Epigallocatechin gallate attenuates adhesion and migration of CD8$^+$ T cells by binding to CD11b

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Background: Although green tea polyphenol catechin has been reported to have antiallergic and anti-inflammatory activities, the precise mechanisms of its effect on the immune system have been poorly investigated.

Objective: In this study, we aimed to elucidate the mechanisms of the anti-inflammatory effect of catechin. For this purpose, we studied the effect of 2 kinds of catechin, epigallocatechin gallate (EGCG) and epicatechin gallate, on peripheral blood CD8$^+$ T cells, which play the key role in immune responses.

Methods: Isolated peripheral blood mononuclear cells or CD8$^+$ T cells were incubated without or with catechin, and the changes in the surface expression of integrin molecules were investigated by flow cytometry and the direct binding of catechin to CD11b molecule by competitive ELISA. Also, the effect of catechin on the ability of CD8$^+$ T cells to bind intracellular adhesion molecule 1 and to migrate in response to chemokines was evaluated by using the adhesion and migration assays.

Results: The 2 catechins directly bound to CD11b expressed on CD8$^+$ T cells, which caused a consequent decrease of flow-cytometric CD11b expression. The effect was more prominent with EGCG than epicatechin gallate, and the impaired expression of CD11b induced by EGCG resulted in decreased ability of CD8$^+$ T cells to adhere intercellular adhesion molecule 1, and consequently decreased migration in response to chemokines.

Conclusion: We concluded that catechin, especially EGCG, by downregulating CD11b expression on CD8$^+$ T cells and, in consequence, inhibiting infiltration of these cells into the sites of inflammation, is a promising new potent anti-inflammatory agent. (J Allergy Clin Immunol 2004;113:1211-7.)

Key words: *Catechin, CD11b, anti-inflammation, CD8$^+$ T cells, adhesion, migration*

Abbreviations used
ECG: Epicatechin gallate
EGCG: Epigallocatechin gallate
FITC: Fluorescein isothiocyanate
ICAM-1: Intercellular adhesion molecule 1
MIP: Monocyte inflammatory protein

It has been reported that catechin, a kind of tea polyphenol, has various physiologic modulative activities, such as antibacterial effects, radical scavenging action, prevention of atherosclerosis, and anticancer effects.[1-5] In addition, epigallocatechin gallate (EGCG), a major component of tea catechin, has an inhibitory effect on allergic reactions.[6-9] Several mechanisms of the EGCG's antiallergic effects have been suggested, such as the inhibition of histamine release from basophilic cells, but the precise mechanisms still remain unclear.[9]

CD11b is a member of α-chain integrin and forms a complex with β2-integrin as Mac-1. CD11b is expressed on neutrophils, monocytes, natural killer cells, and a subset of CD8$^+$ T cells.[10] It plays a central role in mediating migration of leukocytes from peripheral blood to sites of inflammation during the process of host defense.[10,11] CD11b contributes to firm leukocyte adhesion, not only to the endothelium via the intercellular adhesion molecule 1 (ICAM-1) but also to the underlying subendothelium and interstitial extracellular matrix by binding diverse kinds of ligands, such as fibronectin, collagens, and laminins.[12-14] CD8$^+$CD11b$^+$ T cells have been reported to be recently activated effector cells, whereas CD8$^+$CD11b$^-$ T cells are naive or memory T cells.[15,16] Thus, CD8$^+$CD11b$^+$ T cells are assumed to play a crucial role in the inflammatory process, especially in the step of extravasation to the site of inflammation, but the precise mechanisms of CD11b-mediated adhesion and the migration of the CD8$^+$ subset of lymphocytes are poorly investigated as yet. Therefore, elucidation of the effect of catechin on CD8$^+$CD11b$^+$ T cells may lead to a better understanding of the essential nature of the catechin's anti-inflammatory effect.

In the current study, we clearly demonstrated that the 2 main compounds of green tea catechin, EGCG and epicatechin gallate (ECG), selectively downregulated the flow-cytometric expression of CD11b, which is essential for the adhesion to ICAM-1, on CD8$^+$ T cells, with consequent functional changes. This effect was dependent on a direct binding of catechin to CD11b, interfering with the binding of CD11b to the specific mAb. From these findings, we concluded that catechin may exert a potent anti-inflammatory effect by modulating the migration of CD8$^+$ T cells to sites of inflammation.

