Basic and clinical immunology

Epigallocatechin gallate, the main component of tea polyphenol, binds to CD4 and interferes with gp120 binding

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Background: Epigallocatechin gallate (EGCG), the major component of tea polyphenol, has been reported to have various physiologic modulatory activities. Several reports also have shown that catechin has a protective effect against HIV infection, part of which is mediated by inhibiting virions to bind to the target cell surface.
Objective: We investigated the effect of EGCG on the expression of CD4 molecules and on its ability to bind gp120, an envelope protein of HIV-1.
Methods: Peripheral blood CD4+ T cells were incubated in the presence of EGCG, and the expression of CD4 was evaluated by means of flow cytometry. The effect of EGCG on the antibody binding to CD4 was investigated by using a sandwich ELISA, and the effect on the gp120 binding to CD4 was analyzed by means of flow cytometry.
Results: EGCG efficiently inhibited binding of anti-CD4 antibody to its corresponding antigen. This effect was mediated by the direct binding of EGCG to the CD4 molecule, with consequent inhibition of antibody binding, as well as gp120 binding.
Conclusion: The present results suggest a potential preventive effect of EGCG on HIV-1 infection by modulating binding to CD4. (J Allergy Clin Immunol 2003;112:951-7.)

Key words: *CD4, epigallocatechin gallate, gp120, lymphocyte*

It has been reported that the tea polyphenol catechin has various physiologic modulative activities, such as antibacterial effect, radical scavenging action, prevention of atherosclerosis, and antioxidative activities.[1-5] Furthermore, a number of recent studies have described the inhibitory effect of catechin on cancer: catechin inhibits carcinogenesis, tumor growth, invasive ability of cancer cells, and tumor angiogenesis, the latter 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 HIV-1 infection.[11-14] Several mechanisms of the anti-HIV effects of EGCG have been suggested, such as the destruction of virons, the inhibition of reverse transcriptase activities or cellular DNA and RNA polymerases, and the binding of HIV virions to the surface of lymphocytes, but the precise mechanisms still remain unclear.[11-14]

Abbreviations used
ECG: Epicatechin gallate
EGCG: Epigallocatechin gallate
PMA: Phorbol 12-myristate 13-acetate

CD4 is a cell-surface glycoprotein expressed on immature thymocytes and a part of mature T cells. By binding to MHC class II molecules, CD4 serves as a coreceptor during antigen recognition by the T-cell receptors. CD4 plays an important role in T-cell activation because CD4 markedly increases the sensitivity of T cells to antigens presented by MHC class II through transmembrane signal transduction.[15-17]

CD4 also serves as a receptor for HIV-1 because the viral envelope protein gp120 binds to the D1 domain of CD4 with high affinity.[18] Furthermore, increasing evidence indicates that CD4 not only acts as a binding target of HIV vesicles but also plays an important role in the productive infection process. HIV-1 binding to the cell-surface CD4 has been shown to activate a number of transducing proteins, such as nuclear factor κB or mitogen-activated protein kinases, and HIV-1–infected resting CD4+ T cells are triggered to produce HIV-1 in response to this stimulation.[19-21]

In the present study we aimed to investigate the mechanisms of the anti-HIV effect of green tea polyphenols, and for this purpose, we chose 2 different kinds of tea catechin, EGCG and epicatechin gallate (ECG). We clearly demonstrated that EGCG, but not ECG, directly binds to the cell-surface CD4 molecules, competitively interfering with the binding of both anti-CD4 antibodies and the HIV-1 recombinant gp120.

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METHODS

Reagents and antibodies

ECG, EGCG, phorbol 12-myristate 13-acetate (PMA), and anti-β actin mAbs were purchased from Sigma (Saint Louis, Mo). FITC-conjugated or nonconjugated anti-CD11a, anti-CD3, anti-CD54, and anti-CD4 mAbs (clone sk-3) were obtained from BD Pharmingen (San Diego, Calif). The goat polyclonal antibody to CD4 cytoplasmic tail (c-18) was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif), and mouse anti-CD4 mAb (clone no. 34930.111) used for Western blotting and horseradish peroxidase–conjugated rabbit anti-mouse IgG (H+L) used as the secondary antibody for ELISA were from R&D Systems (Minneapolis, Minn).

