assays for apoptosis
Apoptotic cells were quantified by annexin V-FITC and propidium iodide (PI) double staining using a staining kit purchased from PharMingen (San Diego, CA, USA). In addition, induction of apoptosis was detected by a DNA fragmentation assay. The mitochondrial transmembrane potential (Δψm) was determined by flow cytometry (FACS Calibur; Becton Dickinson, San Jose, CA, USA). Briefly, cells were washed twice with PBS and incubated with 1 μg/mL rhodamine 123 (Sigma) at 37°C for 30 min. Rhodamine 123 intensity was determined by flow cytometry.

Cell cycle analysis
Cells (1×106) were suspended in hypotonic solution (0.1% Triton X-100, 1 mM Tris-HCl (pH 8.0), 3.4 mM sodium citrate, 0.1 mM EDTA) and stained with 50 μg/mL of PI. The DNA content was analyzed by flow cytometry. The population of cells in each cell cycle phase was determined using ModifIT software (Becton Dickinson).

Caspase assays
In the caspase inhibitor assay, cells were pretreated with a synthetic pan-caspase inhibitor (20 μM, Z-VAD-FMK) or caspase-3 inhibitor (50 μM, DEVD-CHO), and caspase-8 and -9 inhibitors (50 μM, Z-IETD-FMK and LEHD-CHO, respectively) for 2 h prior to addition of EGCG (100 μM). All inhibitors were purchased from Calbiochem (La Jolla, CA, USA).

Measurement of intracellular generation of ROS
To assess the generation of reactive oxygen species (ROS), control and EGCG-treated cells were incubated with 5 μM DHE (Molecular Probes, Eugene, OR, USA), which is oxidized to the fluorescent intercalator, ethidium, by cellular oxidants, particularly superoxide radicals. Cells (5×106) were stained with 5 μM DHE for 30 min at 37°C, and were then washed and resuspended in PBS. The oxidative conversion of DHE to ethidium 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% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 40 mM Tris-HCl (pH8.0), 150 mM NaCl) at 4°C for 15 min. For the detection of PML/RARα, cells were extracted by the method of Yoshida et al. Mitochondrial and cytosolic fractions were prepared with digitonin-nagarse treatment. Protein concentrations were determined using a protein assay DC system (Bio-Rad, Richmond, CA, USA). Cell lysates (15 μg protein per lane) were fractionated in 12.5% SDS-polyacrylamide gels prior to transfer to the membranes (Immobilon-P membranes, Millipore, Bedford, MA, USA) using a standard protocol. Antibody binding was detected by using an enhanced chemiluminescence kit for Western blotting detection with hyper-electrochemiluminescence film (Amersham, Buckinghamshire, UK). 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-caspase 3, -cytochrome c (PharMingen, San Diego, CA, USA), -Bcl-2, -BAX, -Bcl-XL, -p21CIP1/WAF1, -p27KIP1, -β-actin, -RARα, -Mcl-1, -survivin (Santa Cruz Biotech, Santa Cruz, CA), -cleaved PARP (Cell Signalling Technology, Inc., Beverly, MA, USA), and –Smac/DIA-BLO (MBL, Nagoya, Japan).

Animal model and experimental design
We have established the first human ATRA-resistant APL model in an NOD/SCID mice system using UF-1 cells. Briefly, mice were pretreated with 3 Gy of total body irradiation, which is a sublethal dose that was expected to enhance the acceptance of xenografts. Subsequently, UF-1 cells (1×107 cells) in their logarith-
mic growth phase were inoculated subcutaneously into NOD/SCID mice (Jackson Laboratory, Bar Harbor, ME, USA). Inoculated UF-1 cells formed subcutaneous tumors at the injection site, and cells grew rapidly. Forty days after implantation of the cells, mice with the transplanted cells were randomly assigned to receive water (n=5) or 10 mM EGCG (n=5) as the sole drinking fluid administered daily for 12 days. After 12 days of treatment, mice were sacrificed and dissected to measure tumor weights. When the mice showed severe wasting or when observations were finished, they were sacrificed according to the UKCCCR guidelines, and the day of sacrifice was recorded. Tumors were removed, fixed in 4% paraformaldehyde, and embedded in paraffin. Sections through the tumor were counted from both control and EGCG-treated animals, and expressed as the number of mitoses per field.

