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Inhibitory Effect of (−)-Epigallocatechin 3-Gallate, a Polyphenol of Green Tea, on Neutrophil Chemotaxis in Vitro and in Vivo

KATSUHIKO TAKANO,* KEIKO NAKAIMA, MAKOTO NITTA, FUTOSHI SHIBATA, AND HIDEO NAKAGAWA

Department of Physiological Chemistry, Faculty of Pharmaceutical Sciences, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194, Japan

The effect of (−)-epigallocatechin 3-gallate (EGCG), a major polyphenol of green tea, on neutrophil migration has been studied using multiwell-type Boyden chambers in vitro and a fluorescein isothiocyanate-labeled ovalbumin (FITC−OVA)-induced rat allergic inflammation model in vivo. EGCG inhibited rat neutrophil chemotaxis toward cytokine-induced neutrophil chemoattractant-1 (CINC-1) in a concentration-dependent manner. In addition, CINC-1-induced neutrophil chemotaxis was suppressed by the pretreatment of rat neutrophils with EGCG at the concentration over 15 μg/mL. EGCG caused concentration-dependent suppression of the transient increase in CINC-1-induced intracellular free calcium level in both rat neutrophils and rat CXC chemokine receptor 2 (CXCR2)-transfected HEK 293 cells. EGCG inhibited CINC-1 production by IL-1β-stimulated rat fibroblasts (NRK-49F cells) and lipopolysaccharide-stimulated rat macrophages at the concentration over 50 μg/mL, a comparatively high concentration. Oral administration of EGCG (1.0 mg or 1.5 mg/rat) at 1 h before the challenge with FITC−OVA suppressed neutrophil infiltration into the air pouch (inflammatory site) in the air-pouch type FITC−OVA-induced allergic inflammation in rats. Chemokine levels in the pouch fluids, however, were not influenced by EGCG administration. The results suggest that EGCG suppressed neutrophil infiltration by a direct action on neutrophils, but not by indirect actions, including the suppression of chemokine production at the inflammatory site.

KEYWORDS: (−)-Epigallocatechin 3-gallate; neutrophil; CINC-1; chemotaxis; inflammation

INTRODUCTION

Green tea (leaf extract of *Thea sinensis* L.) is not only taken in Japan, but also in Asia and recently, worldwide. Green tea extract contains catechins, including (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin 3-gallate, and (−)-epigallocatechin 3-gallate (EGCG), which is the major component in green tea. Catechins are thought to be strong antioxidants and EGCG is most effective in reducing reactive oxygen species (*1*, *2*). It has been demonstrated that green tea catechins are effective antioxidants, especially when they interact with the intrinsic α-tocopherol in human low-density lipoprotein (*3*). On the other hand, the anticancer effects of EGCG are manifold, including inhibition of tumor growth, invasion, angiogenesis, and induction of apoptosis in tumor cells (*4*−*7*).

Furthermore, EGCG has been reported to inhibit inflammatory processes (*8*−*11*). Oxazolone-induced mouse type IV allergy was significantly suppressed by a percutaneous administration of EGCG and *O*-methylated EGCG at a dose of 0.13 mg/ear (*10*). Hofbauer et al. (*11*) demonstrated that EGCG is able to significantly inhibit neutrophil transmigration through monolayers of endothelial cells. EGCG has been shown to inhibit histamine release from rat basophilic leukemia (RBL-2H3) cells mainly by inhibiting tyrosine phosphorylation of proteins including pp125^{FAK} (*8*). In addition, the effect of EGCG on inflammatory responses has been demonstrated at molecular levels, such as modulation of nuclear factor κB activation (*12*, *13*). However, relatively few studies concerning the effect of EGCG on neutrophil migration into inflammatory sites have been reported. In previous papers, we demonstrated that neutrophil infiltration in rat inflammation models is highly regulated by rat CXC-chemokines, including cytokine-induced neutrophil chemoattractant (CINC)-1, -2, and -3 (*14*, *15*), and rat neutrophil has a common receptor CXCR2 for three types of CINCs (*16*). In the present study, therefore, we undertook to examine the inhibitory effect of EGCG on the CINC-1-mediated neutrophil chemotaxis in vitro using multiwell-type Boyden chambers. The in vivo effect of EGCG on the neutrophil infiltration into an inflammatory site was also studied by using an air-pouch-type rat allergic inflammation model. We have demonstrated here that EGCG suppresses neutrophil migration through a direct effect on neutrophils.