Isolation of peripheral blood $CD4^+$ T cells

$CD4^+$ T cells were obtained from venous blood drawn from normal healthy volunteers, as described previously.[22] Briefly, PBMCs were isolated by means of centrifugation on a Ficoll-Paque density gradient (Amersham Pharmacia, Uppsala, Sweden). $CD4^+$ T cells were obtained by using a magnetic cell separation system (Miltenyi Biotec, Bergish Gladbach, Germany). For this purpose, PBMCs were incubated with microbead-coupled anti-CD14 mAbs, and the magnetically labeled cells were depleted by retaining them on a magnetic cell separation system column to remove $CD4^+CD14^+$ monocytes. Cells were then incubated with microbead-conjugated anti-CD4 mAbs, and magnetically labeled lymphocytes were obtained by means of positive selection. The purity of the $CD4^+CD14^-$ T cells used in the experiments was 98% to 99%, as analyzed by means of flow cytometry (data not shown). The isolated $CD4^+$ T cells were used immediately after isolation in all experiments.

Cell culture

The human myelomonocytic leukemia cell line U937 and the human promyelocytic leukemia cell line HL-60 were obtained from American Type Culture Collection. Cells were maintained in RPMI-1640 medium supplemented with 5% FCS, 60 mg/mL penicillin G, and 100 mg/mL streptomycin sulfate in a 5% CO_2 and 95% air atmosphere at 37°C.

Detection of cell-surface molecules by means of flow cytometry

$CD4^+$ T cells, U937 cells, and HL-60 cells were incubated in the presence of different concentrations of ECG or EGCG in RPMI-1640 medium supplemented with 0.2% BSA (Sigma) for different times at 37°C. A large amount of cooled (4°C) PBS was then added immediately after incubation to halt the interaction between cells and the catechin present in the medium. Cells were washed twice with PBS and used for the analysis of cell-surface molecules. Unfixed cells were stained with FITC-conjugated mouse mAbs against CD3, CD4 (clone sk-3), CD11a, and CD54. FITC-conjugated mouse IgG mAb of unrelated specificity was always used as a control. Data were collected in a FACS Calibur (Becton-Dickinson, Mountain View, Calif) and analyzed by using CellQuest software (Becton Dickinson).

Detection of endocytosis of cell-surface CD4 by means of flow cytometry

Isolated and unfixed $CD4^+$ T cells were stained with FITC-conjugated anti-CD4 mAbs for 30 minutes at 4°C, and after 2 washings with PBS, cells were incubated without or with either EGCG (50 µmol/L) or PMA (100 ng/mL) in RPMI-1640 containing 0.2% BSA for 1 hour at 37°C. PMA, a strong inducer of CD4 endocytosis, was used as a positive control.[23] After treatment, antibodies bound to the cell surface were removed by incubating cells with acidic PBS (pH 3.0), and cells were again washed twice in PBS. The green fluorescence of the antibodies internalized by means of endocytosis was evaluated in the FACS Calibur.

Western blotting

The isolated $CD4^+$ T cells were incubated with EGCG or ECG (both at 50 µmol/L) in RPMI-1640 containing 0.2% BSA for 1 hour at 37°C and then washed twice with PBS and lysed with 0.5 mL of TS buffer (50 mmol/L Tris-HCl [pH 7.6] and 150 mmol/L sodium chloride) containing protease inhibitors and 1% Triton-X. The protein concentration of the clear supernatant collected by means of centrifugation was evaluated by using the BCA protein assay (Pierce, Rockford, Ill) and was adjusted to give the final concentration of 2 mg/mL. After addition of 2-mercaptoethanol (2%), samples were boiled for 5 minutes and used for the experiments.

SDS-PAGE was performed as described previously[24] by using a Laemmli buffer system and 10% polyacrylamide resolving slab gels. Proteins were transferred electrophoretically to polyvinylidine difluoride (Immunobilon P; Millipore, Bedford, Mass). After blocking with 10% skim milk, the membrane strips were incubated with either mouse anti-CD4 (clone no. 34930.111) or anti-β-actin antibody. Membrane strips were then incubated with biotinylated anti-mouse IgG (Vector Laboratories, Inc, Burlingame, Calif), followed by incubation with avidin-biotin complex (ABC kit, Vector) solution. Color development was carried out with a solution of 3,3´-diaminobenzidinetetrahydrochloride (DAB kit; DAKO, Carpinteria, Calif).

