Epigallocatechin gallate induces apoptosis of monocytes

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Background: Monocytes are the main effector cells of the immune system, and the regulation of their survival and apoptosis is essential for monocyte-involved immune responses. Green tea polyphenol catechin has been reported to have antiallergic and anti-inflammatory activities, but its effect on monocytes has not yet been explored.

Objective: To elucidate the mechanisms of the anti-inflammatory effect of catechin, we studied the effect of catechin, especially epigallocatechin gallate (EGCG), on the apoptosis of monocytes.

Methods: Isolated peripheral blood monocytes were incubated without or with catechin, and apoptosis was evaluated by annexin V and propidium iodide double-staining or terminal deoxynucleotidyl assay. The activation of caspases 3, 8, and 9 was also evaluated by flow cytometry. The influence of GM-CSF or LPS, the known monocyte survival factors, on the EGCG-induced apoptosis of monocytes was investigated.

Results: Among the 4 catechin derivatives tested, EGCG and epicatechin gallate induced apoptosis of monocytes. Caspases 3, 8, and 9, which play a central role in the apoptotic cascade, were dose-dependently activated by EGCG treatment. The EGCG-induced apoptosis of monocytes was not affected by GM-CSF or LPS.

Conclusion: Catechin, especially EGCG, by promoting monocytic apoptosis, may be a new promising anti-inflammatory agent, and should be tested in clinical trials.

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Key words: *Monocyte, apoptosis, catechin, EGCG, inflammation*

Abbreviations used

EC: Epicatechin
ECG: Epicatechin gallate
EGC: Epigallocatechin
EGCG: Epigallocatechin gallate
FITC: Fluorescein isothiocyanate
PI: Propidium iodide
TUNEL: Terminal deoxynucleotidyl

It has been reported that catechin, a kind of tea polyphenol, has various physiological modulative activities, such as an antibacterial effect, a radical scavenging action, a protective effect on gastric mucosa, prevention of atherosclerosis, and antioxidative activities.[1-5] Furthermore, several recent studies have described the inhibitory effect of catechin on cancer. That is, catechin inhibits carcinogenesis, tumor growth, cancer cell invasion, and tumor angiogenesis, the last by suppressing the induction of vascular endothelial growth factor.[6-10] In addition to these antitumor effects, epigallocatechin gallate (EGCG), a major component of tea catechin, has an inhibitory effect on allergic reactions.[11-13] Several mechanisms of EGCG's antiallergic effects have been suggested, such as the inhibition of histamine release from basophilic cells, but the precise mechanisms still remain unclear.[14,15] Recently, we reported that EGCG had an inhibitory effect on T-cell–mediated inflammation.[16] EGCG directly bound to cell surface CD11b of $CD8^+$ T cells and exerted a strong suppression of the adhesion and migration of $CD8^+$ T cells. Although we have clarified the suppressive effect of EGCG on T cells, to our knowledge, nothing has been explored about its effect on monocytes.

Monocytes play an important role in the initiation, development, and outcome of the immune response.[17-20] Immune responses and inflammatory reactions mediated by monocytes are regulated not only positively by cellular recruitment, proliferation, and cross-talk, but also negatively by apoptosis of monocytes.[21,22] Monocytes generated in the bone marrow circulate in the bloodstream for a few days, after which they are programmed to undergo apoptosis in the absence of specific survival signals. Several inflammatory cytokines, such as GM-CSF, TNF-α, and IL-1β, have been reported to antagonize this spontaneous apoptosis of monocytes.[23-25] LPS, a cell wall component of gram-negative bacteria, also has the potential to prevent monocytic apoptosis.[25] On the other hand, several reports have demonstrated that anti-inflammatory cytokines, such as IL-4 or IL-10, and glucocorti-