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METHODS

Reagents and antibodies

Epicatechin gallate and EGCG extracted from green tea were purchased from Sigma (Saint Louis, Mo). The purity of these 2 compounds was >98% and >95%, respectively. Phycoerythrin-conjugated anti-CD11a, anti-CD11b, anti-CD11c, fluorescein isothiocyanate (FITC)–conjugated anti-CD29, purified anti-CD11b, and horseradish peroxidase-conjugated rat antimouse IgG secondary mAbs were obtained from BD Pharmingen (San Diego, Calif). Goat polyclonal antibody to the C-terminus of CD11b was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). Monocyte inflammatory protein (MIP)−1α and MIP-1β were purchased from R&D Systems (Minneapolis, Minn). Purified ICAM-1 was kindly provided by Springer TA (Boston, Mass).

Cell purification and cell culture

In preliminary experiments, blood samples from 5 normal healthy persons were used, and in the subsequent experiments, samples from 3 persons were tested. Representative results are shown in Figs 1 to 7. Informed consent was obtained from all volunteers. $CD8^+$ T cells, granulocytes, and monocytes were obtained from venous blood as described previously.[17,18] Briefly, PBMCs and granulocytes were isolated by centrifugation on a Ficoll-Paque density gradient (Amersham Pharmacia, Uppsala, Sweden). Obtained granulocytes were purified by dextran sedimentation, and contaminating erythrocytes were removed by hypotonic lysis. $CD14^+$ monocytes and $CD8^+$ lymphocytes were obtained by using microbead-coupled mAbs in a magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany). The magnetically labeled $CD14^+$ monocytes were obtained by positive selection. Among the $CD14^-$ cell population—that is, monocyte-depleted, $CD8^+$ T cells were isolated by positive selection. The purity of isolated cells used in the experiments was 98% to 99% as analyzed by flow cytometry (data not shown). The human leukemia (HL) cell line, HL-60, was obtained from American Type Culture Collection. Cells were maintained in RPMI-1640 supplemented with 5% FCS, 60 mg/mL penicillin G, and 100 mg/mL streptomycin sulfate in a 5% carbon dioxide and 95% air atmosphere at 37°C.

Detection of cell surface molecules by flow cytometry

Isolated cells were incubated in the presence of different concentrations of ECG or EGCG in 0.2% BSA/RPMI for different times at 37°C. A large amount of cooled (4°C) PBS was added immediately after incubation to halt the reaction. Cells were stained with FITC-conjugated mAb against CD29 and phycoerythrin-conjugated mAbs against CD11a, CD11b, and CD11c. FITC-conjugated or phycoerythrin-conjugated mouse IgG mAb of unrelated specificity was always used as control. Data were collected and analyzed in FACS Calibur and CellQuest software (Becton Dickinson, Mountain View, Calif).

Competitive ELISA

We speculated that the apparent CD11b downregulation by flow cytometry would be dependent not on the actual loss of its expression but on the binding of EGCG to the CD11b molecule, interfering with antibody binding. To test this hypothesis, we established a competitive ELISA system, as in our previous report,[19] with some modifications. Briefly, HL-60 cells were cultured in the presence of 1.3% dimethylsulfoxide for 48 hours, which increases expression of CD11b (data not shown). HL-60 protein extract was added to a 96-well microtiter plate (Nunc A/S, Roskilde, Denmark) precoated with the goat polyclonal antibody recognizing the C-terminus of CD11b (2 μg/mL) to allow the antibodies catch the CD11b molecules, followed by incubation with various concentrations of ECG or EGCG. Then the mAb recognizing the extracellular domain of CD11b (500 ng/mL) was added, followed by HRP-conjugated rat antimouse IgG secondary antibody. Finally, wells were treated with *O*-phenylenediamine (Nacalai Tesque, Kyoto, Japan) in hydrogen peroxide for color development, and the data were analyzed in an ELISA plate reader at 490 nm.