MATERIALS AND METHODS

Chemicals. EGCG, polymyxin B sulfate and low-endotoxin bovine serum albumin (BSA) were obtained from Sigma-Aldrich Japan, Tokyo.

* Author to whom correspondence should be addressed (telephone +81-76-434-7542; fax +81-76-434-5171; e-mail takanok@ms.toyama-mpu.ac.jp).

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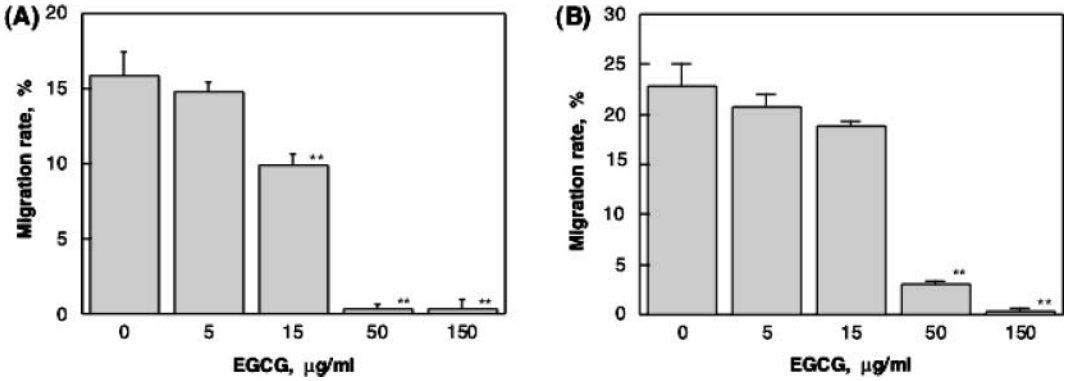

Figure 1. Inhibitory effect of EGCG on CINC-1-mediated neutrophil chemotaxis in vitro. Neutrophil chemotaxis was determined in vitro using 96-multiwell-type Boyden chambers. (**A**) The upper wells of plates contained 300 μL of rat neutrophil suspension (10^7 cells/mL of the medium), while lower wells contained 400 μL of the chemoattractant solution (10^{-8} M CINC-1) containing various amounts of EGCG. (**B**) The upper wells of plates contained the rat neutrophil suspension and various amounts of EGCG, while lower wells contained 10^{-8} M CINC-1. Data are expressed as means ± SEM of 6 determinations. Significant difference from the control; **, $p < 0.01$.

Proteose peptone and lipopolysaccharide (LPS) were purchased from Difco Laboratories. Dulbecco's modified Eagle's medium (DMEM) and sodium carboxymethylcellulose (CMC−Na; Cellogen F3H) were obtained from Nissui Pharmaceutical Co., Ltd., Tokyo, and Daiichi Kogyo Seiyaku, Niigata, Japan, respectively. All other reagents were commercial products of the highest grade available.

Animals. Male Wistar rats (4-week-old) were purchased from Sankyo Lab Service Co. (Tokyo, Japan) and maintained in a temperature controlled room (22 ± 2 °C) with a 12-h light/dark cycle. The present animal experiments were done under the guide for animal experiments, Toyama Medical and Pharmaceutical University.