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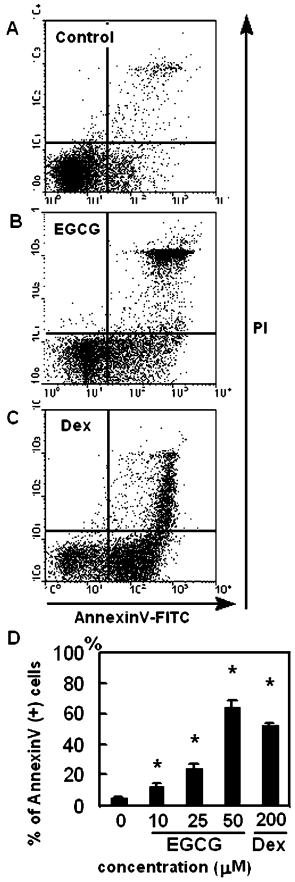

FIG 1. EGCG-induced apoptosis of monocytes. Apoptosis of monocytes was evaluated by flow cytometry. Monocytes isolated from peripheral blood were incubated for 24 hours without **(A)** or with EGCG (**B;** 50 μmol/L) or dexamethasone *(Dex)* (**C;** 200 μmol/L) and stained with annexin V (x-axis) and PI (y-axis). **D,** Percentage of annexin V^+ apoptotic monocytes. Data are expressed as means ± SDs of results from 3 independent experiments, using samples from different donors. *Statistical significance.

coids, such as dexamethasone, promote the apoptosis of monocytes.[23,26,27] Therefore, the homeostasis and apoptotic processes of monocytes were regulated both positively and negatively by multiple stimulations, and this regulation has been postulated to play a pivotal role in the monocytes-related inflammatory responses.

In the current study, we clearly demonstrated that EGCG, a major component of green tea catechin, strongly induced the apoptosis of monocytes. Because the regulation of monocytic apoptosis has a great importance in the regulation of inflammation, this might be one of the mechanisms of EGCG's immunosuppressive effects.

METHODS

Reagents and antibodies

Epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and EGCG, extracted from green tea, were purchased from Sigma (St Louis, Mo). Dexamethasone and LPS (*Escherichia coli*; serotype 026:B6) were also from Sigma, and the anti-CD11b mAb was from BD Pharmingen (San Diego, Calif). GM-CSF was kindly provided by Kirin Brewery Co Ltd (Tokyo, Japan).

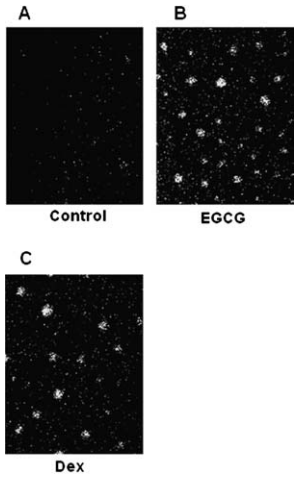

FIG 2. TUNEL staining. Monocytes were incubated for 24 hours without **(A)** or with EGCG (**B;** 50 μmol/L) or dexamethasone (**C;** 200 μmol/L) and stained by the TUNEL method. Apoptotic cells are stained. Similar results were obtained in 3 separate experiments, and the representative one is presented.

Isolation of peripheral blood $CD14^+$ monocytes

$CD14^+$ monocytes were obtained from venous blood drawn from normal, healthy volunteers as described previously.[16] Briefly, PBMCs were isolated by centrifugation on a Ficoll-Paque density gradient (Amersham Pharmacia, Uppsala, Sweden). $CD14^+$ monocytes were obtained by using a magnetic cell separation system (MACS; Miltenyi Biotec, Bergish Gladbach, Germany). For this purpose, PBMCs were incubated with microbead-coupled anti-CD14 mAbs, and the magnetically labeled cells were obtained by positive selection. The purity of the $CD14^+$ monocytes used in the experiments was 98% to 99%, as analyzed by flow cytometry (data not shown). Monocyte-depleted PBMCs were recovered and used as the subset of lymphocytes.