Statistical analysis
Tumor weights and the number of mitotic cells are expressed as mean ± SD. Differences in both parameters were analyzed for significance by Student’s t test. p<0.05 was considered as statistically significant.

Results
Catechin inhibited cellular proliferation of various leukemic cells
We first examined whether the green tea polyphenols and the polyphenolic epicatechin derivatives induced inhibition of the growth of leukemic cells, including NB4, UF-1, HL-60, K562, and U937 cells. The structurally related catechins, i.e., EC, ECG, EGC and EGCG, inhibited the growth of leukemic cells. However, EGCG was the most potent inhibitor among the 4 derivatives (Figure 1). We thus used EGCG for the series of experiments. Treatment with EGCG for 24 h induced a marked inhibition of UF-1 cell growth and, to a lesser but significant extent, led to an inhibition of cellular growth in all other myeloid leukemic cells (Figure 2A). Among the cells tested, UF-1 cells were the most sensitive to EGCG with an IC50 of 50 µM (Figure 2A). Therefore, we used UF-1 cells for the further experiments.

EGCG induced G1 cell cycle arrest and subsequent apoptosis
The effects of EGCG on cell cycle progression were investigated using UF-1 cells. The cells were treated with 100 µM EGCG for 24 h and analyzed for cell cycle distribution by means of flow cytometry. Culture with EGCG increased the population of cells in the G1 phase from 69.9% to 91.0%, with a reduction of cells in the S phase from 27.7% to 7.5% (Figure 2B). In addition, a strong induction of apoptosis was shown by the appearance of a haplodiploid DNA peak with sub-G1 DNA contents at 24 h after treatment (Figure 2B). These results indicate that EGCG led to cell cycle arrest at the G1 phase followed by apoptosis. We thus confirmed the induction of apoptosis by EGCG by means of DNA ladder formation and annexin V/PI staining. Interestingly, DNA ladder formation was confirmed at a time point as early as 3 h by electrophoresis of genomic DNA extracted from UF-1 cells treated with 100 µM EGCG (Figure 2C). Consistent with these results, the numbers of annexin V-positive cells increased after incubation with EGCG for 3 h (Figure 2D), thus indicating that EGCG rapidly induced apoptosis in UF-1 and NB4 cells.

Effects of EGCG on caspase-3 activity
To address the apoptotic pathway in EGCG-treated UF-1 cells, we next examined the activation of caspase-3 by Western blot analysis using antibody that recognizes both active and inactive forms of caspase-3. The 32-kDa procaspase-3 was cleaved into active forms (17kDa) after just 1 h of exposure to EGCG (100 µM) (Figure 3A). In addition, significant PARP cleavage was detected by 6 h of treatment with EGCG (Figure 3A). Furthermore, to elucidate the functional role of caspases in EGCG-induced apoptosis, experiments were performed with a series of caspase inhibitors. UF-1 cells were treated with 100 µM EGCG for 24 h, either alone or in combination with Z-VAD-FMK (pan caspase inhibitor), DEVD-CHO (caspase-3-specific inhibitor),
Z-IETD-FMK (caspase-8-specific inhibitor), or LEHD-CHO (caspase-9-specific inhibitor). EGCG-induced apoptosis was completely blocked by treatment with Z-VAD-FMK, DEVD-CHO, and LEHD-CHO, but not caspase-8-specific inhibitor, Z-IETD-FMK (Figure 3C). These results suggest that EGCG-induced apoptosis is associated with the activation of caspase-3 and -9, but not of caspase-8.