Effect of EGCG on CINC-1-Induced Neutrophil Chemotaxis in Vitro. Neutrophils were harvested about 16 h after intraperitoneal injection of Krebs/Ringer bicarbonate solution containing 1% (w/v) casein (120 mL/kg body wt) to male Wistar rats (body wt, 300−400 g). Rat neutrophils were washed and finally suspended at 10^7 cells/mL of medium (RPMI-1640 medium containing 0.1% low-endotoxin BSA). Chemotaxis assay in vitro was carried out by using two plates of 96-multiwell-type Boyden chambers that were separated by a polycarbonate filter with pores 2 μm in diameter according to a minor modification (*17*) of the method of Watanabe et al. (*18*). EGCG and CINC-1 were dissolved in the medium, and three types of experiments were designed to estimate the effect of EGCG on the CINC-1-induced neutrophil chemotaxis in vitro. In the first experiment, the upper wells of the plates contained 300 μL of rat neutrophil suspension (10^7 cells/mL of the medium), while lower wells contained 400 μL of the chemoattractant solution (10^{-8} M CINC-1) containing various amounts of EGCG. In the second experiment, upper wells contained the neutrophil suspension together with EGCG, while lower wells contained 10^{-8} M CINC-1 alone. In the third experiment, neutrophils were incubated with various amounts of EGCG at 25 °C for 20 min, and washed twice with the medium to remove EGCG. The upper wells contained the EGCG-treated neutrophils, while lower wells contained 10^{-8} M CINC-1. As an index of chemotaxis, the number of neutrophils migrating into the lower wells during an 80-min incubation at 37 °C in a CO_2 incubator was expressed as a percentage (migration rate) of the neutrophils (3×10^6 cells/well) applied to the upper wells.

Measurement of Intracellular Free [Ca^{2+}] Levels. Rat neutrophils were collected from male Wistar rats as described above. HEK 293 cells stably expressing rat CXC chemokine receptor 2 (CXCR2) were prepared as described in a previous paper (*16*) and maintained in DMEM supplemented with 10% (v/v) fetal calf serum. Using rat neutrophils and CXCR2-transfected HEK 293 cells, intracellular free [Ca^{2+}] levels of both cells were measured by a procedure described previously (*19*). Briefly, rat neutrophils (10^7 cells/mL) or CXCR2-transfected HEK 293 cells (5×10^6 cells/mL) were incubated with the calcium indicator fura-2 acetoxymethyl ester (2.5 mM) in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing 10 mM HEPES, 0.25% (w/v) BSA and 10 mM glucose (pH 7.4) for 30 min at 37 °C. After incubation, the neutrophils and CXCR2-transfected HEK 293 cells were washed twice and resuspended at 10^6 cells/mL and 5×10^5 cells/mL, respectively, with the buffer. In the presence of 1 mM $CaCl_2$, fluorescence changes were monitored by a fluorescence spectrophotometer at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. Fura-2-loaded cells were stimulated with 10^{-8} M CINC-1 in the presence of EGCG, then lysed with 0.1% (v/v) Triton-X100; finally Ca^{2+} was chelated by 6 mM EGTA. Intracellular free [Ca^{2+}] levels were calculated as described by Grynkiewicz et al. (*20*).

Effect of EGCG on Chemokine Production in Vitro. The effect of EGCG on chemokine production was studied using interleukin (IL)-1β-stimulated fibroblasts (NRK-49F cells) and LPS-stimulated rat macrophages in culture. Normal rat kidney fibroblasts (NRK-49F cell line) were cultured in DMEM supplemented with 5% (v/ v) fetal calf serum, 25 mM HEPES, penicillin 0.1 mg/mL, and streptomycin 0.1 mg/mL in 96-multiwell dishes. When the NRK-49F cells reached a confluent monolayer, the dish was washed with serum-free medium, and cultured with various amounts of EGCG for 24 h in DMEM containing recombinant human IL-1β (10^{-9} M), polymyxin B sulfate (10 μg/mL) and 0.1% (w/v) low-endotoxin BSA.

Macrophages were collected from male Wistar rats (body wt, 250−300 g) by peritoneal lavage with PBS 4 days after intraperitoneal injection of 10% (w/v) proteose peptone (35 mL/kg body wt) in sterilized 0.9% (w/v) NaCl. Cells were washed and suspended in DMEM supplemented with 5% (v/v) fetal calf serum at $1-2.5 \times 10^6$ cells/mL. The cells were cultured for 24 h in 96-multiwell dishes at 37 °C in a CO_2 incubator, and nonadherent cells were removed. Adherent cells (macrophages) were washed with serum-free medium and cultured with various amounts of EGCG for 24 h in DMEM containing LPS (3 μg/mL) and 0.1% (w/v) low-endotoxin BSA.

The conditioned medium was centrifuged at 700 *g* for 20 min at 4 °C, and the CINC-1 level in the supernatant was determined by a specific enzyme-linked immunosorbent assay (ELISA), as described in a previous paper (*21*).