Induction of apoptosis

Monocytes or lymphocytes, prepared as discussed, were suspended in 5% FCS/RPMI 1640, without or with different concentrations of catechin, dexamethasone (200 μmol/L), GM-CSF (1000 U/mL), and/or LPS (10 ng/mL). After 24-hour incubation in plastic flasks at 37°C, nonadherent cells were collected, and the adherent cells were incubated in PBS containing 0.02% EDTA for 5 minutes at 4°C to reduce the adherence. Cells were then detached and collected by vigorous pipetting. Collected adherent cells, together with the nonadherent ones, were washed twice with PBS and used for further examinations. In another experiment, monocytes were pretreated with 2 μg/mL anti-CD11b mAb before incubation with EGCG to block the binding of EGCG to CD11b.

Detection of apoptosis by flow cytometry

Monocytes or lymphocytes were prepared and treated as described, and then stained with fluorescein isothiocyanate (FITC)–conjugated annexin V and propidium iodide (PI) for 5 minutes at

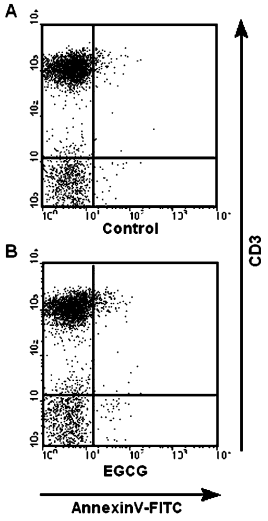

FIG 3. EGCG did not affect the survival of lymphocytes. Apoptosis of lymphocytes was evaluated by flow cytometry. Monocyte-depleted PBMCs were incubated without **(A)** or with **(B)** EGCG (50 μmol/L) for 24 hours. *X-axis* represents annexin V–FITC, and *y-axis* represents CD3-PE. Similar results were obtained in 3 separate experiments, and the representative one is presented.

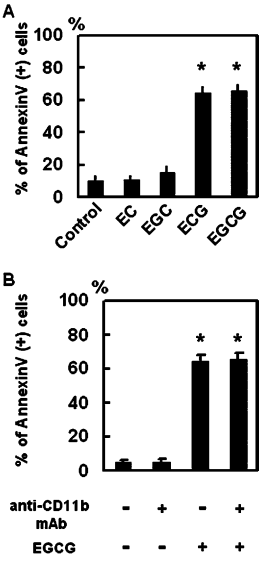

FIG 4. A, Comparison of monocytic apoptosis induced by different catechin derivatives. Monocytes were incubated for 24 hours without or with 50 μmol/L EC, EGC, ECG, or EGCG. **B,** Effect of CD11b blocking on the EGCG-induced apoptosis. Monocytes, preincubated with anti-CD11b mAb or isotype-matched control mAb, were treated without or with EGCG (50 μmol/L). The percentages of apoptotic cells were evaluated by annexin V staining. Data are expressed as means ± SDs of results from 3 independent experiments, using samples from different donors. *Statistical significance.

room temperature. The population of annexin V^-PI^- viable cells and annexin V^+ apoptotic cells was evaluated by flow cytometry. Data were collected in a FACS Calibur (Becton-Dickinson, Mountain View, Calif) and analyzed by using the CellQuest software (Becton Dickinson).

Terminal deoxynucleotidyl staining

Terminal deoxynucleotidyl (TUNEL) staining was performed by using the in situ cell death detection kit (Roche Diagnostics, GmbH, Mannheim, Germany). Monocytes treated with EGCG or dexamethasone were fixed by 4% paraformaldehyde and permeabilized with 0.5% Tween 20. Cells were washed twice and resuspended in the solution containing TUNEL transferase and FITC-conjugated nucleotides. Free 3′-OH DNA ends of the cells were labeled at 37°C for 1 hour, and fluorescein labels incorporated in nucleotide polymers were visualized by using a laser confocal microscope (Fluoview, Olympus, Japan).

Caspase activity

Caspases 3, 8, and 9 activities were evaluated by using caspase 3, 8, and 9 detection kits (Oncogene, San Diego, Calif). Monocytes treated with EGCG or dexamethasone were washed twice and suspended in PBS containing FITC-conjugated specific caspase inhibitors DEVD-FMK (Asp-Glu-Val-Asp-fluoromethyl-ketone), IETD-FMK (Ile-Glu-Thr-Asp-fluoromethyl-ketone), or LEHD-FMK (Leu-Glu-His-Asp-fluoromethyl-ketone) for the detection of caspases 3, 8, or 9, respectively. Caspase inhibitors were allowed to permeate into the cells and bind irreversibly to the activated caspases for 1 hour. The fluorescence retaining in the cells was analyzed by flow cytometry.