Detection of EGCG binding to CD4 by a competitive ELISA assay

A competitive ELISA system was applied for the detection of mutual binding between EGCG and the CD4 molecule. Initially, protein extracts of U937 containing CD4 molecules were obtained by means of repeated freeze-thawing and sonication. Flat-bottomed 96-well microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with the goat polyclonal antibody recognizing the carboxy-terminus of CD4 (2 µg/mL). For blocking nonspecific binding, the plate was incubated with 1% BSA/PBS, and the U937 cell lysate was added to allow the antibodies to catch the CD4 molecules of the lysate. Wells were then incubated with various concentrations of ECG or EGCG for 1 hour at 37°C, followed by means of incubation with the mAb recoginizing the extracellular domain of CD4 (clone sk-3, 500 ng/mL) for 1 hour at 37°C. After incubation with horseradish peroxidase–conjugated rabbit anti-mouse IgG secondary antibody, wells were then treated with *o*-phenylenediamine (Nacalai Tesque, Kyoto, Japan) in H_2O_2 for color development. The reaction was stopped with 2 mol/L H_2SO_4 and analyzed with an ELISA plate reader at 490 nm.

Gp120 binding assay

$CD4^+$ T cells, prepared as mentioned above, were incubated for 1 hour at 37°C with differing concentrations of ECG or EGCG and dissolved in RPMI-1640 containing 0.2% BSA. After the incubation, cells were washed with PBS and incubated with 1 µg/mL FITC-conjugated recombinant gp120 (Immunodiagnostics, Woburn, Mass) for 30 minutes at room temperature. The fluorescence intensity of gp120-FITC bound to the surface of lymphocytes was measured with the FACS Calibur.

Statistical analysis

The unpaired Student *t* test was used to determine statistical significance. Differences at a *P* value of less than .05 were considered statistically significant.