Adhesion assay

A 96-well microplate (Iwaki, Tokyo, Japan) was coated with purified ICAM-1 (10 μmol/L) for 24 hours at 4°C and blocked for nonspecific binding. Isolated $CD8^+$ T cells were labeled with 1% Calcein-AM solution (Dojindo, Kumamoto, Japan) and incubated with different concentrations of ECG or EGCG for 30 minutes at 37°C. T cells, 2×10^5, were seeded and the plates incubated for 1 hour. After gentle washing to remove nonadherent cells, the number of adherent T cells was determined by measuring the fluorescence intensity at 525 nm in a microscope photometer (Terrascan VP; Minervatech, Tokyo, Japan). Wells without washing were always adopted as positive controls and wells without cells as negative controls. The percentage of cell adhesion was determined as the ratio to the positive control wells after subtraction of the negative control value.

Migration assay

The cell migration assay was conducted by using transwell migration chambers (5 μm pore size; Corning, Tokyo, Japan). Isolated $CD8^+$ T cells were suspended in 0.2% BSA/RPMI with different concentrations of catechins, and 2×10^5 cells were transferred to the upper chamber. The lower chamber contained the same medium without or with MIP-1α (100 ng/mL) or MIP-1β (100 ng/mL) as the chemoattractant. Cells were allowed to migrate for 3 hours and then were recovered from the lower chambers. The number of migrating cells was counted in the FACS Calibur. Alternatively, migrating cells were stained with phycoerythrin-conjugated anti-CD11b mAb, and the percentage of $CD11b^+$ cells was analyzed in the FACS Calibur.

Statistical analysis

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

RESULTS

Epigallocatechin gallate treatment attenuates the CD11b expression of $CD8^+$ T cells

Initially, isolated PBMCs were incubated for 30 minutes in the absence (Fig 1, *A*) or presence of either EGCG (100 μmol/L; Fig 1, *B*) or ECG (100 μmol/L; Fig 1, *C*). In control samples—that is, without catechin, the population of $CD8^+CD11b^+$ cells accounted for ~20% to 40% of the $CD8^+$ cell population, consistent with previous reports[20] (data not shown). EGCG treatment caused a remarkable decrease in the CD11b expression in both $CD8^+$ and $CD8^-$ cell populations, whereas ECG treatment had no evident effect. Only a small population of $CD4^+$ T cells expressed CD11b (data not shown); the majority of $CD8^-CD11b^+$ cells were assumed to be $CD19^+$ B cells. $CD8^+$ T cells, but not B cells, play a critical role at the site of inflammation; therefore, we focused on the $CD8^+CD11b^+$ T-cell population for the evaluation of