Determination of Cell Viability. Neutrophils, macrophages, and NRK-49F cells were incubated with various concentrations of EGCG under an appropriate condition described above. After incubation, the cells were removed from wells by the addition of PBS containing 0.375% (w/v) trypsin and 0.03% EDTA, and the cell viability was determined by dye exclusion test.

Effects of EGCG on FITC−OVA-Induced Allergic Inflammation in Rats. Rat air-pouch type fluorescein isothiocyanate-labeled ovalbumin (FITC−OVA)-induced allergic inflammation was induced as described previously (*15*). Briefly, male Wistar rats (body wt, 140−180 g) were immunized by intradermal injection of 0.5 mL of the FITC−OVA (1 mg/mL)/Freund's complete adjuvant (FCA) emulsion at five sites (0.1 mL/site). On day 7 after the immunization, the rats were reimmunized by intradermal injection at the basal site of the tail with 0.1 mL of the emulsion, and 10 mL of air was injected subcutaneously on the dorsum to make an ellipsoid-shaped air pouch. On the fourth day after the first air injection, 10 mL of air was again

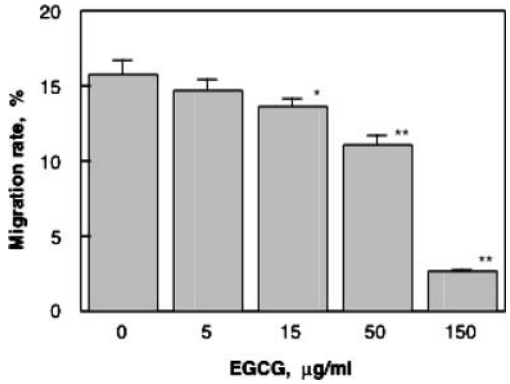

Figure 2. Effect of pretreatment of neutrophils with EGCG on CINC-1-mediated neutrophil chemotaxis in vitro. Neutrophils were incubated with various amounts of EGCG at 25 °C for 20 min and washed twice with the medium. Upper wells contained the neutrophils pretreated with EGCG, while lower wells contained 10^{-8} M CINC-1. Data are expressed as means ± SEM of 6 determinations. Significant difference from the control; *, $p < 0.05$; **, $p < 0.01$.

injected into the preformed air pouch to maintain the pouch. On day 14 after the first immunization, FITC−OVA (7 mg) dissolved in a sterilized solution (7 mL) of 1% (w/v) CMC−Na in saline was injected into the preformed air pouch to induce allergic inflammation. Control rats were injected with 1% (w/v) CMC−Na solution (7 mL) alone. After a 16-h fast, EGCG solution (0.5, 1.0, or 1.5 mg dissolved in 1 mL of distilled water/rat) was orally administered 1 h before the challenge with FITC−OVA, while the vehicle (1 mL of distilled water/rat) was orally given to control rats. The body weights of rats were 206 ± 3 g (means ± SEM) on day 14, and the doses of 0.5, 1.0, or 1.5 mg/rat of EGCG were equivalent to those of about 2.4, 4.9, or 7.3 mg/kg body weight, respectively. At appropriate times after the challenge, a portion (0.3 mL) of the pouch fluid was collected and used for a count of infiltrated cells and determination of chemokine concentrations. The pouch fluid (0.1 mL) was diluted 4−20 times with 0.1% (w/v) trypan blue dissolved in PBS, and then the number of cells was counted microscopically. The remaining fluid (0.2 mL) was diluted 5 times with PBS containing 1% (w/v) BSA and centrifuged at 10,000 *g* for 15 min at 4 °C. The concentrations of CINC-1 and rat macrophage inflammatory protein (MIP)-1α in the supernatant were determined by ELISA specific for each chemokine as described previously (*21*, *22*).

Statistical Analysis. Data are expressed as means ± SEM, and their significances were analyzed by the Student's *t* test.

RESULTS

Inhibitory Effect of EGCG on CINC-1-Induced Neutrophil Chemotaxis in Vitro. Effects of green tea catechins including (+)-catechin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin 3-gallate, and EGCG on CINC-1-induced neutrophil chemotaxis were studied using multiwell-type Boyden chambers in vitro. When catechins together with 10^{-8} M CINC-1, a potent rat neutrophil chemoattractant, were added to lower chambers, (−)-epicatechin 3-gallate significantly inhibited the neutrophil chemotaxis only at a high dose (150 μg/mL), while (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin did not have an effect (data not shown). On the other hand, EGCG inhibited rat neutrophil chemotaxis toward CINC-1 in a concentration-dependent manner (**Figure 1A**) and, therefore, we continued the research in focusing only on EGCG.