Statistical analysis

The unpaired Student *t* test was used to determine statistical significance. Differences at $P < .05$ were considered statistically significant.

RESULTS

Apoptosis of monocytes induced by EGCG

Initially, the effect of EGCG on the survival of monocytes was evaluated by using annexin V–PI staining (Fig 1). Culture in the presence of EGCG for 24 hours resulted in a significant increase in the population of annexin V^+ cells (Fig 1, *A and B*; 4.9% vs 63.0%; control vs EGCG). Although both populations of annexin V^+PI^- and annexin V^+PI^+ cells were significantly increased, no increase in the population of annexin V^-PI^+ cells was observed, suggesting that the cell death induced by EGCG was through induction of apoptosis but not necrosis. The apoptosis of monocytes induced by EGCG was dose-dependent and even the lowest concentration tested (10 μmol/L) could induce significant apoptosis (Fig 1, *D*). Treatment with dexamethasone also resulted in the significant increase of apoptotic cells, as reported previously (Fig 1, *C*). Both EGCG-treated and dexamethasone-treated monocytes showed a strong fluorescence by TUNEL staining, corroborating the result of annexin V staining (Fig 2).

EGCG does not affect the survival of lymphocytes

To evaluate whether EGCG induced apoptosis of other leukocyte subsets, the effect of EGCG on lymphocyte survival was investigated. Monocyte-depleted PBMCs

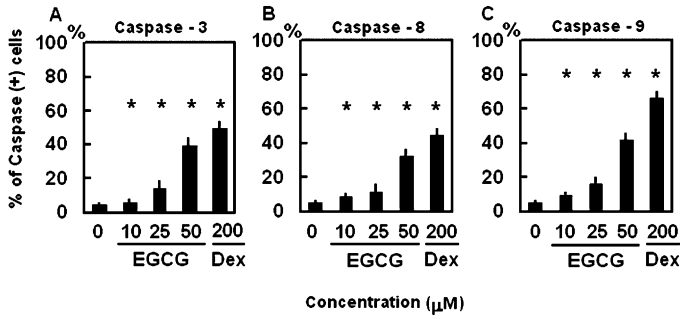

FIG 5. Activation of caspases 3, 8, and 9. Monocytes were incubated without or with different concentrations of EGCG or dexamethasone. The percentages of caspase-activated cells were evaluated by flow cytometry. The data are expressed as means ± SDs of results from 3 independent experiments, using samples from different donors. *Statistical significance.

were double-stained with annexin V and anti-CD3 mAb (Fig 3). In both the $CD3^+$ and $CD3^-$ lymphocyte subsets, the population of annexin V^+ cells did not increase by the treatment with EGCG for 24 hours, even with the highest concentration (50 μmol/L). In addition, EGCG did not induce apoptosis of $CD3^-$ cells, suggesting that the apoptosis-inducing effect of EGCG was monocyte-specific.

Comparison of catechin derivatives on induction of monocyte apoptosis

We examined different catechin derivatives for their capacity to induce apoptosis of monocytes. Four green tea catechins—EC, EGC, ECG, and EGCG—were tested at 50 μmol/L. The percentage of annexin V^+ monocytes after 24-hour incubation was as follows: control, 10.5%; EC, 10.9%; EGC, 15.2%; EGC, 64.0%; and EGCG, 65.4% (Fig 4, *A*). Although no induction of apoptosis was observed with EC or EGC treatments, incubation in the presence of ECG resulted in a significant induction of monocytic apoptosis, an effect comparable with EGCG.