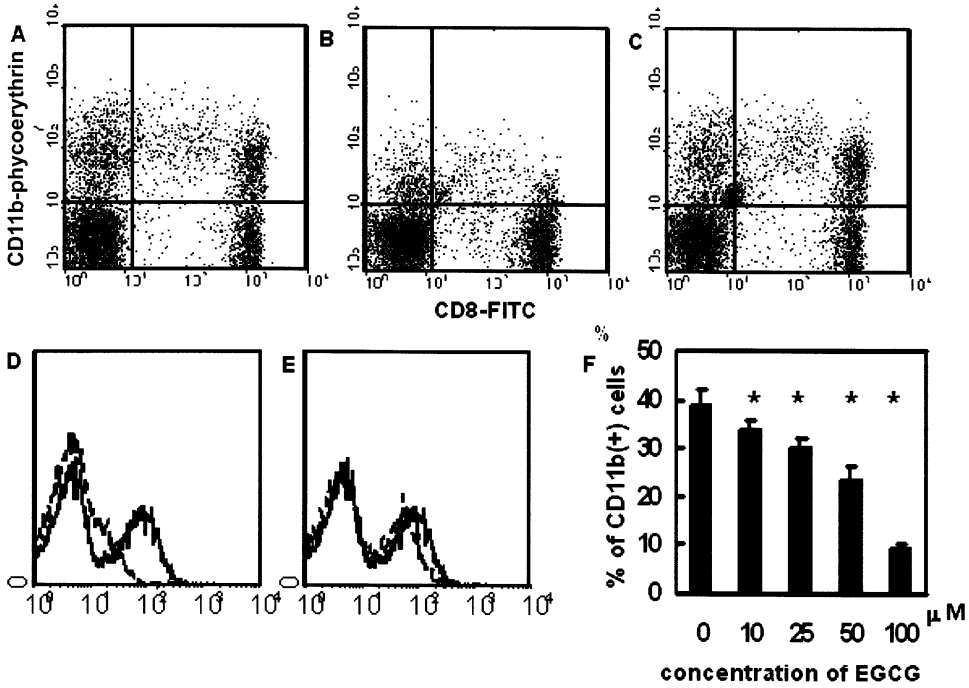

FIG 1. EGCG downregulates CD11b expression on $CD8^+$ T cells. **A-C,** Flow-cytometric analysis of PBMCs. PBMCs isolated from peripheral blood were incubated without (*A*) or with 100 μmol/L EGCG (*B*) or ECG (*C*) and double-stained with anti-CD8 (FITC) and anti-CD11b (phycoerythrin) mAbs. **D** and **E,** CD11b expression on isolated $CD8^+$ T cells. Isolated $CD8^+$ T cells were incubated without (*solid line*) or with (*dotted line*) 100 μmol/L EGCG (*D*) or ECG (*E*) and stained with anti-CD11b mAb (phycoerythrin). **F,** Alternatively, the percentage of $CD11b^+$ cells after treatment with different concentrations of EGCG was evaluated by flow cytometry. The data are expressed as means ± SDs of results from 3 independent experiments. *Statistical significance compared with control.

the possible anti-inflammatory effect of EGCG. For this purpose, $CD8^+$ T cells were purified and the changes of CD11b expression induced by catechin investigated. EGCG treatment caused a significant decrease in the expression of CD11b (Fig 1, *D*), similar to the effect in nonpurified $CD8^+$ T cells. However, ECG also exerted a significant effect (but weaker than that of EGCG) on purified $CD8^+$ (Fig 1, *E*), unlike the effect on nonpurified cells. This CD11b downregulation was dose-dependent, and even with the lowest concentration of EGCG tested (10 μmol/L), a small but significant decrease in CD11b expression was observed (Fig 1, *F*). The percentage of $CD11b^+$ cells decreased from 38.8% in the control to 9.1% in the EGCG treatment at 100 μmol/L.

Effect of catechin on the CD11b expression of monocytes and granulocytes

To elucidate whether this CD11b downregulation induced by EGCG was T-cell specific, we examined the changes in the CD11b expression of monocytes and granulocytes. As shown in Fig 2, EGCG treatment significantly decreased the expression of CD11b on both monocytes and granulocytes in a dose-dependent manner, suggesting that it is not T-cell specific. However, the effect of EGCG was weaker on these 2 subsets of leukocytes compared with $CD8^+$ T cells, and ECG did not affect their CD11b expressions. Therefore, we focused on the $CD8^+CD11b^+$ T-cell subpopulation in the following experiments.

Effect of catechin on the expression of other integrins

The β1-integrin and β2-integrin families play an important role in the extravasation of T cells. Here, we investigated the changes in the expression of the other members of the β2 family, namely CD11a and CD11c, as well as the β1-integrin (CD29), induced by EGCG. CD11a was strongly expressed on $CD8^+$ T cells, but CD11c was absent, and EGCG treatment (100 μmol/L) did not affect their expressions (Fig 3, *A*, *B*). CD29 was strongly expressed on $CD8^+$ T cells, and its expression was also not affected by EGCG (Fig 3, *C*). ECG also caused no change in the expression of CD11a, CD11b, and CD29 (data not shown). Double staining of $CD8^+$ T cells with CD29 and CD11b mAbs revealed that this subset of lymphocytes could be divided into 3 subgroups (Fig 3, *D*): CD29-dim/CD11b-negative, CD29-bright/CD11b-negative, and CD29-bright/CD11b-positive cells, with almost all of the CD29-bright cells also $CD11b^+$. As shown in Fig 3, *E*, the expression of CD11b on CD29-bright $CD8^+$ cells was