When EGCG together with neutrophil suspension was added to upper chambers, EGCG inhibited the CINC-1-induced neutrophil chemotaxis in a concentration-dependent manner (**Figure 1B**). Similarly, the neutrophil chemotaxis was concentration-dependently suppressed by the pretreatment of rat neutrophils with EGCG at a concentration over 15 μg/mL (**Figure 2**).

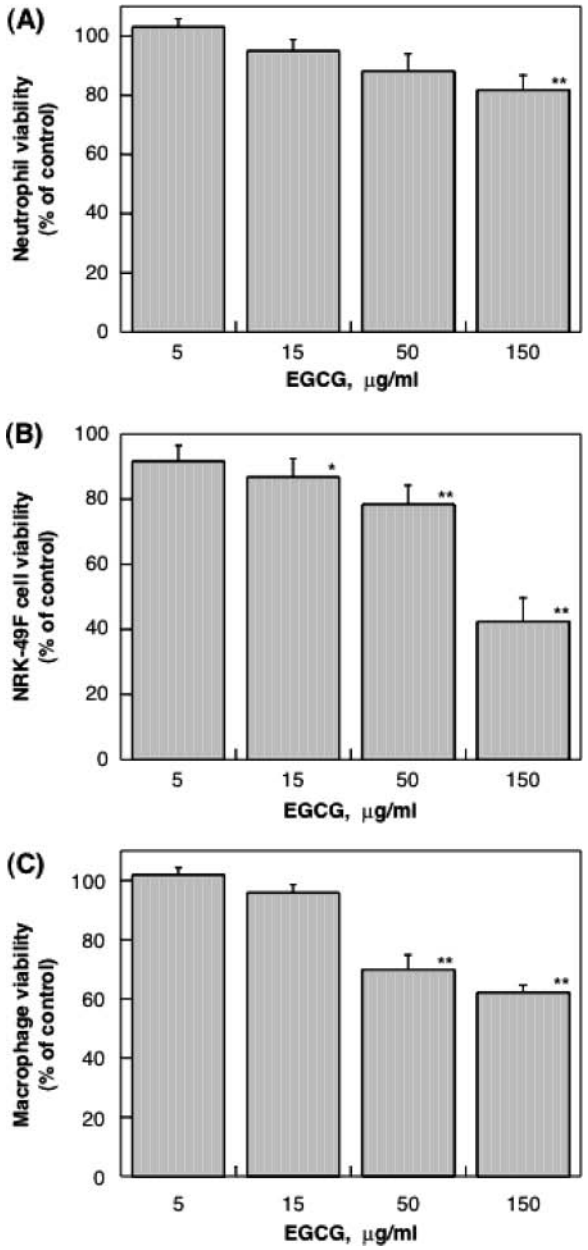

Figure 3. Effect of EGCG on cell viability. Cells were incubated with various concentrations of EGCG, and viabilities of neutrophils (**A**), NRK-49F cells (**B**), and macrophages (**C**) were determined by dye exclusion assay. Data are expressed as means ± SEM of 5 determinations. Significant difference from the control; *, $p < 0.05$; **, $p < 0.01$.

Effect of EGCG on Cell Viability. EGCG-mediated toxicity to rat neutrophils was studied. As shown in **Figure 3A**, EGCG at a concentration of 150 μg/mL slightly reduced neutrophil viability, whereas CINC-1-induced neutrophil chemotaxis was almost completely suppressed by the concentration (150 μg/mL) of EGCG (**Figure 1**). The results suggest that EGCG-mediated suppression of neutrophil chemotaxis is not highly dependent on toxicity to rat neutrophils.

On the other hand, EGCG at the concentration over 50 μg/mL was significantly toxic to NRK-49F cells and rat macrophages (**Figure 3B**,**C**).