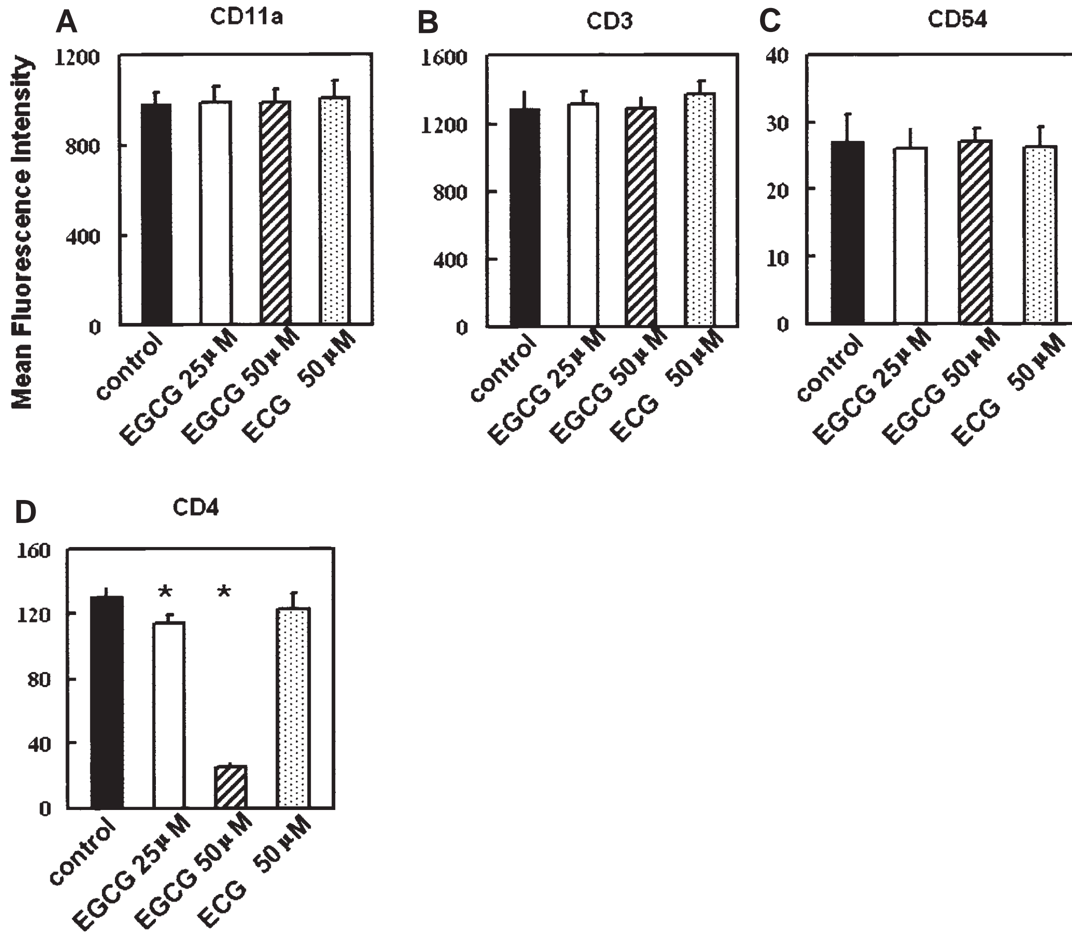

FIG 1. Flow-cytometric analysis of the expression of CD11a **(A)**, CD3 **(B)**, CD54 **(C)**, and CD4 **(D)** on lymphocytes treated without *(filled box)* or with catechins at various concentrations (25 μmol/L EGCG *[open box]*, 50 μmol/L EGCG *[shaded box]*, or 50 μmol/L ECG *[dotted box]*). The data are expressed as the means ± SD of results from 3 independent experiments. *Statistical significance.

RESULTS

EGCG treatment attenuates the CD4 expression of T cells and leukemia cell lines

As shown in Fig 1, EGCG caused a significant decrease in the expression of CD4 in a dose-dependent manner. The effect was evident with EGCG at 25 μmol/L but more prominent at 50 μmol/L ($P < .05$ for both doses). Thus the dose of 50 μmol/L EGCG was chosen for the subsequent experiments. The expressions of CD3, CD54, and CD11a, however, were not significantly affected by EGCG. CD54 and CD11a were chosen here because they are important costimulatory molecules in the T-cell activation by antigen-presenting cells, as well as in the T-T and T-B cell interaction, in which the CD4 molecule plays the central role.

EGCG-treated T cells were stained with trypan blue or double-stained with FITC-conjugated AnnexinV and propidium iodide to exclude the possibility that the CD4 downregulation was dependent on the cytotoxic effect of EGCG on lymphocytes. The trypan blue exclusion test result was negative (data not shown), and also, no increase in either the Annexin V–positive or propidium iodide–positive cell populations was observed (data not shown), suggesting that the decrease of CD4 expression is not dependent on cell death.

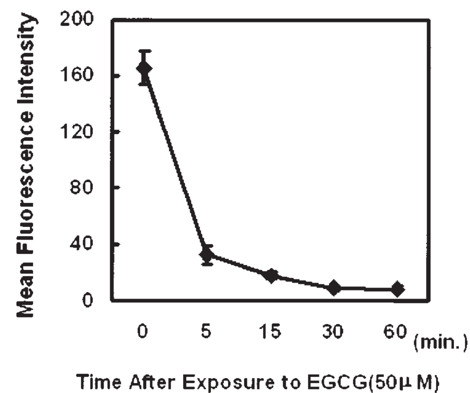

FIG 2. The time course of CD4 downregulation after exposure to EGCG. Lymphocytes were exposed to EGCG at 50 μmol/L, and the expression intensity of CD4 was measured at the various time points by means of flow cytometry. The data are expressed as the means ± SD of results from 3 independent experiments.