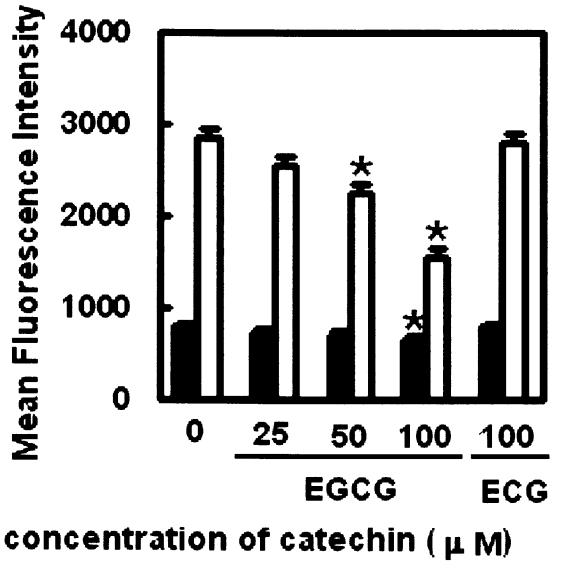

FIG 2. Effect of EGCG treatment on the CD11b expressions of granulocytes and monocytes. Isolated granulocytes (*filled box*) or monocytes (*open box*) were treated with different concentrations of EGCG or ECG. Cells were washed twice and stained with anti-CD11b mAb (phycoerythrin), and the expressions of CD11b were evaluated by flow cytometry. The data are expressed as means ± SDs of results from 3 independent experiments. *Statistical significance compared with control.

selectively downregulated, without affecting the expression of CD29.

Time kinetics of CD11b downregulation

Next, the time course change of CD11b expression induced by EGCG was evaluated. Only 5-minute exposure to EGCG was enough to cause a marked decrease in the expression of CD11b (17.6% of control), and after 2-hour incubation, the impaired CD11b expression was restored to ~37.0% (Fig 4); after 5 hours, ~66.5%.

Detection of catechin binding to CD11b by competitive ELISA

As shown in Fig 5, by using a competitive ELISA, we could clearly demonstrate that EGCG markedly inhibited the binding of the specific antibody to the captured CD11b molecule. Even with the lowest concentration tested (50 μmol/L), the inhibition rate was as high as 85.8%, whereas ECG, even at the highest concentration (200 μmol/L), showed a significant but lower inhibitory effect (40.7%). These data correlate well with the findings of flow

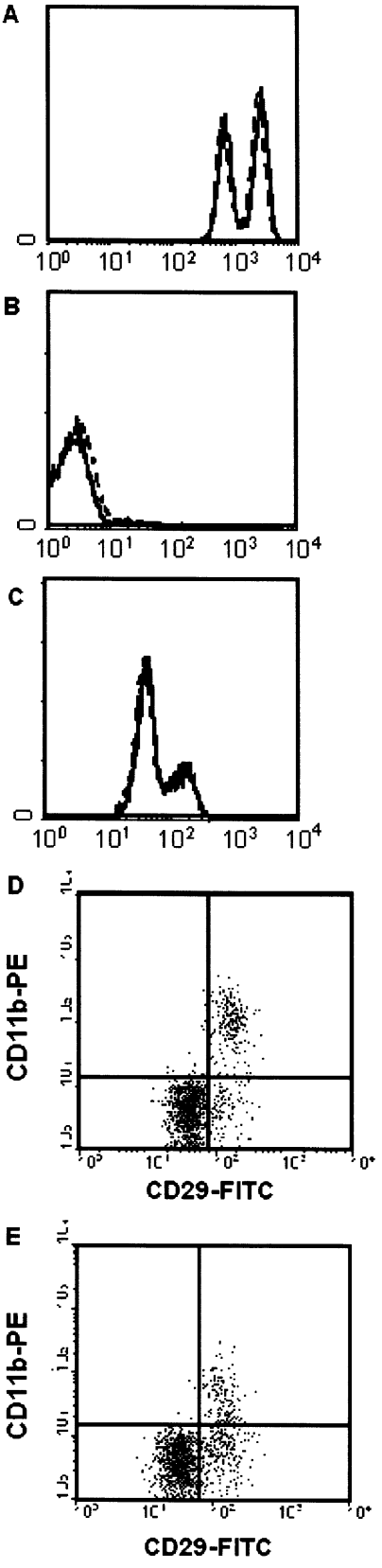

FIG 3. EGCG treatment does not affect the expression of other integrins. **A-C,** Flow-cytometric analysis of CD11a (*A*), CD11b (*B*), and CD29 (*C*) expression. Isolated $CD8^+$ T cells were incubated without (*solid line*) or with (*dotted line*) 100 μmol/L EGCG and stained with anti-CD11a or anti-CD11c mAbs (phycoerythrin). **D** and **E,** $CD8^+$ T cells were incubated without (*D*) or with (*E*) 100 μmol/L EGCG and stained with anti-CD29 (FITC) and anti-CD11b (phycoerythrin) mAbs. Representative results of 3 independent experiments are shown. These results are representative of 3 independent studies.