Suppression of CINC-1-Induced Calcium Mobilization by EGCG. EGCG concentration-dependently suppressed the CINC-

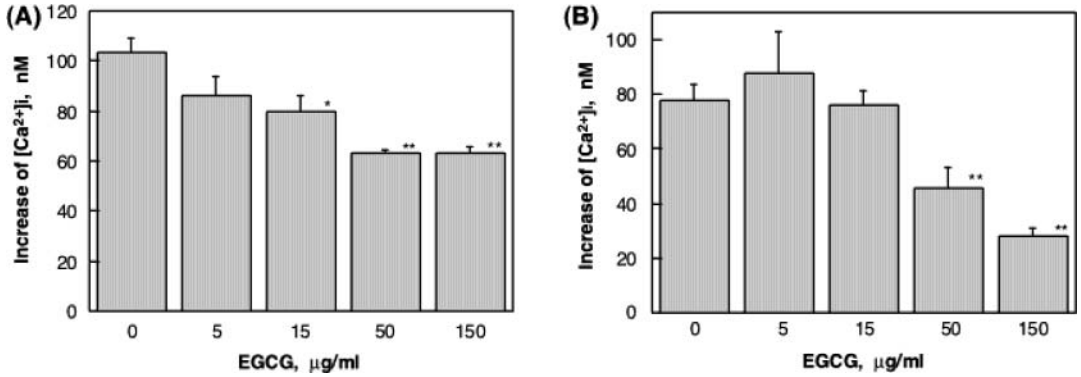

Figure 4. Suppression of CINC-1-induced calcium mobilization by EGCG. Increased intracellular Ca^{2+} levels were measured after stimulation of rat neutrophils (**A**) or CXCR2-transfected HEK 293 cells (**B**) with CINC-1 (10^{-8} M) together with various amounts of EGCG. Data are expressed as means ± SEM of 3 determinations. Significant difference from the control; *, $p < 0.05$; **, $p < 0.01$.

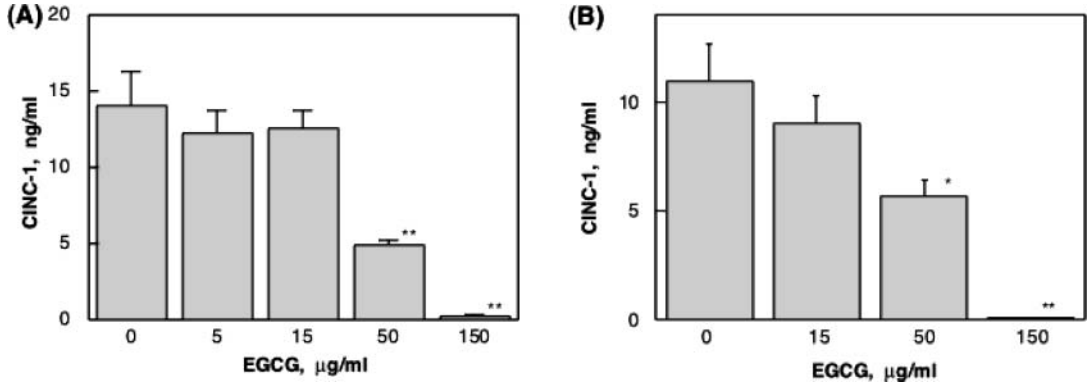

Figure 5. Inhibitory effect of EGCG on CINC-1 production by fibroblasts and macrophages. (**A**) Normal rat kidney fibroblasts (NRK-49F cell line) were cultured with various amounts of EGCG in the presence of human IL-1β (10^{-9} M), polymyxin B (10 μg/mL), and 0.1% (w/v) low-endotoxin BSA at 37 °C for 24 h. (**B**) Rat peritoneal macrophages were cultured with various amounts of EGCG in the presence of LPS (3 μg/mL) and 0.1% (w/v) low-endotoxin BSA at 37 °C for 24 h. The conditioned media were centrifuged, and CINC-1 levels in the resulting supernatants were determined by a specific ELISA. Data are expressed as means ± SEM of 6 determinations. Significant difference from the control; *, $p < 0.05$; **, $p < 0.01$.