Basic and clinical immunology

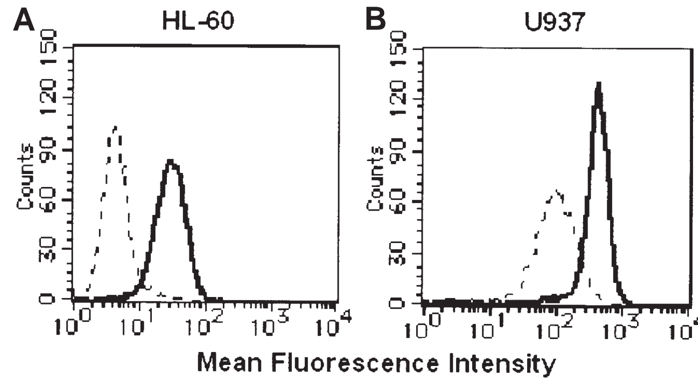

FIG 3. The CD4 expression of HL-60 **(A)** and U937 **(B)** incubated without *(solid line)* or with *(dashed line)* EGCG (50 µmol/L) evaluated by means of flow cytometry.

Next, we evaluated the time course of the CD4 downregulation after exposure to EGCG. As shown in Fig 2, 5-minute exposure was enough to cause an 80.7% ($P < .05$) decrease of CD4 surface expression on lymphocytes, and exposure for more than 30 minutes caused an almost complete disappearance of the cell-surface CD4. Experiments were repeated with 4% paraformaldehyde instead of cooled PBS to confirm that the addition of cooled PBS was able to halt the interaction between EGCG and cells, and exactly the same results were obtained (data not shown).

The $CD4^+$ human leukemia cell lines U937 and HL-60 were also investigated to evaluate whether this inhibitory effect of EGCG on CD4 expression is T-cell specific. Both HL-60 and U937 expressed a substantial amount of CD4 on the cell surface, and incubation with EGCG at 50 µmol/L considerably decreased CD4 expression on both cell lines (Fig 3). Although this effect was common to both cell lines, it was more pronounced on HL-60 cells, with an almost complete loss of the CD4 expression. The effect on U937 cells was rather small.

EGCG does not induce CD4 endocytosis

CD4 was strongly expressed on T cells (Fig 4, *A*), and acid removal of the anti-CD4 antibodies resulted in the decrease of the fluorescence intensity to the same levels of cells treated with isotype-matched control IgG (Fig 4, *B*). Interestingly, although no internalized fluorescence was observed when antibody removal was performed immediately after incubation with anti-CD4 antibodies, a small amount of internalized fluorescence was detected when cells were incubated in medium for 1 hour at 37°C without any treatment and before antibody removal (Fig 4, *C*). This can be attributed to the constitutive CD4 endocytosis, which is consistent with previous reports.[25,26] Incubation with 100 ng/mL PMA, a well-known potent inducer of CD4 endocytosis, significantly increased the acid-resistant intracellular fluorescence ($P < .05$; Fig 4, *D*).[23] Treatment with EGCG, however, did not result in a significant increase in the intracellular fluorescence, indicating that the downregulation of CD4 was not the consequence of CD4 endocytosis (Fig 4, *E*).

Total CD4 volume is not affected by EGCG treatment

The total volume of CD4 was evaluated by means of Western blotting after EGCG treatment and compared with that of untreated cells to exclude the possibility that the decreased CD4 expression was dependent on shedding of CD4 molecules from the cell surface. Bands of 55-kd molecular weight corresponding to CD4 were detected in the untreated, as well as catechin-treated, cell samples, and no significant difference in the expression intensity was observed among these 3 samples (Fig 5). These data indicate that the decreased CD4 expression is not dependent on loss of CD4 protein from lymphocytes.

EGCG competes with and inhibits binding of anti-CD4 mAb to CD4

To investigate the possibility that EGCG directly binds to cell surface CD4 and competitively inhibits the binding of anti-CD4 antibodies, we established a sandwich competitive ELISA assay. EGCG markedly inhibited the binding of the sk-3 antibody to the captured CD4 molecule. Even with the low concentration tested (50 µmol/L), the inhibition rate was up to 67.1% ± 6.9%, and the efficiency of inhibition further increased in a dose-dependent manner (Fig 6). ECG, however, had no significant inhibitory effect on sk-3 binding to captured CD4 when tested at 50 and 100 µmol/L, and only a small effect (28.0% ± 8.9%) was observed when tested at an extremely high concentration (200 µmol/L). These data correlate well with the findings of the flow-cytometric analysis, indicating that the observed decrease of CD4 expression is not due to the actual loss of CD4 molecules but results from a competitive inhibition of EGCG on binding of the anti-CD4 antibody to the corresponding molecule.