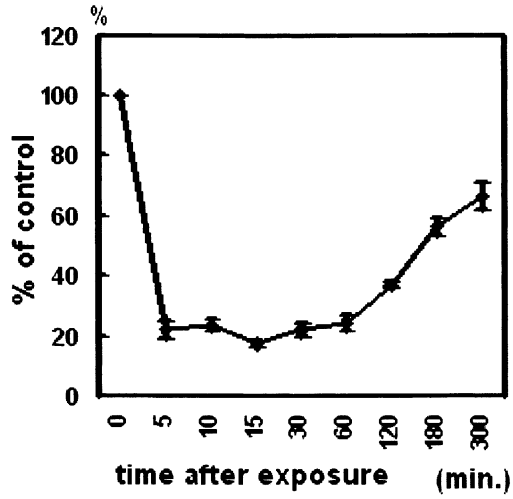

FIG 4. Time course experiment of CD11b expression. $CD8^+$ T cells were incubated with or without 100 μmol/L EGCG for different times. After incubation, a large amount of cooled PBS was added to halt the interaction, and cells were stained with anti-CD11b mAb (phycoerythrin). Mean fluorescent intensity was evaluated by flow cytometry. Values represent the ratio to mean fluorescent intensity of control cells. The data are expressed as means ± SDs of results from 3 independent experiments.

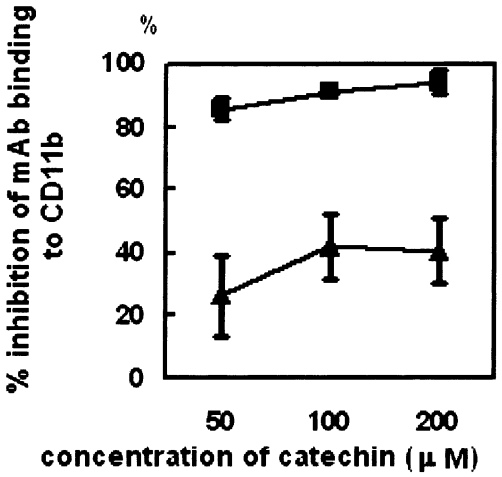

FIG 5. Direct binding of EGCG and ECG to CD11b. EGCG (*triangle*) and ECG (*square*) dose-dependently inhibited the binding of specific mAb to CD11b in a competitive ELISA. The effect of EGCG was more pronounced than that of ECG. Data are expressed as the means ± SDs of results from triplicate wells.

cytometry and quite resemble our previous findings with the CD4 molecule.[19]

Adhesion assay

Preincubation of isolated $CD8^+$ T cells with EGCG resulted in the significant decrease in the number of ICAM-1 adherent cells in a dose-dependent manner (Fig 6). ECG also caused a significant and dose-dependent decrease of adherent cells, but the effect was weaker. These results correlated well with the alteration of CD11b induced by EGCG and ECG.

Migration assay

Initially, the migratory property of $CD11b^+CD8^+$ cells was analyzed in the absence of catechin (Fig 7, *A*). Although only 32.3% of purified $CD8^+$ cells were $CD11b^+$, migrating cells were predominantly $CD11b^+$ cells. In the control condition, that is, without any chemoattractant in the lower chamber, 82.0% of the migrating cells were found to be $CD11b^+$. Addition of MIP1-α or MIP-1β, although it caused an increase in total migrating cells, did not alter the percentage of $CD11b^+$ cells. These results led us to conclude that CD11b expressed on $CD8^+$ T cells is closely associated with their migratory property. Then, we evaluated the effect of catechins on the migration of $CD8^+$ T cells. In all experimental conditions, that is, without or with MIP-1α or MIP-1β, EGCG markedly and dose-dependently decreased the number of migrating cells (Fig 7, *B-D*). ECG, even with the highest concentration (100 μmol/L) tested, did not alter the number of migrating cells, in contrast with the fact that ECG effectively decreased the T-cell adhesion to ICAM-1.