1-induced transient increase in intracellular free calcium level ($[Ca^{2+}]_i$) that is one of the functions of neutrophil chemoattractant CINC-1 (**Figure 4A**). In addition, EGCG suppressed the CINC-1-induced calcium mobilization in CXCR2-transfected HEK 293 cells in a concentration-dependent manner (**Figure 4B**).

Inhibitory Effect of EGCG on Chemokine Production in Vitro. IL-1β-stimulated rat fibroblasts (NRK-49F cells) and LPS-stimulated rat macrophages were cultured in the presence of EGCG for 24 h. As shown in panels **A** and **B** of **Figure 5**, EGCG inhibited CINC-1 production by IL-1β-stimulated NRK-49F cells and LPS-stimulated macrophages at a comparatively high concentration (>50 μg/mL).

Effects of EGCG on FITC−OVA-Induced Allergic Inflammation in Rats. EGCG (0.5, 1.0, or 1.5 mg/rat) was orally administered to FITC−OVA-immunized-rats at 1 h before the antigen challenge (**Figure 6**). Oral administration of EGCG (1.0 mg and 1.5 mg/rat) significantly suppressed the infiltration of neutrophils into the air pouch of FITC−OVA-induced allergic inflammation in rats, while EGCG at a dose of 0.5 mg/rat was without effect (**Figure 6**). On the other hand, EGCG (1 mg/rat) had no effect on chemokine concentrations in the pouch fluid (**Figure 7**), suggesting that chemokine production by inflammatory cells was not influenced by EGCG at a dose of 1 mg/rat, which suppressed neutrophil infiltration.

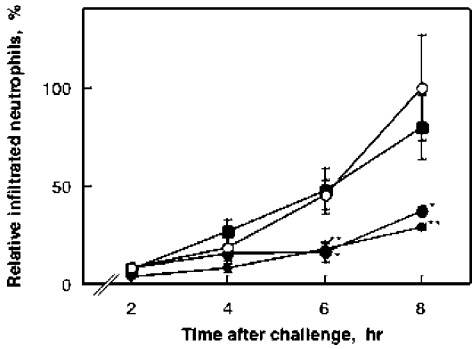

Figure 6. Inhibitory effect of EGCG on neutrophil infiltration in rat air-pouch/FITC–OVA-induced allergic inflammation. At 1 h after oral administration of EGCG (0.5 mg/mL/rat, ■; 1 mg/mL/rat, ●; 1.5 mg/mL/rat, ◆) or vehicle (1 mL water/rat, control, ○), 7 mL of FITC–OVA solution (1 mg/mL of 1% CMC–Na solution) was injected into the preformed air pouches on the backs of rats immunized with FITC–OVA. The pouch fluids were collected at 2, 4, 6, and 8 h after injection of FITC–OVA solution, and the number of neutrophils was determined. These experiments were done independently twice, and the number of neutrophils infiltrating into the air pouch was expressed as a percentage of the number of neutrophils (3.3×10^7 cells/mL of pouch fluid) of control rats at 8 h after challenge. Data are expressed as means ± SEM of 6 or 7 rats. Significant difference from the control group; *, $p < 0.05$; **, $p < 0.01$.

DISCUSSION

In the present study, the effect of green tea catechins on neutrophil chemotaxis in vitro has been determined using a potent rat CXC-chemokine, CINC-1, that was demonstrated to play an important role in neutrophil recruitment in rats (*15*). We have found that EGCG among green tea catechins is most