Interference of EGCG with gp120 binding to lymphocytes

To investigate whether the binding of EGCG to the CD4 molecule on human lymphocytes is capable of inhibiting the binding of gp120 to CD4 in a similar way

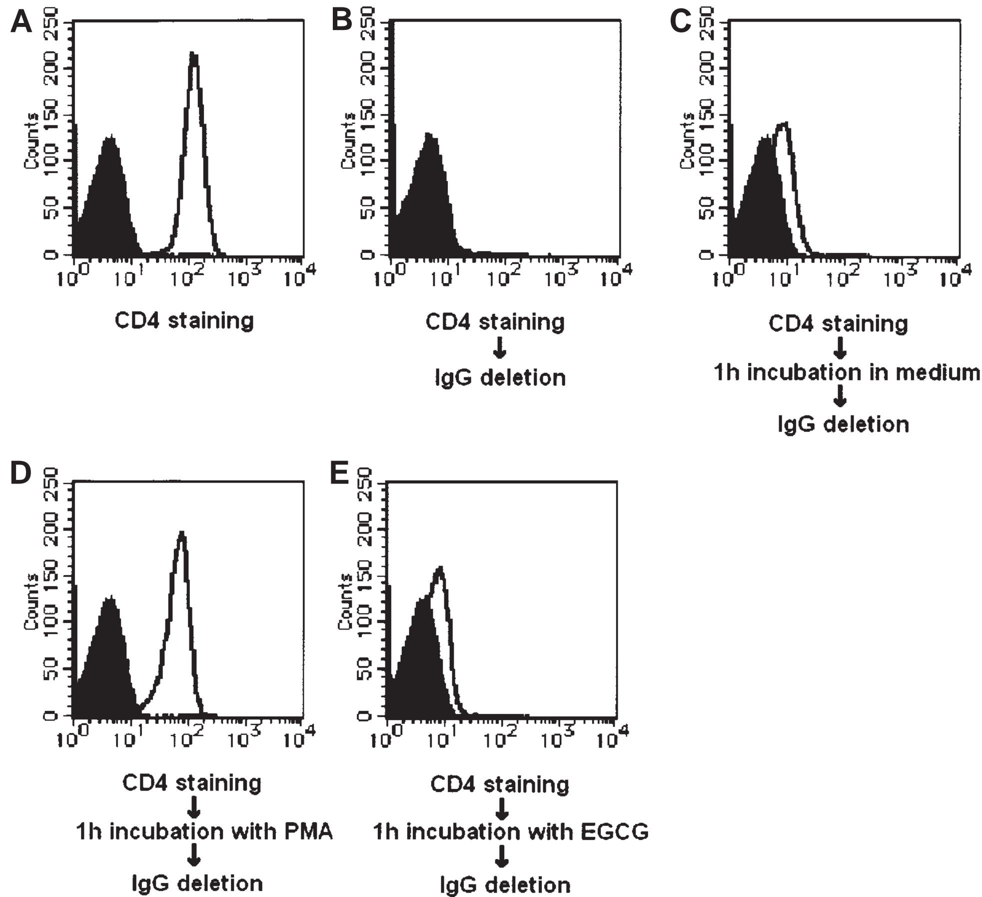

FIG 4. Detection of CD4 endocytosis: CD4 expression on normal human T lymphocytes **(A)**; acid treatment immediately after staining with FITC-conjugated anti-CD4 **(B)**; 1-hour incubation of lymphocytes in medium before acid treatment **(C)**; lymphocytes incubated with PMA **(D)** or EGCG **(E)** before acid treatment. The *filled line* represents the negative control.

to the anti-CD4 antibody, we analyzed the binding ability of FITC-conjugated recombinant gp120 to the EGCG-treated and EGCG-untreated CD4+ T cells. Isolated lymphocytes were treated with various concentrations of EGCG and then incubated with the FITC-conjugated gp120. As shown in Fig 7, EGCG markedly inhibited the binding of gp120 to lymphocytes in a dose-dependent manner, but complete inhibition of gp120 binding could not be achieved, even with the highest concentration of EGCG tested (100 µmol/L). In contrast, treatment with ECG did not alter the binding capacity of gp120 to the cell surface, even with the highest concentration.