Blocking of CD11b signaling

We previously reported that EGCG bound to the cell surface CD11b, and monocytes strongly expressed CD11b.[16] To elucidate whether the apoptosis in monocytes induced by EGCG was dependent on the binding of EGCG to CD11b, the competitive blocking assay was performed. As we previously reported, anti-CD11b mAb (clone ICRF44) and EGCG competitively bound to CD11b. Initially, the ability of the CD11b-specific mAb to induce apoptosis of monocytes was evaluated. As shown in Fig 4, *B*, specific antibody binding to CD11b did not induce apoptosis. Then we examined whether blocking of EGCG binding to CD11b with the specific MAb could affect the ability of EGCG to induce apoptosis. CD11b-mAb–treated monocytes were incubated with EGCG, but the percentage of apoptotic cells did not change.

Caspase activities

Because caspases 3, 8, and 9 play central roles in the apoptotic cascade, the activation of these 3 enzymes during the induction of apoptosis was investigated. As shown in Fig 5, caspases 3, 8, and 9 were all activated by EGCG treatment in a dose-dependent manner. Incubation with dexamethasone also significantly increased the activities of these caspases.

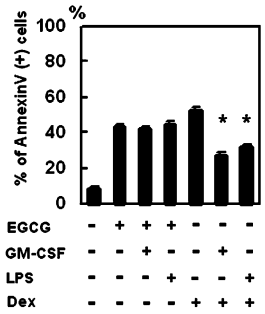

FIG 6. Effect of survival factors. Monocytes were incubated with different combinations of EGCG (50 μmol/L), dexamethasone (200 μmol/L), GM-CSF, and LPS. The percentages of apoptotic cells were evaluated by annexin V staining. The data are expressed as means ± SDs of results from 3 independent experiment, using samples from different donors. *Statistical significance.

Effect of survival factors on apoptosis of monocytes

GM-CSF and LPS have been reported to have the ability to antagonize apoptosis of monocytes.[23,25] We investigated whether the presence of GM-CSF or LPS affected the apoptosis induced by EGCG or dexamethasone. As shown in Fig 6, addition of GM-CSF or LPS did not affect the apoptosis induced by EGCG. In contrast, the apoptosis induced by dexamethasone was significantly inhibited by both GM-CSF and LPS.

DISCUSSION

In recent years, catechin has been focused on as the main component of green tea, which has many biomodulative properties.[1-5] Among these bioactivities, several reports have demonstrated the antiallergic and anti-inflammatory effects of tea catechins *in vivo*.[11-13] Few reports on the

mechanisms of the inhibitory effect of catechins to the immune system are found in the literature.[14,15] Recently, we reported a new mechanism of the suppressive effect of EGCG on lymphocytes. That is, EGCG bound to CD11b expressed on $CD8^+$ T cells and strongly suppressed the adhesion and migratory capacity of lymphocytes.[16] However, little has been investigated about the effect of catechin on other leukocyte subsets. In this study, we clearly demonstrated another mechanism of the anti-inflammatory effect of catechins—that is, the induction of apoptosis on monocytes.

Glucocorticoids such as dexamethasone have been reported to induce apoptosis of monocytes.[26,28] The systemic monocytopenia after glucocorticoid administration is supposed to be an important mechanism in the treatment of chronic inflammatory diseases.[29,30] In this study, we investigated the apoptosis of monocytes induced by dexamethasone and compared with that induced by EGCG. For the detection of apoptotic cells, annexin V–PI double-staining was performed. EGCG induced a significant and dose-dependent increase of annexin V^+ apoptotic monocytes, whereas the population of annexin V^-PI^+ necrotic cells did not change. Treatment with dexamethasone also significantly increased the number of apoptotic monocytes. Apoptosis was further confirmed by TUNEL staining. TUNEL was strongly positive after treatment with both EGCG and dexamethasone. This apoptosis-inducing effect of EGCG was monocyte-specific, because EGCG did not affect the survival of other leukocyte subsets.