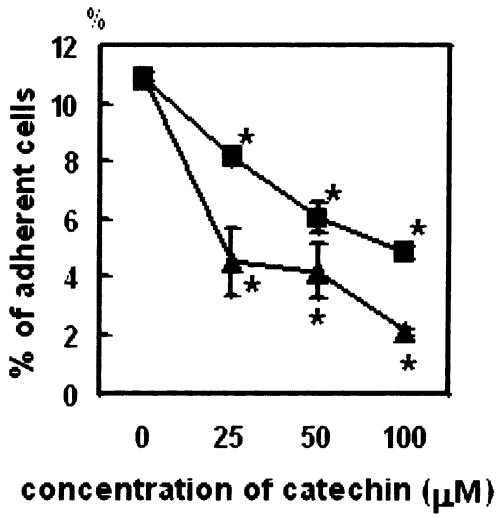

FIG 6. EGCG and ECG inhibit adhesion of $CD8^+$ T cells to ICAM-1. A 96-well microplate was coated with purified ICAM-1. Isolated $CD8^+$ T cells prestained with Calcein-AM solution were preincubated with different concentrations of EGCG (*triangle*) or ECG (*square*). Cells were then added to the microplate and were allowed to adhere to the microplate precoated with ICAM-1 for 1 hour. The microplate was washed twice and the fluorescence of adherent cells measured in a photometer. The percentage adhesion was calculated as the ratio to positive control wells. Data are expressed as the means ± SDs of results from 3 wells. *Statistical significance compared with control.

DISCUSSION

Several reports have referred to the antiallergic and anti-inflammatory properties of tea catechins, such as the

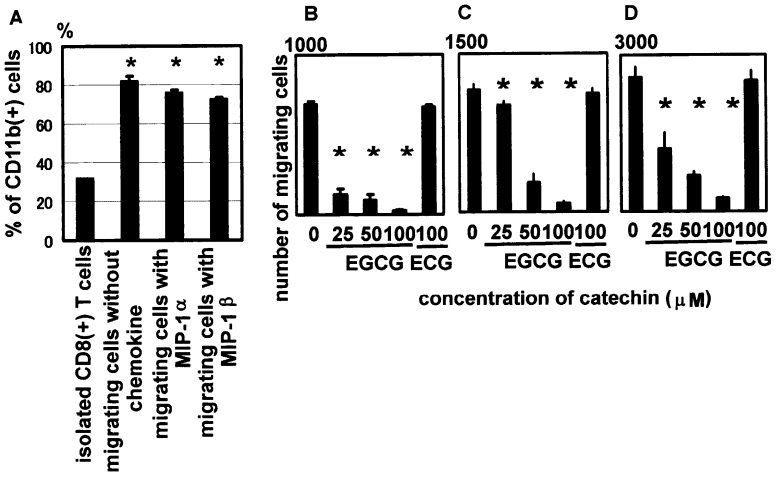

FIG 7. EGCG inhibits migration of $CD8^+$ T cells. $CD8^+$ T cell populations, before and after migration, were stained with anti-CD11b mAb, and the percentage of $CD11b^+$ cells was evaluated by flow cytometry **(A)**. Alternatively, catechin was added to the upper chamber and PBS **(B)**, MIP-1α (100 ng/mL; **C**), or MIP-1β (100 ng/mL; **D**) to the bottom chamber. The number of cells migrating to the bottom chamber was evaluated by counting cells from duplicate wells. Data are expressed as the means + SDs of results from 2 wells. *Statistical significance compared with control.

inhibition of mouse type IV allergies and protection against the artificially induced inflammatory injuries caused by UV radiation.[7,8] The mechanisms of the catechin's anti-inflammatory effect, however, still remain unclear. Green tea contains several isomers of catechin, and EGCG accounts for approximately half of them. It is the most potent compound among these isomers because it possesses both pyrogallol and galloyl groups, which contribute to the biological activities of catechin.[21]

Incubation of the total lymphocyte population with EGCG caused a rapid disappearance of the subset of $CD8^+CD11b^+$ cells. It is known that a minor portion of $CD8^+$ T cells expresses CD11b, which has been reported to be a marker of T-cell activation.[16,22] Naive/memory $CD8^+$ T cells express CD28, but not CD11b, and effector T cells, which have high migratory ability, lose CD28 expression and acquire CD11b upregulation during their activation. CD11b is the α-chain of β2-integrin family and is, in addition to $CD8^+$ T cells, expressed on granulocytes and monocytes. Several studies have demonstrated that CD11b/CD18 contributes to the firm granulocytic and monocytic adhesion to the endothelium via ICAM-1 and mediates the subsequent transendothelial migration of adherent cells.[10,11,23,24] However, currently, little has been investigated of CD11b expression on $CD8^+$ T cells and its functional implications.