DISCUSSION

Previous reports have demonstrated that EGCG inhibits HIV-1 infection of lymphocytes in multiple manners, and obstruction of viral attachment to the target cells is one of the suggested mechanisms of the anti-HIV-1 effect of EGCG.[13,14] Because the CD4 molecule is the receptor for HIV-1 and the regulation of CD4 expression has an important role in the viral life cycle, in the present study we investigated the mutual interaction between CD4 and EGCG.

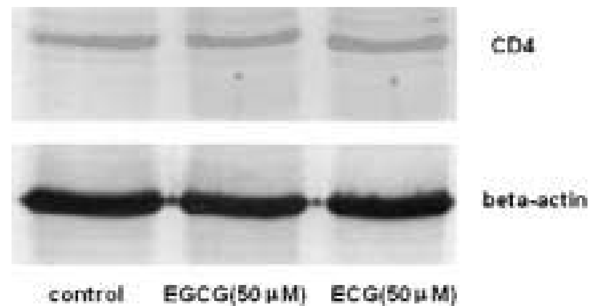

FIG 5. Western blot analysis of lymphocytes incubated without *(left lane)* or with ECG *(middle lane)* or EGCG *(right lane)*. Membranes were stained with either anti-CD4 *(upper)* or anti-β-actin *(lower)*.

Initially, we demonstrated that incubation of CD4+ T cells with EGCG, but not ECG, resulted in a significant decrease in the binding of anti-CD4 antibodies to the cell surface, which means that EGCG seemed to down-modulate the cell-surface expression of CD4. This down-regulation was observed not only on lymphocytes but also on other CD4+ leukemia cell lines, suggesting that it is not a lymphocyte-specific effect. No changes in the expression of other cell-surface molecules were

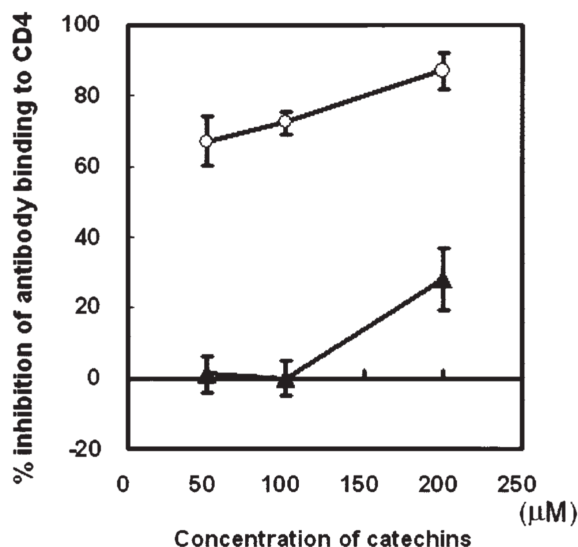

FIG 6. The competitive binding of EGCG and anti-CD4 antibody to the CD4 molecule analyzed by mean of ELISA. The *vertical axis* represents the percentage inhibition of the binding of anti-CD4 mAb to the CD4 molecule and was obtained on the basis of the ratio to the control wells. Data are expressed as the mean ± SD of results from 3 wells.

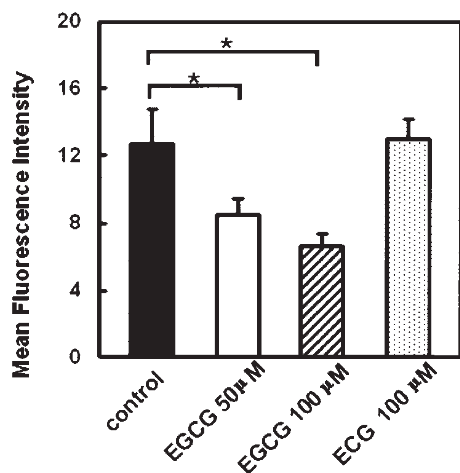

FIG 7. EGCG interference with GP120 binding to CD4. The *vertical axis* represents the mean fluorescence intensity of FITC-conjugated gp120 bound to lymphocytes obtained by means of flow cytometry. The data are expressed as mean ± SD of results from 3 independent experiments. *Statistical significance.

observed, leading us to conclude that the downregulation was a CD4-specific event.