Green tea is reported to contain several derivatives of catechin: EC, EGC, ECG, and EGCG. EGCG accounts for approximately half of the green tea catechins and is the most potent compound among these derivatives.[31] We evaluated the apoptosis-inducing effect of these 4 compounds. ECG and EGCG strongly induced apoptosis of monocytes, whereas EC and EGC had no effect. Because the former 2 isomers possess a galloyl group and the latter 2 do not, the galloyl group is supposed to play an important role in the apoptosis induction. Pyrogallol group, which is present in EGC and EGCG but not EC and ECG, is also reported to contribute to the biological activities of catechin. However, the percentage of annexin V^+ cells was not different in ECG-treated and EGCG-treated monocytes, suggesting that the pyrogallol group is not essential for the induction of apoptosis.

As we reported previously,[16] EGCG binds to the cell surface CD11b and suppresses its function, ie, the ability to bind its ligands. EGCG could bind to CD11b expressed on not only lymphocytes but also monocytes; therefore, 2 possible mechanisms of the EGCG-induced apoptosis of monocytes can be suggested. One is that the binding of EGCG to CD11b interferes with the binding to its ligands, leading to inhibition of the survival intracellular signaling. The other is that EGCG itself, by binding to and activating CD11b, exerts an apoptosis-inducing signaling. To test these hypotheses, we used the anti-CD11b mAb (clone ICRF44), which was shown in our previous report to block the binding of EGCG to CD11b. Blocking the binding of EGCG to CD11b molecule did not affect the induction of apoptosis. Therefore, neither of the 2 mechanisms seemed to be responsible for apoptosis induction, suggesting another mechanism independent of CD11b. Although the binding affinity of EGCG for CD11b was significantly higher than that of ECG (data not shown), the ability of both catechins to induce monocytic apoptosis was quite similar, facts that taken together suggest the independency of CD11b in the apoptosis induction by catechins.

Next, the caspase activities were investigated. Two major pathways of apoptosis have been identified according to the initiator caspase: the death receptor pathway, involving caspase 8, and the mitochondrial pathway, in which various signals can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase 9. Both pathways result in the downstream activation of caspase 3.[32] Incubation with EGCG invoked a dose-dependent activation of caspase 3. Both caspase 8 and caspase 9 were also dose-dependently activated, suggesting the involvement of both death receptor and mitochondrial pathways. EGCG has anticancer activities and induces apoptosis of some tumor cells.[33] Several mechanisms by which EGCG induces apoptosis has been postulated, including EGCG's binding to and activation of Fas, suppression of bcl-2 family proteins, and activation of NFκB signaling pathway.[34-36] Because both caspase cascades were activated, induction of apoptosis of monocytes by EGCG seems to be a multiple event, similar to that of tumor cells. The Fas signaling and consequent activation of caspase 8 was also shown to be essential for the dexamethasone-induced apoptosis of monocytes, but the activation of caspase 9 has not been investigated.[28] Interestingly, we could demonstrate that dexamethasone, in addition to caspase 8, activated caspases 3 and 9. Consequently, monocytic apoptosis induced by glucocorticoid is also invoked by multiple mechanisms.

GM-CSF and LPS act as an antagonist for the monocytic apoptosis.[25,28] However, their preventive effects on glucocorticoid-induced apoptosis of monocytes have not yet been explored. Interestingly, we could demonstrate that GM-CSF and LPS effectively antagonized apoptosis of monocytes induced by dexamethasone, whereas they had no effect on the apoptosis by EGCG. The depletion of monocytes is supposed to be an important mechanism of the anti-inflammatory effect of glucocorticoids. However, inflammatory cytokines released from infiltrating leukocytes at the site of inflammation could reduce the effect of glucocorticoid. In contrast, EGCG-induced apoptosis is not affected by these survival factors, and consequently, EGCG may be promising as an anti-inflammatory drug. In previous reports, the highest concentration of EGCG achieved after oral ingestion was 7 μmol/L.[37,38] Therefore, further investigation on how to achieve higher serum concentrations, either by improving the oral absorption of catechins or developing other strategies, such as the use intravenous administration, is required.

In conclusion, for the first time, we demonstrated that tea polyphenol EGCG exerted an anti-inflammatory effect by inducing apoptosis of monocytes. Further investigation to develop EGCG as a clinical agent is desirable.

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