In the flow-cytometric experiments using purified $CD8^+$ cells, we could clearly demonstrate that EGCG treatment caused a significant downregulation of CD11b expression in a dose-dependent manner. Similarly, EGCG significantly decreased CD11b expression of granulocytes and monocytes in a dose-dependent manner. Although $CD8^+$ T cells, granulocytes, and monocytes all infiltrate to the inflammatory site and play a pivotal role in the inflammatory response, the effect of EGCG was the most prominent on $CD8^+$ T cells, followed by monocytes and granulocytes, in this sequence. Therefore, in the following experiments, we focused on this poorly recognized $CD8^+$ T-cell subpopulation and investigated the changes induced by catechins.

The CD11b downregulation induced by EGCG is not dependent on cell apoptosis, as confirmed by annexinV staining (data not shown). Evaluation of the time course of the EGCG effect showed that it occurs within few minutes, suggesting that the interaction between EGCG and lymphocytes is a quite rapid event. Interesting, however, was the finding that after 2 hours of culture in the presence of EGCG, the impaired expression of CD11b began to increase. The instability of EGCG in culture medium and its consumption by treated cells, as well as the transitoriness of the effect, are the possible reasons for this CD11b restoration. Hong et al[25] have described a short half-life of 130 minutes for EGCG, which rapidly decreased in cell culture conditions.

We also investigated the effect of EGCG on the other 2 α-subunits of the β2-integrin family, CD11a and CD11c, as well as the β1-chain of the β-1 integrin family, because all of these molecules play an important role in the adhesion, and consequently migration, of leukocytes. Catechin treatment had no effect on the expression of these integrins, suggesting that it is CD11b-specific.

In a previous report, we demonstrated that EGCG bound to CD4, blocking specific antibody binding as well as the binding of glycoprotein 120.[19] EGCG binding to cell surface CD4 is a rapid reaction, completed within few minutes. The flow-cytometric downregulation of CD11b induced by catechin was also a quite rapid reaction, leading us to hypothesize that it could be dependent on a mechanism similar to that for CD4. By using a competitive ELISA, we could clearly demonstrate that EGCG bound the CD11b molecule, similar to CD4, inhibiting the binding of the specific mAb.

Our observation that the migrating $CD8^+$ T cells were predominantly of $CD11b^+$ phenotype confirmed the importance of CD11b in the migration of $CD8^+$ T cells. Next, to evaluate whether the masking of CD11b resulted in attenuation of its activity, we focused on the main functions of β2-integrins, adhesion and migration, by using catechin-treated $CD8^+$ T cells. Both the adhesive and migratory capabilities of $CD8^+$ T cells significantly decreased after EGCG treatment, and the effect was also dose-dependent. Corroborating our findings, Katiyar and Mukhtar,[8] by using a mouse model of UV-induced inflammation, demonstrated that catechins significantly inhibited the infiltration of $CD11b^+$ leukocytes to sites of inflammation.

Although several investigations have reported the upregulation of CD11b expression, in situations such as cell activation,[26] little has been described of CD11b downregulation, and to our knowledge, the only effector reported to cause downregulation of CD11b on monocytes is the tumor cell-derived prostaglandin E2.[27,28] Although nonsteroidal anti-inflammatory drugs, which inhibit prostaglandin synthesis by inhibition of cyclooxygenase, are also known to inhibit adhesion and migration of T cells,[29] the involvement of CD11b in this inhibition has not been investigated.

In summary, for the first time, we demonstrated that tea polyphenol EGCG directly binds to CD11b on $CD8^+$ T cells, with functional consequences. Because it causes a decrease in the adhesive and migratory properties of $CD8^+$ T cells to sites of inflammation, EGCG should be considered a promising anti-inflammatory agent, and further investigation to develop it as a clinical agent is desired.

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