**Expression of apoptosis-associated proteins**

The expression of Bax protein was increased in a time-dependent manner by treatment with EGCG in UF-1 cells (Figure 4A). In contrast, EGCG did not modulate the levels of anti-apoptotic Bcl-2 and Bcl-X family proteins in UF-1 cells. However, Bcl-2 family Mcl-1 and survivin were down-regulated after 6 h of EGCG treatment (Figure 4A). Expression of cdk inhibitors (p21(CIP1/WAF1) and p27(KIP1)) was rapidly increased after 1 h exposure of EGCG in UF-1 cells (Figure 4B). UF-1 is a cell line from a patient with APL who had the chromosomal abnormality t(15;17), resulting in a PML/RARA fusion gene; this chimeric gene and its product may play important roles in leukemogenesis and development of this specific type of leukemia. Therefore, we
Catechin induces apoptosis of leukemic cells

Figure 3. Effects of EGCG on caspase activation. A. Western blot analysis of caspase-3 and cleaved PARP. Total cellular proteins (15 µg per each lane) were separated on 12.5% SDS-polyacrylamide gels and transferred to the membrane. Protein levels of caspase-3 were detected by Western blot analysis using antibody against caspase-3. Treatment with EGCG (100 µM) induced processing of caspase-3 (32 kDa), indicated by the appearance of a 17 kDa cleaved active form. The anti-cleaved PARP antibody recognized cleaved PARP (84 kDa) in EGCG-treated UF-1 cells. Coomassie brilliant blue stain was used to confirm that each lane contained equal amounts of protein. B. Effects of caspase inhibitors on EGCG-treated UF-1 cells. Inhibition of EGCG-induced apoptosis of UF-1 cells was estimated in a co-culture with a series of caspase inhibitors. Cells were preincubated with each caspase inhibitor for 2 h prior to addition of 100 µM EGCG. Results are expressed as the mean ± SD of three different experiments. Z-VAD-FMK, pan caspase inhibitor; DEVD-CHO, caspase-3 inhibitor; Z-IETD-FMK, caspase-8 inhibitor; and LEHD-CHO, caspase-9 inhibitor.

Figure 4. Expressions of the apoptosis-associated proteins and CDK inhibitors. A. UF-1 cells were treated with 100 µM EGCG for the indicated time. Cell lysates (15 µg per each lane) were fractionated on 12.5% SDS-polyacrylamide gels and analyzed by Western blotting with antibodies against Bcl-2, Bcl-XL, Mcl-1, survivin, Bax and β-actin proteins. B. The protein level of CDK inhibitors (p21CIP1/WAF1, p27KIP1) was detected by Western blotting. Re-blotting with β-actin staining demonstrated that equal amounts of protein were present in each lane.

Figure 5. EGCG-induced apoptosis occurred via the mitochondrial pathway. A. Flow cytometric analysis of mitochondrial ΔΨm, as estimated by rhodamine 123 intensity. UF-1 cells were cultured with 100 µM EGCG for 3 hours, and rhodamine 123 fluorescence was analyzed by flow cytometry. B. Western blot analysis of cytochrome c in EGCG-treated UF-1 cells. Cells were incubated with 100 µM EGCG for the indicated time. The arrow indicates the expression of cytosolic cytochrome c. C. Effects of antioxidant and MRC inhibitors on the release of cytochrome c and Smac/DIABLO from the mitochondria to the cytosol during EGCG-induced apoptosis in UF-1 cells. UF-1 cells were pre-treated with NAC (10 mM), rotenone (1 µM), myxothiazol (100 nM) for 1 h, followed by the treatment of 100 µM EGCG for 3 h. The release of cytochrome c and Smac/DIABLO was analyzed by Western blotting.
analyzed the effect of EGCG on this disease-specific chimeric protein. EGCG did not induce degradation of the PML/RARα chimeric protein (data not shown), suggesting that EGCG-induced apoptosis in UF-1 cells is not involved in the PML/RARA signaling pathway.