Cell-surface CD4 expression is regulated in multiple manners, such as CD4 endocytosis, intracellular retention of the molecular complex, and shedding from the cell surface.[27,28] Many reports have shown that various stimuli, such as antigen presentation or T-cell receptor cross-linking, and especially HIV infection can induce CD4 downregulation.[29,30] The reduction of CD4 is supposed to prevent superinfection of HIV-1, contributing to cohabitation of the virus without killing the host cells.[31]

Endocytosis of CD4 is known to be inducible by various kinds of stimuli. For instance, exposure of lymphocytes to gangliosides or phorbol esters, such as PMA, results in rapid endocytosis of CD4, which occurs in a few minutes.[29,32] In our experiment the CD4 downregulation induced by EGCG treatment also required only a few minutes to occur, but no increase in the internalized CD4 level was observed. Another reported mechanism of the CD4 downregulation is the retention of CD4 molecules into the endoplasmic reticulum. Gp160, an intracellular precursor of gp120, interferes with CD4 transportation to the cell surface, and consequently, CD4 down-regulation occurs.[33] However, this reaction requires the spontaneous disappearance of CD4 from the cell surface. Because EGCG-induced CD4 downmodulation is an event that occurs within a few minutes, this mechanism was also excluded.

Under certain circumstances, CD4 is released from the cell surface and circulates in the plasma as soluble CD4. The precise mechanisms of CD4 shedding from the cell membrane are, however, still poorly understood. Although several reports have described the increase of soluble CD4 in the plasma of patients with various infectious diseases, especially during HIV-1 infection,[34-36] at present, no substances capable of inducing CD4 shedding in vitro have been reported. In our study the total amount of CD4 protein contained in the cell lysates, as detected by means of Western blotting, did not decrease after treatment with EGCG, suggesting that the CD4 degradation is not likely to be dependent on CD4 shedding from the cell surface.

Another possibility is that the reduction of CD4 detected by means of flow cytometry is not due to the actual loss of the molecule itself. That means that EGCG might have the ability to bind to the CD4 molecule and competitively inhibit the binding of antibodies to CD4. Using a sandwich ELISA assay, we clearly demonstrated that EGCG, but not ECG, binds to CD4 and blocks antibody binding. Several peptides or nonpeptic organic compounds, such as gp120, of HIV-1 have been reported to be potential ligands of CD4.[27] Huang et al[37] made a computational screening of approximately 150,000 nonpeptic organic compounds in a chemical database for potential ligands of CD4 and developed a compound named TJU103 as a novel CD4 ligand and also a specific inhibitor of $CD4^+$ T-cell–mediated responses. Although they examined a quite large number of chemical compounds, they did not refer to EGCG or any kind of polyphenol as the ligand of CD4. Furthermore, the structure of TJU103 did not resemble that of EGCG.

We then examined whether EGCG can antagonize gp120 binding as a ligand for CD4 and could demonstrate that EGCG effectively interfered with gp120 binding to lymphocytes in a dose-dependent manner. These findings are consistent with those of the previous report, which demonstrated that EGCG can inhibit the binding of HIV-1 vesicles to lymphocytes.[13] Thus the blocking effect of EGCG on gp120 as a ligand for CD4 might be one of the principal mechanisms of the anti-HIV-1 properties of EGCG.

In summary, for the first time, we demonstrated that EGCG binds to CD4 and blocks antibody and gp120 binding. It opens new doors for the clinical application of EGCG as a new anti-HIV-1 drug. However, we should be careful to avoid false expectations of a single compound. Although in addition to the anti-HIV effect, many other health benefits of EGCG, such as cancer prevention and antioxidative properties, have been reported, doses higher than that achievable in patients' sera were used in most of the reports, as well as in ours. To our knowledge, in previous reports[38,39] the serum concentration achieved after oral ingestion of EGCG was approximately 1 μmol/L. Consequently, further investigation is required on how to achieve the serum concentrations necessary for prevention of gp120 binding to CD4, either by improving the absorption of catechins or by developing other strategies, such as the use intravenous administration.

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