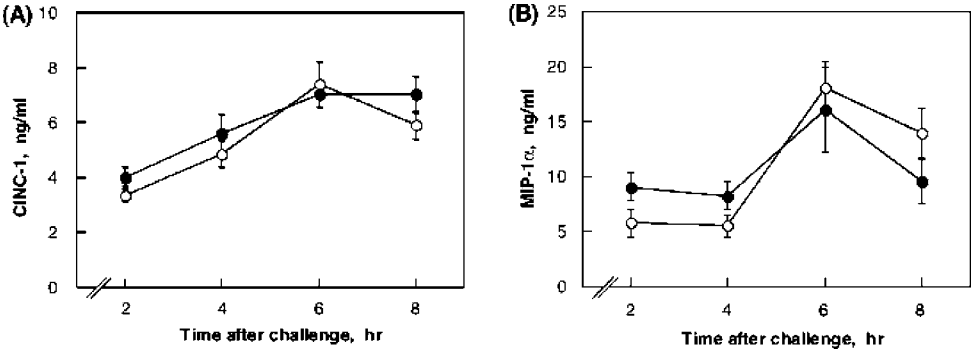

Figure 7. Time course of chemokine concentrations in the pouch fluids in rat air-pouch/FITC–OVA-induced allergic inflammation. At 1 h after oral administration of EGCG (1 mg/mL water/rat, ●) or vehicle (1 mL water/rat; control groups, ○), 7 mL of FITC–OVA solution (1 mg/mL of 1% CMC–Na solution) was injected into the preformed air pouch on the back of rats immunized with FITC–OVA. The pouch fluids were collected at 2, 4, 6, and 8 h after injection of FITC–OVA solution, and the concentrations of CINC-1 (A) and MIP-1α (B) in the pouch fluids were determined by specific ELISA for each chemokine. Data are expressed as means ± SEM of 6 or 7 rats.

effective in reducing neutrophil chemotaxis toward CINC-1. In addition, pretreatment of neutrophils with EGCG inhibited CINC-1-mediated neutrophil chemotaxis in the absence of EGCG (**Figure 2**). The results suggest that once neutrophils contact EGCG in blood, the neutrophils would have a low response to chemokines produced at inflammatory sites. In a previous paper (*16*), we reported that CINCs, including CINC-1, induced a transient increase in intracellular free Ca^{2+} in CINC receptor (rat CXCR2)-transfected HEK 293 cells. We have demonstrated in the present study that EGCG inhibits the calcium mobilization, not only in neutrophils, but also CXCR2-transfected HEK 293 cells (**Figure 4**). However, EGCG may not be a selective inhibitor to the effect of CINC-1 to neutrophils (between chemokine and target cells), because EGCG has been shown to suppress neutrophil chemotaxis induced by other chemoattractants including fMLP (*11*) and IL-8 (*23*). These inhibitory effects of EGCG might be based on its cytotoxicity for neutrophils, because EGCG is well-known as potent inducer of apoptosis in cancer cells (*5*). To exclude this possibility, we have estimated the effect of EGCG on neutrophil viability (**Figure 3**). Our results suggest that EGCG showed toxicity to neutrophils significantly but slightly, suggesting that the inhibitory effects of EGCG depend highly on its anti-inflammatory character, but not on toxicity.

It has been reported that EGCG inhibits endotoxin-mediated tumor necrosis factor-α production by blocking nuclear factor-κB activation through inhibiting IκB kinase activity (*13*, *24*). On the other hand, in a previous paper (*15*), we reported that chemokine levels in the pouch fluids significantly increased 4 h after the FITC−OVA challenge, the number of infiltrated neutrophils had a good correlation with total chemokine levels in the pouch fluids in the air pouch/FITC−OVA-induced allergic inflammation, and the neutrophil infiltration was suppressed by neutralization of chemokines with anti-CINC antibodies, indicating that neutrophil recruitment is also strongly dependent on chemokine levels in the allergic inflammation. However, in the present study, although the concentrations of CINC-1 (a CXC-chemokine) and rat MIP-1α (a CC-chemokine) in the pouch fluids were not influenced by oral administration of EGCG at a dose of 1.0 mg/rat (**Figure 7**), neutrophil infiltration was suppressed in the air pouch/FITC−OVA-induced allergic inflammation (**Figure 6**). It was demonstrated that rat plasma concentration of EGCG began to decrease 1−2 h after oral administration and low bioavailability of EGCG was observed as a result of slow absorption, high first pass effect, and wide tissue distribution (*25*, *26*). We can assume that, at a dose (1 mg/rat) of EGCG, concentrations of EGCG and its biologically active metabolite are unable to reach a level that can inhibit chemokine production at the inflammatory site of rat allergic inflammation. Alternatively, EGCG might act on neutrophils in the circulating blood preferentially before reaching the inflammatory site (in the air-pouch). As a result of this assumption, EGCG might suppress neutrophil infiltration without reducing chemokine levels in the pouch fluids in the allergic inflammation.

In conclusion, our results suggest that EGCG suppresses neutrophil migration by a direct action on neutrophils, but not by indirect actions including the inhibition of chemokine production at the inflammatory site.

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