**EGCG-induced death signaling is mediated through the mitochondrial pathway**

Mitochondrial changes, including permeability transition pore opening and the collapse of ∆ψm, result in the release of cytochrome c into the cytosol, which subsequently causes apoptosis by the activation of caspases. After treatment with 100 µM EGCG for 3 h, low Rh123 staining in UF-1 cells indicated an increase in the loss of ∆ψm (Figure 5A). The loss of ∆ψm appeared in parallel with the activation of caspase-3 and -9, as well as with apoptosis. In addition, EGCG induced a substantial release of cytochrome c from the mitochondria into the cytosol in a time-dependent manner in UF-1 cells (Figure 5B). These results suggest that mitochondrial dysfunction causes the release of cytochrome c into the cytosol; caspase-9 and -3 were then activated, thereby propagating the death signal. The major sources of ROS are components of the mitochondrial respiratory chain (MRC). We also examined the role of the MRC components in EGCG-induced apoptosis in UF-1 cells by using MRC inhibitors, rotenone and myxothiazol. Antioxidant, NAC, completely inhibited the release of cytochrome c and Smac/DIABLO from the mitochondria into the cytosol, but MRC inhibitors caused partial inhibition (Figure 5C).

**ROS production triggers EGCG-induced apoptosis**

Several investigators have reported that EGCG-induced apoptosis is often associated with the generation of ROS. We therefore analyzed the production of intracellular ROS in control and EGCG-treated cells. Treatment of UF-1 cells with 100 µM EGCG for 3 h caused dramatic oxidation of DHE to ethidium, and resulted in the induction of intracellular ROS (Figure 6A). Furthermore, treatment of UF-1 cells with a thiol antioxidant, NAC, an excellent supplier of glutathione (GSH), completely blocked generation of intracellular ROS and EGCG-induced apoptosis in UF-1 cells (Figure 6A and data not shown). Our data indicate that the modulation of molecules involved in the redox system, particularly that of the GSH, may determine the sensitivity of leukemia cells to EGCG. Furthermore, we examined the effect of EGCG on induction of apoptosis and ROS production in fresh leukemia cells from 6 patients with AML. These results were consistent with the results obtained from the previously mentioned cell lines, i.e., sensitivity to EGCG-induced apoptosis was
observed in fresh leukemia cells from patients, and similarly increased production of intracellular ROS was also observed (Figure 7A and B). ROS production was 4.0 and 1.9-fold increased in EGCG-sensitive and -less sensitive fresh myeloid leukemic cells from the patients (patients 1 and 2, respectively) (Figure 7B).

**EGCG induces apoptosis in vivo**

Our *in vitro* data prompted us to examine whether the effects of EGCG are equally valid *in vivo*. After 40 days of implantation of UF-1 cells into NOD/SCID mice, water, used as a control (n=5), or 10 mM EGCG (n=5) treatment were administered orally as the sole drinking fluid ingested daily for 12 days. We found that tumor weight was significantly lower in the mice that were given EGCG compared to the mice treated with water as a control (*p*<0.05, mean weight: 1.52g in the EGCG-treated group vs. 2.20g in the control group) (Figure 8A). During the treatment, the EGCG-treated mice appeared healthy and continued to eat. In addition, pathologic analysis at autopsy revealed no EGCG-induced tissue changes in any of the organs (*data not shown*). These results suggest that EGCG had no toxic effects on mice during the treatment. Tumor cell proliferation was evaluated by counting the number of mitoses. Comparing the number of mitoses by hematoxylin and eosin staining in the same fields, a significantly lower number was observed in the EGCG group (*p*<0.001, Figure 8B), suggesting that cell proliferation was more inhibited in the EGCG-treated mice than in the control mice.

**Discussion**

Extensive *in vitro* cell culture studies, as well as *in vivo* studies in animal models, have verified the cancer preventive effects of green tea, and specifically, of its individual polyphenols. Epidemiological studies, though inconclusive, have suggested that green tea may reduce the risks associated with many cancers including bladder, prostate, esophageal and gastric carcinomas. Green tea extracts, especially its major polyphenolic component EGCG, are capable of inhibiting the growth of a variety of mouse and human cancer cells via the induction of *in vitro* apoptosis. However, the precise mechanism of EGCG-induced apoptosis remains to be elucidated. In this study, we demonstrated that one green tea component in particular, EGCG, suppressed the cellular growth of various fresh leukemic cells and myeloid leukemia cell lines, especially promyelocytic UF-1 cells. The observed growth inhibition of leukemic cells was due to the induction of apoptosis. Mitochondria play an essential role in death signal transduction such that permeability tran-
sition pore opening and collapse of the $\Delta \psi_m$ resulted in the rapid release of caspase activators such as cytochrome c into the cytoplasm. We demonstrated that the loss of the $\Delta \psi_m$ increased within 3 h of treatment, a time frame parallel to that of induced apoptosis. Furthermore, caspase-3 was activated by EGCG, and caspase-3 and caspase-9 inhibitors suppressed the apoptotic effects of EGCG. Taken together, these results suggest that EGCG-induced apoptosis in leukemic cells is associated with the loss of $\Delta \psi_m$ and with the activation of caspases, probably via the cytochrome c/Apaf-1/caspase-9 pathway. We further demonstrated that EGCG had no influence on the expression of Bcl-2 and Bcl-XL, but it up-regulated the levels of Bax protein, and down-regulated the levels of Mcl-1 and survivin in UF-1 cells in a time-dependent manner. We detected that EGCG-induced apoptosis in UF-1 cells and in certain fresh leukemia samples was associated with an increase in the levels of intracellular ROS. It has been suggested that the generation of ROS is a common mechanism in one of the representative pathways of apoptosis. Oxidants are capable of depleting GSH and damaging the cellular antioxidant defense system, and can directly induce apoptosis. On the other hand, antioxidants such as NAC can inhibit apoptosis. Interestingly, a recent study using cDNA microarrays has identified that superoxide dismutases (SOD) are target molecules of estrogen-induced apoptosis in leukemic cells, and inhibition of SOD causes an accumulation of ROS and leads to the release of cytochrome c from the mitochondria. Although EGCG is generally well-known as an antioxidant, it can also behave as a pro-oxidant under certain conditions. Therefore, we hypothesized that EGCG-induced apoptosis is also related to the GSH redox system, in a manner similar to the As$_2$O$_3$-induced apoptosis in APL cells. An antioxidant, NAC, increased intracellular GSH contents and completely blocked EGCG-induced apoptosis. In contrast, MRC inhibitors, rotenone and myxothiazol, partially blocked EGCG-induced apoptosis. In this work, we also examined whether EGCG-induced apoptosis is associated with an increase in the levels of intracellular ROS and the loss of cell cycle checkpoints in cancer cells confers a growth advantage. Therefore, EGCG suppresses cell growth by imposing cell cycle checkpoints in leukemic cells. To clarify the mechanisms of EGCG effects on the cell cycle, we examined the expression of cell cycle-associated proteins, including CDK, CDK inhibitors and various cyclins by Western blot analysis. Expression of p21$^{kip1}$ and p27$^{kip1}$ increased in a time-dependent manner, whereas the expression of cyclin D1, cyclin E, CDK2, and CDK4 were not altered by EGCG (data not shown). Up-regulation of p21$^{kip1}$ and p27$^{kip1}$ may thus play a pivotal role in inducing cell cycle arrest and apoptosis in EGCG-treated UF-1 cells. Recent studies have also indicated that green tea is an effective inhibitor of angiogenesis in vivo.

Catechin, a component of green tea, is a natural compound, and it appears to be safer than current chemotherapeutic drugs. Since we could not observe any organ damage in vivo, catechin might be developed as a new potent anti-cancer agent for the management of hematologic malignancies. In particular, it might be useful in older patients or in immunocompromised patients because of its safety and lack of known toxicity. Since green tea extracts have already entered phase I trials in patients with solid tumors in the USA, it would be useful to design similar clinical trials with leukemic patients to evaluate its anti-leukemic effects. In conclusion, this component of green tea may have potential as a novel therapeutic agent to replace or augment the more cytotoxic agents currently used to treat the patients with leukemia.
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